RESEARCH ARTICLE

Sokoto Journal of Veterinary Sciences

(P-ISSN 1595-093X: E-ISSN 2315-6201)

http://dx.doi.org/10.4314/sokjvs.v22i4.4

Shettima *et al.*/*Sokoto Journal of Veterinary Sciences*, **22**(4): 259 - 266.

Molecular characterization of chicken infectious anaemia virus isolated from village chickens in Maiduguri, Nigeria

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Publication History:
Received: 04-03-2024
Revised: 30-09-2024
Accepted: 10-10-2024

Abstract

The presence and genetic variability of chicken infectious anaemia virus (CIAV) among apparently healthy village chickens in Maiduguri, Nigeria, were investigated using conventional polymerase chain reaction (PCR) and Sanger's sequencing, respectively. A total of 100 tissue samples (thymus, liver, bursa of Fabricius, and spleen) were collected from 100 apparently healthy village chickens of 1-13 weeks old. The tissues from each bird were pooled and then frozen at -20°C. Chicken infectious anaemia virus DNA was extracted from the pooled tissues and subjected to PCR and DNA sequencing. The PCR results showed that a total of 42/100 (42%) of the pooled tissue samples were positive for CIAV. The result of the sequencing indicated genetic variations among the field CIAVs detected in the pooled tissues of the village chickens. The evolutionary relationship inferred between six of the isolates (CIAV 15, 41, 42, 80, 43, and 37) obtained in this study formed a cluster with isolates from India, Thailand, Japan, South India, Taiwan, China, USA, and Australia, while CIAV 14 and CIAV 36 (with 87% homology) diverged to formed a sub-clade. Two other Nigerian isolates, CIAV 79 (with 52% homology with the six isolates) and CIAV 39 (57% homology with CIAV 79) formed a separate clade. Therefore, CIAV had been found in apparently healthy village chickens in Maiduguri, and some of the isolates formed a cluster with isolates from other parts of Asia, America, and Europe. There is a need to investigate the interactions between CIAV and immune cells of village chickens so that accurate preventive measures against the disease will be taken.

Keywords: Anaemia, CIAV, PCR, Sequencing, Village chicken

Introduction

Chicken infectious anaemia virus (CIAV) is a non-enveloped, negative-sense single-stranded DNA virus with icosahedral symmetry belonging to the family *Anelloviridae* (Schat, 2003; Li *et al.,* 2017; Rosario *et al.,* 2017). It is a small (25nm) virus of about 2300 base pairs with three open reading frames (McNulty *et al.,*1990). The virus is the only member of the genus *Gyrovirus* (Li *et al.,* 2017; Rosario *et al.,* 2017), and encodes viral proteins VP1, VP2, and VP3 (Ducatez *et al.,* 2008). The CIAV was first recognized and isolated from commercial chickens during an investigation of Marek's disease vaccine accident in Japan in 1974 (Yuasa *et al.,* 1979). The virus is highly resistant to most disinfectants in nature and hence ubiquitous (Miller *et al.,* 2003).

The disease caused by CIAV usually affects chicks less than 3 weeks of age and is associated with high mortality characterized by severe anaemia, lymphoid depletion, yellowish to whitish bone marrow, atrophy of bursa of Fabricius, thymus, and haemorrhages (Yuasa *et al.,* 1979; Miller *et al.,* 2003). Concurrent infections caused by CIAV and other avian viruses are known to caused reduction of the cortical thermocytes which in turn caused immunosuppression in chickens (Noteborn & Koch, 1995). The CIAV causes serious economic challenges, especially to the broiler industry and the producers of specific pathogen-free eggs (Oluwayelu, 2010; Shettima *et al.,* 2017). Loses in CIAV infections are due to poor growth, high mortality, and the cost of antibiotics used to control secondary bacterial infections (McNulty, 1991). Despite the economic consequences caused by this virus to the poultry industry as reported in different parts of the world, there is a paucity of information about the virus, including its genetic variability in the study area.

