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Molecular epidemiology of chicken anemia virus in Nigeria

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Summary. Between February 2002 and May 2004, chicken anemia virus (CAV) was detected by PCR in organ samples from 14 flocks of poultry farms in Lagos, Ogun and Oyo States in Southwestern Nigeria. The farms reported low (<5%) to high mortalities (up to 100%) with various lesions at necropsy. The complete VP1 gene of 30 of these positive strains was sequenced. Strains that diverged by up to 4.4% on a nucleotide level differed only by up to 2.5% at the amino acid level (7 aa) as a result of clustered silent mutations. No amino acid substitutions specific for Nigerian strains were observed. Some birds had a CAV mixed infection. Genetic clustering of the VP1 gene did not correlate with differences in flock mortality but the co-infection of CAV with IBDV may be particularly lethal. This first molecular epidemiological study of CAV in Africa shows that the Nigerian strains cluster with viruses from very diverse geographic origins and were almost as diverse (4.4%) as all other strains combined (5.8%).

Introduction

Chicken anemia is caused by a virus that was first isolated in specific-pathogen-free (SPF) chicks in Japan in 1979 [20]. Characteristic symptoms are aplastic anemia paired with hemorrhagic lesions. Other lesions include watery blood, pale bone marrow, atrophy of thymus and bursa, and swollen and discolored liver. Generalized lymphoid atrophy and immune-suppression are often associated with secondary viral, bacterial or fungal infections [14]. Increased consumption of antibiotics and depressed weight gain represent an economic threat especially for the broiler industry and SPF-egg producers, even if direct mortality caused by chicken anemia virus (CAV) is usually relatively low.

CAV has been found in many countries with a poultry industry [14]. Although the virus is thought to be ubiquitous, it has not been reported from Africa with

the exception of South Africa, where CAV was diagnosed only by pathology and serology in young chicks [18] and in Nigeria, where a serology study was recently carried out in commercial chicken flocks [12]. Only a limited number of CAV strains from the US, Asia, Australia and Europe and none from Africa were genetically characterized.

CAV, the only member of the genus *Gyrovirus* (family *Circoviridae*) is a non-enveloped, icosahedral virus of about 25 nm in diameter with a negative-sense single-stranded circular DNA genome. The viral genome consists of 2.3 kb with 3 partially overlapping open reading frames (ORFs) [10] for VP1, the major viral structural protein (51.6 kDa), VP2, a scaffolding protein (24 kDa) and VP3, a non-structural protein named apoptin (13.6 kDa) for its ability to induce apoptosis. VP1 and VP2 are the main targets of neutralizing antibodies.

In most cases, the VP1 gene, which seems to present the highest variability of the 3 ORFs was submitted to Genbank. So far all viruses seem to belong to the same worldwide serotype [14], but the emergence of new serotypes cannot be excluded and would have important consequences for vaccine efficacy and serodiagnosis.

Chicken farming provides an important source of animal protein, but chicken meat is expensive in most parts of Africa, whereas it is one of the cheapest meat in Europe. In Nigeria, 211000 metric tons of chicken meat and 476000 metric tons of eggs were produced in 2004 (http://faostat.fao.org). Production could be further increased and costs reduced if disease outbreaks could be properly controlled. In a recent serological study, we reported that in Nigeria most commercial flocks become infected soon after maternally derived antibodies disappear [12]. Chicken anemia is normally not recognized or diagnosed in Nigerian farms. Here we report for the first time the detection and molecular characterization of CAV in African poultry.

Material and methods

Organ samples

Between February 2002 and May 2004, some farms in Lagos, Ogun and Oyo States in Southwestern Nigeria reported low (<5%) to high mortalities (up to >60% in commercial chicken flocks, and up to 100% in an experimental flock) with and without lesions at necropsy. Some flocks showed signs of co-infection with infectious bursal disease virus (Table 1). 128 organ samples (113 thymuses, 5 livers and 10 bursa of Fabricius) were collected during necropsy from 23 flocks of 7 commercial farms in Lagos, Ikire, Lanlate and Ibadan (Lagos, Ogun and Oyo States) and a naturally infected experimental flock at the Department of Veterinary Medicine, University of Ibadan (Nigeria). Flocks counted between 189 and 26000 chickens with an average of about 8200 birds. Samples were collected from 3 to 36 weeks old cockerels, broilers, pullets and breeders and stored for up to 2 years at −20 °C until further processing. None of the farms vaccinated against CAV.

