



## Genetic Differences and Pathogenicity of Chicken Anemia Virus Strains in Broiler's Baby Chicks



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### Abstract

**T**HIS STUDY aims to molecular characterization of the Chicken Anemia Virus (CAV) in young broilers in the Giza governorate, Egypt in 2021, assess the genetic diversity among the proliferating viruses, and vaccine strains, and evaluate their pathogenicity to improve viral management. Viral protein 2 (VP2) gene was amplified and subsequently conducted molecular-level characterization. In five out of the ten flocks sampled CAV was detected. Furthermore, the examination of the phylogenetic connections performed according to the sequences of the VP2 gene demonstrated the classification of the observed field strains within genogroups I, II, and IIIb. In silico analysis was performed on Cux-1 and the detected five strains to investigate the secondary structures of VP2 proteins. The findings indicated a significant resemblance in the distribution and quantity of  $\alpha$ -helical and  $\beta$ -strand topologies. To assess the pathogenicity of the virus, two isolates from genotype III (MZ574096.1) and genotype II (MZ477000.1) were administered intramuscularly to sets of day-old, specific pathogen-free chicks. At 28 days following inoculation, the Red Blood Corpuscle count (X106 $\mu$ L) was (1.4  $\pm$  0.39 and 1.8  $\pm$  0.12).

**Keywords:** CAV, Genetic characterization, VP2, Young chicken.

### Introduction

Chicken anemia virus (CAV) belongs to the genus Gyrovirus, which is the only genus in the Anelloviridae family [1]. The CAV genome is made up of a single-stranded circular DNA that contains three overlapping open reading frames (ORFs). These ORFs code for three different viral proteins (VPs): VP1, the primary protein of the capsid; VP2, a non-structural protein phosphatase; and VP3, an additional non-structural protein called apoptin [2, 3]. It has been documented that VP1 has hypervariability in its sequence. VP1 is an important protein in viral replication and pathogenicity, and it also enhances the generation of the host antibody

[4,5]. To ensure the proper translation of VP1, the VP2 protein is considered a "brickwork protein". There are reports indicating that exposure to VP3 can cause programmed cell death in thymic cells and infected lymphoid T cells of chickens [6].

Gyrovirus Avian Infectious Anemia (CAV) is known to spread in countries with a significant broiler chicken industry [7]. The virus can cause symptoms within 15 to 21 days or remain latent for over three weeks [8]. The primary targets of the virus are bone marrow, hematopoietic stem cells (including red and white blood cells), the thymus, and T lymphocytes [9]. Chicks below two to three weeks of age exhibit symptoms that include anemia,

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(Received 20/12/2023, accepted 07/05/2024)

DOI: 10.21608/EJVS.2024.256791.1736

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stunted growth, lethargy, drooping wings, untidy feathers, paleness of the soft tissues, crest, wattles, and beak [10]. Additionally, blood loss (intramuscular and subcutaneous) and abnormalities of the thymus and bone marrow have been observed [11].

The severity of a disease in chicks is determined by their immunological condition or their blood levels of maternal antibodies. If a chick above two weeks old is infected subclinically, it may suffer from CAV [12]. CAV suppresses the immune system and makes the chick vulnerable to secondary infections from various infectious agents [13]. It may also weaken the chick's immunological response to vaccinations. In addition, poultry populations affected by CAV may suffer from infectious bursal disease, Marek's disease, and respiratory and skin conditions caused by bacteria and viruses [14]. To prevent CAV, breeders in Egypt administer a live attenuated virus vaccination to flocks aged between 9 and 15 weeks. Vertical transmission of CAV can be reduced by maternal anti-CAV antibodies in chickens [15].

In Egypt, CAV was initially noted in 1990 [16]. Then studies continued and reported that the virus has spread widely off farms throughout Egypt, even though vaccination. As well as monitoring the evolution of sequences and molecular characteristics of the virus in Egypt [17,18].

The purpose of this study conducted in Egypt in 2021 was to examine CAV (Chicken Anemia Virus) in young chicks. The investigation included an assessment of the genetic variation between the propagating viruses and vaccine strains, with the aim of improving virus management. Furthermore, the study also evaluated the pathogenicity of these viruses in chickens.

## **Material and Methods**

### *Ethical approval:*

This examination was conducted under the regulations of the National Research Centre, Egypt, and licensed under license number 14712012022.

