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RESEARCH

Molecular and pathological study on Chicken Anemia Virus

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ABSTRACT

Background: Chicken Anemia Virus (CAV) is an infectious disease that is causing major economic losses in the poultry industry and these losses are attributed to high mortalities, reduced production and cost for preventive medications.
Objective: The aim of this study was to identify CAV obtained from farms with problems associated with decreased body weight and immunosuppression. Also to determine their relationship with vaccine used in the field and other reference strains. Then to observe microscopic lesions and to detect CAV antigens by immunohistochemically in thymus and bone marrow tissues of SPF chicks infected with CAV.
Methods: In this study, 30 layer farms of age 40 to 100-days-old from different governorates (Alexandria, Gharbia, Giza and Qalubia) were examined for CAV with real-time PCR and histopathological examination. The positive samples were prepared and inoculated in SPF chicks' one-day-old then at aged 10-days post-inoculation collected organs for PCR, sequencing and immunohistochemically examination.
Results: There's one positive farm of age 100-days-old from Qalubia governorate. The histopathological examination of positive sample showing severe depletion of hematopoietic cells in bone marrow replaced by adipocytes. Spleen exhibited depletion of lymphocytes with focal coagulative necrosis in addition to congested blood vessels. PCR was done on the positive real-time PCR sample, which separate amplified band at 418 bp then the sequences was submitted to the GenBank under accession number MH260568 for the VP1 gene. Our strain has 99% identity with nucleotides of Indian and Japan strains, 97% amino acid identity with the same strains. Our circulating strain has low nucleotides identity 37%, 38% and amino acid identity 15% for both with vaccinal strains (Cux-1N/Germany and CAV/Nobilis®P4) respectively. The high percentage of similarity of nucleotides identity with Egyptian isolates and our strain were with Egypt I/Giza 2009, Egypt II/Fayoum I, Egypt III/Fayoum II that was 86% for all, as well as amino acid identity was 86%, 87% and 86% respectively.
Conclusion: The obtained results indicate that our circulating strain has great differences from vaccinal strains which used in the field, so we need periodic characterization of circulating CAVs in Egypt to improve methods of virus control and to usually determine the relationship of circulating CAV with vaccine strains and other CAV strains. The most obvious thing in this study, that's we need to make our vaccine from our strain which circulating in the field with periodic updating.

Keywords: Chicken Anemia Virus, SPF chicks, real-time PCR, PCR, histopathology, IIFT and Immune peroxidase.

BACKGROUND

Chicken Anemia Virus (CAV) is one of the most common infectious diseases in the world. In 1979, it was first reported in Specific-Pathogen-Free (SPF) chicks in Japan (Yuasa *et al.*, 1979), during an investigation of Marek's disease outbreaks. The infection with CAV poses a serious economic threat especially to the producers of specific-pathogen-free (SPF) eggs and the broiler industry. Economic losses due to CAV infections come from poor growth, increased

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mortality and the money cost of antibiotics used to control secondary bacterial infections (McNulty, 1991). McIlroy *et al.,* (1992) reported a loss of about 18.5% due to decreased weight at processing and increased mortality in CAV-infected birds. In the poultry industry, there are losses due to production losses, condemnation, mortality and the cost of preventive medication.

CAV is an important pathogen with a worldwide distribution. It is a small DNA virus with a closed circular, negative, single stranded DNA genome and it belongs to genus Gyrovirus of family Circoviridae (Schat, 2009). CAV is a small, non-enveloped, icosahedral virus measuring 25-26.5 nm in diameter with single negative stranded circular DNA genome (Pringle, 1999). The viral genome consists of 2.3 kilobases, with 3 partially overlapping open reading frames (McConnell *et al.,* 1993) which encoding three viral proteins: VP1 (51.6 kDa), the major viral capsid protein, VP2 (24 kDa) a novel dual specificity protein phosphatase (Noteborn *et al.,* 1998 & Peters *et. al.,* 2002) that also probably acts as scaffolding protein during virion assembly and VP3 (13.6 kDa) also called apoptin, which has been shown to have apoptotic activity in transformed cell lines (Noteborn *et. al.,* 1991& Peters *et al.,* 2001). VP1 shows the highest nucleotide variability; therefore, it is usually used for genetic characterization and molecular studies of CAV (Craig *et. al.,* 2009).