Materials and Methods

Sample collection and storage

A total of 100 tissue samples were conveniently collected from the thymus, liver, bursa of Fabricius, and spleen of village chickens. This includes 14 chickens from Maiduguri Monday market; 12 chickens from Maiduguri Custom Market; 11 chickens from Chad Basin ward; 12 chickens from Mairi ward; 12 chickens from University of Maiduguri staff quarters; 9 chickens from 303 Housing Estate; 12 chickens from Dalori Quarters; 10 chickens from Malari ward; and 8 chickens from Gwange ward, all in Maiduguri Metropolis, Borno state, Nigeria. The samples were placed in sample bottles and stored at -20 ^{0}C refrigerator at the Virology Laboratory, Faculty of Veterinary Medicine, University of Maiduguri, Nigeria.

DNA extraction, concentration, and purity

Tissue samples from each bird were pooled, and DNA extraction was carried out from each homogenized pooled tissue sample using innuPREP DNA Mini kit 2.0 (Aj innuscreen, GmbH, Berlin, Germany), according to the manufacturer's protocol. The concentration and purity of the extracted DNA described by Hamisu *et al*. (2022) and stored at -20^{o}C.

Conventional polymerase chain reaction (PCR) and agarose gel electrophoresis

The primers that can specifically detect CIAV were searched using the BLASTn online resource. Table 1 outlines the details of the primers used for the PCR. Before the commencement of the PCR, a positive control of chicken infectious anaemia virus (Cux-1 DNA) was obtained from the Influenza Laboratory of the National Veterinary Research Institute (NVRI) Vom, Plateau State, Nigeria.

The conventional PCR was performed using innuPREP DNA Mini kit 2.0 and the samples were run on Eppendorf Mastercycler (Nexus, Hamburg, Germany), with a final concentration of 25µl. The reaction mixture consists of nuclease free H_2O (8.5µl), F-Primer (10µM) 0.5µl, R-Primer (10µM) 0.5µl, 2×Master mix 12.5µl and template DNA 3.0 µl. The cycling conditions included an initial denaturation step of 30 seconds at 95^{o}C; annealing for 45 seconds at 64^{o}C; and elongation for 5 minutes at 65^{o}C. Two percent agarose was prepared, then, 2µl of loading dye and 5µl of PCR amplicons were mixed, and then loaded into the wells of the agarose. The gel tank was connected to the power source at 120V for 25 minutes. The result was viewed using a UV trans-illuminator, gel documentation system (Cleaver Scientific, UK).

Sanger's sequencing

Ten of 42 samples that were PCR-positive for CIAV were identified for sequencing. DNA from these samples was extracted and purified using Wizard® SV Gel and PCR Clean-Up System purification kit (Promega, USA). The purified PCR products were then sent to Inqaba® Biotech (Inqaba® Biotech, South Africa) for sequencing. After the sequencing, the data were forwarded for further bioinformatics analysis.

Data analysis

Sequence data were analysed using BioEdit and MEGA 7 software (Kumar *et al.,* 2016) to compare the relatedness between sequences from this study and other sequences of CIAV in the Genbank. Other demographic data were presented on tables.

Table 1: Primers used to amplify and sequence the gene of CIAV obtained in naturally infected village chickens in Maiduguri, Nigeria

Primer name	Sequence (5′-3′)	Size	Position	Amplicon size	Reference
Cux-standard	O3F - CAAGTAATTTCAAATGAACG	20	452-471	387	Cardona *et al.* (2000)
	O3R- TTGCCATCTTACAGTCTTAT	20	819-838		

Results

The conventional PCR successfullify detected CIAV DNA in tissue samples collected from village chickens in Maiduguri. Specifically, the CUX-1 primer set amplified the expected 387-base pair (bp) DNA fragment, which was visualized as a distinct band on an agarose gel following electrophoresis (Figure 1). Out of the 100 tissue samples analyzed using the conventional PCR approach, 42 (42%) samples were positive for CIAV nucleic acid and 10 were conveniently used for sequencing.