*Tissue samples:* A total of ten healthy chicken flocks (aged 1-7 days old) from Giza governorate from June to September, which were vaccinated against live attenuated CAV (CUX-1 and Nobilis P4), were examined. The chicks were raised using the deep litter method in open system farms and received IB and ND vaccines on the first and fifth days, respectively.

We collected tissues from the bursa, bone marrow, thymus gland, and liver, and combined them in a 20% w/v saline solution with 2000 units/mL penicillin and 200 µg/mL streptomycin. The mixture was then centrifuged at 3000 rpm for 15 minutes, and a sample was sent to our laboratory for examination.

The clear supernatant was collected and stored at -80°C for further use [19].

*DNA extraction and CAV detection:* Total genomic DNA was obtained using the PathoGene-spin® DNA/RNA (iNtRON Biotechnology, Seongnam) following the guidelines provided by the manufacturer. Partial VP1 gene amplification was performed using Taqman assays CAV Q5:5'-GCCCGGTACGTATAGTGTGAG-3' CAV probe 5'-(6FAM)-CTGCCGAACCCCAATCTACTATGACTATCC-(TAMRA)-3' Cux-1 specific 5'-CCGTGAGAAAAGATGACCCCTT-3' [20] performed in 2.2.2 s in AB Applied Biosystems. The cycle was 95 °C/10 min after 95° C/15 s and 60° C/1 min for 40 cycles for a mixture consisting of 25 picomoles of each primer combined with 10 picomoles of CAV probe and 5 µL of sample DNA and DNase-free water was added until the mixture volume reached 50 µL.

The samples that tested positive underwent a standard assay utilizing VP2 primers (F: 5'-CTAAGATCTGCA ACTGCGGA-3' and R: 5'-CCT TGG AAG CGG ATAGTC AT-3') to produce a 418 bp segment of the CAV genome. [21]. The PCR mixture was prepared within a 25 µL reaction volume, comprising 12.5 µL of WizPure PCR 2X Master, 1 µL of each primer within 7.5 µL of nuclease-free water, and 3 µL of the DNA template (extracted from the sample). The cycling process started at 95° C for 5 min, succeeded by 50 cycles at 95° C for 30 sec, 50° C for 30 sec, and concluded with a 30-sec at 72° C. Finally, the positively identified bands were submitted for sequencing.

### *Sequencing of the VP2 amplicons*

After obtaining PCR amplicons for the VP2 gene, double-strand sequencing was performed using the ABI 3730xl DNA sequencer. The positively identified samples were then sent to Macrogen® Company for further analysis. The sequencing data was analyzed using NCBI Blast and was assembled, edited, and visualized through Jalview software version 1.8.3-1.2.9-JAL. To determine the conservation of the VP2 protein, the PSIPRED 4.0 tool and Jalview software version 1.8.3-1.2.9-JAL were used to analyze the secondary structures. Additionally, neighbor-joining phylogenetic analyses were executed using MEGA X: Molecular Evolutionary Genetics Analysis on various computational platforms [22, 23].

### *Pathogenicity appraisal of CAV*

At just one-day old, around 60 Specific Pathogen-Free (SPF) chicks were divided into three groups, with 20 chicks in each group. Two of the groups were given intramuscular injections of 0.2 mL of the supernatant (organ suspension) [24] of the selected isolates (MZ574096.1 and MZ477000.1). The

remaining group was used as the negative control. Each group was placed in a separate environmentally regulated isolation unit, where they had unrestricted access to food and water throughout the experiment. Blood was collected every week without a coagulant for serum separation, which was used for the ELISA (Enzyme-Linked Immunosorbent Assay) test. At 28 days post-inoculation, heparinized blood samples were taken for hematological examination (3 birds/group).

#### *Hematological examinations:*

Blood smears were stained using Wright's stain for differential leukocyte count, and relative values were calculated based on Schalm's method [25].

**ELISA (Enzyme-Linked Immunosorbent Assay) test:** A commercial ELISA test kit from Biochek® was used to measure CAV-specific antibody titer in experimental groups.

#### *Statistical analysis*

The significant differences in hematological values were determined by the t-test using Excel (Microsoft 2017).

### **Results**

#### *Detection of CAV*

Out of the ten flocks tested, Taqman and traditional methods both confirmed that 50% of them were CAV-positive.

#### *Genebank registry*

The five CAV isolates were assigned accession numbers MZ574095.1 to MZ477001.1 and were submitted to Genebank.