DNA isolation

15–50 mg of tissue were homogenized in a manual tissue grinder (Bioblock, Tournai, Belgium) containing 300 µl of double distilled water (DDW) to a liquid paste. Tissue grinders were

Table 1. Origin of organ samples from which viruses were sequenced

Farm id	Flock id	Location	Chicken type	Flock size	Age of bird (weeks)	Signs at necropsy	Signs of IBDV[a] infections	Mortality	Sample number[b]
U	1	Lanlate	broiler	26000	6	no specific lesion	no gross lesion	<5%	12
U	2	Lanlate	broiler	21000	3	no specific lesion	no gross lesion	<5%	28
U	3	Lanlate	broiler	18000	7	no specific lesion	no gross lesion	<5%	71, 73
C	4	Ibadan	broiler	10000	5	hyperaemic and swollen thymus	?	<5%	100, 101, 102, 103
E	5	Ejioku	broiler	4200	4	cloudy air sacs, slightly swollen hyperaemic thymus	?	8%	105, 106, 107, 108
T	6	Ikire	pullet	3985	6	minor muscular hemorrhages	pathology +	11% (in 9 days)	9, 11
S	7	Lagos	pullet	6000	5	hemorrhages on thigh muscles, enlarged and hemorrhagic bursa	histopathology	35–40%	20
S	8	Lagos	pullet	5200	5	hemorrhages on thigh muscles, enlarged and hemorrhagic bursa	histopathology	35–40%	30, 31, 34
S	9	Lagos	pullet	5200	5	hemorrhages on thigh muscles, enlarged and hemorrhagic bursa	histopathology	35–40%	38
G	10	Ibadan	cockerel	189	5	loss of weight, no specific lesion	no gross lesion	53%	111, 112, 113, 114, 115
A	11	Abeokuta	young breeder	6400	12	gout, intestinal and gastric hemorrhages	PCR −	>60%	117, 118
A	12	Abeokuta	young breeder	3600	6	gout, enlarged gall bladder	PCR −	>60%	119, 120
A	13	Abeokuta	breeder	5200	36	bung eye, ectoparasite infestation, pedunculate follicle	PCR −	>60%	121
B	14	Ibadan	layer	10	21	dullness, diarrhea, muscular hemorrhages and swollen bursa	PCR +	100%	116

B: experimental birds naturally infected by CAV
[a]IBDV: Infectious bursal disease virus
[b]Corresponds to last digit of the WHO designation

rinsed twice with 10% sodium hypochlorite, 3 times with double distilled water and once with 70% isopropanol before being dried and reused for another sample of the same flock. Between flocks, tissue homogenizers were kept for at least 6 hrs in 10% sodium hypochlorite at room temperature before further rinsing. Homogenates were stored for less than 3–4 days at −20 °C until further use for DNA isolation. DNA was extracted using the QIAamp DNA Blood mini kit (Qiagen, Leusden, The Netherlands), following the instructions of the manufacturer. DNA was eluted in 60 µl elution buffer and stored at −20 °C until further use.

Polymerase Chain Reaction (PCR)

The extracted DNA was first screened for CAV genome using a highly sensitive nested detection PCR of a constant region of the VP2–VP3 overlapping region (nucleotides 516 to 725, numbering according to Meehan et al. [9]). Sensitivity of detection was $1.7 * 10^{-2}$ $TCID_{50}$ of a commercial CAV vaccine (Nobilis® CAV P4; Intervet B.V., Boxmeer, The Netherlands). The VP1 gene was amplified from CAV-positive samples either in a nested or a semi-nested format. The gene was sequenced in 3 partially overlapping fragments: S1, S2 and S3 (Table 2). All PCRs were performed in 25 µl with 0.5 U Platinum® Taq DNA Polymerase (Invitrogen, Merelbeke, Belgium) per reaction for the 1st round PCR and 1 U of Platinum® Taq per reaction for the nested PCR. The equivalent of 0.5 µl of the reaction mix of the first PCR was transferred to a new tube for the nested reaction. The primers used (Eurogentech, Seraing, Belgium) as well as the specific PCR conditions are summarized in Table 2. All programmed cycling was performed in a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). PCR amplicons were analyzed in a 1.5% agarose gel (Ultrapure, Invitrogen, Merelbeke, Belgium), using 1× TAE as electrophoresis running buffer and stained with ethidium bromide (15 µg in 100 ml of agarose gel). The above commercial vaccine was used as positive PCR control and to optimize the different PCR.

Cloning

Some PCR products were cloned before sequencing using the Topo TA cloning® kit for sequencing (Invitrogen, Merelbeke, Belgium). PCR products were inserted into a pCR®4-TOPO® plasmid and transformed into TOP10 *E. coli*. Colonies were then selected and analyzed by PCR and PCR products of plasmids containing the inserts of interest were sequenced.

Sequencing

PCR products were purified using the Jetquick PCR purification kit (Genomed, Loehne Germany). Purified products were quantified with Pico Green (Molecular Probes, Leiden, The Netherlands) using a Tecan Genios Reader (Mechelen, Belgium). 10 ng DNA were used for sequencing in both directions with the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Nieuwerkerk, The Netherlands) on a capillary sequencer (model 3100 avant, Applied Biosystems, Nieuwerkerk, The Netherlands) using the PCR primers as sequencing primers (Table 2). In case of nucleotide ambiguity, sequencing was repeated. Nigerian VP1 sequences were submitted to GeneBank under the accession numbers AJ888519 to AJ888532. Identical sequences were submitted only once. Nobilis® CAV P4 vaccine sequence was also submitted to Genbank under the accession number AJ890284. Strains were designated following a nomenclature recommended by WHO for measles or influenza virus for instance (CAV/location.WHO country code/week.year/sample number).

