Full genome sequences of chicken anemia virus demonstrate mutations associated with pathogenicity in two different field isolates in Egypt

Sara Abdel-Mawgod¹ · Amany Adel¹ · Abdel-Satar Arafa¹ · Hussein A. Hussein²

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Abstract Chicken anemia virus (CAV) is an important pathogen associated with immunosuppression in chicken. In this study, out of samples collected from 115 commercial poultry farms, 12 samples were CAV positive by PCR. Partial sequence and phylogenetic analysis of VP1 gene revealed that the detected viruses were clustered to genotype I (n = 3) and genotype II (n = 9). Motifs of both low (E144) and high pathogenic strains (T89, I125, Q141) were found in the three viruses of genotype I. Whereas genotype II viruses demonstrated the characteristic motifs of highly pathogenic strains (I75, T89, I125, Q141, and Q144). Three isolates representative of both genotypes (CAV/CA1, CAV/GZ1 and CAV/SK4) were selected for full genome sequencing and results revealed that the VP2 gene had two substitutions at V153 and E 175, while VP3 gene had only one substitution at C118. To evaluate virus pathogenicity, two isolates from each genotype (CAV/SK4 of genotype I and CAV/CA1 of genotype II) were intramuscularly inoculated in two groups of one-day-old specific pathogen free chicks. Eighteen days post inoculation, PCR detected CAV in 75 and 90% of chicks in group I and II; respectively. Mortalities in inoculated chicks were 5 and 20% and packed cell volume values were 0.21 and 0.19;

respectively. CAV/CA1 and CAV/SK4 isolates showed pathogenic evidences at the level of genetic (Q141 and 394Q) with variable degree of virulence. In conclusion, the study reports the circulation of at least two genotypes of CAV among chicken population with mutation associated with pathogenicity.

Keywords CAV · Full genome sequence · Genetic characterization · Pathogenicity evidence · Egypt

Introduction

CAV is an economically important pathogen with a worldwide distribution. It was first isolated in 1979 in Japan which was the major agent responsible for a disease causing severe anemia and immunosuppression [34]. It has a small negative single stranded circular DNA genome. It belongs to genus *Gyrovirus* of family *Circoviridae* [21]. The CAV genome has three major partially overlapping open reading frames (orfs) which named VP1, VP2 and VP3 with different lengths of nucleotide sequences 1350,651 and 366 respectively. These three overlapping orfs encode for three viral proteins of different molecular weights VP1 (51.6 KDa), VP2 (211 KDa) and VP3 (13.6 KDa) [17]. The VP1 is the major viral structural protein, the VP2 is a scaffolding protein and the VP3 is a non-structural protein named apoptin. The VP1 and VP2 are the protective proteins that induce neutralizing antibodies [15]. The VP1 shows the highest nucleotide variability; therefore, it is usually used for genetic characterization and molecular studies of CAV [24].

CAV has genetically three distinct genotypes (I, II, and III) which have been recognized through phylogenetic analysis based on the VP1 gene. Only minor variations in

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✉ Sara Abdel-Mawgod
drsara_vet2006@yahoo.com

¹ Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, P.O. Box 264, Dokki, Giza 12618, Egypt

² Virology Department, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt

predicted amino acid sequences have been noted in CAV isolates from different parts of the world [7, 14]. Previous studies had identified only a single serotype [19] until the emergence of a putative serotype 2 strain (strain CAV-7) from the United States [27].

The incidence of CAV infection is high; however, the clinical disease is very uncommon. The clinical disease occurs in very young chicks which get infected with vertically transmitted virus from breeders [2]. While Infection occurring between 1 and 4 days of age result in clinical symptoms and lesions, observed mainly in 2–4 week-old flocks [5]. Typical symptoms observed including weakness, depression, anorexia and stunting [34]. The virus replicates in lymphoid cells and induces cytopathic effect [20]. Cell death is believed to occur by apoptosis following expression of VP3 [13]. Lymphocyte depletion results in immunosuppression and increases susceptibility to various viral and bacterial pathogens [31].

