Molecular Characterization of Infectious Bronchitis Virus from Chickens in Sokoto State, Nigeria

Mungadi, H.U.[1*], Mera, U.M.[2], Daneji, A.I[2], Musa, U.[3], Alkali, B.R [4], Bande, F.[5], Shuaibu, A.B.[4], Garba, S.[1] and Yakubu Y.[6]

[1]*Veterinary Teaching Hospital, Usmanu Danfodiyo University Sokoto, Nigeria*
[2] *Department of Veterinary Medicine Faculty of Veterinary Medicine Usmanu Danfodiyo University Sokoto, Nigeria*
[3]*Department of Veterinary Pathology, Faculty of Veterinary Medicine Usmanu Danfodiyo University Sokoto, Nigeria*
[4] *Department of Veterinary Microbiology, Faculty of Veterinary Medicine Usmanu Danfodiyo University Sokoto, Nigeria*
[5] *Department of Veterinary Services, Ministry of Animal Health and Fisheries Development Usman Faruk Secretariat, Sokoto State, Nigeria*
[6]*Department of Veterinary. Public Health and Preventive Medicine, Faculty of Veterinary Medicine Usmanu Danfodiyo University Sokoto, Nigeria*
**Corresponding author*

Abstract: **- The aim of this research was to apply molecular technique to characterize infectious bronchitis virus (IBV) isolates and determine evolutionary relationship and genetic diversity of IBV strains from chickens in Sokoto State, Nigeria. Tracheal and cloacal swabs were taken from 150 chickens showing some clinical signs of infectious bronchitis (IB) for reverse transcription polymerase chain reaction (RT-PCR), gene sequencing and Phylogenetic analysis. Sequencing and phylogenetic analysis showed four out of the strains isolated were close to NGA/A11637/2006 strain previously reported in Northern Nigeria while one sequence showed closeness to a Chinese patridge/GD/S14/2003 strain which has not been reported previously in Nigeria. It was therefore concluded that,this study has provided an update on some circulating IBV strainsand the identification of two new strains of which are genetically distinct from all those earlier reported. The strains might have been introduced through migratory birds as well mutation or recombination of the previously reported IBADAN strain.**

The recommendations were, there is need to know the pathogenicity of the circulatingIBV strains in the area and test the efficacy of available vaccines, also there is need to impose strict biosecurity measures in poultry houses in the study area to prevent contracting poultry diseases through migratory birds.

The Gen-Bank accession numbers for the full *S1* gene sequences of TR/B/SK/05 and CL/YM/IS/15 are MK659577 and MK659578 respectively.

Key words: **Molecular characterization, infectious bronchitis virus, S1 gene hypervariable region, Chickens, Sokoto state.**

I. INTRODUCTION

The disease infectious bronchitis (IB) is an acute, highly contagious disease of chickens caused by infectious bronchitis virus (IBV), which is classified as an enveloped, single-stranded RNA virus of the genus *gamma Coronavirus*, family *Coronaviridae*[1]. More than 50 serotypes within the IBV are recognized worldwide [2, 3].In the past, IBV was thought to be primarily a disease of chickens; however, other non galliforms were later identified to be infected with the virus though without significant clinical disease [4].The virus is spread via the respiratory route in droplets and expelled during coughing or sneezing by infected chickens, likewise infection may occur through ingestion of contaminated feed or water [5]. IBV causes significant economic losses, mostly because of reduced productivity rather than bird mortality [6].Although no pathognomonic signs in IB, the disease is characterized by respiratory signs such as gasping, coughing, sneezing, tracheal rales and nasal discharge [7,1].Other signs include depression, swollen face and frothy exudate in the eyes. In layers, there are respiratory distress, decrease in egg production and loss of internal egg quality and egg shell quality [1]. Lesions associated with IB include consolidation of the lungs, inflammation of the trachea, and the ureters and presence of urates in the ureters [8].