The sexes of the village chickens' samples tested showed 20/44 (45.5%) males and 22/48 (45.8%) females were positive for CIAV DNA, while none of those whose sex were not determined tested positive for CIAV 0/8(0%) (Table 2).

The age distribution of the CIAV DNA-positive samples showed that 13/41 (31.7%) were for the age group chicks and 29/59 (49.2%) for growers (Table 3). Table 4 presents the distribution of CIAV DNA-positive samples based on the location of the birds sampled. The result shows a variation in prevalence rates between locations- Maiduguri Monday Market live bird market: 13/14 (92.9%), Chad Basin: 8/11 (72.7%), Dalori quarters: 7/12 (58.3%), Gamboru live bird market: 5/12 (41.7%), 303 housing estate: 3/9 (33.3%), Unimaid staff Quarters: 4/12 (33.3%), Gwange backyard farm: 0/8 (0%) and Malari ward: 0/10 (0%). Using a neighbour-joining tree, the evolutionary relationship amongst the 10 sequences of CIAV VP1gene revealed that eight of the isolates (CIAV 41, CIAV 43, CIAV 42, CIAV 15, CIAV 80, CIAV 14, CIAV 37, and CIAV 36) formed a major cluster, which are further subdivided into three distinct sub-clusters formed by CIAV 36; CIAV 41 and CIAV 43; and CIAV 42, CIAV 15, CIAV 80, CIAV 14, CIAV 37. Furthermore, an additional cluster is formed by CIAV 39 and CIAV 79 (Figure 2). The sequences were deposited in Genbank, and accession numbers were provided (MW051846- MW051855).

The estimates of evolutionary distance between ten sequences using the Jukes-Lantor Model at 1 decimal place showed that the five isolates (CIAV 14, CIAV 37, CIAV 80, CIAV 15, and CIAV 42) are identical to each other, while isolates CIAV 36, CIAV 41 and CIAV 43 also appeared to be identical. However, isolates CIAV 39 and CIAV 79

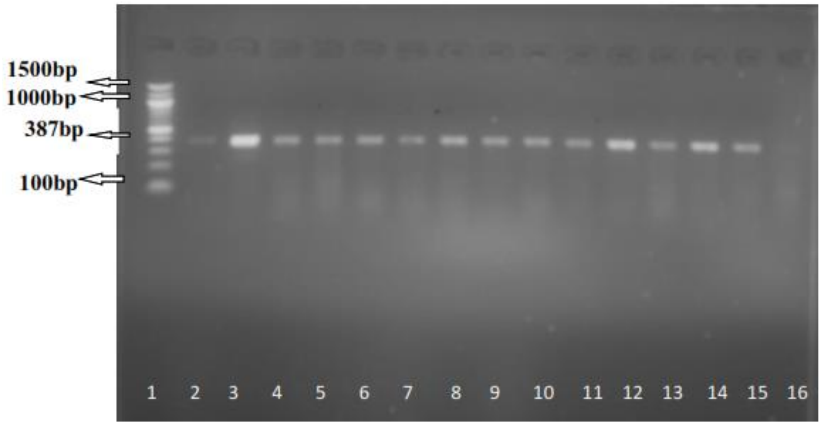

Figure 1: Agarose gel electrophoresis of CIAV. The size of the target gene is 387bp; Lane: L1= 100bp molecular weight marker, L16 = negative control, L3=positive control, L4-15= +ve samples, L2= -ve sample