In vitro, CAV can be propagated in embryonated eggs and lymphoblastoid cell lines but not in chicken embryo fibroblasts, chicken kidney cells or other primary cells [33]. Most molecular studies on the virus have used suspension cultures of the Marek's disease virus-transformed chicken T cell line MDCC-MSB1 for propagation [3]. Since the isolation of the virus in SPF chicks or in cell cultures (MDCC-MSB1 cells) is laborious and time consuming, the virus infection has been diagnosed rapidly by detecting the virus DNA by hybridization or by PCR in tissue samples from infected birds [32].

In Egypt, El-Lethi [8] reported the suspicion of CAV in dressed poultry and serological investigations have been proved the intensive exposure of commercial chicken to CAV [4]. Hussein reported the isolation of CAV for the first time in Egypt [11]. Later on, many studies described the molecular diagnosis of CAV in chicken population [1]. In this study, we aim to genetic characterize CAV circulating in Egypt and evaluating their pathogenicity in chickens.

Materials and methods

Sampling

During the period of 2015–2017, samples were collected from a total of 115 broilers and layers poultry located in 7 different governorates (Dakahlia, Ismailia, Sharkia, Kalio-beya, Giza, Al Wadi Al-Gadeed and Cairo). The sampling was based on notifications from suspected cases from those governorates. The samples were collected from different tissues (liver, spleen, thymus and bone marrow). Preparation of tissue samples were carried out by grinding with a mortar and pestle in PBS with addition of antibiotic

mixture (1000 I.U. penicillin/ml + 1 mg streptomycin sulphate/ml) to prepare a 20% tissue homogenate. Freezing and thawing were applied three times and then the homogenates were centrifuged at 3000 rpm for 20 min. The supernatant was transferred into new tubes for PCR and genetic characterization.

PCR detection of CAV

DNA was extracted from examined tissue homogenates using QIAamp DNA extraction kit (Qiagen, Germany Cat. No 51304). The oligonucleotide primers 5'-GAC TGT AAG ATG GCA AGA CGA GCT C-3' and 5'-GGC TGA AGG ATC CCT CAT TC-3' were used to amplify a 675 bp DNA fragment of Vp1 gene [28]; from nucleotides 823–1498, numbering is corresponding to the Del-Ros strain (GenBank AF313470). The PCR assay was performed by Emerald Amp[®] GT PCR master mix (cat no. RR310A) in the final volume of 50 µL, with the following components: 25 µL master mix, 15 µL PCR grade water, 2 µL for each primer, and 5 µL templates. The amplification was performed under the following conditions: one initial cycle of denaturation step at 95 °C for 15 min followed by 40 cycles of 95 °C for 45 s., 56 °C for 45 s., and 72 °C for 1 min and one final extension cycle at 72 °C for 10 min. The amplification products were analyzed by electrophoresis on 1.5% agarose gel, stained by ethidium bromide, examined under transilluminator and the size of the PCR products were confirmed by visualization on an ethidium bromide stained agarose against DNA ladder (GelPilot[®] 1 kb Ladder, Qiagen, Germany).

The positive samples were amplified for full genome sequence by amplification of PCR by using designed primers for this study as shown in supplementary Table S1. The PCR reactions for full genome amplification were carried out as the same previous conditions that were described above.

Partial and full genome nucleotide sequence and phylogenetic analysis

The PCR fragments for both partial and full genes were purified with the QIAquick[®] Gel Extraction Kit (Qiagen, Germany) according to the manufacturer instructions.

The purified PCR products of 12 CAV positive and 3 full samples were sequenced directly using the ABI PRISM[®] BigDye[®] Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM[®] 3130 genetic analyzer (Applied Biosystems) with 80 cm capillaries. Out of the partially sequences viruses 3 were selected for full genome sequencing. The sequences were edited with SeqScape[®] Software Version 2.5 (Applied Biosystems), assembly of the consensus sequences

and alignment trimming was performed by Bioedit software (version 7), using Clustal W method. The phylogenetic tree was drawn using MEGA7 software with other representative viruses from the GenBank and in comparison with other Egypt and international strains. Finally, the identity between all viruses was carried out using Bioedit software. The partial and complete sequences for the CAV of this study were submitted and annotated on GenBank of the National Center for Biotechnology Information (NCBI), and their accession no. were recorded in supplementary table (Table S2).