As conventional virus isolation technique has beentoo cumbersome, RT-PCR of reverse transcribed RNA has replacedit as a rapidand highly sensitive technique for the detection of IBV viral RNA [9]. Samples for IBV isolation are obtained as soon as clinical signs of the disease are evident. Tracheal swabs are preferred and are placed directly into cold media with antibiotics to suppress bacterial growth and preserve the viability of the virus [10]

Four virus-specific proteins have been identified; the spike (S) glycoprotein, the membrane or matrix (M) glycoprotein, the envelope (E) protein and the nucleocapsid (N) protein [11]. The nucleocapsid (N) protein of IBV is closely associated with the genomic RNA, and has highly conserved amino acid and nucleotide sequences, i.e. very little variation in the N-gene sequence is seen between various strains of IBV. The spike glycoprotein (S) is anchored in the viral envelope and is post translationally cleaved into two proteins designated S1 and S2. In contrast to the N protein, the S1 segment protein is very diverse in terms of both nucleotide sequence and deduced amino acid sequences [12, 13]. It is

believed that important neutralizing antibody-inducing epitopes are situated in the S1 protein, thus it is essential for development of protective immunity[12]. This region is also the most variable region among various IBV strains, thus, genotypic characterization largely depends on the hyper variable region of S1 glyco-protein [12]. It is important to isolate and type the IBV virus strains prevailing in a geographical area regularly and vaccinate birds accordingly with vaccine strains that offer maximum cross protection against the circulating/prevailing field virus. Combination of different strains may provide broad protection in comparison with single strain based vaccine. Similarly, vaccines can be developed from recently isolated IBV field strain(s) from a particular region [14]. Ducatez*et al.* [15]reported the presence of IBV strains in poultry in some West African countries (Niger and Nigeria) using virus cross-neutralization test and RT-PCR. The results showed not onlythat common IBV strains such as 793/B-, Massachusetts-, D274-and B1648-like strains circulate in the region but also a newstrain designated as "IBADAN" NGA/A116E7/2006 which is a QX-like IBV variant with unusual genetic and serological characteristics was identified. Since the reports of Ducatez*et al*. [15] on the strains of IBV in some states in Nigeria (Sokoto inclusive), there was dearth of information on the prevailing strains of the virus in this environment which necessitates carrying out research to know the current situation of the virus.

II. MATERIALS AND METHODS

2.1 Sampling frame

The study was carried out in Sokoto state Nigeria which has four agricultural zones namely; Sokoto, Isa, Gwadabawa and Tambuwal. The research was designed to be a prospective cross sectional study. Samples were conveniently collected from 150 chickens, these comprised of indigenous and exotic chickens from all of the four agricultural zones of the state. Indigenous chickens were sampled from live bird markets and back yard poultry, while exotic chickens which comprised of broilers, pullets and layers were sampled from commercial poultry houses and backyard poultry flocks situated across the state. Types of chickens, age and sex were used as sampling frame in this research. The study covered a period from November 2016 to February 2017. Table 2.1 and 2.2 show the sampling frame used in the study and areas where samples were collected respectively.

Table 2.1: Sampling frame

Variables	**Number of pooled samples**
Type	
Exotic	40
Indigenous	60
Age	
Adults	42
Young	58
Sex	
Male	42
Female	58
Zone	
Sokoto	54
Tambuwal	10
Gwadabawa	16
Isa	20

Table 2.2: Areas where samples were collected

Local Chickens	**Commercial Chickens**
Sokoto meat and vegetables market	Tabanki farms Sokoto
UnguwarRogo poultry market	Maska farms Sokoto
Achida Market	Dan Uda Farms Sokoto
Illela Market Tambuwal market	Ambarura farms GuiwaLowcostSokoto A backyard Poultry MaberaSokoto
Two households in Achida	Two backyard Poultry in LowcostSokoto
Two households in Illela	Three backyard Poultry in TamajeSokoto
Two households in Tambuwal	Three backyard poultry in Achida
	Three backyard poultry in Illela
	Three backyard poultry in Tambuwal

2.2 Sampling Method

Chickens with manifestations of IB were sampled (purposive sampling). One hundred and fifty (150) tracheal and cloacal swabs each were collected making 300 samples for viral RNA isolation and characterization. Each swab was placed in a sterile 1.5 micro tube containing 1mL of Phosphate buffered saline (PBS) as described by Martin *et al.* [16] and transported to Central Veterinary Research Laboratory, of the Usmanu Danfodiyo University, Sokoto in cold conditions. The swabs were then scrapped on the side of the tube to facilitate removal of the contents from the swab head. Swabs from three chickens of same location, sex, age category and from same route were pooled together in order to increase viral RNA concentration and to reduce the number of samples (100 pooled tracheal and cloacal swabs) and stored at -20^0C until analysis as described by Jonathan [17].

