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Deciphering the genetic characteristics of VP1, Vp2 and VP3 genes of chicken anemia virus isolated in Egypt during 2016-2020.

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Abstract: Chicken anemia virus (CAV) is a self-limiting infectious virus of young chicks' results in sever immunosuppressive effect. In the current study, liver, bursa, thymus samples were taken from apparently healthy and diseased young chicks less than 25 days old from four different Egyptian governorates during the period from Dec 2016 to March 2020. Real time PCR revealed that infection rate is about 3.33 % in the diseased chicks while healthy chicks were negative. Sequencing analysis of VP1 gene revealed the presence of several point mutation on nucleotide level resulted in six amino acid mutation, yet none of these SNPs alters either the nuclear export motifs or nuclear localization motifs. Sequence analysis of both VP2 and VP3 genes revealed minimal point mutation and the common signature motifs of virulence were maintained in all studied isolates.
[Soliman, Y.A.; Maha, A.N. Gamal; Eman, M. S. El-Nagar; and Heba, M. Salim. **Deciphering the genetic characte ristics of VP1, Vp2 and VP3 genes of chicken anemia virus isolated in Egypt during 2016-2020.** *Nat Sci*2023,23 (3):18-29]. ISSN 1545-0740 (print); ISSN 2375-7167(online). http://www.sciencepub.net/nature 03. doi:10.7537/marsnsj210323.03.

Keywords: chicken anemia virus, VP1, VP2, VP3, apoptin, PCR, sequencing.

Introduction:

Chicken infectious anemia virus is a single-stranded negative-senseDNA, an icosahedral non-enveloped virus belonging to the family *Anelloviridae* genus *Gyroviris* (ICTV, 2022, Noteborn et al., 1992; Todd et al., 1990) that triggers a self-limited yet serious immunosuppressive disorderand anemia that jeopardize the infected chicken to viral infections and causes vaccination failure (Yao, et al., 2019) that might lead to dramatic financial impact.

CAV was first identified in Egypt in 1991 (El-Lethi 1990) in commercial chicken flocks. Subsequent reports indicate that the virus has spread throughout Egypt as a result of extensive off-farm transmission and a lack of effective vaccination (Hussein et al., 2016), and that has a serious economic impact due to the immunosuppressive effect and subsequent infection with other bacterial and viral pathogens, particularly NDV and IB virus (Samy and Naguib, 2018).

The 2390bp circular and covalently linked CAV genome encodes three partially overlapping ambisense open reading frames (*ORF*s) (Noteborn et al., 1991) coding for three polypeptide antigens and replicated by a rolling-circle mechanism (Prasetyo et al., 2009).

The 1200bp VP1gene which codes for the sole major structural protein (52 kDa capsid protein). The 650 bp VP2 that codes for replicase enzyme (24 kDa) with dual-specificity phosphatase activity (Peters et al., 2002) and acts as a scaffold protein in the capsid assembly (Cheng, 2019); VP1 and VP2 are protective proteins that induce neutralizing antibodies (Koch et al., 1995). Finally, the 360 bp VP3codes for the apoptin protein (16 kDa) which is a non-structural protein that implicated in viral cytotoxicity and apoptosis induction in host cells (Schat 2009 and Natesan 2006). Based on the VP1 gene sequence analysis, there are three different genotypes of CAV (Snoeck et al., 2012). Genotypes II and III were found worldwide, only Australian isolates make up genotype I. (Kim et al., 2010).

Sequence analysis of VP1 revealed that the gene contain 2 functional nuclear localization motifs (NLS) and 3 nuclear export sequences (NES) that shuttle VP3 between both nucleus and cytoplasm, maintain the VP2 protein within the nucleus to regulate viral replication and viral protein synthesis (Peters et al., 2006, Peters et al., 2002 and Yamaguchi et al., 2001). The NLS1 spanning the position 3-18 and NLS2 at position 23-47 (Cheng et al., 2019) while the NES at position 76-84, 109-119, and 375-387 for the three NES respectively.

The second viral protein is the VP2 with possess dual specificity phosphatase activity (Peters et al., 2006) it has both protein-tyrosine and protein-threonine or protein-serine phosphatase activity, the signature motif found at amino acid position 94-103

with ICNCGQFRKH sequence (Peters et al., 2002).The phosphatase activity is crucial for the viral replication within the infected T-cells and hemocytoblast of infected chickens that might be the cause of the regularity dysfunction and consequently immunosuppression.

The VP3 protein antigen is coded by *orf*3 of chicken anemia virus and has an apoptotic function through binding to anaphase-promoting complex/cyclosome (APC/C) causing cell cycle inhibition and inducing arrest in G2/M phase (Feng et al., 2020).

The current study is directed to investigate the presence of CAV infection in 4 governorates with a special focus on the genetic sequence of the three CAV genes to find the degree of genetic diversity among those isolated as a priory to developing a DNA vaccine for controlling the infection.

Material and Methods:

Clinical Samples:

Liver, thymus, and bursa (*n*=90) from diseased (*n*=30) and apparently healthy (*n*=60) chicken farms were collected from four governorates during the period from Dec 2016 to March 2020. The diseased chickens showed signs of retard growth, and weakness, with a mortality rate ranging from 2-9%, and P/M lesions revealed extensive petechial hemorrhage in the breast and thigh muscles, thymus gland, and peritoneal cavity and extensive hemorrhages in the kidneys, pale bone marrow, and a swollen discoloration of the liver at necropsy. All tested samples were fromchickensless than 25 days old. Samples were kept in sterile containers and stored at -20°C until processed within 24 h after collection.