#### *Molecular characterization and phylogenetic analysis of VP2*

According to Quaglia et al. [26] the identified CAV strains from various flocks were classified into four genogroups: I (MZ574095.1), II (MZ477000.1), IIIb (MZ574096.1 and MZ477001.1), and IV (MZ476999.1). Notably, the MZ477001.1 strain was grouped in genogroup IIIb with vaccine strains 26P4 and Nobilis P4 (Fig.1). The nucleotide sequences of the identified CAV strains also showed similarities to previously identified CAV strains from Egypt with bootstrap values of 70% (Fig. 2). There was significant conservation of the VP2 protein between the Egyptian CAV and the five CAV isolates (Fig. 3). Moreover, the detected CAV strains' nucleotide sequences were similar to strains from Pakistan, while they showed less homology with strains from Taiwan, India, the USA, Brazil, and China (Fig. 4).

Patterns of amino acid substitution were identified at two distinct sites in the VP2 protein of Cux-1, which is a standard strain. These sites are located at document\_number\_1 (GCG---GTG) and document\_number\_2 (GAT---GGC). Table 1

provides more information on this. An in-silico analysis was also carried out to compare the secondary structure of VP2 protein derived from Cux-1 (the reference strain) and five CAV strains. The analysis revealed that the  $\alpha$ -helix and  $\beta$ -strand architectures were arranged similarly across all six strains. Furthermore, there were no insertions or deletions in the sequenced portion of VP2 from the five CAV isolates compared to Cux-1, as shown in Fig. 5.

#### *Results of the pathogenicity*

The post-mortem findings: Thymus gland atrophy and yellow, fatty bone marrow were observed, along with a bursa of Fabricius shrinkage, swollen pale liver and spleen, and significant ossification delay of the keel bone (at 28 days post-infection (dpi)).

#### *Hematological results:*

The groups infected with CAV showed signs of hypochromic anemia, which was characterized by a significant reduction in the RBC count ( $X10^6/\mu L$ ) in both the MZ574096.1 and MZ477000.1 infected groups ( $1.4 \pm 0.39$  and  $1.8 \pm 0.12$ ), respectively. In addition, there was a significant decrease in PCV% ( $29 \pm 1.6$  and  $31 \pm 1.1$ ) and Hb % ( $11.77 \pm 0.52$  and  $11.79 \pm 0.11$ ) in all samples (Fig. 6). It was also observed that although there was a significant increase in lymphocytes in the two infected groups compared to the control group, the total WBCS count ( $X10^3/\mu L$ ) and the percentage of each of its derivatives (heterophile, lymphocyte, monocyte, and eosinophil) significantly decreased in the infected groups compared to the control group.

The results of the ELISA assay for CAV-infected SPF chicks are displayed in Fig.7 as Geometric Mean Titers (GMT). The production of CAV-specific antibodies began after the 7th-day post-infection (dpi) in the chicks belonging to the virus-infected group, and the antibody levels peaked at 28 dpi across all groups. Throughout the testing period, serum samples from the negative control group consistently yielded negative results in the ELISA assessments (Fig. 7).

### **Discussion**

The VP2 and VP3 genes are known to cause apoptosis within infected cells. In addition, the VP2 protein is used as a target antigen to produce monoclonal antibodies. However, research on the VP2 gene is limited [27]. Therefore, a better understanding of this protein could aid in managing CAV infection more effectively. Studies of partial sequences of the VP2 gene revealed that the identified strains belonged to different genogroups (I, II, and IIIb). This highlights the diversity of CAV strains circulating in Egypt and affecting young chicks. One particular strain MZ477001 was found to cluster with vaccine strains 26P4 and Nobilis P4 in genogroup IIIb. This observation has already been

noted in Italy [26], where a strain with a similar nucleotide sequence to the vaccine strain (Del Ros vaccine) was detected. This suggests that a vaccination strain or a field strain with a vaccine-like sequence could spread. CAV vaccines are made by introducing field strains into tissue culture cells or embryonated chicken eggs to make the virus less vigorous [28]. Moreover, the degree of attenuation does not necessarily prevent the vaccine from being transmitted to and among young chicks, either vertically or horizontally [17]. Although attenuated CAV strains can revert to virulent phenotypes after chick-to-chick transmission, vaccinating young chicks with such strains may pose a threat [29].

It was also observed that all of the sequences that were evaluated shared conservation in the R-PTPase  $\alpha$  domain (95CNCGQF100). This was also mentioned previously in Egypt [30].