2.3 Viral RNA Extraction

Viral RNA extraction Mini kits QIAamp® (QIAGEN® Sample and Assay Technologies products sourced from Germany) with product code 52904 were used for RNA extraction as described by the manufacturer and were stored at room temperature before use. RNA extraction was done for the 100 pooled samples. Purity and concentration of the 100 extracted viral RNA were determined using a J.P Selecta series 2005® UV spectrophotometer.

2.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Commercial live IBV vaccine-strain H120 was used as positive control for RT-PCR optimization.

i First Reverse transcription (cDNA synthesis) and PCR

The extracted RNAs were reverse transcribed to form cDNA. This was performed with **5**µL of denatured RNA sample, 2µL of gDNA remover and 7uL of RNAse free water (Qiagen). The mixture was incubated at 42^0C for 2 minutes. This was then added to another mixture containing 1µL of reverse primer UTR 41, 4µL of quantiscript® RT-buffer and 1µL of quantiscript® reverse transcriptase and incubated at 42^0C for 30 minutes and inactivated at 95^0C for 3 minutes. The cDNA was stored at -20^0C.

Integrated DNA Technologies IDT® primers (Coral vial, IOWA, USA) were used for the PCR. The cDNA was screened for IBV using PCR targeted to the highly conserved 3'UTR of the virus genome with primers designed by Cavanagh*et al.*, [18].Forward **(**Sense specific) primer UTR11 was first used in combination with reverse (anti-sense specific) primer UTR 41to amplify a 266 base pair DNA fragment.

For the first PCR, IBV vaccine-strain H120 was used as positive control for RT-PCR optimization after which 5µL of cDNA was used with 12.5µL QIAGEN® master mix (MgCl2, dNTP) 2X (Coral vial, IOWA, USA) 1µL each of forward and reverse primers and 5.5µL of RNAse-free water to a final volume of 25µL in 0.2ml micro tubes. The tubes transferred into AB®thermocycler (Applied biosystem). The thermal profile for the PCR was 94 ˚C for 3 minutes, followed by 35 cycles of 94 ˚C for 30 seconds, 48 ˚C for 30 seconds and 72˚C for 1 minute, followed by 72^0 C for 10 minutes.To visualize the amplified PCR products, electrophoresis was carried out using 1.5% agarose gel and stained with ethidium bromide DNA Gel stain (Sigma Aldrich® Missouri USA) and 1X TBE buffer according to standard (85V, 3.00A, 300W) for 45 minutes. The products were screened for the presence of a specific fragment in comparison with a Gene Ruler 100 bp DNA Ladder (Thermo specific). Positive bands were checked on the gel under UV-light using BioRad® gel imager.

ii Detection of viral S1 gene

The second RT-PCR assay, was carried out to amplify the viral S1 gene. In this regards cDNA was synthesized from RNA of only UTR positive samples. While detection of S1 was carried out with the transcribed RNA, GBR1 as forward primer and GBR2 as reverse primer according to Fang *et al*., [19] (Integrated DNA Technologies IDT® primers). Sequences of the forward primer (GBR1) and reverse primer (GBR2) are shown in table 2.3. The anticipated amplicon was about 1800bp, encompassing the entire *S1* region. The PCR reaction (25 uL) contained 3uL of cDNA (about 100 ng), 12.5uL of 10x PCR master mix (qiagen) 2 uL of 10 mMol of each of the two primers (forward and reverse),7.5 uL of RNAse- free water. The PCR conditions for amplification were 94^0 C for 5 minutes, 30 cycles of 94^0 C for 40 seconds, 60^0 C for 40 seconds, and 72^0 C for 2 minutes, followed by 72^0 C for 10 minutes.