CAV causes severe aplastic anemia, subcutaneous and intramuscular hemorrhages, generalized lymphoid atrophy of all hematopoietic and lymphoid organs and intramuscular, subcutaneous hemorrhages destruction of erythroblastoid cells in the bone marrow and depletion of certain lymphoid organs in young chickens (Noteborn *et. al.,* 1991). The clinical signs are mainly noticed in young chicks of 10–14 days of age, which acquire the infection vertically. Chickens older than 2-3 weeks of age are also susceptible to infection but only develop a subclinical disease evidenced by poor vaccine response (Schat, 2003). The virus is transmitted either vertically from hens infected for the first time during lay or horizontally in chicks devoid of maternal antibodies (Bulow and Schat, 1997). Mortalities and morbidities due to CAV infection may reach 55% and 80% respectively (Lai *et. al.,* 2013).

The diagnosis of CAV infection is based on clinical signs, gross lesions in affected birds, detecting infectious virus DNA and virus-specific antibodies. However, a confirmatory diagnosis required isolation and identification of the CAV. The isolated viruses originating from different parts of the world are belongs to the same serotype and are antigenically indistinguishable by serum neutralization tests (McNulty, 1991&Yuasa and Imai, 1986). For rapidity and suitability testing a large numbers of serum samples enzyme-linked immunosorbent assay (ELISA) is the best for investigating the epidemiology of CAV infection in poultry farms (Schat, 2003; Dhama *et al.,* 2008).

In Egypt, El-Lethi, (1990) reported the suspicion of CAV in dressed poultry and serological investigation have been proved the intensive exposure of commercial chicken to CAV (Zaki and El-Sanousi, 1994; Sabry *et al.,* 1998; Amin *et al.,* 1998). The molecular diagnosis of the CAV for the first time in Egypt Hussein *et al.,* 2001 and the isolation of the CAV from clinical and subclinical infected broiler-breeder flocks. Many previous publications have reported detection of CAV in chicken population (Hussein *et al.,* 2002& Hegazy *et al.,* 2010& Mohamed, 2010& Eid Hussein *et al.,* 2016 and Erfan *et al.,* 2018). The aim of this study was to identify CAV obtained from farms with problems associated with decreased body weight and immunosuppression. Also to determine their relationship with vaccine used in the field and other reference strains. Then to observe microscopic lesions to detect CAV antigens by immunohistochemically in thymus and bone marrow tissues of SPF chicks infected with CAV.

MATERIALS AND METHODS

Collected samples:

About 30 farms from different governorates (Alexandria, Gharbia, Giza and Qalubia) were examined 5 chickens from each to detect CAV, these farms had main complains of immunosuppression, loss of body weight and significant death percent. Thymus loops, bone marrow, bursa of Fabricius, liver and spleen were collected aseptically from freshly dead and sacrificed bird. The age of examined farms ranged from 40 to 100-days representing different breeds (Lohmann layer, Balady, white LHL layer, ISA and Arbor Acres layer), which showing clinical signs and post mortem lesions suggestive to be chicken anemia virus.

Tissue Sample Suspension Preparation for extraction:

The collected tissues have been homogenized in saline containing 2000 iu/ml Penicillin and 200 mcg/ml Streptomycin. These organs were ground making homogenized (20% W/V) then centrifuged at 3000 rpm for 15 minutes. After centrifugation the clear supernatant fluid put at -20°C until used (Laboratory Manual for the Isolation and Identification of Avian Pathogens, 1998).

DNA Extraction Methods:

Tissue suspensions supernatants were treated with the PathoGene-spin™ DNA/RNA Extraction Kit following the manufacturer's instructions (iNtRON Biotechnology, Seongnam, Korea). After measuring DNA concentrations using the NanoDrop ND-1000 (NanoDrop, Wilmington, DE) the samples were stored at -70°C.