Isolation and titration procedures of CAV

Two samples were selected according to the genetic characters for isolation and titrated in SPF chicks. To confirm that the two samples were free from mixed infection with Influenza (H5 and H9), Infectious bursal disease and Newcastle disease viruses, the tissue homogenates tested by PCR for these viruses. For isolation, two groups of 1-day-old SPF chicks (10 chicks/group) were intramuscularly inoculated by 0.1 ml of each CAV positive samples [9]. Chicken Infectious Dose (CID₅₀) was measured by inoculation of other two groups of 1-day-old PSF chicks (25 chicks/group) with tenfold serial dilution (10^1 – 10^5) with 5 chicks/each dilution using Reed and Meunch method [22].

Pathogenicity evaluation of CAV

Sixty SPF chicks of one-day-old were divided into three groups (20 chicks/group). Two groups were inoculated intramuscularly with 0.1 ml of each selected isolate containing 10^2 CID₅₀ [9] and the third group was kept as negative control. After 18 days, body weight and blood samples with and without anticoagulant were collected from all live chicks for PCV and ELISA. Then the birds were euthanized for PCR assay. PCV was determined by using the microhematocrit method. CAV antibodies were measured by the Commercial CAV antibodies ELISA kit (ProFLOk PLUS, Synnbiotics, San Diego/USA).

Statistical analysis

The significant differences of data from body weight and hematocrit value were determined by one way analysis of variance using SPSS program.

Results

Results of PCR detection of CAV

Amplification of the extracted DNA from 115 tissue samples yielded 12 positive DNA bands of corrected size at 675 bp by conventional PCR, with the incidence of 10.4%. The highest CAV incidence was in Sharkia with positivity rate 17.4% while Ismalia and El-Wady Al-Gadeed were negative for CAV. Two samples out of the twelve positive samples were detected from layers with incidence 16.7% and ten samples were detected from broilers with incidence 83.3%.

Nucleotide and amino acid sequence analysis

The obtained nucleotide and amino acid sequences of twelve CAV partial VP1 gene in this study were compared with other CAV reference strains in GenBank by multiple alignments with the ClustalW included in Bioedit software. The nucleotide sequence analysis showed that nine (CAV/Dk1, CAV/Dk2, CAV/SK1, CAV/GZ3, CAV/SK2, CAV/SK3, CAV/CA1, CAV/KL2 and CAV/GZ2) viruses were closely related to each other with identity 97–100%. They were similar to the previous field strains isolated from Egypt (Egy-1) and also they were similar to CAV strain from Malaysia, Bangladesh and Japan with identity 95–100%, while the other three (CAV/KL1, CAV/GZ1 and CAV/SK4) viruses were closely related to each other with identity 97–98% and were similar to reference strains from USA, China and Australia with identity 97–99%. The obtained amino acid sequences had variable substitutions in the VP1 as clarified in Table 1. In addition, CAV/GZ1 had a unique substitution H21Q. CAV/KL1 showed additional substitution C149M, while all Egyptian CAV showed M157V substitution.

The full genome sequences of three selected Egyptian CAV (CAV/CA1, CAV/GZ2 and CAV/SK4) under this study were aligned with other reference strains that obtained from the Genbank. The amino acid sequence showed additional substitution in VP1 where CAV/SK4 contain S287, G370, L376, Q394, S413 and CAV/CA1 and CAV/GZ2 contain T287, S370, L376, Q394, A413. VP2 amino acid sequence also showed substitution at residues V153 and E175 for the three CAV strains, in addition to P16A and S180T in both CAV/CA1 and CAV/GZ2, while VP3 showed only one substitution C118 in all of them.