Table 2. Experimental conditions of nested detection PCR and nested sequencing PCR

PCR		Primer sequence	[Primer] (nM)	Annealing (°C)	[$MgCl_2$] (mM)	Amplicon (bp)	PCR programme: denaturation, annealing, elongation (s), number of PCR cycles	Primer source
1st round detection		Fwd: 03F: 5′ CAAGTAATTTCAAATGAACG 3′ Rev: 03R: 5′ TTGCCATCTTACAGTCTTAT 3′	500	54	4	386	30 s, 30 s, 60 s, 35 cycles	Cardonna et al. (2000)
Nested detection		Fwd: N3: 5′ CCACCCGGACCATCAAC 3′ Rev: N4: 5′ GGTCCTCAAGTCCGGCACATTC 3′	500	60	4	209	30 s, 30 s, 60 s, 35 cycles	
1st round sequencing	S1 fragment	Fwd: OS1F: 5′ CCCTCGAAGAAGCGATCCTG 3′ Rev: OS1R: 5′ AAAGCGGGTTTCAATGTGTG 3′	500	58	4	751	30 s, 30 s, 60 s, 35 cycles	this study
	S2 fragment	Fwd: OS2F: 5′ CCGGTCAGTTGATTGCGGA 3′ Rev: OS2R: 5′ TTTGTGCCTTGCGCTACGTA 3′	500	60	2	782	30 s, 30 s, 60 s, 35 cycles	
	S3 fragment	Fwd: S3F2: 5′ GCTTTGCAACACTCACAG 3′ Rev: S3R7: 5′ CCCTTTCAGGGCTGC 3′	500	55	2	422	30 s, 30 s, 60 s, 35 cycles	
Nested sequencing	S1 fragment	Fwd: S1F: 5′ CCCCGAACCGCAAGAAGGTGTAT 3′ Rev: S1R: 5′ CCGTGGGCTGCATCATCATT 3′	500	60	2	563	60 s, 60 s, 60 s, 40 cycles	this study
	S2 fragment	Fwd: S2F: 5′ GGCCTAATTGCTGGCTGCCGCT 3′ Rev: S2R: 5′ GGGGTCCCCGGCTCCTCTCG 3′	500	69	2	573	60 s, 60 s, 60 s, 40 cycles	
	S3 fragment	Fwd: S3F: 5′ TTTTCCTCCAGGGCAACGTTCA 3′ Rev: S3R7: 5′ CCCTTTCAGGGCTGC 3′	200	60	4	403	20 s, 20 s, 40 s, 20 cycles	

Data analysis

Sequences were analyzed using the Bioedit program [5]. This program was also used to read the sequencing electropherograms, to exclude nucleotide ambiguity and to identify double peaks as indication of potential mixed infections. To ensure the reliability of sequences, forward and reverse sequences were aligned with ClustalW [1]. Phylogenetic analysis was based on the entire VP1 gene sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 [8]. Phylogenic analysis of nucleic acid and deduced amino acid sequences was done with the Neighbor Joining method, Kimura 2 parameters. Amino acid sequences were also analyzed with the Neighbor Joining method, with the Poisson correction. Bootstrap values (1000 replications) were indicated on each tree. Nucleotides were numbered according to Meehan et al. [9].

Results

Nucleic acid alignment and phylogenic analysis

From 53 organs (47 thymuses, 5 livers and 1 bursa) positive for CAV in the nested detection PCR, 30 complete sequences of VP1 gene were obtained from 14 different flocks and 8 farms (Lanlate, Ibadan, Ejioku, Ikire, Lagos and Abeokuta, Table 1). Partial sequences were obtained from 7 additional samples. All 30 sequences were 1350 nucleotides long and had no insertions or deletions. In half of the sequences, the nucleotides in positions 975 and 1032 could not be unequivocally determined because of repeated conflicting results in the forward and reverse sequences: forward sequences gave a C and a G; reverse sequences displayed a T and an A in these nucleic acids positions. Both mutations were also found in the other strains: the Del-Ros strain (AF313470) displays for instance ^{975}T and ^{1032}A and TR20 (AB027470) ^{975}C and ^{1032}G. These synonymous nucleotides had no influence on the amino acid level. Nigerian VP1 sequences presented 21 nucleotide mutations that had not been observed before in strains from other parts of the world (nt positions 36, 180, 210, 225, 246, 306, 345, 348, 540, 747, 753, 810, 840, 849, 900, 1028, 1077, 1149, 1188, 1275, and 1278, Table 3). Samples from a given flock had identical sequences (e.g. CAV/Ikire.NIE/11.02/9 and CAV/Ikire.NIE/11.02/11) with the exception of CAV/Ibadan.NIE/11.02/102 which differed from other strains (CAV/Ibadan.NIE/11.02/100, CAV/Ibadan.NIE/11.02/101 and CAV/Ibadan.NIE/11.02/103) of the same flock by a single nucleotide (C1094G) and CAV/Abeokuta.NIE/19.04/117 which differed from CAV/Abeokuta.NIE/19.04/118 by 15 nucleotides (positions 36, 414, 717, 747, 849, 900, 975, 1028, 1077, 1080, 1110, 1116, 1263, 1278, and 1338).

Nucleotide diversity of the 27 Nigerian VP1 of Fig. 1 was 4.4% in comparison to 5.8% for the 30 relevant CAV VP1 sequences from other parts of the world listed in GenBank. The Nigerian sequences did not increase the latter diversity.