In the sequenced segment of VP2, the five investigated strains exhibited three nucleotide alterations: at position 458 from C to T, at position 506 from A to G, and at position 507 from T to C. These alterations in nucleotides were also identified in previous Turkish isolates. Amino acid mutation analysis revealed that the isolates displayed mutations at positions A153V and D169G, in comparison to the reference strains Cux-1 and Cuxhaven 1. The A153V mutation is frequently encountered in Turkish isolates. Yet, the D169G mutation is not commonly observed in Turkish isolates [31].

In general, the autopsy showed an identical pattern of distribution in the tropic organs (thymus, spleen, liver, and bone marrow) with variation in intensity. This aligns with the previous findings that severity dictates the extent of lesions [32]. For further pathological appraisal, SPF one-day-old chicks were infected with two Egyptian isolates (MZ574096.1 and MZ574096.1). By the end of the study (28th day), the identified antibodies confirmed the experimental CAV infection on the first day of age. The indirect ELISA system is characterized by its speed, high sensitivity, and specificity for the identification of serum antibodies against CAV. It is suitable for the serological diagnosis of CAV in

specific pathogen-free (SPF) and commercial poultry populations. These outcomes correspond with the observations of some researchers [18] but differ from the results obtained by other investigators, who have proposed that sera collected during the later stages of CAV infection could produce negative outcomes in serological tests [33].

The hematological profile of the infected groups showed hypochromic anemia characterized by a decrease in PCV, erythrocyte count, and hemoglobin content, which could potentially be attributed to the direct link to the virus's direct effect on the bone marrow, resulting in aplasia and a detrimental impact on the erythrocytic and granulocytic lineages of bone marrow cells, consequently affecting erythropoiesis and myelopoiesis [34]. The leukocytic finding of infected groups showed leukocytopenia, which could be attributed to the fact that the primary cells affected during CAV infection's progression involve hematopoietic progenitor cells in the bone marrow and thymic precursor cells in the thymus cortex [35].

#### *Conclusion and recommendations*

Based on the partially sequenced VP2 gene pedigree, it was determined that the CAV strains detected in young Egyptian chicks are members of genogroups I, II, and IIIb. This study coupled one verified strain with the Nobilis P4 and 26P4 vaccine strains. It is essential to periodically check CAVs for genetic alterations and study their evolutionary history.

#### *Acknowledgment*

We thank all staff members of the Poultry Diseases Department, and Biotechnology Department Animal Health Research Institute (AHRI), Poultry Diseases Department, and Department of Parasitology and Animal Diseases Veterinary Research Institute, National Research Centre.

#### *Conflicts of interest*

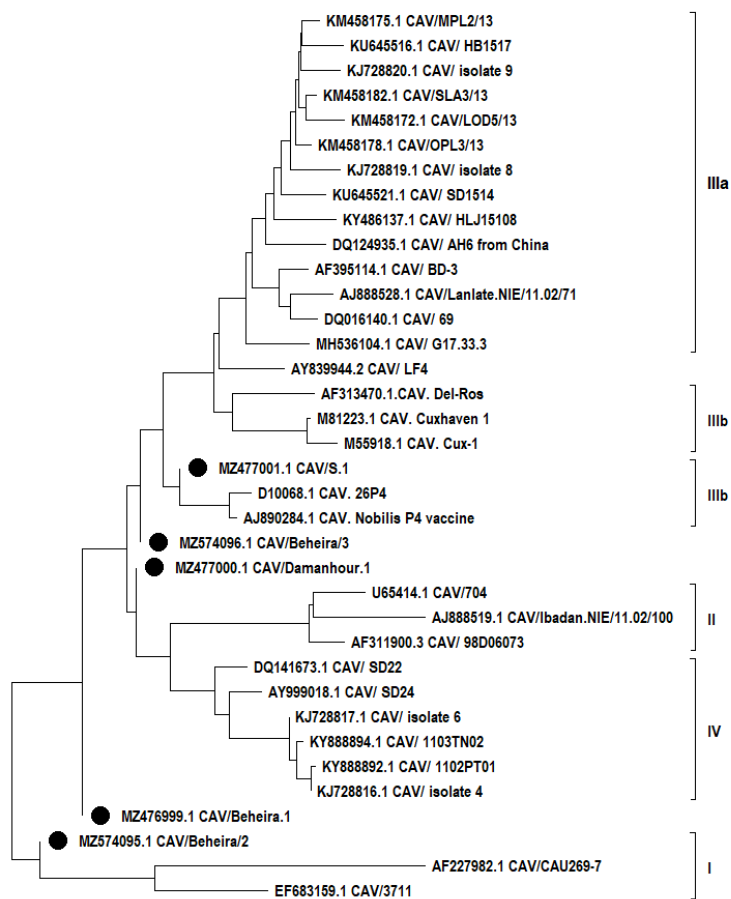
The authors declared no competing interests.