Phylogenetic analysis of CAV genome

The phylogenetic tree of partial amino acids sequence of the VP1 gene revealed the presence of two groups that

Table 1 Amino acid substitution comparison of VP1 gene among the twelve Egyptian CAVs and other reference CAVs

Virus name	Genotype	22	75	89	97	125	139	141	144	157
GD-1-12	I	H	V	T	M	L	K	Q	E	M
TJBD40	I	–	–	–	–	–	–	–	–	V
98D02152	I	–	–	–	–	I	–	–	–	V
3-1P60	I	–	–	–	–	I	–	–	–	–
A2	I	–	–	–	–	I	–	–	–	–
LF4	II	–	I	–	–	–	Q	–	Q	V
BD-3	II	–	–	–	L	I	Q	–	Q	V
98D06073	II	–	I	–	L	I	Q	–	Q	V
Australia 704	II	–	I	–	L	I	Q	–	Q	V
TR20	II	–	I	–	L	I	Q	–	Q	V
EGY1	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Dak1/2015	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Dak2/2016	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Sha1/2015	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Giz3/2016	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Sha2/2016	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Sha3/2016	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Cai1/2015	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Giz2/2016	II	Q	–	–	L	I	Q	–	Q	V
CAV/Egypt/Kal2/2017	II	Q	I	–	L	I	Q	–	Q	V
CAV/Egypt/Giz1/2015	I	Q	–	–	–	–	–	–	–	V
CAV/Egypt/Sha4/2017	I	–	–	–	–	I	–	–	–	V
CAV/Egypt/Kal1/2016	I	Q	–	–	–	I	–	–	–	V

were defined in this study as I and II. The three (CAV/GZ1, CAV/SK4, CAV/KL1) belonged to group I clustered together with (GD-1-12, TJBD40, CUX-1, 3-1P60 and A2), while the other nine (CAV/Dk1, CAV/Dk2, CAV/SK1, CAV/GZ3, CAV/SK3, CAV/SK4, CAV/CA1, CAV/KL2 and CAV/GZ2) belonged to group II with reference strain (LF4, BD-3, TR20 and Australia 704) (Fig. 1). The phylogenetic tree of the complete genome of CAV/CA1, CAV/GZ2 and CAV/SK4 revealed the same classification of the partial VP1 sequence (Fig. 2).

Isolation and titration of (CAV/CA1) and (CAV/SK4) viruses

CAV isolation was confirmed by PCR and the weekly collected sera were tested serologically using ELISA test to confirm CAV seropositivity. Titration of both (CAV/CA1) and (CAV/SK4) revealed that CID_{50} was $10^{3.8}$ and $10^{3.3}$; respectively.

Pathogenicity evaluation of (CAV/CA1) and (CAV/SK4) viruses

Clinical signs (emaciation, depression and ruffled feather) and post mortem findings (congested and fragile bone

marrow, markedly atrophied thymus gland and enlarged liver and spleen) associated with CAV infection were varied according to inoculated strain and the day post inoculation. The CAV was detected in group of CAV/CA1 with positive percent 90% by PCR, 81.25% seropositive by ELISA and PCV values was 0.19 in comparison to CAV/SK4 group that was 75, 63.16, 0.21%; receptively. Mortality rates of both were 20 and 5%; receptively. There are significant differences ($P > 0.0001$) between means of the 2 groups that having the same capital and small letter in the same column (Table 2).