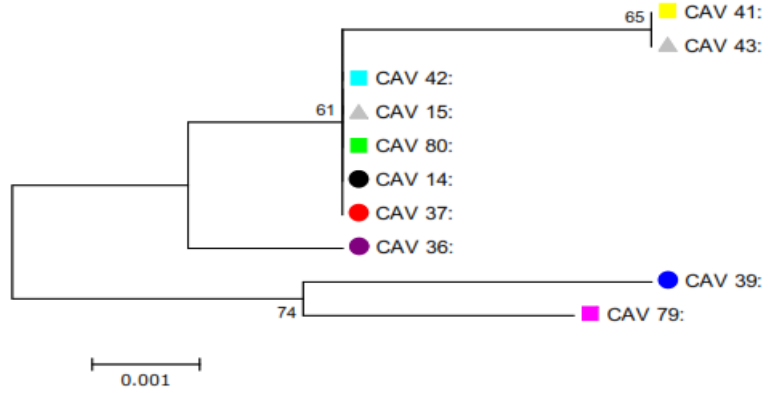

Figure 2: Phylogenetic tree of 10 CIAV VP1- gene sequences obtained from village chickens in Maiduguri Nigeria. The tree was inferred using the Neighbour-Joining method, with 1000 bootstrap replicates

Table 2: Sex distribution of village chicken tested for CIAV DNA in Maiduguri, Nigeria

Sex	Number tested	Number (%) positive
Male	44	20 (45.5)
Female	48	22 (45.8)
Not sexed	8	0 (0)
Total	100	42 (42)

Table 3: Age distribution of village chickens tested for CIAV DNA in Maiduguri, Nigeria

Age group	Number of Samples Tested	Number Positive
Chicks	41	13[a] (31.7)
Growers	59	29[a] (49.2)
Total	100	42 (42)

Table 4: Spatial distribution of village chickens tested for CIAV DNA in Maiduguri, Nigeria

Location	Number of samples tested	Number (%) positive
Malari ward	10	0[a] (0)
Chad basin (backyard)	11	8[a] (72.7)
Unimaid staff quarters	12	4[a] (33.3)
Custom market	12	5[a] (41.7)
Monday market	14	13[a] (92.9)
303 housing estate	9	3[a](33.3)
Mairi (backyard)	12	2[a] (16.7)
Dalori quarters	12	7[a] (58.3)
Gwange (backyard)	8	0[a] (0)
Total	100	42 (42)

Table 5: Pairwise distance estimation of 10 CIAV VP1- gene sequences obtained from village chicken in Maiduguri, Nigeria

	1	2	3	4	5	6	7	8	9	10
CIAV_14:		0.003	0.000	0.005	0.003	0.005	0.000	0.000	0.000	0.003
CIAV_36:	0.003		0.003	0.005	0.004	0.004	0.003	0.003	0.003	0.004
CIAV_37:	0.000	0.003		0.005	0.003	0.005	0.000	0.000	0.000	0.003
CIAV_39:	0.009	0.012	0.009		0.006	0.004	0.005	0.005	0.005	0.006
CIAV_41:	0.003	0.006	0.003	0.012		0.006	0.003	0.003	0.003	0.000
CIAV_79:	0.009	0.006	0.009	0.006	0.012		0.005	0.005	0.005	0.006
CIAV_80:	0.000	0.003	0.000	0.009	0.003	0.009		0.000	0.000	0.003
CIAV_15:	0.000	0.003	0.000	0.009	0.003	0.009	0.000		0.000	0.003
CIAV_42:	0.000	0.003	0.000	0.009	0.003	0.009	0.000	0.000		0.003
CIAV_43:	0.003	0.006	0.003	0.012	0.000	0.012	0.003	0.003	0.003	

showed some degree of divergence (Table 5). The evolutionary relationship inferred between the isolates from this study compared with other geographically dispersed CIAV isolates using the neighbour-joining method showed that ten of the Nigerian isolates from this study, CIAV 1 CIAV 36, CIAV 37, CIAV 15, CIAV 41, CIAV 42, CIAV 80, CIAV 43, CIAV 79 and CIAV 39 form a cluster and a major clade with those isolates from India, Thailand, Japan, South India, Taiwan, China, USA and Australia, while CIAV 14 and CIAV 36 appear to diverge a little further. However, two of the Nigerian isolates CIAV 79 and CIAV 39 appeared to diverge singly and form a sub-clade (Figure 3). It is important to note that other isolates from southeastern Nigeria appeared to distance themselves from all other isolates and clustered to form a separate clade.