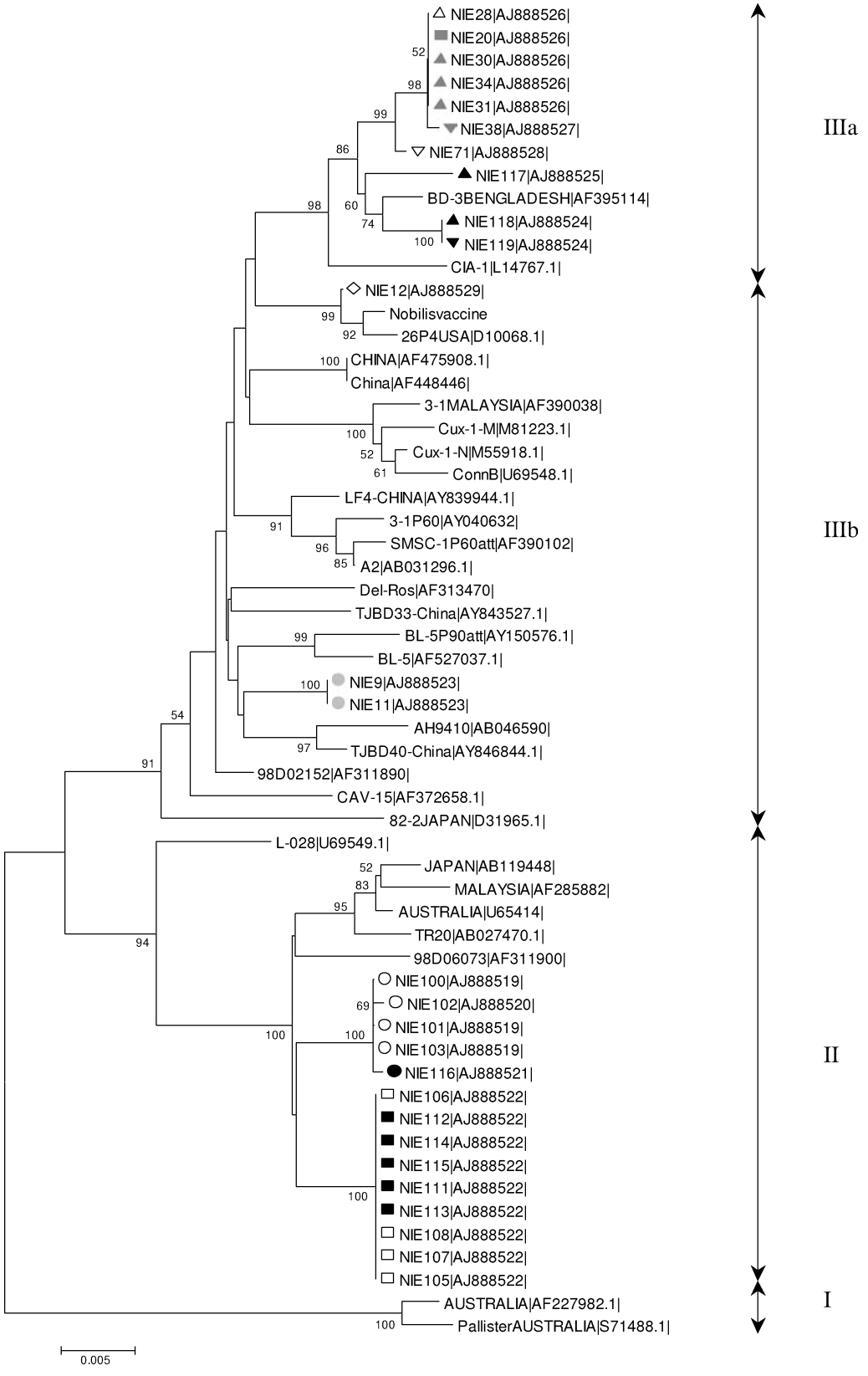
Phylogenic analysis of 27 complete Nigerian VP1 sequences revealed 2 major groups with either 2 or 4 subgroups. The complete phylogenetic tree obtained by aligning the 58 VP1 sequences shows three major clusters (Fig. 1): one with only the 2 Australian strains (Pallister and AF227982); a second one with some Nigerian strains, and strains from the USA, Japan, Australia and