Discussion

CAV is an infectious agent that affects commercial poultry and has a worldwide distribution. The first report of CAV in Egypt was in 1990 [8]. The presence of CAV antibodies in Egyptian poultry flocks provided strong evidence for the extensive exposure of the chicks to such virus [4]. In this study, Out of 115 tissue homogenate were tested for the presence of CAV DNA, twelve samples were positive with percentage 10.4% where Sharkia was the highest province in CAV incidence. But Al Wadi Al-Gadeed and Ismalia were negative for CAV. It confirmed that CAV is widely

Fig. 1 Phylogenetic tree of partial VP1 amino acids showed that the Egyptian CAV cluster into two groups (I and II) where the three (CAV/GZ1, CAV/SK4, CAV/KL1) belonged to group I and the other nine (CAV/Dk1, CAV/Dk2, CAV/SK1, CAV/GZ3, CAV/SK3, CAV/SK2, CAV/CA1, CAV/KL2 and CAV/GZ2) belonged to group II

Fig. 2 Phylogenetic tree of the Egyptian CAV complete genome showed that CAV/CA1, CAV/GZ2 and CAV/SK4 clustered into two groups with other CAV reference strains

distributed among chicken flocks in Sharkia governorate [10]. The incidence of CAV according to flock breed showed that two of the twelve positive samples were layer chicken (16.7%) and ten samples were broilers (83.3%) which indicated that the both breed were at risk of CAV infection.

Three genetically distinct genotypes (I, II, and III) have been recognized in classification of CAV based on the phylogenetic analysis of the VP1 gene [7, 14]. The three (CAV/GZ1, CAV/SK4 and CAV/KL1) belonged to group I which include (GD-1-12, TJBD40). The other nine (CAV/Dk1, CAV/Dk2, CAV/SK1, CAVGZ3, CAV/SK2, CAV/SK3, CAV/CA1, CAV/KL2 and CAV/GZ2) belonged to genotype II with other reference strains (LF4, BD-3).

On the base of deduced amino acid sequence of VP1, residues at positions of 75, 97, 139, and 144 of amino acid sequence had specific pattern among each genotype where (CAV/Dk1, CAV/Dk2, CAV/SK1, CAVGZ3, CAV/SK2, CAV/SK3, CAV/CA1 and CAV/KL2) viruses showed I75, L 97, Q139 and Q144 that similar to those belonged to group II and CAVGZ2 have the same genotype II pattern except V⁷⁵, while (CAV/GZ1, CAV/SK4 and CAV/KL2) viruses showed V75, M 97, K139 and E144 that similar to those belonged to group I [16]. These results indicated that both genotype I and II circulating in Egypt with different incidences. The incidence of CAV according to Genotypic groups indicate the genotype II (75%) is more spreading than genotype I (25%), Sharkia is the highest incidence for

Table 2 Comparison between pathogenicity indices of the two Egyptian isolates

Virus ID	PCR	ELISA		PCV	Mortality %	Body weight
		Titer	Positive %			
CAV/CA1	90%	2215	81.25%	0.193 ± 0.017 ^a	20	76.7 ± 4.75 ^b
CAV/SK4	75%	1685	63.16%	0.210 ± 0.016 ^a	5	85.4 ± 5.01 ^b
Neg control	Neg	Neg	Neg	0.361 ± 0.02 ^A	0	126.26 ± 4.8 ^B

The statistical analysis was performed using SPSS with Mean ± SD for PCV and Body weight. The means having the same capital and small letter in the same column with significant differences ($P < 0.0001$)

genotype II (3/4), while Cairo and Dakahlia has only genotype II.

The genetic variations of CAV is determined depending on the sequence of the VP1 gene which contains the hypervariable region in between the amino acid residues 139–151 [16], in addition to presence of the genetic determinates of CAV virulence. Previous studies recorded the amino acid motif of the highly pathogenic CAV as I75, T89, I125, Q141, and Q144 strains [12]. In comparison with VP1 sequence of Egyptian CAV in this study, it was found that the viruses (CAV/Dk1, CAV/Dk2, CAV/SK1, CAV/GZ3, CAV/SK2, CAV/SK3, CAV/CA1, CAV/KL2 and CAV/GZ2) contain the motif of highly pathogenic viruses. Whereas the (CAV/GZ1, CAV/SK4 and CAV/KL1) contain the amino acid E144 of low pathogenic viruses, beside the presence of amino acid motif T89, I125, Q141 which are related to the highly pathogenic strains. The nine viruses were characterized with the presence of Glutamine (Q) at both amino acid residues 139 and 144, these motif were found in the highly pathogenic strains which are associated with the reduction of the virus spread in tissue culture. On the other hand, (CAV/GZ1, CAV/SK4 and CAV/KL1) had Glutamic acid (E) at position (144E) which is always accompanied by lysine (139 K) as it was recorded in previous studies [23]. All of the Egyptian CAVs characterized in this study had the pathogenic determinant glutamine (Q 394), therefore the viruses of this study may be pathogenic [10].