Real-time PCR primers:

Oligonucleotide sequences for the detection of chicken anemia virus DNA using real-time Taqman assays

CAV Q5:5'-GCCCCGGTACGTATAGTGTGAG-3'

CAV probe 5' - (6FAM)-CTGCCGAACCCCCAATCTACTATGACTATCC-(TAMRA)-3'

Cux-1* specific 5'-CCGTGAGAAA**G**ATGA**CC**CCTT-3'

* GenBank accession no. L14767.

The reactions were carried out in an AB Applied Biosystems. The conditions consisted of 95 °C for 10 min then 40 cycles consisting of 95 °C for 15 sec and 60 °C for 1 min. The real-time PCR data acquisition and analysis were performed using Analysis computer system, V 2.2.2 software (AB AppliedBiosystems (Carrie *et al.,* 2002).

Histopathological Examination:

Collected organs (bone marrow, thymus and spleen) were fixed in 10% buffered neutral formalin solution, processed in normal way; paraffin sections (5 microns thickness) were prepared and stained with haematoxylin and eosin (H&E) (Fischer *et al.,* 2006).

Production of CAV antiserum:

Anti- CAV antiserum was prepared in rabbits with vaccinal strain (Nobilis®, CAV P4). The serum was harvested when titers were high and used hyper immune serum for immunohistochemistry (Laboratory Manual for the Isolation and Identification of Avian Pathogens, 1998).

SPF chicks' inoculation:

- **Inoculum preparation:**

Tissue samples were prepared according to the method of Zhou *et* al., 1997. Briefly, tissue samples were ground and processed with a mortar and pestle in PBS with addition of antibiotics to prepare a 20% tissue homogenate. The homogenate was mixed with an equal volume of chloroform for 15 min in a shaker. Three times of repeated freezing and thawing were applied and then the homogenate was centrifuged for 20 min at 3000 rpm. The supernatant was aliquot and stored at - 70 ^{0}C till used for SPF chick's inoculation.

- **Chicks' inoculation:**

The positive samples from real-time PCR was prepared and carried out to inoculation at 1-day-old SPF chicks intramuscularly in the thigh with 0.2 ml of the supernatant fluids/chick. At 10 days post inoculation, the birds were bleeded and liver, thymus loops, bone marrow and spleen were collected and used for preparation of homogenate for DNA extraction for conventional PCR then sequencing. Another set of previous organs were collected for histopathological examination and immunohistochemistry examination (Hussein *et al.*, 2001).

- **Conventional PCR primers:**

The primers (F: 5'-CTA AGATCT GCA ACTGCG GA-3'and R: 5'-CCT TGG AAG CGG ATAGTC AT-3') were used to amplify a fragment of 418 bp of CAV genome (Hussein *et al.*, 2002).

A total volume of 50 µl PCR reaction containing 5µl of the extracted DNA and 45 µl of PCR reaction mixture. Following an initial cycle at 95°C for 5 min, 50 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec was conducted. The amplification products were analyzed by electrophoresis on 1.5% agarose gel, stained by ethidium bromide and PCR products were confirmed by visualization ethidium bromide stained agarose against DNA ladder (100 bp, NEW ENGLAND *BioLabs*).

- **Sequencing of the amplified part of VP1gene:**

The GATC Company by using ABI 3730xl DNA sequencer by using forward and reverse primers combining the traditional Sanger technology with the new 454 used for performing gene sequencing using an Applied Bio systems 3130 genetic analyzer (ABI, USA) for sequencing the CAV (418 bp) PCR product of the VP1 gene. The sequencing data were checked by NCBI Blast search and assembled edited chromatographed using Bio Edit software version 7.1.5 program. To assess the genetic relatedness among the CAV and the phylogenetic tree was generated by the distance-based neighbor-joining (NJ) method using MEGA version 6.

- **Immunohistochemistry Examination:**

***Immune Peroxidase:**

Small pieces of spleen and thymus tissues were fixed and processed for paraffin embedding, cut at thickness of 5µm and put on coated slide (Naish, 1989). The sections were de-waxed in xylene and re-hydrated in PBS for 5 min and stained for CAV antigen localization by using the non-biotin system and utilize a poly HRP (horseradish peroxidase) conjugate to locate where the rabbit antibody is bound to the target CAV antigen. The technique was done according to the manufacturer's instructions (Genemed, Biotechnologyies, Inc.).