Molecular detection of the CAV by real-timePCR

Virus DNA was extracted from all samples using GeneJet DNA purification kit (Thermos Scientific cat # K0722) according to the manufacturer's instructions. Briefly, 5mg of the sample tissues were homogenized by mechanical homogenizer (PRO 200homogenizer - Pro Scientific USA) using a Saw-tooth generator 10mm (dia.) x 115mm (L) at 4°C. Homogenized tissues and digested with 20uL of proteinase K in 180uL of digestion buffer for 10 min at 56 °C, samples were lysed with 200 µL of lysis buffer for an additional 5 min, 400uL of 50% ethanol was added and the whole mixture was centrifuged at 6000 rpm/2min to remove the coarse particles. The supernatant was transferred to the GeneJET column and centrifuged as before. The column was washed once with washing buffers I and then with washing buffers II. DNA was eluted in 50uL of elution buffer. The DNA was stored at -20C until used.

The presence of the CAV virus in the samples was examined by real-timePCR (Kaffashi et al., 2021), 5 ng of the purified DNA was mixed with the 10µl of 2xQuantiNova Probe Master Mix (QuantiNova Probe PCR Kit Qiagen cat# 208252), 50 pmole of each primer, and 100pmole of the probe (Table 1), 0.1µl of 1:20 dilution of ROX reference dye in a 20µl final reaction volume. The reaction condition was one cycle at 95°C/2min and 40 cycles at 95 °C/10sec denature and annealing and extension at 60 °C/10 sec.

Sequencing analysis of the full length VP1, VP2 and VP3 genes:

Five ng of DNA from samples that gave positive amplification in qPCR were mixed with 5 µL of 10X high fidelity buffer (High Fidelity Hot Start Core Kit Jena Bioscience Cat # PCR 235S) and 1µl of the dNTPs mix (2.5mM each) and100pmole of each of primer sets that amplify the full-lengthVP1, VP2, and VP3 (Table 1). Finally, 0.5 µl of high fidelity hot start *pfu* polymerase was added and the PCR tubes were spinned to collect down the fluid. The reaction was adjusted at 95°C/3 min for initial denature and 35 cycles of denaturing at 95°C/20 sec, annealing at 60°C/30 sec, and extension at 68°C/3min using T professional 3000 thermal cycler (Biometra, Germany). The PCR product was then electrophoresed on 1% agarose, stained with ethidium bromide, and visualized under the U.V. transilluminator.

Sequencing:

For the preparation of the gene for sequencing, the PCR product was separated on 1%low melting agarose. The bands were sliced off and purified with the biospin PCR purification kit (Biobasic cat # BSC03S1) as described by the manufacturer. Briefly, the gel slices were melted at 65°C for 7 min, mixed with 500 ml of gel extraction buffer and placed on the spin column provided with the kit, centrifuged at 4500 rpm/2min/4°C, and washed twice with the washing solution. Finally, the amplicon was eluted in 30 µL of the elution buffer and stored at -20 °C until sequenced.

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primer used for PCR amplification. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Analysis:

Nucleotide sequence analysis were conducted with the Laser Genes sequence analysis software package (LaserGene, version 10; DNAStar, Inc.). Alignments of the sequences were performed using *Clustal W* module.

Phylogenetic analysis was created using BioNJ clustering method as described by Tamura and Nei (1993)

Table (1) the sequence of the primers and probes used in the current study

For detection of CAV in samples	Primer/probe	Sequence
	CAV-F	5'-AGCTCGTCTTGCCATCTTACA-3'
	CAV-R	5'-AAAGCTTGATTACCACTACTCCCA-3'
	CAV Probe	5'-FAM-ACCTTCTTGCGTTCGGGGTC-TAMR-3'
For amplification of the full length CAV - VP1 gene	VP1-CL.F	5'-ATGGCAAGACGAGCTCGCAGAC-3'
	VP1-CL.R	5'-GGTGCTGTTCGCCCAGTTGAC-3'
For amplification of the full length CAV – VP2 gene	VP2-Cl.F	5'-ATGCACGGGAACGGCGGAC-3'
	VP2-Cl.R	5'-CACTATACGTACCGGGGCG-3'
For amplification of the full length CAV – VP3 gene	VP3-CL.F	5'-ATGAACGCTCTCCAAGAAGATAC-3'
	VP3-CL.R	5'-CAGTCTTATACACCTTCTTGCGG-3'

Results:

Screening the samples for the presence of CAV using quantitative Real time PCR:

A total of 90 samples from four governorate (collected during 2016 – 2020) were tested for the presence of CAV in all specimens (Liver, bursa and thymus) using real time PCR; only 6 out of 90samples tested were positive (fig 1), all the positive samples were from diseased farms, none of the apparently healthy chicken farms gave positive C_q. All the tested organs in the diseased chicken farms were positive although the C_q were vary from 15.56 to 36.68 indication different viral load per each organ within the tested farms.

These samples were used for further analysis and sequencing of the full-length VP1, VP2 and VP3 genes. The strains were designated and the accession number of each gene sequence was addressed as in table 2.

Fig (1) the amplification plot of the positive CAV tested liver samples, the Cq values vary from 15.56 - 36.68 for the samples CAV-YA.2019. 3. and CAV-YA.2019. 72. Respectively