#### *Funding statement*

No funding.

**TABLE 1. partial amino acid sequence VP2 comparison among the five detected Egyptian CAV strains in the study and Cux-1 (reference strain).**

	Number of amino acid in VP2 gene	
	153	169
M55918.1 CAV/ Cux-1	A	D
M81223.1 CAV/ Cuxhaven 1	V	D
MZ476999.1 CAV/ Beheira.1	V	G
MZ477000.1 CAV/ Damanhour.1	V	G
MZ477001.1 CAV/ S.1	V	G
MZ574095.1 CAV/ Beheira/2	V	G
MZ574096.1 CAV/ Beheira/3	V	G



**Fig. 1. Genogroup classification tree Phylogenetic tree based on the partial VP2 nucleotide sequence of five CAV strains (marked with a black circle) and reference CAV strains.**

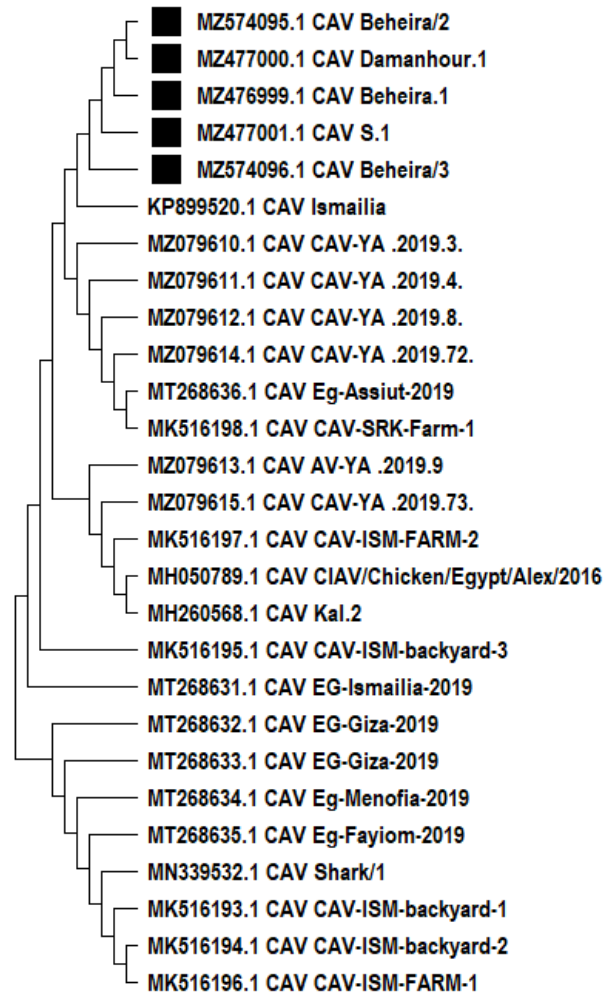


Fig. 2. Phylogenetic tree based on the partial VP2 nucleotide sequence of five CAV strains (marked with a black circle) and Egyptian CAV strains previously detected. bootstrap values  $\geq 70\%$  are reported.

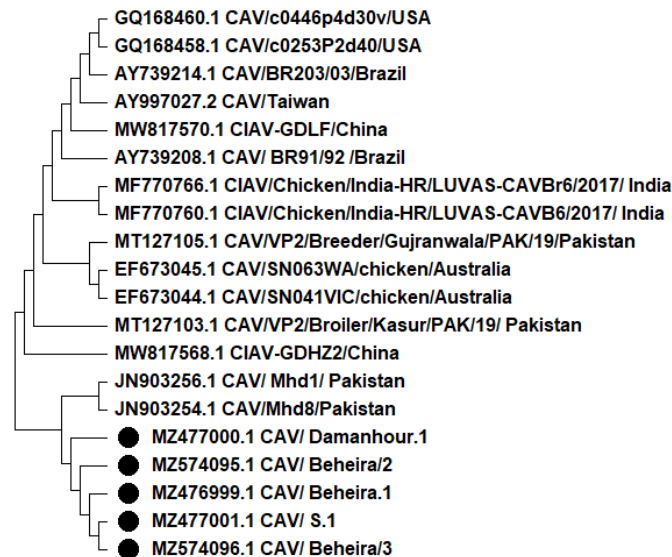


Fig. 3. Phylogenetic tree based on the partial VP2 nucleotide sequence of five CAV strains (marked with a black circle) and other countries' CAV strains previously detected. bootstrap values  $\geq 70\%$  are reported.