The estimation of evolutionary divergence for all the Nigerian and foreign isolates revealed that CIAV 14 and CIAV 36 appear to be identical, while CIAV 37, CIAV 41, CIAV 80, CIAV 15, CIAV 42 and CIAV 43 are identical to isolates from India, Thailand, Japan, Taiwan, South India, China and the USA. However, two of the Nigerian isolates from Oyo and Abeokuta (south) appear to be identical. It is important to note that other Nigerian isolates including CIAV 79 appear not to be identical to all other isolates. Isolates from this study, CIAV 39 appear to be identical to an isolate from Australia, while two isolates from Thailand and China appear to be identical to each other (Table 6).

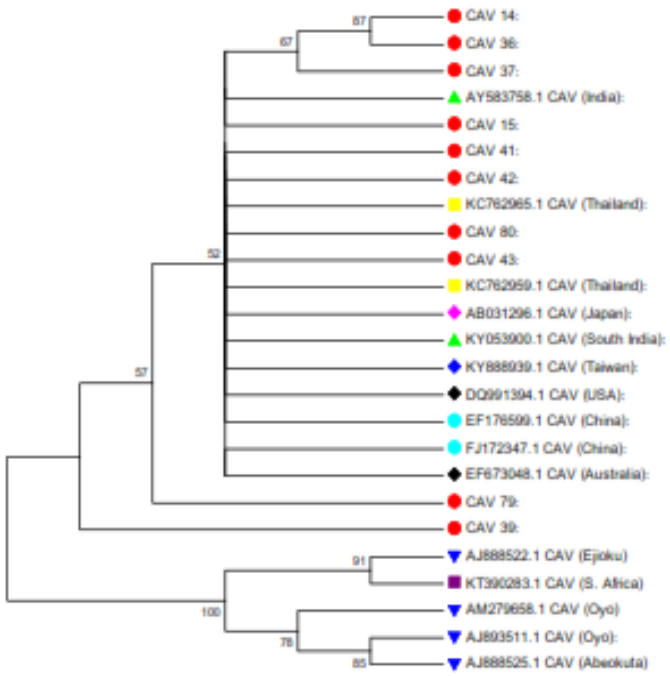

Figure 3: Phylogenetic relatedness of the CIAV isolates (CIAV 14,15,36,37,39,41,42,43,79 and 80) obtained in this study with other geographically dispersed CIAV isolates. The tree was inferred using the Neighbor-Joining method, with 1000 bootstrap replicates

polymerase chain reaction (PCR) have the major advantage of providing a faster and sensitive detection of more fastidious viral pathogens. The presence of CIAV DNA at the expected base pairs of 387bp in the 42% of tissues from apparently healthy village chickens tested in Maiduguri, Nigeria, supports the report of Adedeji *et al.* (2024) who detected CIAV in 45.8% of the twenty-four pulled tissue samples collected from backyard poultry flocks in Jos, Nigeria. Recently, a study conducted in Korea reported that all 28 tissue samples from chickens collected for PCR were positive for CIAV (Song *et al.,* 2024). However, a previous study by Mohamed (2010) in Egypt reported CIAV DNA in only 26.6% of 7-week-old broilers. Similarly, Kulkarni *et al*. (2024) reported that out of the 50 pooled tissue samples collected from layer chickens in India, 20% were positive for CIAV by PCR. Therefore, the result from this study can be considered high when compared with some previous findings, thus, reflecting an attention-demanding situation. Backyard or free-range chickens may play a role in the epidemiology of CIAV. Knowledge of their infection status is important for adopting

Table 6: Pairwise distance estimation (4 decimal places) between the 10 CIAV VP1- gene sequences obtained from village chicken in Maiduguri, Nigeria and other foreign isolates