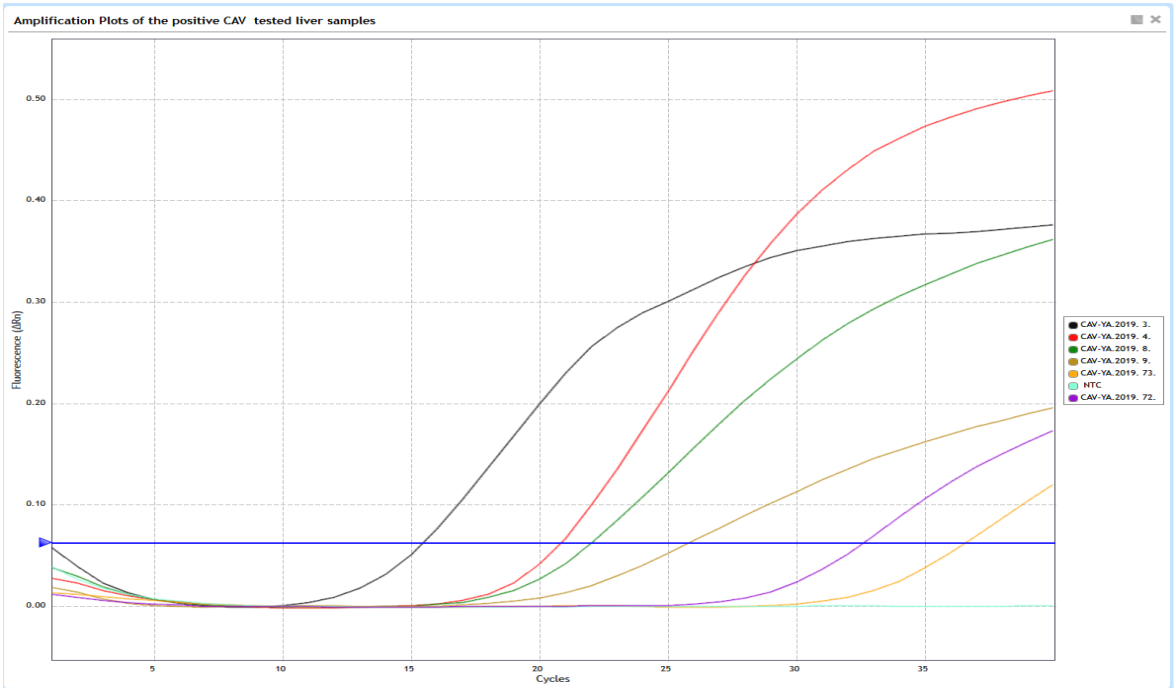


Table (2) the accession number of the full-length VP1, VP2, and VP3 genes and the origin of the six sequenced isolates of chicken anemia virus.

Accession #	Strain name	governorate	description
Mq089610	CAV-YA.2019. 3.VP1	El-Beheira	capsid protein (VP1) gene
MT621393	CAV-YA.2019. 4.VP1	El-Beheira	
Mq089611	CAV-YA.2019. 8.VP1	El-Arish	
Mq089612	CAV-YA.2019. 9.VP1	El-Arish	
MT621392.1	CAV-YA.2019.72.VP1	El-Sharkia	
MT621391.1	CAV-YA.2019. 73.VP1	El-Dakahlia	
MZ152236	CAV-YA.2019.3.VP3	El-Beheira	Apoptosis-like (VP3) gene
MZ152237	CAV-YA.2019.4. VP3	El-Beheira	
MZ152238	CAV-YA.2019.8. VP3	El-Arish	
MZ152239	CAV-YA.2019.9. VP3	El-Arish	
MZ152240	CAV-YA.2019.72.VP3	El-Sharkia	
MZ152241	CAV-YA.2019.73.VP3	El-Dakahlia	
MZ079610	CAV-YA .2019.3.VP2	El-Beheira	Dual-specificity protein phosphatase (VP2) gene
MZ079611	CAV-YA .2019.4.VP2	El-Beheira	
MZ079612	CAV-YA .2019.8 VP2	El-Arish	
MZ079613	CAV-YA .2019.9.VP2	El-Arish	
MZ079614	CAV-YA .2019.72.VP2	El-Sharkia	
MZ079615	CAV-YA .2019.73.VP2	El-Dakahlia	

Sequence analysis of full-length VP1

The full-length VP1 gene was amplified from all 6 tested samples using high-fidelity *pfu* polymerase (Fig 2A). Sequence analysis revealed that there were several point mutations (SNPs) distributed all over the entire sequence (fig 3 and 4). The phylogenetic tree constructed based on the nucleotide sequence (fig 5) revealed that the isolates were divided into 3 main groups with strain CAV/VP1-YA.2019.3 and CAV/VP1-YA.2019.4 in the first group and CAV/VP1-YA.2019.8 and CAV/VP1-YA.2019.9 in the second group whereas the third group contains CAV/VP1-YA.2019.72 and CAV/VP1-YA.2019.73. The diversity between the 6 isolates was very minimal (0.05 and 0.01) based on the nucleotide and deduced amino acid sequence respectively. These SNPs had very little impact on the deduced amino acid as it gave rise only to 6 amino acid substitutions at positions 22, 25, 97, 287, 290 and 343.

It was also noticed that the two NLS and the three putative NES were conserved among the 6 sequenced isolates, and the common signature profile for the pathogenic type was conserved along the VP1 deduced amino acid sequence (table 3) when compared to the German cuxhaven-1 strain which is a low pathogenic vaccine strain. The hypervariable region spanning amino acid 139-151 that affects the viral replication and spread was also conserved to the pathogenic type (amino acid Q_{139} and Q_{144}) where that vaccine strain has amino acid E in these two positions.

Fig (2): **(A)** amplification of 1300 bp full-length VP1. **(B lane 1, 2)** 650 bp full-length VP2. **(B lane 3, 4)** 360 bp full-length VP3 gene from representative samples that were positive in the real time PCR. M is the 100bp plus DNA ladder.