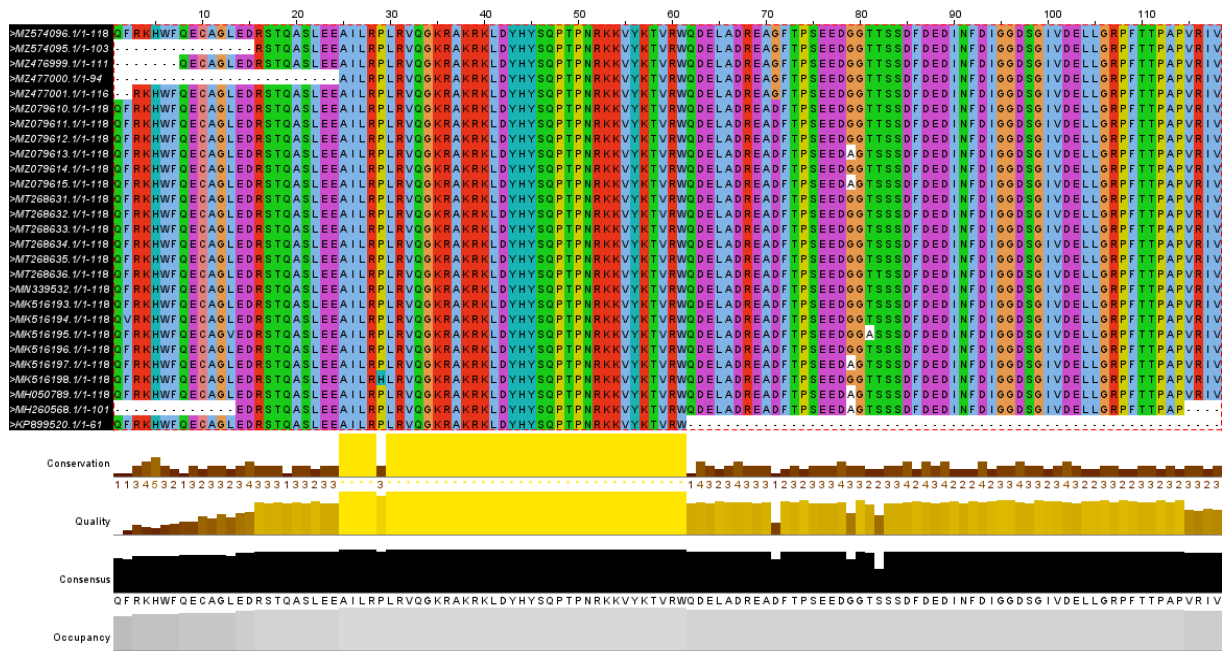


Fig. 4. Conservation score of VP2 protein between five CAV isolates and Egyptian CAV strains previously detected.