Electrophoresis using agarose gel was done as after the first PCR and viewed under UV light for positive bands. Amplicons of the positive samples were sent to Inqaba Biotechnical Industries West Africa, Ibadan which were further transported to the Industry's main branch in Pretoria, South Africa for sequencing.

Table 2.3Primers used in the study

Name	Sequence (5′ to 3′)	Target Region
GBR1	ATGTTGGTGAAGTCACTGTTTATA	*S1* gene
GBR2	ATACGCGTTTGTATGTACTCATCTG	*S1* gene

GBR: Fang *et al*. [19]

All the above mentioned procedures were carried out in Central Research Laboratory, Faculty of Veterinary Medicine,City Campus UDUS.

2.5 Sequencing and Phylogenetic Analysis

i. Sequencing

The PCR products were purified using a Monarch® PCR & DNA clean up kit (Biolabs®) following the instructions of the manufacturer.DNA (10–100 ng) was sequenced in both directions with a Big Dye™ Terminator version 3.1 cycle sequencing kit (Applied Biosystems™) Sequence studio genetic analyzer; (Applied Biosystems) using the PCR primers (IDT®) as sequencingprimers.

2.6 Data Analysis

Simple percentages were used to analyze PCR result.Basic Local Alignment Search Tool (BLAST) was used to determine sequence identities and query cover. The obtained sense and anti-sense sequences were submitted for quality evaluation using Phil's Read Editor (Phred) online application [20]. The sequences were assembled together with the Cap-Conting application in Bioedit 7.0.9.0 software. Multiple Sequence Comparison with Log Expectation (MUSCLE) was used to align the sequences with the reference sequences downloaded from National Center for Biotechnology Information (NCBI) database as recommended by Chenna *et al*.,[21] using MEGA 7 software [22] The software was used to construct a nucleotide Phylogenetic tree (Neighbor- joining, 1,000 bootstrap replications). IBV sequences from all relevant reference strains available in GenBank (NCBI) were used for comparison. The tree was used to determine the genotype of the sequenced IBV strains. The evolutionary distances were computed using the Maximum Composite Likelihood method [23]. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 80 positions in the final dataset.

III. RESULTS

After RNA extraction, 84 RNA passed purity and concentration test. From the gel image in the first round of PCR where 266bp was amplified using UTR primers and Biolabs® 100bp ladder, there was appearance of bands corresponding to the targeted position on DNA ladder, indicating the amplicons that were positive (figure. 3.1).

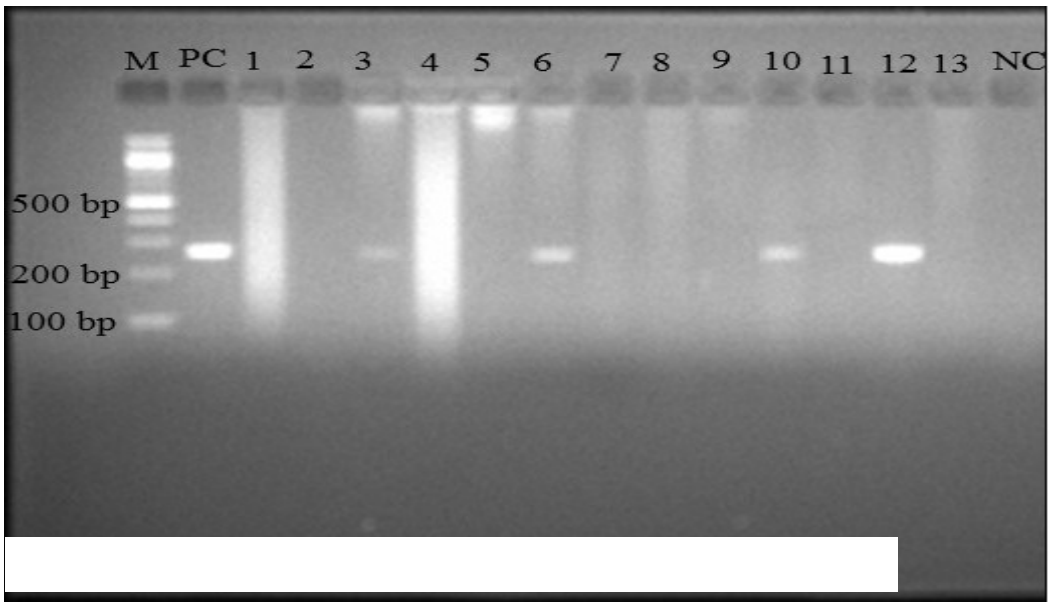

Figure 3.1Representative of elecrophoresis gel images of thefirst PCR (using UTR primers)