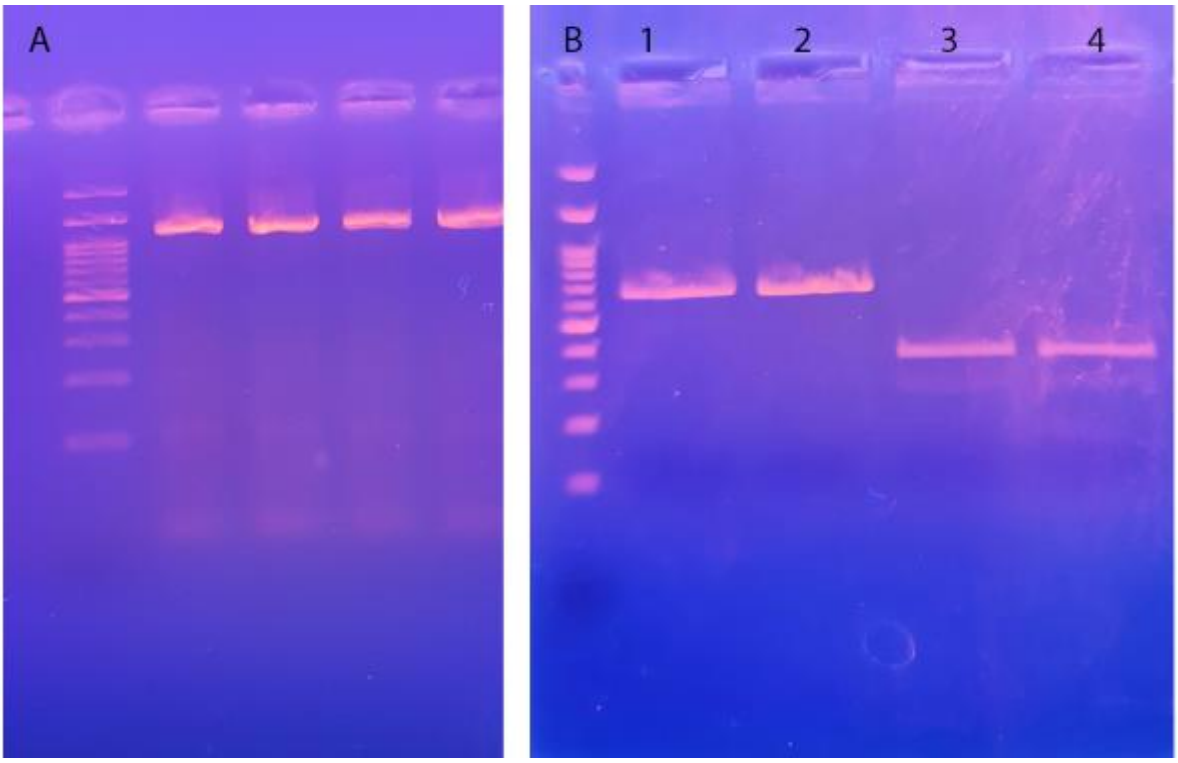


Table (3) the deduced amino acid position diversity of VP1 among the 6 sequenced isolates of CAV in the current study when compared to the vaccine and virulent strains. (Santen et al., 2001 and Hailemariam et al., 2008)

Strain	**Deduced amino acid substitutions in the VP1**										
Amino acid position	22	75	89	97	125	139	141	144	370	394	413
CAV-YA.2019.3	H	I	T	M	I	Q	Q	Q	S	Q	A
CAV-YA.2019.4	H	I	T	M	I	Q	Q	Q	S	Q	A
CAV-YA.2019.8	N	I	T	L	I	Q	Q	Q	S	Q	A
CAV-YA.2019.9	N	I	T	L	I	Q	Q	Q	S	Q	A
CAV-YA.2019.72	Q	I	T	L	I	Q	Q	Q	S	Q	A
CAV-YA.2019.73	Q	I	T	L	I	Q	Q	Q	S	Q	A
Ref genome of Cux-1 vaccine strain	H	V		M		K		E	G	H	

Fig (3) VP1 nucleotide sequence alignment of the six isolates showing multiple sequence mutation. The nucleotide sequences were aligned using clastal *W* method, and point mutations were recorded for each strain.