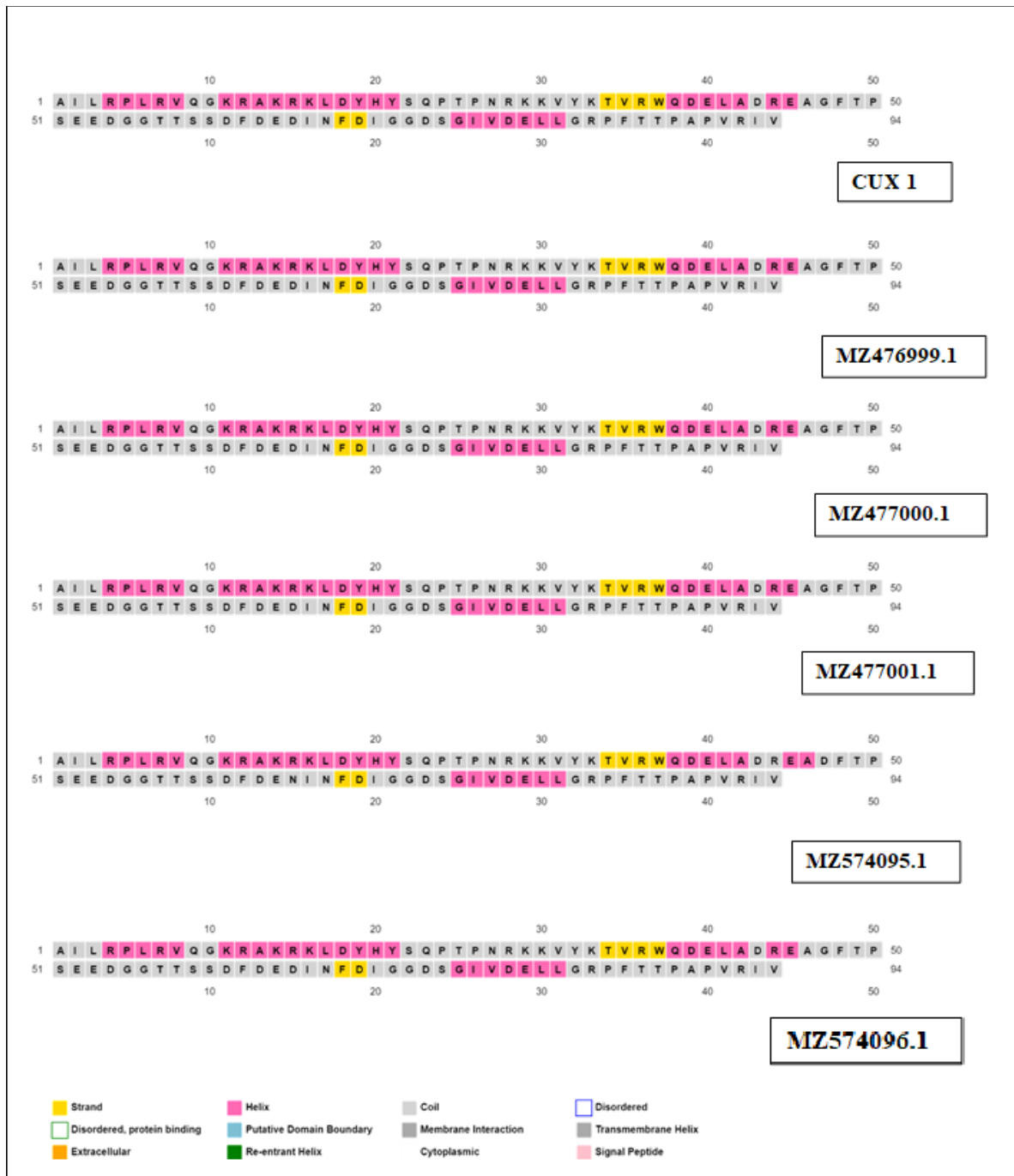


Fig. 5. Secondary structures of the VP2 protein of Cux-1 (reference strain) and five CAV strains. A: alanine, C: cysteine, D: aspartate, E: glutamate, F: phenylalanine, G: glycine, H: histidine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: arginine, S: serine, T: threonine, V: valine, W: tryptophan, Y: tyrosine.



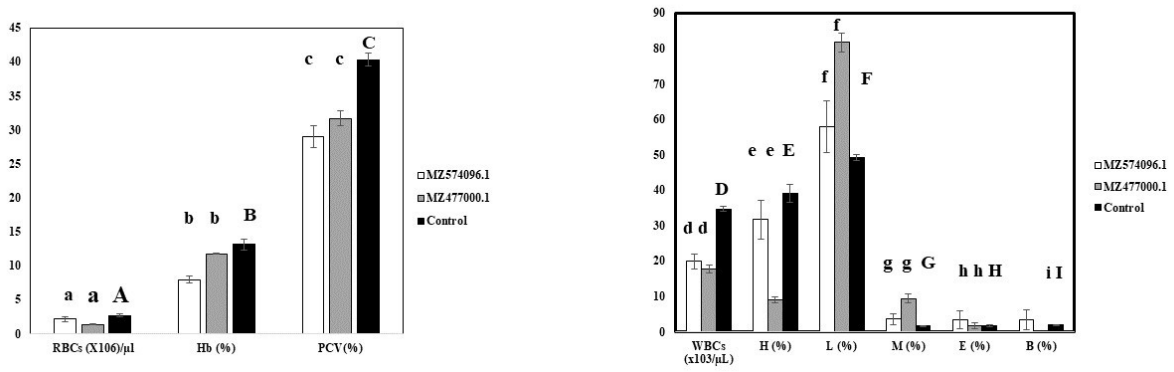


Fig. 6. Hematological examination of blood from CAV-infected groups and control negative group.