On the level of the complete sequence for 3 Egyptian viruses, there are amino acid changes in the VP2 and the VP3 genes in comparison with different reference strains. The VP2 showed two substitutions at V153 and E175 in the three Egyptian CAV isolates, in addition to unique substitutions as P16 A and S 180 T in both of CAV/Cai1 and CAV/Giz1. While the VP3 gene showed only one substitution at C118 in all of them. Hence, it means that the genome sequences of VP2 and VP3 are relatively conserved in comparison with VP1 [35].

For further pathological evaluation, two Egyptian isolates (CAV/CA1 and CAV/SK4) were inoculated in SPF one-day-old chicks. All the chicks developed the expected clinical symptoms included weakness, ruffled feathers and stunted growth after 7–10 days. Generally, postmortem

examination showed identical distribution pattern in organs of tropism (thymus, spleen, liver and bone marrow) with differences in their severity. This agree with the previously observation that the lesions depend on the degree of severity [25]. At the end of study (18th day), CAV infectivity was confirmed by PCR test where (CAV/CA1) and (CAV/SK4) showed positivity 90 and 63.16%. Infected chicks in the two study groups, proved seropositive against CAV with positivity 75 and 81.25% by ELISA receptively. The detected antibodies proved the experimentally infection with CAV at 1 day age. The indirect ELISA system is rapid, sensitive and specific for the detection of serum antibodies to CAV and it is well-suited for the serological diagnosis of CAV in SPF and commercial chicken flocks. These results are in agree with some researchers' results [26] and they are not in agree with other researchers who suggested that sera collected at late stages of CAV infection may give negative results when serologically tested [30].

The means of PCV values for the infected chicks with CAV/CA1 and CAV/SK4 were 0.19 and 0.21; receptively, with a significant reduction ($P < 0.0001$) in comparison with the negative control group (0.36). Therefore, these results supported the hypothesis that the CAV is directly cytotoxic for bone marrow hematopoietic precursor cells in young chicks [18]. That is in agree with previous studies which reported that CAV causes reduction in the production of mature red blood cells (erythropoiesis) and myelopoiesis leading to hypoplasia, anaemia and panleukopenia [6]. Mortality rates of CAV/CA1 and CAV/SK4 were 20 and 5%; receptively, in addition to a significant decrease in weight gain in almost all the inoculated chicks that is typical to CAV infection. Based on the previous results of this study, both the Egyptian viruses CAV/CA1 and CAV/SK4 showed pathogenic evidences at the level of genetic and pathogenic parameters with different degree of virulence. Both the Egyptian CAV viruses are pathogenic and could produce typical symptoms and lesions of anemia, but CAV/CA1 was more pathogenic than the CAV/SK4 as there are a significant reduction in PCV and body weights in the chicks were infected with the CAV/SK4 virus in comparison with the chicks were infected with the CAV/CA1 virus. Also, CAV/SK4 virus

had the amino acid motif of low pathogenic strains (E144) and pathogenic marker (Q141 and Q394) [29, 32]. In Conclusion the recent study shows that CAV is still circulating in the Egyptian poultry farms. The results also indicate that the genetic variability in local CAV isolates can be divided into two genotypic groups (genotype I and genotype II). Full genes sequence analysis shows a little divergence in most coding regions. This study shows that the two CAV viruses have variable pathogenicity in chickens; however the CAV/CA1 is more pathogenic than the CAV/SK4. Further studies are required for investigation of the pathogenicity difference among CAV strains in different ages of chickens.