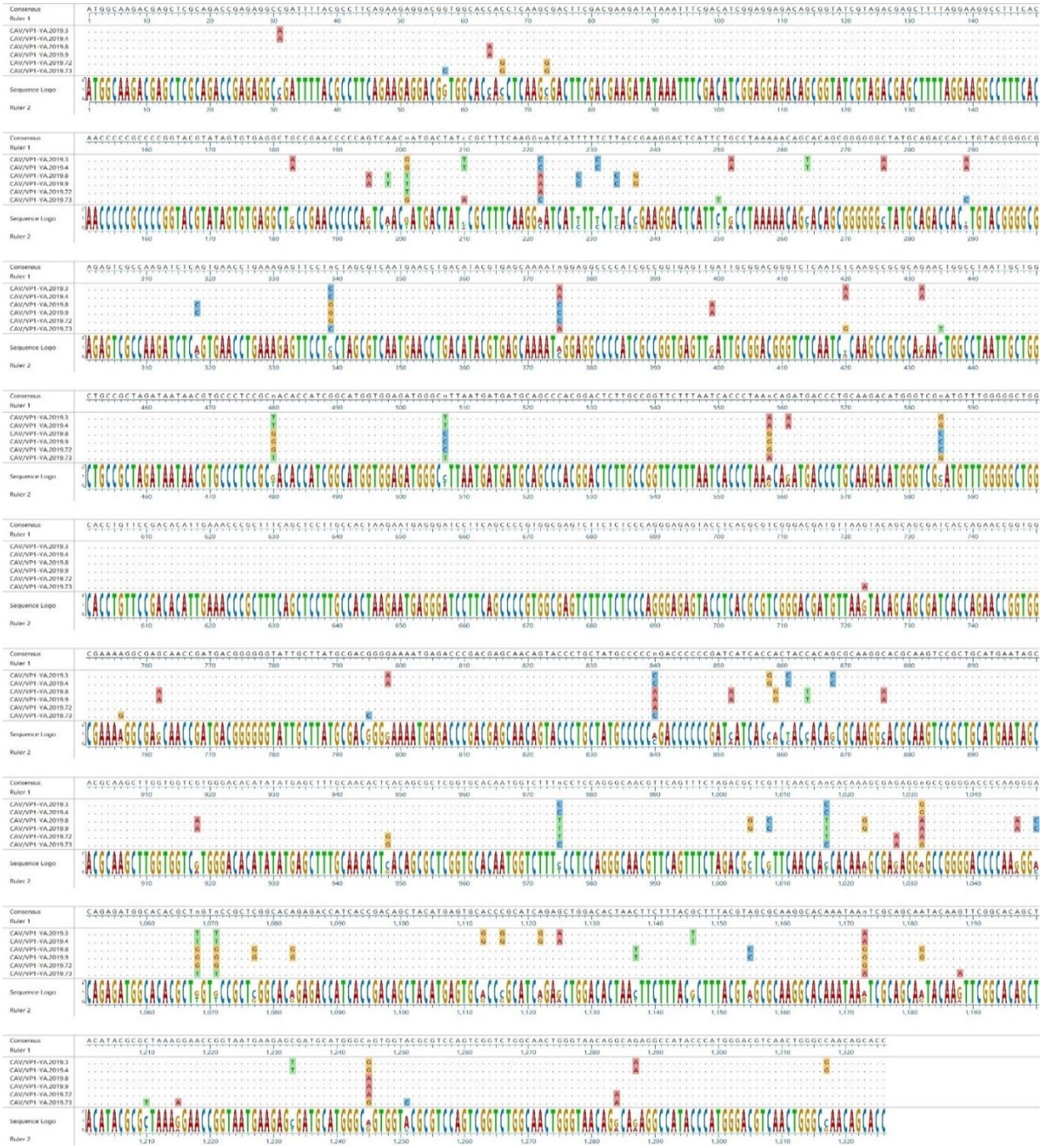


Fig (4) VP1 deduced amino acid sequence alignment of the six isolates showing the 6-point mutations at position 22, 25, 97, 287, 290 and 343

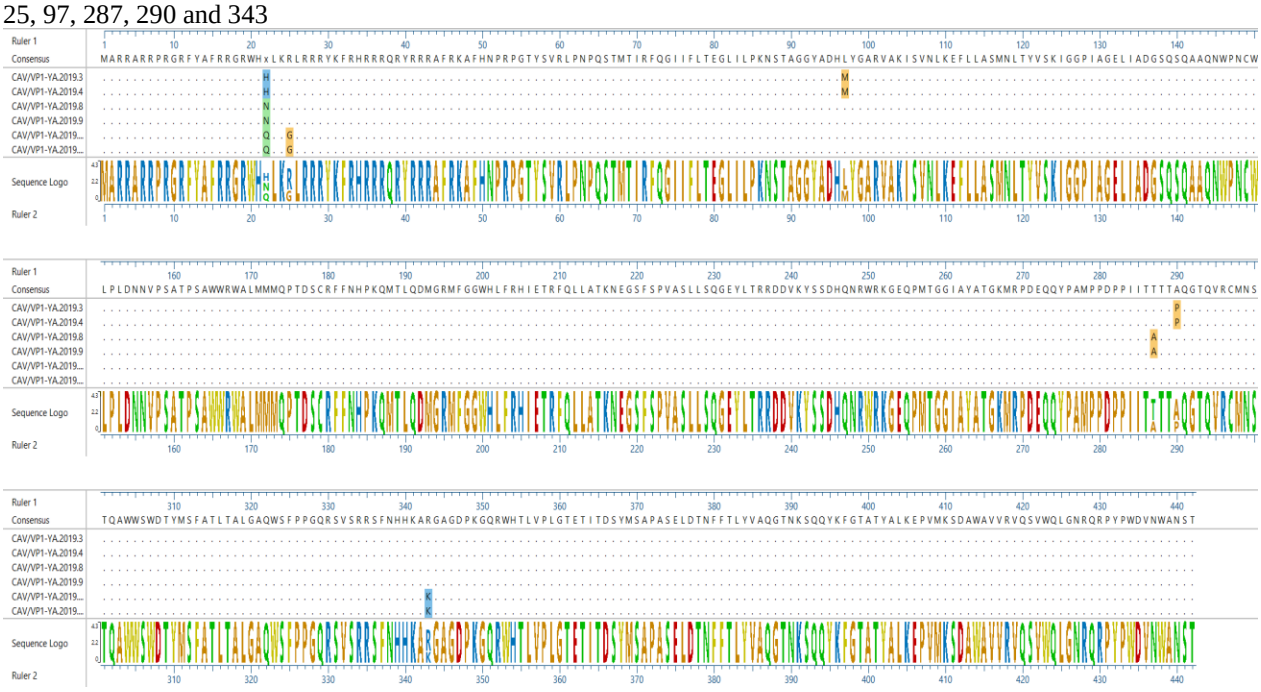
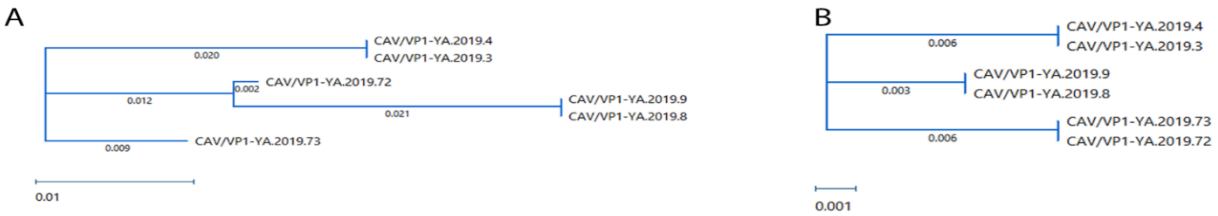


Fig (5) the phylogenetic tree of VP1 gene of the 6 studied CAV isolates constructed on the bases of the nucleotide sequence (A) or the deduced amino acid sequence (B).