M= Molecular ladder, PC= Positive control, 1-13= well number, NC=Negative control.

Eleven positive samples were obtained at the end of first PCR (13%). The Samples were designated as route/type/location/sample ID number. TR=Trachea; CL=Cloaca; B=Broiler; P= pullet; (L= layer but none was positive); AF=Adult female (Local); AM=Adult male (Local); YM=Young male (Local); YF=Young female (Local); SK=Sokoto; GW=Gwadabawa; and IS=Isa. viz;TR/B/SK/05, TR/B/SK/06, TR/B/SK/23, TR/AF/SK/07, CL/AM/SK/06, TR/YM/IS/15, CL/YM/IS/15, CL/P/SK/05, CL/YM/GW/08, TR/AF/IS/04 and TR/YF/SK/16.

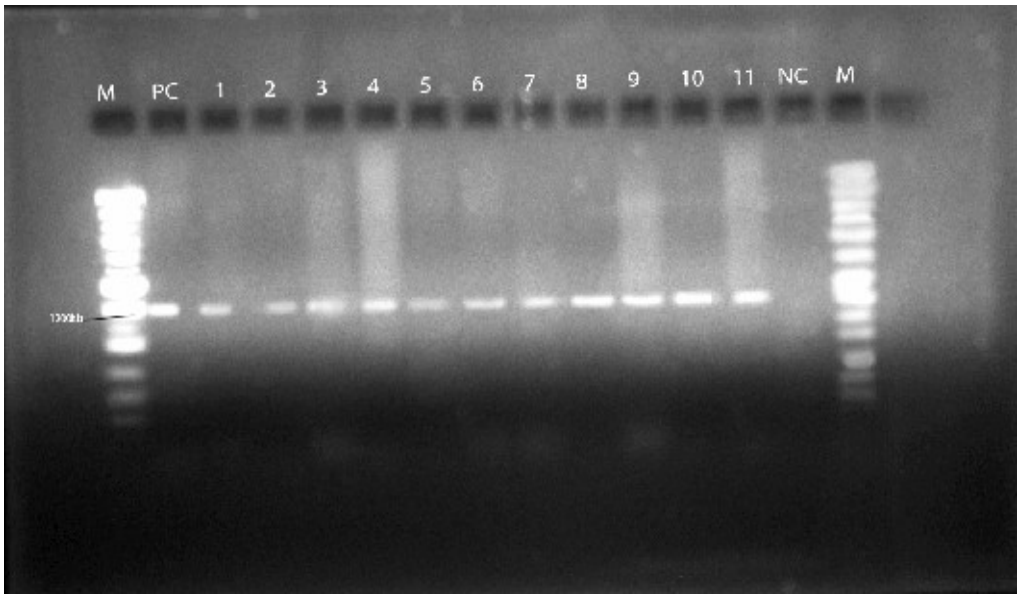

Figure 2.2Elecrophoresis gel image of second round of PCR (using *S1* primers)

M= Molecular ladder, PC= Positive control, 1-11= well number, NC=Negative control.

IV. SEQUENCING AND PHYLOGENETIC ANALYSIS

After processing the positive samples, the hyper variable region of the *S1* gene was sequenced and compared with reference IBV sequences available in GenBank (Fig. 4.1). Five (5) DNA sequences were obtained. The samples from which the sequences were obtained are TR/B/SK/05, TR/B/SK/O6, TR/AF/SK/07, TR/YM/IS/15 and CL/YM/IS/15.