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References

1. AboElkhair M, Abd El-Razak AG, Metwally AY. Molecular characterization of chicken anemia virus circulating in chicken flocks in Egypt. *Adv Virol.* 2014;797151–6.
2. Adair BM. Immunopathogenesis of chicken anemia virus infection. *Dev Comp Immunol.* 2000;24:247–55.
3. Akiyama Y, Kato S. Two cell lines from lymphomas of Marek's disease. *Biken J.* 1974;17:105–16.
4. Amin AA, Hassan MK, Mona M, Abdel-Zaher, A. A serological study of the prevalence of chicken anaemia in commercial flocks. In: Proceedings of the 5th science conference, Egypt veterinary poultry association; 1998. p. 69–75.
5. Dhama K, Kataria JM, Senthilkumar N, Dash BB, Tomar S. Standardization and application of polymerase chain reaction and indirect immunofluorescent technique for detection of chicken infectious anaemia virus. *Indian J Comp Microbiol Immunol Infect Dis.* 2002;23:11–22.
6. Dhama K, Mahendran M, Somvanshi R, Chawak MM. Chicken infectious anaemia virus: an immunosuppressive pathogen of poultry—a review. *Indian J Vet Pathol.* 2008;32:158–67.
7. Ducatez MF, Owode AA, Abiola JO, Muller CP. Molecular epidemiology of chicken anemia virus in Nigeria. *Arch Virol.* 2005;151:97–111. <https://doi.org/10.1007/s00705-005-0609-7>.
8. El-Lethi AM. Viral contamination of dressed poultry. M.V.Sc. thesis, Cairo University, Egypt; 1990.
9. Ezzi A, Shoushtari A, Marjanmehr H, Toroghi R, Tavasoly A, Bahmani-nejad MA. Experimental studies of pathogenesis of chicken infectious anaemia virus (3 isolates) in Iran Archives of Razi Institute, Vol. 67, No. 1, June 13–19; 2012.
10. Hegazy AM, Abdallah FM, Abd-El Samie LK and Nazim AA. Incidence of Chicken Anemia Virus in Sharkia governorate chicken flocks. *Assiut Vet Med J.* 2014;752–61(142).
11. Hussein HA, Sabry MZ, El-Ebiary EA, El-Safty M, Abdel-Hady AL. Chicken infectious anemia virus in Egypt: molecular diagnosis by PCR and isolation of the virus from infected flocks. *Ar J Biotechnol.* 2002;5(2):263–74.
12. Islam MR, Johne R, Raue R, Todd D, Muller H. Sequence analysis of the full-length cloned DNA of a chicken anaemia virus (CAV) strain from Bangladesh: evidence for genetic grouping of CAV strains based on the deduced VP1 amino acid sequences. *J Vet Med B Infect Dis Vet Public Health.* 2002;49:332–7.
13. Jeurissen SHM, Wagenaar F, Pol JMA, van der Eb AJ, Noteborn MHM. Chicken anemia virus causes apoptosis of thymocytes after in vivo infection and of cell lines after in vitro infection. *J Virol.* 1992;66:7383–8.
14. Kim HR, Kwon YK, Bae YC, Oem JK, Lee OS. Molecular characterization of chicken infectious anemia viruses detected from breeder and broiler chickens in South Korea. *Poult Sci.* 2010;89:2426–31.
15. Koch G, van Roozelaar DJ, Verschueren CA, van der Eb AJ, Noteborn MH. Immunogenic and protective properties of chicken anaemia virus proteins expressed by baculovirus. *Vaccine.* 1995;8:763–70.
16. Kye S-J, Kim J-Y, Seul H-J, Kim S, Kim S-E, Lee H-S, Sorn S, Choi K-S. Phylogenetic analysis and genetic characterization of chicken anemia virus isolates from Cambodia. *Poult Sci.* 2013;92(10):2681–6.