Table 3. Nucleic acid substitutions specific to Nigerian CAV VP1

Strains[a]	Nucleic acid position[b]																								
	36	180	210	225	246	306	345	348	540	708	747	753	789	810	840	849	900	1028	1077	1149	1188	1275	1078	1299
JAPAN(AB119448), MALAYSIA(AF285882), AUSTRALIA(U65414), TR20(AB027470.1), 98D06073(AF311900), CAV-15(AF372658.1)	T	G	C	C	C	C	G	A	G	T	G	A	T	C	C	G	C	G	C	T	G	G	T	A
NIE105, NIE106, NIE107, NIE108, NIE111, NIE112, NIE114, NIE115	.	.	.	.	A	.	.	.	T	.	.	T	.	.	.	.	.	.	.	C	.	C	.	.
NIE100, NIE101, NIE102, NIE103, NIE116	.	A	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	G
L-028(U69549.1), SMSC-1P60att(AF390102), A2-JAPAN(AB031296.1), 3-1P60(AY040632), NobilisVaccine, CHINA(AF475908.1), CHINA(AF448446), BL-5P90att(AY150576.1), BL-5(AF527037.1), 98D02152(AF311990), 82-2JAPAN(D31965.1), TGB40China(AY846844.1), LF4China(AY839944.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
BD-3BANGLADESH (AF395114), 26P4USA(D10068.1), **NIE12**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.
CIA-1(L14767.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.
NIE9, NIE11	.	A	.	G	.	T	.	C	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.
AH9410(AB046590), Del-Ros(AF313470)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
Cux-1-M(M81223.1), Cux-1-N(M55918.1), ConnB(U69548.1), 3-1MALAYSIA(AF390038)	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
NIE20, NIE28, NIE30, NIE31, NIE34	.	.	A	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	A	.	.	.
NIE38	.	.	A	A	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	A	.	.	.
NIE71	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	
AUSTRALIA(AF227982.1), PallisterAUSTRALIA (S71488.1)	.	.	.	.	.	.	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
TGB33China(AY843527.1)	.	.	.	G	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
NIE117	C	.	.	.	.	.	.	.	.	.	C	.	.	.	A	C	T	A	.	C	.	.	.	G
NIE118, NIE119	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	T	C	.	.	A	G

[a]Nigerian strains in bold

[b]Numbering according to Meehan et al. [9]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NIE71|AJ888528|
NIE117|AJ888525|
BD-3BENGLADESH|AF395114|
NIE118|AJ888524|
NIE119|AJ888524|
CIA-1|L14767.1|
NIE12|AJ888529|
Nobilisvaccine
26P4USA|D10068.1|
CHINA|AF475908.1|
China|AF448446|
3-1MALAYSIA|AF390038|
Cux-1-M|M81223.1|
Cux-1-N|M55918.1|
ConnB|U69548.1|
LF4-CHINA|AY839944.1|
3-1P60|AY040632|
SMSC-1P60att|AF390102|
A2|AB031296.1|
Del-Ros|AF313470|
TJBD33-China|AY843527.1|
BL-5P90att|AY150576.1|
BL-5|AF527037.1|
NIE9|AJ888523|
NIE11|AJ888523|
AH9410|AB046590|
TJBD40-China|AY846844.1|
98D02152|AF311890|
CAV-15|AF372658.1|
82-2JAPAN|D31965.1|
L-028|U69549.1|
JAPAN|AB119448|
MALAYSIA|AF285882|
AUSTRALIA|U65414|
TR20|AB027470.1|
98D06073|AF311900|
NIE100|AJ888519|
NIE102|AJ888520|
NIE101|AJ888519|
NIE103|AJ888519|
NIE116|AJ888521|
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NIE115|AJ888522|
NIE111|AJ888522|
NIE113|AJ888522|
NIE108|AJ888522|
NIE107|AJ888522|
NIE105|AJ888522|
AUSTRALIA|AF227982.1|
PallisterAUSTRALIA|S71488.1|
0.005
IIIa
IIIb
II
I

Malaysia; the third group included some other Nigerian strains as well as 23 strains from many other countries. The inter-group divergence was 4.9 to 5.7% for the groups I–II, 5.3 to 5.8% for the groups I–III and 2.6 to 4.6% for the groups II–III. Maximal intra-group diversities were 0.6, 2.7 and 3.6% for groups I, II and III respectively.

Amino acid alignment and phylogenic analysis

When the amino acid sequences of 28 Nigerian VP1 genes were aligned, the maximal amino acid diversity was 2.5%, only half of the maximal nucleotide diversity. The maximal amino acid diversity of 4.1% of the 31 non-Nigerian sequences was not increased by the Nigerian strains.