Samples 1 and 2 belonged to broiler chickens from same back yard poultry in Mabera area Sokoto. Sample number 3 was from local adult hen from Sokoto meat and vegetable market. Samples 4 and 5 were obtained from local young cocks from Achida market.

Following the results of sequencing, BLASTing and Phylogenetic analysis in this study, it showed that nucleotide sequences obtained from four of the isolates; TR/B/SK/05, TR/B/SK/06, TR/AF/SK/07 and TR/YM/IS/15 are closely related to 'IBADAN' strain (NGA/A116E7/2006) accession number FN430415 a variant unique to Nigeria reported by Ducatez *et al*. [15] named accordingto the location where the reference virus was foundwith 82% nucleotide sequence similarity. While nucleotide sequences obtained from CL/YM/IS/15 is most closely related to IBV (patridge/GD/S14/2003 strain accession number AY636283 (GenBank) reported from Asia with82% nucleotide sequence similarity. The strain was also found to be closely related to Chinese IBV strains gamma CoV/ck/china/1101/16 and IBV strain YX10 with accession numbers KY62011 and JX840411 respectively which clustered on the Phylogenetic tree both with 80% nucleotide sequence similarity.

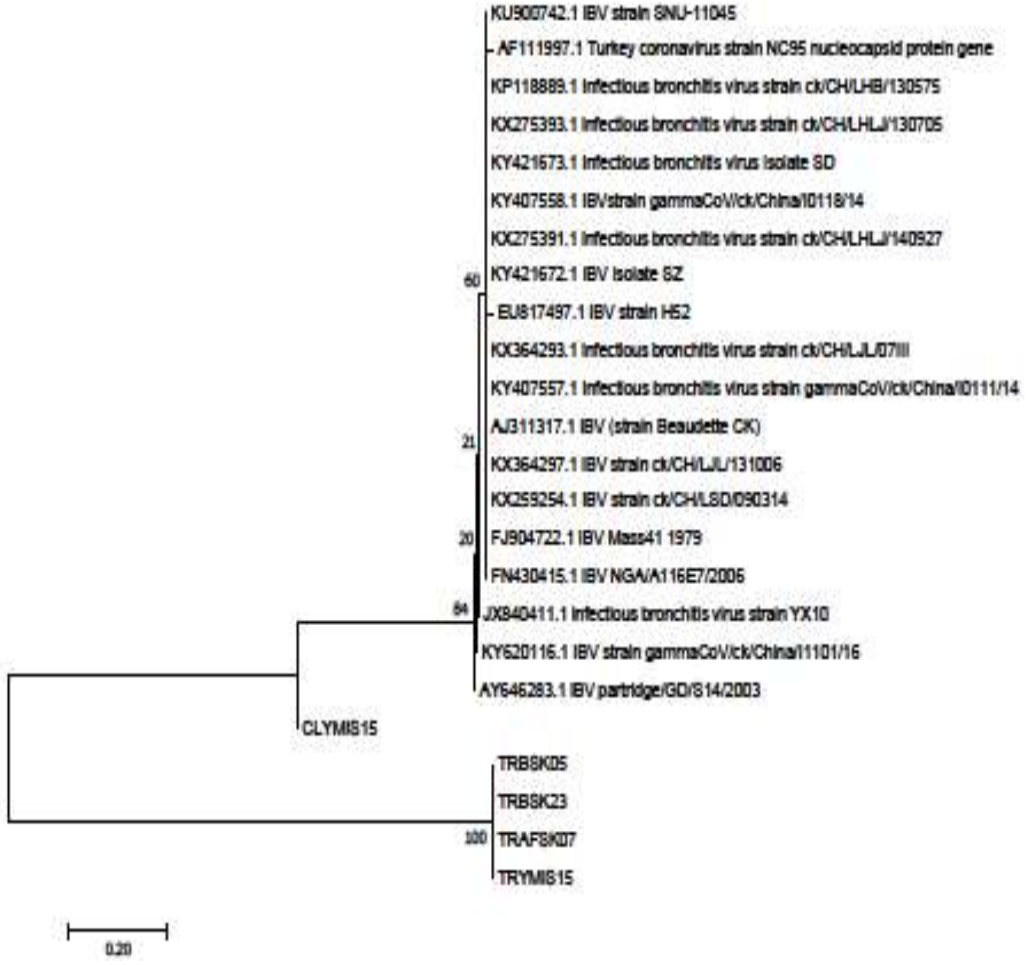

Fig.4.3 Phylogenetic Analysis of the Full-length IBV *S1* Gene Strains from SokotoState at Nucleotide Level.