Nucleotide sequence analysis of full-length VP2 gene:

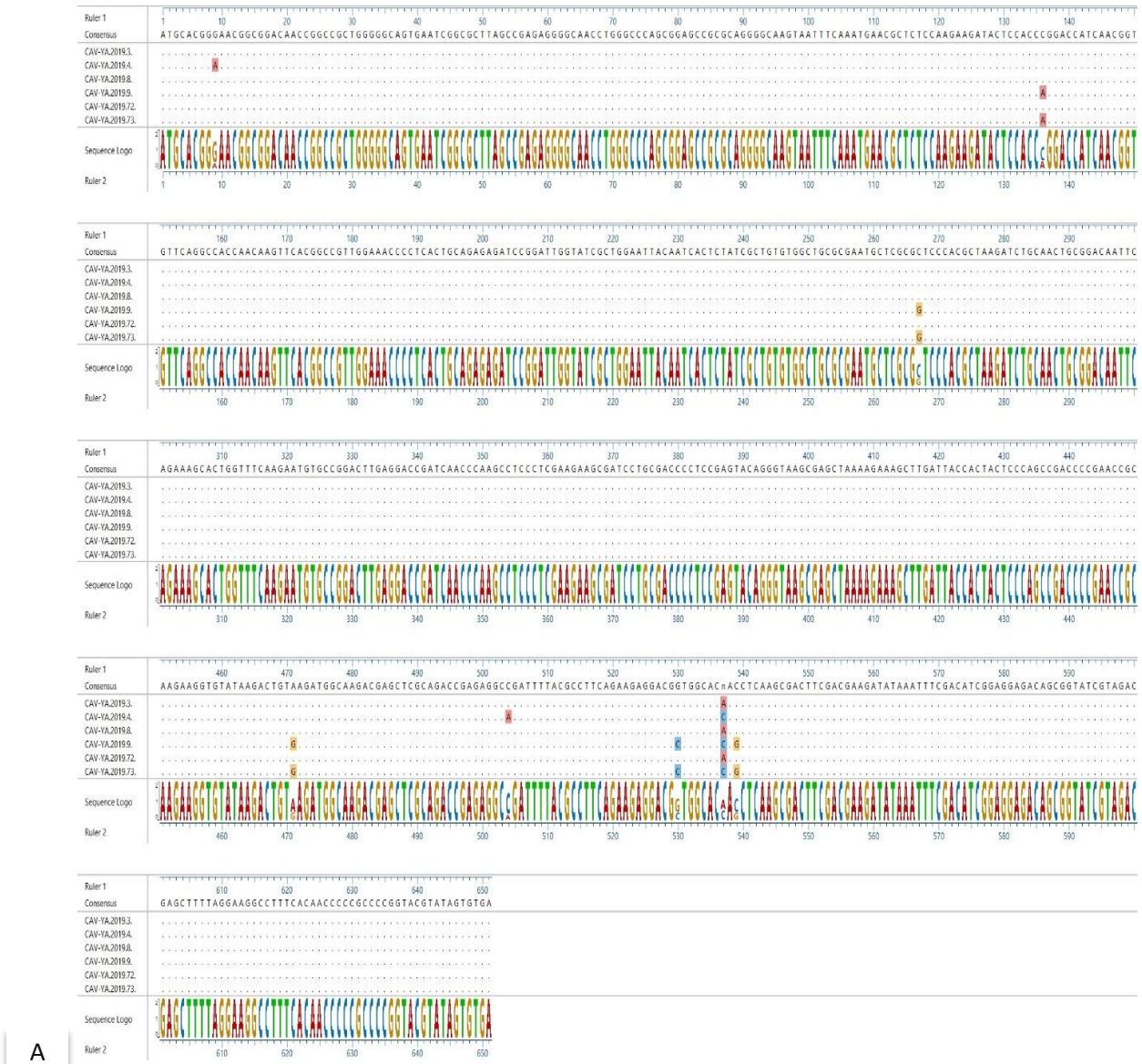
Thefull-length650 bp VP2 gene was amplified from the 6 isolated strains of CAV (**Fig 2 B lane 1, 2)**. Minimal SNPs have been recognized in the analysis of the nucleotide sequence of the 6 strains under study with only two mutationsin the amino acid sequence at position 177 (G>A) and 180 (T>S) (Fig 6) in only 2 isolates (CAV-YA,2019.9 and CAV-YA.2019.73) these 2 point mutations have no effect on the pathogenesis of the isolates. The similarity index showed that the differences between the 6 isolates is very minimal with the most divergence occurring between CAV-YA2019.9 and CAV-YA.2019.72 (0.00928).

phylogenetic tree constructed based on nucleotide sequences grouped isolates CAV-YA, 2019.3, CAV-YA, 2019.8 and CAV-YA, 2019.72 together while CAV-YA, 2019.73 and CAV-YA, 2019.9 were widely apart.

Nucleotide sequence analysis of full-length VP3 gene:

Amplification of the 360bp full-length VP3 gene (**Fig 2 B lane 3, 4)** from the 6 CAV isolated followed by sequence analysis revealed that this gene was the most conservative among the 3 genes of CAV (fig 8). Only one amino acid substitution was recorded at position 54 where A>G in only two isolates (CAV-YA.2019.9 and CAV-YA.2019.73) which were group together in the constructed phylogenetic tree (fig 9)

Fig (6) Nucleotide sequence (A) and deduced amino acid sequence alignments of the VP2 gene of the 6 tested strains of CAV. Notice there were 8 positions with point mutations on nucleotide level only two positions of point mutation had impact on the amino acid level.