***Immune Fluorescent:**

A tissue from bone marrow was fixed and processed for paraffin embedding, cut at five-micron thickness and put on coated slide. The protocol itself was adapted from one obtained by

(Maria and Peter, 2003) by using Fluorescein-Labeled Antibody to Rabbit IgG (H+L) produced in Goat Catalog No. 172-1506 (KPL).

RESULTS

Real-time PCR results:

One sample was positive from 30 farms examined by real-time PCR, the one positive farm of age 100-days-old of ISA type from Qalubia governorate with CT reading 26.

Histopathological Results:

The positive farm with real-time PCR showing severe depletion of hematopoeitic cells in bone marrow replaced by adipocytes (fig.1A, X 200) and this is due to the destruction of hemocytoblasts in bone marrow leading to severe depletion of myeloid and erethroid cells producing anemia. Spleen exhibited depletion of lymphocytes (fig.1B, X 200) with focal coagulative necrosis (fig.1C, X 200) in addition to congested blood vessels due to the infection of mature lymphocytes with CAV. Focal starry sky depletion in cortex of thymus (fig.1D, X200) with cellular necrotic foci and severe hemorrhage in the medulla (fig.1E, X 200). Moreover, widening of few hussal's corpuscles forming cysts containing necrotic debris and granulocytes (fig.1F, X400). Thymic lesions were destruction of T lymphocytes progenitor cells in thymus results in depletion of helper and cytotoxic cells inducing severe atrophy and depletion of lymphocytes.

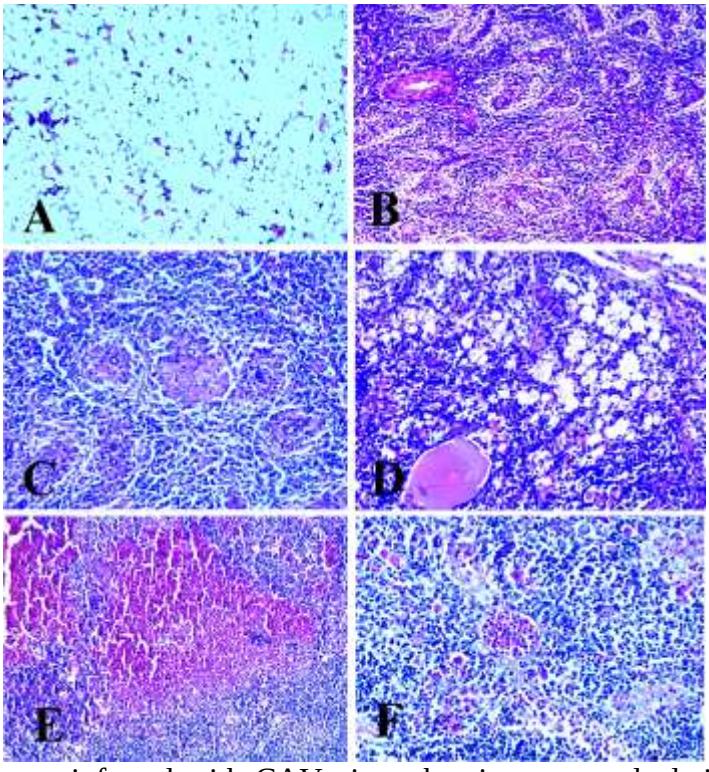

Fig. 1: A: Bone marrow infected with CAV virus showing severe depletion of hematopoeitic cells replaced by adipocytes (X 200). **B:** Spleen showing depletion of lymphocytes (X 200). **C:** Spleen infected with CAV virus showing focal coagulative necrosis (X200). **D:** Thymus infected with CAV virus showing focal starry sky depletion in cortex of thymus (X200). **E:** Thymus infected with CAV virus showing severe hemorrhage in the medulla (X 200). **F:** Thymus infected with CAV virus showing widening of few hussal's corpuscles forming cysts containing necrotic debris and granulocytes (X400).

SPF chick's inoculation results:

- **Conventional PCR:**

A DNA fragment of 418 bp amplified VP1gene by PCR using DNA extracted from SPF organs suspension was detect limit of this part of common CAV.