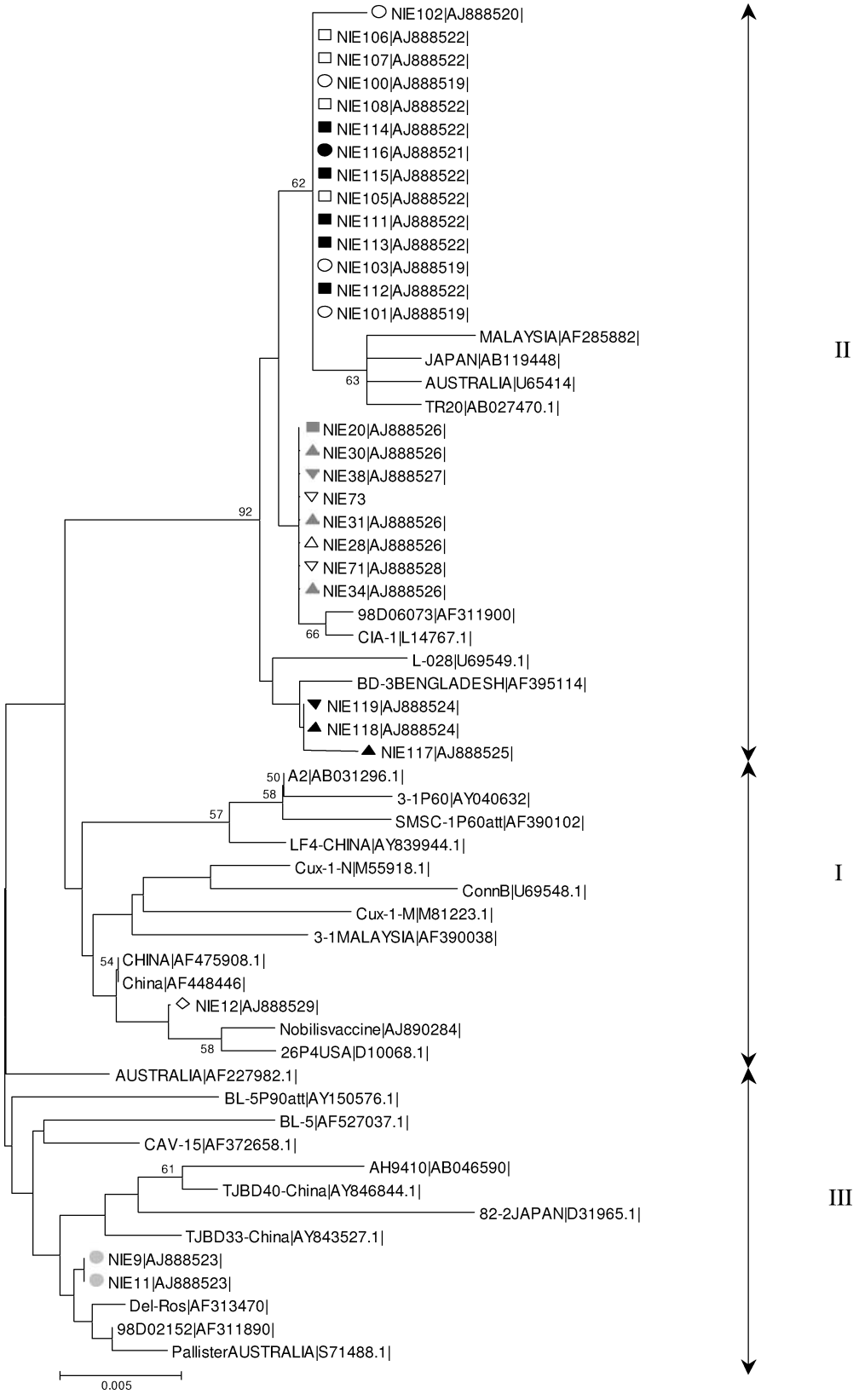
Only 7 variable amino acids positions were detected in more than one Nigerian strain (positions 75, 97, 139, 144, 287, 413 and 370, the latter with 2 distinct amino acids; numbering according to Meehan et al. [9]). Four additional mutations were detected in a single strain only (E254G and S447T for CAV/Lanlate.NIE/11.02/12, T365S for CAV/Ibadan.NIE/11.02/102 and R343K for CAV/Abeokuta.NIE/19.04/117). Thus only 2 of these mutations were found in the hypervariable region (aa 139–151, [13]). Apart from the T365S for CAV/Ibadan.NIE/11.02/102 and R343K for CAV/Abeokuta.NIE/19.04/117, no amino acid mutation was only found in Nigerian strains.

The phylogenetic tree showed 3 major clusters, each of which contained Nigerian strains (Fig. 2). Interestingly, except for CAV/Ikire.NIE/11.02/9-11 and CAV/Lanlate.NIE/11.02/12, all Nigerian strains as well as a strain from Bangladesh and one from the USA that clustered in group III at the nucleotide level (Fig. 1) became closely related at the amino acid level to nucleotide group II sequences as a result of synonymous nucleotides. Similarly, the groups of CAV/Ibadan.NIE/11.02/100-like and CAV/Ejioku.NIE/11.02/107-like viruses (Fig. 1) differed only by silent mutations to become identical at the amino acid level (Fig. 2).

The inter-group divergence was 1.4 to 3.4% (group I–II), 1.2 to 2.5% (group I–III) and 1.8 to 4.1% (group II–III). Maximal intra-group diversities were 2.9, 1.6 and 2.9% for group I, II and III respectively.

When all published strains were considered, amino acid substitutions were observed in 52 positions (CAV/Ibadan.NIE/11.02/100 as reference strain), 28 of which were found each in only one strain. In the "hypervariable" region (aa 139–151, [13]), 5 positions were mutated in at least 2 strains and another 3 positions in a single strain (data not shown). The probability of mutations within the

Fig. 1. Phylogenic analysis of 27 new complete CAV VP1 nucleic acids sequences from Nigeria, the Nobilis® P4 vaccine and 30 complete VP1 sequences currently available on GenBank. Numbers at nodes correspond to bootstrap values >49. Nigerian strains from the same flock are given the same symbol. Open, gray and closed symbols correspond to low, intermediate and high mortality, not necessarily related to CAV infection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NIE107|AJ888522|
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NIE113|AJ888522|
NIE103|AJ888519|
NIE112|AJ888522|
NIE101|AJ888519|
MALAYSIA|AF285882|
JAPAN|AB119448|
AUSTRALIA|U65414|
TR20|AB027470.1|
NIE20|AJ888526|
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NIE34|AJ888526|
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TJBD33-China|AY843527.1|
NIE9|AJ888523|
NIE11|AJ888523|
Del-Ros|AF313470|
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54
58
61
0.005
II
I
III