Ruler 1
Consensus
CAV-YA.2019.3.
CAV-YA.2019.4.
CAV-YA.2019.8.
CAV-YA.2019.9.
CAV-YA.2019.72.
CAV-YA.2019.73.
Sequence Logo
Ruler 2

A

B

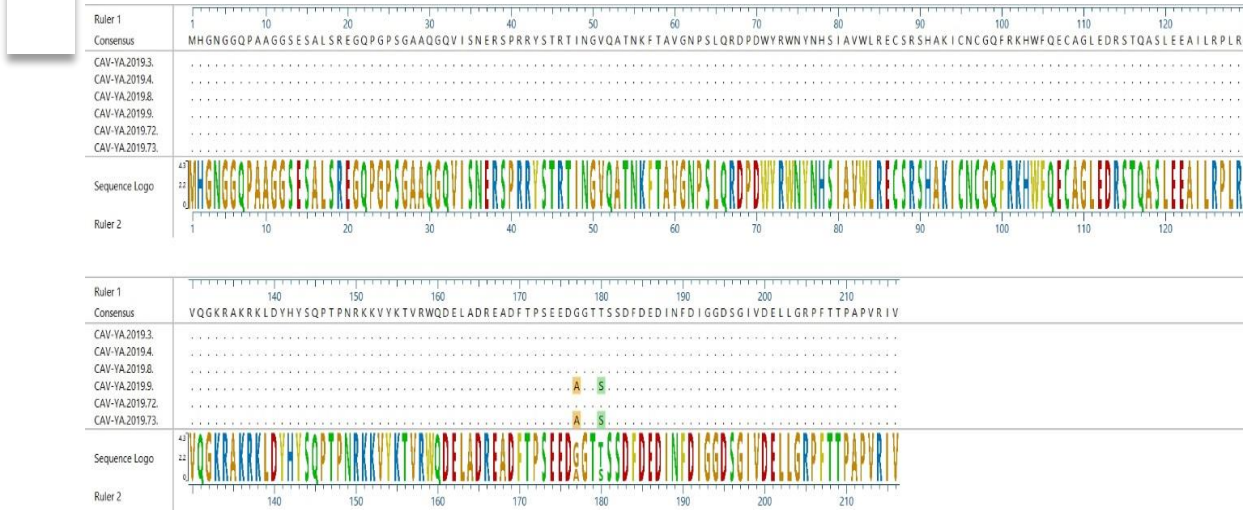
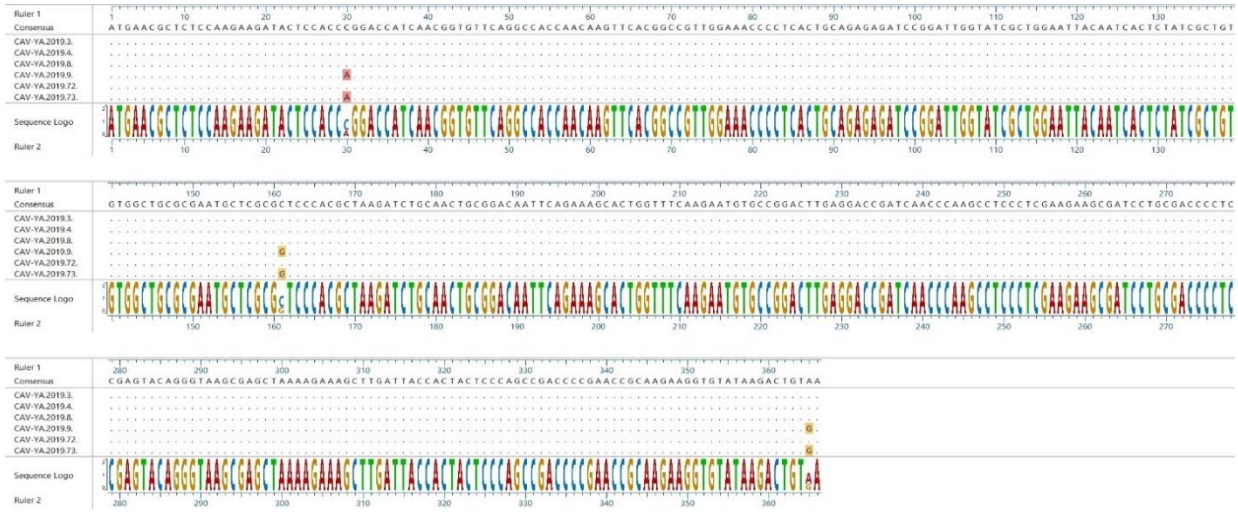

Ruler 1
Consensus
CAV-YA.2019.3.
CAV-YA.2019.4.
CAV-YA.2019.8.
CAV-YA.2019.9.
CAV-YA.2019.72.
CAV-YA.2019.73.
Sequence Logo
Ruler 2

Fig (7) phylogenetic tree of the 6 tested isolates based on the nucleotide sequence of the full length VP2. Note that the 6 isolates clustered in 3 groups with group one contain 3 isolates, 72, 8 and 3 showed 100% similarity, group 2 contain isolate 73 and 9 (with similarity of 100%) were the most distance (0.016). Group 3 contain one isolate (isolate 4).

Fig (8) Nucleotide sequence (A) and deduced amino acid sequence alignments of the VP3 gene of the 6 tested strains of CAV. Notice there were 3 positions with point mutations in nucleotide level only one position of point mutation had impact on the amino acid level.