The tree was constructed with the Neighbour joining method (Maximum composite likelihood) with 1000 bootstrap replicates. TR/B/SK/05, TR/B/SK/06, TR/AF/SK/07, TR/YM/IS/15, CL/YM/IS/15 and some IBV reference strains were included. Bar 0.20 nucleotide substitutions per site. The evolutionary history was inferred using the Neighbor-Joining

method [24]. The optimal tree with the sum of branch length = 2.07563859 is shown. 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.

V. DISCUSSION

In this study, positive samples were obtained from all the categories of chickens sampled (variables) except for Tambuwal zone where no positive sample was obtained. It should be considered that some of the extracted RNA failed purity and concentration test which eventually were not used in running the PCR. Also positive samples were obtained from both tracheal and cloacal swabs. IB is disease of all ages and the virus could be retrieved from organs of all the systems it affects [1].

The sequenced isolates TR/B/SK/05, TR/B/SK/06, TR/AF/SK/07 and TR/YM/IS/15 (Sq1) that clustered together in the phylogenetic tree in this study might have evolved as a result of mutation of NGA/A116E7/2006 (IBADAN) strain considering the high similarity of about 82%, or recombination of the IBADAN strain with vaccine strain M41 where they are evident to have clustered in the phylogenetic tree. This clearly showed that the characterized IBV strains have close relationship with the M41 commonly used for vaccination. This finding supports the report of Ducatez*et al.*, [15] where they reported that from the IBV strains they isolated, 3 clustered with vaccine strains and wild type strain D274 which they attributed to recombination between Mass like and D274 strains. They mentioned that in Nigeria, breeder farms vaccinate 10-day-old chicks with asingle dose of live-attenuated IBV vaccine (e.g. Massachusetts-likestrains H120 and H52) which therefore could not fully excludethat some of the strains detected 'IBADAN strains' were vaccine-derived. The 'IBADAN' like strains in this study could belong to respiratory pathotypes considering that they were obtained from tracheal swabs of chickens manifesting clinical signs of IB even though Ducatez*et al*. [15] reported by that there were absence of obvious clinical signs in chickens infected with the IBADAN strain.

One of the strains 'CL/YM/IS/15' (Sq2) was found to be closely related to patridge/GD/S14/2003,CoV/ck/china/1101/16 and IBV-YX10 virus strains obtained from cloacal swab of young male indigenous chicken in Isa zone. The strain might have been introduced through migratory birds as birds that are in contact with or reared close to chickens infected with IBV could serve as carriers. There is no particular clinical sign previously attributed to patridge/GD/S14/2003, CoV/ck/china/1101/16 and IBV-YX10 virus strains. Our findings also confirms that there are differences in nucleotide sequences between the strains sequenced in this study and the previously reported strains deposited in GenBank that were used for comparison. It is well known that IBV strains have great diversity in their *S1* gene which is responsible for the virus classification; this complicates the establishment of vaccine strategies to control the disease.With continuous mutation of the virus and lack of well established vaccination strategy against the disease in the study area, it could be believed that the virus would continue to spread among chickens in the environment.

The sequences of TR/B/SK/05 (Sequence1) and CL/YM/IS/15 (Sequence2) were deposited in GenBank and were assigned MK659577 and MK659578 accession numbers respectively.

VI. CONCLUSION

From this work it was concluded that, the sequenced isolates are believed to be new IBV strains in chickens in Sokoto State which are genetically distinct from all reported IBV strains. The strains might have been introduced through migratory birds as well mutation or recombination of the previously reported IBADAN strain. This is the first study where Chinese like strains patridge/GD/S14/2003, CoV/ck/china/1101/16 and IBV-YX10 are reported in Nigeria.

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