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
CIAV_14:		0.000	0.004	0.006	0.004	0.005	0.004	0.004	0.004	0.004	0.004	0.004	0.007	0.102
CIAV_36:	0.000		0.004	0.006	0.004	0.005	0.004	0.004	0.004	0.004	0.004	0.004	0.007	0.102
CIAV_37:	0.006	0.006		0.006	0.003	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.006	0.100
CIAV_39:	0.015	0.015	0.012		0.005	0.003	0.005	0.005	0.005	0.005	0.005	0.005	0.007	0.096
CIAV_41:	0.006	0.006	0.003	0.009		0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.098
CIAV_79:	0.012	0.012	0.009	0.003	0.006		0.004	0.004	0.004	0.004	0.004	0.004	0.007	0.098
CIAV_80:	0.006	0.006	0.003	0.009	0.000	0.006		0.000	0.000	0.000	0.000	0.000	0.005	0.098
CIAV_15:	0.006	0.006	0.003	0.009	0.000	0.006	0.000		0.000	0.000	0.000	0.000	0.005	0.098
CIAV_42:	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000		0.000	0.000	0.000	0.005	0.098
CIAV_43:	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000	0.000		0.000	0.000	0.005	0.098
AY583758.1_CIAV_(India):	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000	0.000	0.000		0.000	0.005	0.098
KC762959.1_CIAV_(Thailand):	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000	0.000	0.000	0.000		0.005	0.098
AB031296.1_CIAV_(Japan):	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.098
KC762965.1_CIAV_(Thailand):	0.009	0.009	0.006	0.012	0.003	0.009	0.003	0.003	0.003	0.003	0.003	0.003	0.006	0.100
KY053900.1_CIAV_(South_India):	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.098
KY888939.1_CIAV_(Taiwan):	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.098
DQ991394.1_CIAV_(USA):	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.098
FJ172347.1_CIAV_(China):	0.009	0.009	0.006	0.012	0.003	0.009	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.098
EF176599.1_CIAV_(China):	0.006	0.006	0.003	0.009	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.098
EF673048.1_CIAV_(Australia):	0.015	0.015	0.012	0.018	0.009	0.015	0.009	0.009	0.009	0.009	0.009	0.009		0.098
AJ893511.1_CIAV_(Oyo):	0.991	0.991	0.980	0.958	0.968	0.968	0.968	0.968	0.968	0.968	0.968	0.968	0.968	
AM279658.1_CIAV_(Oyo)	0.947	0.947	0.936	0.915	0.926	0.926	0.926	0.926	0.926	0.926	0.926	0.926	0.926	0.031
AJ888525.1_CIAV_(Abeokuta)	0.991	0.991	0.980	0.958	0.968	0.968	0.968	0.968	0.968	0.968	0.968	0.968	0.968	0.034
AJ888522.1_CIAV_(Ejioku)	0.958	0.958	0.947	0.926	0.936	0.936	0.936	0.936	0.936	0.936	0.936	0.936	0.936	0.080
KT390283.1_CIAV_(S._Africa)	0.968	0.968	0.958	0.936	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.083

Discussion

successful prevention and control strategies, such as biosecurity and vaccination. Chicken Infectious Anaemia Virus has been identified using different virological, serological (Oluwayelu *et al.,* 2005; Shettima *et al.,* 2017) and molecular techniques (Schat, 2003; Oluwayelu, 2010). Molecular techniques like polymerase chain reaction (PCR) have the major advantage of providing a faster and sensitive detection of more fastidious viral pathogens. Therefore, the detection of CIAV DNA in village chicken tissues by PCR in this study confirms the susceptibility of these chickens to CIAV infections since DNA presence is evidence that they were actually infected with the virus. Since vaccination measures against CIAV is not practiced among backyard chickens in Nigeria (Diaz, 2014), the detection of CIAV DNA in the apparently healthy, free-roaming Nigerian backyard chickens in this study indicates natural exposure to the virus and implicates them as potential sources of the infection to commercial chickens. This agrees with the findings of Simeonov *et al.* (2014) in Bulgaria, Hegazy *et al.* (2014) in Germany, Hussein *et al.* (2016) in Sharkia Egypt and Shettima *et al.* (2017) in Maiduguri, Nigeria. The sex distribution of the samples tested revealed no difference between the sexes. This finding contradicts the results of Shettima *et al.* (2017), who reported a higher prevalence of the disease in males compared to females. Notably, unlike Shettima *et al.* (2017) who collected a higher number of samples from male birds, in this study, higher number of females birds were sampled. The higher prevalence observed among female birds may be attributed to this disparity in sampling representation.