A

B

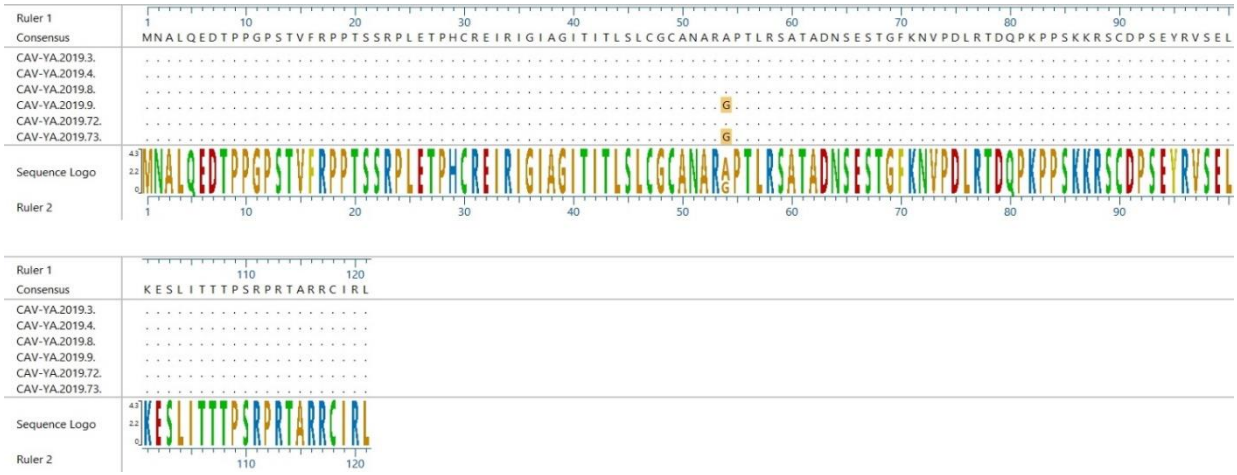
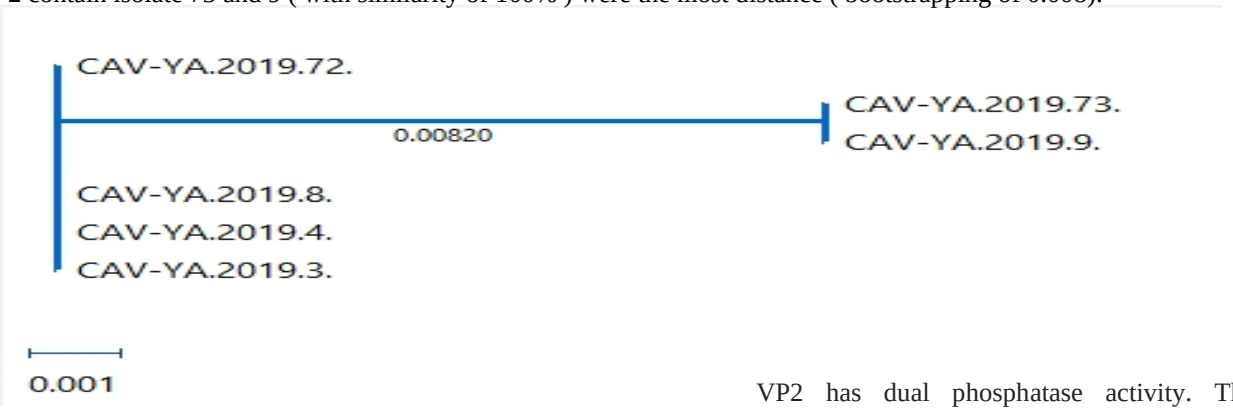


Fig (9) phylogenetic tree of the 6 tested strains based on the nucleotide sequence of the full length VP3. Note that the 6 isolates clustered in 2 groups with group one contain 4 isolates, 72 , 8, 4 and 3 showed 100% similarity , group 2 contain isolate 73 and 9 (with similarity of 100%) were the most distance (bootstrapping of 0.008).



Discussion:

Chicken anemia virus infection considered self-limiting infection. However, the immunosuppression caused by the virus results in sever dramatic effect on the immune status of the chicken either against subsequent viral infection or in the vaccination status of the birds. The ambisense 2.3kbp ssDNA genome lacks the nano-nucleotide stem-loop structure at the replication origin. The virus has only three proteins with only VP1 considered the structure protein. These three proteins are transcribed from 4 intermediate transcripts with sizes of 2Kb, 1.3 Kb, 1.2 Kb and 0.8 Kb (Kamada et al., 2006).

VP1 gene is about 1326 bp coding for 440 amino acid long polypeptide with a molecular mass of 51.6 KDa. It is the only structural protein with the basic amino terminal sequence (Hu et al., 2018 and Meehan et al., 1998). VP1 gene has 2 essential motifs for viral replication and localization interchangeably in both nucleus and cytoplasm(Cheng et al., 2019). The first NLS1found at nucleotide position 3 -21 with conserved sequence at position 1-10 (RRARRPRG) and NLS2 at position 24-47 while the three NES found at positions 76-84, 109-119 and 375 to 387 (ELDTNFFTLYVAQ). Both NLS and NES motifs were found to be conserved among the six Egyptian isolates of CAV in the current study despite the fact that VP1 is most variable sequence among the genome of the virus, (Ducatez et al., 2006 and Islam et al., 2002). This NLS is found in the arginine-rich(amino acid 3 to 46) region of the N terminal region (first 40 amino acids).Different virulence determination sequences (hypervariable regions) have been found in the VP1 amino acid sequence (Aşkar , 2019 , Zhang et al., 2012) that differentiate the virulent from the vaccine strains table (3) , with all the six sequenced Egyptian isolates in the current study having the virulent pathogenicity profile when compared with the vaccine strains

VP2 has dual phosphatase activity. The catalytic motif signature was found to be cysteine at amino acid position 97(Cheng et al., 2012). Mutations at these residues to serine reduce viral replication in host T- lymphocytes (Peters et al., 2002), and all the sequenced VP2 genes of the six CAV isolates of the current study were found to have C_{97} denoting the virulent nature of the strains. It was also noticed also that, the R-PTPase α domain ($_{95}$CNCGQF$_{100}$) was conserved among all tested sequences

VP2 also known as a CRM-1 independent nuclear localization protein with simple NLS motif spanning positions 133-138 that were found to be conserved among the six studied isolated. (Heilman et al., 2006)

VP3 protein is known as an apoptin protein antigen that induces apoptosis in the infected host immune cells and in human cells and thus used in anti-oncogenic studies and anti-tumor treatment (Feng et al., 2020).

The apoptin protein VP3 showed very minimal mutations (only 2 mutations on the amino acid level) that had no impact on the function of the protein or the virulence of isolates

In conclusion, the six studied CAV isolates had the amino acid signature of virulent strains. The most variable gene found to be VP1 while both VP2 and VP3 showed very minimal changes.

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3/11/2023