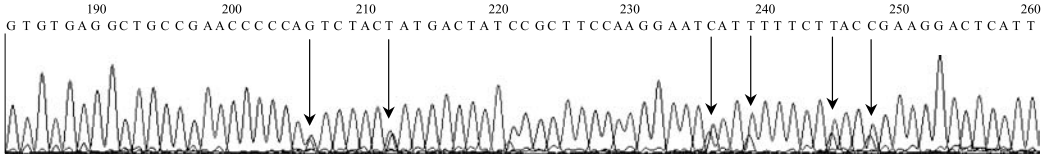

Fig. 3. Electropherogram of CAV/Nigeria.NIE/11.02/73 forward sequence from nucleic acid positions 173 to 250. Arrows indicate double peaks

13 amino acids of the "hypervariable" region was 62% (8/13) whereas the probability of mutation within the whole VP1 amino acid sequence was only 12% (52/450) confirming the hypervariable nature of the amino acid domain 139–151.

Mixed infection

The above nucleotide sequence analysis did not include CAV/Lanlate.NIE/11.02/73, CAV/Abeokuta.NIE/19.04/120, or CAV/Abeokuta.NIE/19.04/121 because of ambiguous nucleotides in respectively 34, 58 and 49 positions, despite repetitive sequencing. The electropherograms show that ambiguity was caused by clear double peaks (Fig. 3). Cloning experiments of CAV/Lanlate.NIE/11.02/73 VP1 segments confirmed 2 distinct CAV strains at the nucleic acid level (data not shown). The 2 cloned nucleotide sequences were also distinct from the CAV/Lanlate.NIE/11.02/71 sequence obtained from the same flock (data not shown) while these 3 strains were identical at the amino acid level (Fig. 2).

Discussion

This is the first molecular analysis of CAV from the African continent. The study is based on the genetic diversity of the VP1 gene of 30 new strains. The comparison by Islam et al. [7] of 13 amino acid sequences of VP1 and of the 10 available complete sequences of VP2 and VP3 showed that with a maximal diversity of 4%, VP1 was more diverse than VP2 (1.4%) and VP3 (2.2%). A similar observation was made in 14 CAV strains from Alabama, where 7 amino acid substitutions were found in the N-terminal half of VP1 and only 1 in the C-terminal halves of VP2 and VP3 [17]. When we repeated this analysis on a nucleotide level, we found maximal diversities of 5.8%, 1.6% and 2.2% for VP1 (1350 nt), VP2 (651 nt) and VP3 (369 nt) for the strains compared by Islam et al. [7]; and 5%, 1.2% and 1.2% for the sequenced parts of VP1 (642 nt), VP2 (357 nt) and VP3 (178 nt) for the 14 Alabama strains. Thus VP1 is clearly more diverse also on the nucleotide level than the other two CAV genes.

Fig. 2. Phylogenic analysis of the predicted amino acids sequences of Fig. 1. Numbers at nodes correspond to bootstrap values >49. The three major groups were identified as I, II and III

Although 53 organs samples were positive for CAV in the nested detection PCR, full-length VP1 sequences were obtained only for 30 samples probably because of the longer fragments targeted in the sequencing PCR (up to 573 bp versus 209 bp for the nested detection PCR, Table 2) and partial DNA degradation as a result of extended storage. On the other hand, we cannot rule out that some strains may have been missed because of mutations within the primer locations.

On a nucleotide level the Nigerian strains cluster with viruses from very diverse geographic origins and were almost as diverse (4.4%) as all other strains combined (5.8%). Islam et al. [7] identified common signatory amino acids ^{75}I/T, ^{97}L, ^{139}Q and ^{144}Q for their group II with strains from Australia (704), Japan (TR-20), USA (CIA-1 and L-028), and Bangladesh (BD-3). These 5 strains were also member of our group II (Fig. 2), which included in addition a Malaysian (AF285882), and other US (98D06073) and Japanese strains (AB119448) as well as 26 of our Nigerian strains. All of these strains have the above group II amino acid profile (Fig. 2). The strains from Alabama [17] having a ^{75}I, ^{97}L, ^{139}Q and ^{144}Q profile also clustered with our group II strains at the amino acid level.

Three clusters were identified in the phylogenetic tree derived from the deduced amino acid sequences of 58 VP1. Although Islam et al. [7] observed also 3 clusters when they compared 13 VP1 sequences, their grouping has been reshuffled. Interestingly some strains (members of groups IIIa and II: CAV/Ibadan.NIE/11.02/100-like and CAV/Ejioku.NIE/11.02/107-like strains on the one hand, CAV/Lagos.NIE/11.02/20-like, CAV/Lagos.NIE/11.02/38 and CAV/Lanlate.NIE/11.02/71 on the other hand) differed only by silent mutations. As a consequence, most Nigerian strains are almost identical at the amino acid level, despite an up to 4.4% difference at the nucleotide level (Fig. 2). Similar strains from other countries do not undergo this reshuffling. The 40 silent mutations were unevenly distributed throughout the VP1 genome: 13/40 (32.5%) silent mutations were found between amino acids 335 and 372 representing only 8.4% (38/450 aa) of the VP1 gene. Another 17.5% (7/40) of the described silent mutations were between amino acids 65 and 79 (corresponding to only 3.3% of the total aa sequence), just after the end of the overlapping VP2–VP1 ORFs (aa 59/60 of VP1), suggesting that the additional constraints due to the overlapping ORFs seems to limit the accumulation of mutations.