17. Lai GH, Lin MK, Lien YY, Fu JH, Chen HJ, Huang CH, Tzen J, Lee MS. Expression and characterization of highly antigenic domains of chicken anemia virus viral VP2 and VP3 subunit proteins in a recombinant *E. coli* for sero-diagnostic applications. *BMC Vet Res.* 2013;9:161.
18. Liu ZG, Zheng SM, Xu YB, Zhou ZY, Wang XR, Yang LP. Immunopathological changes of chicken infectious anemia. *Sci Agric Sin.* 1997;30:74–82.
19. McNulty MS, Connor TJ, McNeilly F, McLoughlin MF, Kirkpatrick KS. Preliminary characterization of isolates of chicken anaemia agent from the United Kingdom. *Avian Pathol.* 1990;19:67–73.
20. Noteborn MHM, Koch G. Chicken anaemia virus infection: molecular basis of pathogenicity. *Avian Pathol.* 1995;24:11–31.
21. Pringle CR. Virus taxonomy at the XI international congress of virology, Sydney, Australia. *Arch Virol.* 1999;144:2065–70.
22. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg.* 1938;27:493–7.
23. Renshaw RW, Some C, Weinkle T, O'Connell PH, Ohashi K, Watson S, Lucio B, Harrington S, Schat KA. A hypervariable region in VP1 of chicken infectious anemia virus mediates rate of spread and cell tropism in tissue culture. *J Virol.* 1996;70:8872–8.
24. Schat KA. Chicken infectious anemia. In: Saif YM, editor. Diseases of poultry. Iowa: Iowa State Press; 2003. p. 182–202.
25. Schat KA, van Santen V. Chicken infectious anemia. In: Saif YM, Barns HJ, Gilsson JR, Fadly AM, McDougald LR, Swayne DE, editors. Disease of poultry. 11th ed. Ames: Iowa State University Press; 2009.
26. Sommer F, Cardona C. Chicken anemia virus in broilers: dynamics of the infection in two commercial broiler flocks. *Avian Dis.* 2003;47:1466–73.
27. Spackman E, Cloud SS, Rosenberger JK. Comparison of a putative second serotype of chicken infectious anemia virus with a prototypical isolate II. Antigenic and physicochemical characteristics. *Avian Dis.* 2002;46:956–63.
28. Todd D, Niagro FD, Ritchie BW, Curran W, Allan GM, Lukert PD, Latimer KS, Steffens WL III, McNulty MS. Comparison of three animal viruses with circular single-stranded DNA genomes. *Arch Virol.* 1991;117:129–35.
29. Todd D, Scott AN, Ball NW, Borghmans BJ, Adair BM. Molecular basis of the attenuation exhibited by molecularly cloned highly passaged chicken anemia virus isolates. *J Virol.* 2002;76:8472–4.
30. Toro H, Ramirez AM, Larenas J. Pathogenicity of chicken anaemia virus (isolate 10343) for young and older chickens. *Avian Pathol.* 1997;26:485–99.

31. Von Bulow V. Infectious anemia. In: Calnek BW, editor. Diseases of poultry. 9th ed. Ames: Iowa State University Press; 1991. p. 690–9.
32. Yamaguchi S, Imada T, Kaji N, Mase M, Tsukamoto K, Tanimura N, Yuasa N. Identification of a genetic determinant of pathogenicity in chicken anaemia virus. *J Gen Virol*. 2001;82:1233–8.
33. Yuasa N. Propagation and infectivity titration of the Gifu-1 strain of chicken anemia agent in a cell line (MDCC-MSB1) derived from Marek's disease lymphoma. *Natl Inst Anim Health Q*. 1983;23:13–20.
34. Yuasa N, Taniguchi T, Yoshida I. Isolation and some characteristics of an agent inducing anemia in chicks. *Avian Dis*. 1979;23:366–85.
35. Zhang X, Liu Y, Wu B, Sun B, Chen F, Ji J, Ma J, Xie Q. Phylogenetic and molecular characterization of chicken anemia virus in southern China from 2011 to 2012. *Sci Rep*. 2013;3:3519.