There was no difference in the age distribution in the detection rates of CIAV DNA among village chickens in the study area. This does not tally with the report by Al-Ajeeli *et al.* (2020) who observed higher seropositivity in older birds (28-30 weeks). This finding is also not consistent with previous findings that indicated an increase in seropositivity rate at older ages (Owoade *et al.,* 2004; Sharma *et al.,* 2014). Similar findings to the result of this study were reported in India (Bhatt *et al.,* 2011) and China (Zhou *et al.,* 1996). The higher CIAV DNA detection rate reported in Maiduguri live bird markets could be due to the fact that birds are kept in close contact with higher degree of congestion coupled with transport stress. Similar findings were also reported in Ibadan (Oluwayelu *et al.,* 2008) and Maiduguri, (Shettima *et al.,* 2017).

Two of the isolates, (CIAVs 14 and 36) in this study appeared to be identical when compared with other global isolates. However, the remaining isolates (CIAV 37, CIAV 41, CIAV 80, CIAV 15, CIAV 42 and CIAV 43) correspond (identical) to isolates from India, Thailand, Japan, Taiwan, South India, China and USA, while CIAV 39 appeared to correspond (identical) to an isolate from Australia. It is also important to note that CIAV 79 appears not to correspond (identical) to all other isolates from this study, this is an indication that CIAV 79 could be a different strain of CIAVs. This supports the findings of Van Santan *et al.* (2001) and Ducatez *et al.* (2006) on the existence of CIAV mixed infections or quasi-species and the earlier report of Oluwayelu & Todd (2008) that indigenous chickens probably harbour a mixed population of CIAV strains. Also, co-infections with at least two CIAV strains were reported in indigenous chickens in southern Nigeria (Oluwayelu, 2010). The origin of the CIAV DNA observed in this study maybe due to natural exposure and transmission could be vertical or horizontal. Eight of the CIAV DNA from this study had the same nucleotide sequence with CUX-1 which was different from that obtained for the other two isolates (CIAV 79 and CIAV 39). This suggests that Nigerian backyard chicken CIAVs differ at the nucleotide sequence level (Oluwayelu, 2010).

Generally, the CIAVs are diverse belonging to genotypes II and III (Adedeji *et al.,* 2024), and the genetic study of the virus in Nigeria and some African countries confirmed that CIAV both genotypes II and III were circulating in poultry in these countries (Ducatez *et al.,* 2006; Snoeck *et al*., 2012).

Therefore, the findings of this study showed that chicken anaemia virus is widely distributed among village chickens of different sexes and age groups. It is also important to note that CIAV 79 is distinct from all other isolates from this study, an indication that CIAV 79 is a different strain of CIAVs. The study indicated that circulating CIAV in Maiduguri show nucleotide variations with the sequences of some CIAVs from other parts of the world. This also proves the existence of CIAV mixed infections in indigenous chickens. Further, lower productivity and poor performance generally associated with backyard chickens in Nigeria, among other factors, could also be related to CIAV infections. In addition, backyard chickens provide a rich milieu for the generation of novel genotypes of CIAV that may alter the epidemiologic picture of this virus in the future.

Acknowledgements
The authors wish to thank the staff of the North-East Biotechnology Centre of Excellence, University of Maiduguri for assisting the researchers during the molecular aspect of the work.

Funding
No funding was received.

Conflict of Interest
The authors declare that there is no conflict of interest.

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