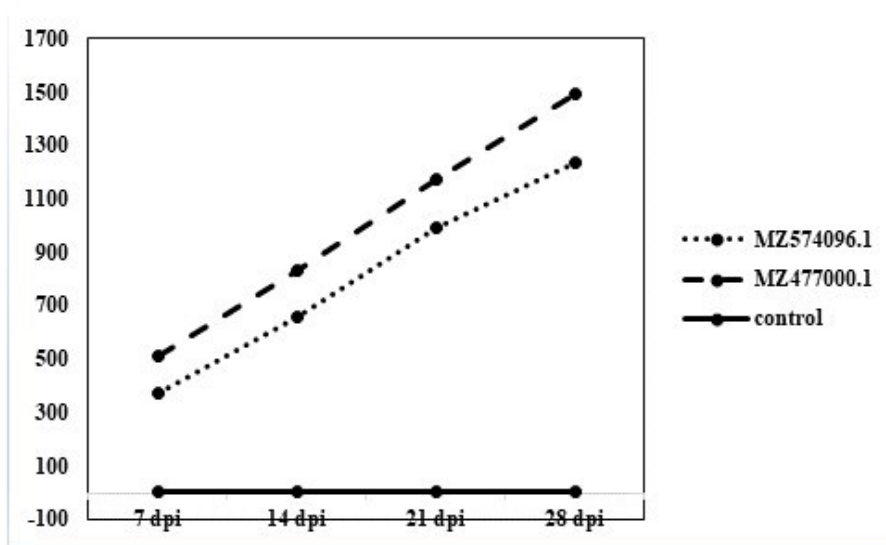


Fig. 7. The examined serum, which was collected from the SPF chicks with two infected strains of CAV, summarises the GMT (Geometric mean Titer).

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## الاختلافات الوراثية والإمراضية بين بعض السلالات المكتشفة من فيروس فقر الدم في الدجاج في فراخ اللحم الصغيرة

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### المستخلص

تهدف هذه الدراسة إلى توصيف فيروس فقر الدم في الدجاج في دجاج التسمين الصغير في مصر عام 2021، وتقييم التنوع الوراثي بين الفيروسات التي تم العثور عليها، وسلالات اللقاحات، وتقييم قدرتها المرضية لتحسين التعامل مع اصابها الفيروس و القضاء عليه.

تم تضخيم جين البروتين الفيروسي وإجرائه بعد ذلك للتوصيف على المستوى الجزيئي. في خمسة من أصل عشرة قطعان تم اكتشاف CAV التي تم أخذ عينات منها. علاوة على ذلك، أظهر فحص الروابط التطورية التي تم إجراؤها وفقًا لتسلسل جين VP2 تصنيف سلالات الحقل المرصودة ضمن المجموعات الجينية الأولى والثانية والثالثة ب. في تحليل السيليكو تم إجراء تحليل على Cux-1 والسلالات الخمس المكتشفة لفحص الهياكل الثانوية لبروتينات VP2. أشارت النتائج إلى وجود تشابه كبير في توزيع وكمية طوبولوجيا  $\alpha$ -helical و  $\beta$ -strand لتقييم قدرة الفيروس على الأمراض، تم إعطاء عزلتين من النمط الجيني III (MZ574096.1) والنمط الجيني II (MZ477000.1) في العضل لمجموعات من الكتاكيت المحددة الخالية من مسببات الأمراض بعمر يوم واحد. بعد 28 يومًا من التفقيح، كان عدد كريات الدم الحمراء (X106 $\mu$ L) (1.4  $\pm$  0.39) و (0.12  $\pm$  1.8). يعد تحليل التطور الجيني المتسق للفيروس فقر الدم أمرًا بالغ الأهمية، لأنه يركز على دراسة آثار التغيرات الجينية على الفوعة وفعالية اللقاحات.

الكلمات الدالة: فيروس فقر الدم في الدجاج - البروتين الفيروسي 2 - الكتاكيت الصغيره - التوصيف الجيني