In some of the sequencing electropherograms double peaks suggested mixed infections or quasi-species. Similar to observations in the Alabama strains [17], we confirmed co-infections of at least 2 strains by cloning and sequencing of individual clones of CAV/Lanlate.NIE/11.02/73 VP1 segments. Similar double peaks were also seen in CAV/Abeokuta.NIE/19.04/120 and CAV/Abeokuta.NIE/19.04/121 but were not further investigated. Thus CAV does not seem to develop extensive quasi-species and even co-infection seems to be relatively infrequent. In a few sequences discrepant nucleotides were reproducibly found in the forward and reverse strands. Additional cloning experiments would be required to distinguish between mixed infections/quasi-species and other explanations such as disparate secondary structures that could bias certain nucleotide positions.

The flocks included in this study displayed a variety of diverse symptoms and flock mortality ranged from <5% to 100% (Table 2). All Nigerian strains sequenced in this study had a threonine in position 89 (instead of an alanine), which was thought to be associated with attenuation and a weaker reactivity with the monoclonal antibody 2A9 [15]. However, Todd et al. [16] suggested that the above mutation would only attenuate in combination with ^{75}I, ^{125}L, ^{141}L and ^{144}E. Few Nigerian strains showed some of these mutations (^{89}T and ^{144}E for CAV/Ikire.NIE/11.02/9, CAV/Ikire.NIE/11.02/11 and CAV/Lanlate.NIE/11.02/12, all associated with a lower mortality, ^{89}T and ^{75}I for all the other Nigerian strains). A lower pathogenicity was also thought to be associated with a Q394H [19], a substitution absent in the Nigerian strains. *In vitro*, ^{139}Q and/or ^{144}Q phenotypes have been proposed to be responsible for a reduced spreading capacity [13]. All the Nigerian isolates had these mutations except for CAV/Ikire.NIE/11.02/9, CAV/Ikire.NIE/11.02/11 and CAV/Lanlate.NIE/11.02/12 (^{139}K and ^{144}E profile).

The CAV/Lanlate.NIE/11.02/12 strain, the only Nigerian strain in the cluster I (amino acid level), is not a vaccine strain since Nigerian flocks are normally not vaccinated against CAV. The low mortality associated with the corresponding flock (Table 1) could perhaps be seen in the context of its similarity with the live-attenuated vaccine Nobilis® CAV P4 (Fig. 2; 5 nt substitutions responsible for only 2 aa substitutions), although other strains were also associated with low mortality. Viruses from flocks with different mortalities are found both in cluster II and III at the nucleotide level as well as the amino acid level. Virtually identical viruses have been found in flocks with vastly different mortalities (e.g. CAV/Ejioku.NIE/11.02/106 and CAV/Ibadan.NIE/11.02/112 in cluster II on an amino acid and nucleotide level; Figs. 1 and 2). Thus, the different levels of mortality cannot be unequivocally assigned to certain mutations or genetic clustering (of VP1).

Furthermore, the multiple concurrent infections found in Nigerian flocks [4, 11, Owoade et al., manuscript in preparation] complicate any attempt to correlate symptoms and mortality with a given pathogen. Thus CAV may be endemic in Nigeria, and may contribute or exacerbate pathology without necessarily being the main cause of clinical signs and mortality observed here. Table 1 suggests that high mortality may be partially explained by infectious bursal disease virus (IBDV) co-infection, although in the field CAV and IBDV symptoms can in some cases be similar. Islam et al. [7] also reported a >50% mortality in an IBDV co-infected flock in Bangladesh. In this study, most infected flocks were 4–12 weeks old and CAV was found in birds up to 36 weeks of age (Table 1). Immunocompetent chickens become normally resistant to chicken infectious anemia by 3 weeks of age [14]. It was reported that chickens infected with both viruses had a prolonged acute phase of disease [2, 3] and higher CAV titers could be detected for longer periods post-infection [6]. We reported previously that in Nigeria, 56% of chickens had antibodies against CAV and that antibodies developed normally as soon as the chickens became susceptible after waning of maternally derived antibodies [12]. Although in older birds infections without disease seems to be possible [12],

immunosuppression due to IBDV infection could further explain the prolonged susceptibility to CAV and vice versa.

This first molecular epidemiological study of CAV in Africa shows that the limited diversity of the VP1 gene, which cannot explain differences in flock mortality, translates into highly homologous amino acid sequences. The co-infection of CAV with IBDV may be particularly lethal. To understand the economic burden of CAV, poultry farmers and veterinarians should be more sensitive to clinical signs and pathology of CAV in Nigeria. Further investigations are necessary to evaluate the specific damages caused by CAV to Nigerian poultry industry.

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