- **GenBank accession numbers:**

The generated Sequences were submitted to the GenBank under accession number MH260568 for the VP1 gene fragments.

- **Sequencing of VP1 gene and phylogenetic analysis:**

The tree (Fig.2) was mad with our strain (MH260568 CAV/Kal.2) and other representative viruses from the GenBank. The comparison was done with some Egyptian strains (Zag.1, 2, 3, 4-2015& Egy1, 2, 3, 4 & Egypt I /Giza2009, Egypt II / Fayoum l, Egypt III / Fayoum II), vaccinal strains (CAV/Nobilis® P4, CAV/Del-Rose/USA, Cux-1M/Germany and Cux-1N/Germany) and some CAV strains from China, USA, Taiwan, Indian and Japan.

The phylogenetic tree (Fig.2) for the CAV/Kal.2 with other Egyptian CAV strains indicate grouping of most Egyptian viruses in one cluster with vaccinal strains. The CAV/kal.2 is in one cluster with 3 Egyptian strains (Egypt I/ Giza2009, Egypt II/Fayoum I and Egypt III/ Fayoum II), Indian and Japanizes strains. Our strains very close to CAV/LUVAS-CAVT9/India and CAV/Fukushima-2/2014/Japan with 99% nucleotides identity for both and 97% amino acid identity for both strains. The Egypt I/ Giza2009, Egypt II/Fayoum I and Egypt III/ Fayoum II have nucleotides identity 86% for all strains and amino acid identity 86%, 87% and 86% respectively with the CAV/Kal.2. The vaccinal strains (Cux-1M/Germany, Cux-1N/Germany, CAV/Nobilis®P4, CAV/26P4/USA and CAV/Del-Rose/USA,) have great difference from our strain, the nucleotides identity are 38%, 38%, 37%, 37% and amino acid identity are 15% for all 5 vaccinal strains. The other Egyptians strains (Zag.1, 2, 3, 4-2015& Egy1, 2, 3, 4) in table 1 have nucleotides identity with our strain ranged from 37% to 40% and amino acid identity ranged from 15% to 17%.

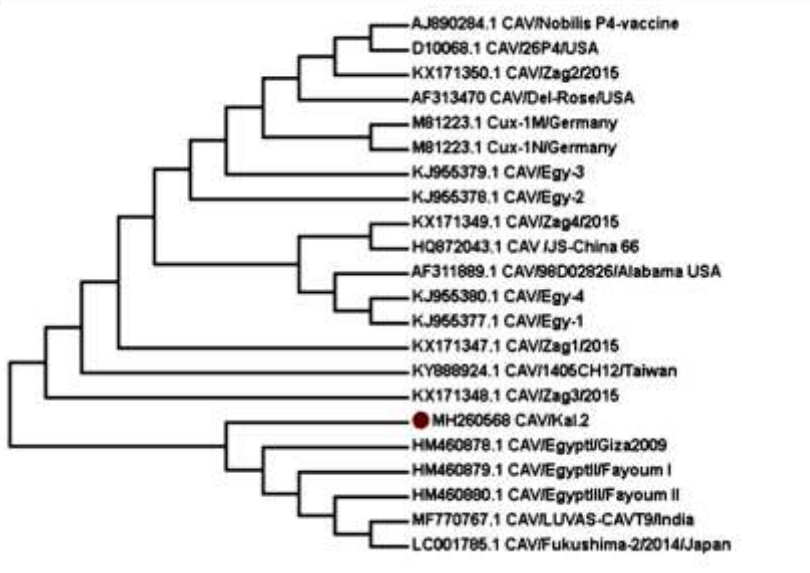

Fig. 2: Phylogenetic relationships among CAV strain based on the alignment of the partial nucleotide sequences of amplifiedVP1 and the used tree was constructed by the Neighbor Joining algorithm.

Table 1: Nucleotide and amino acid identities of MH260568 CAV/Kal.2 with selected references of Egypt strains, other world country strains and different vaccinal sequence

Strain name Nucleotide identity % (upper right) Amino acid identity % (lower left)	>MH260568 CAV/Kal.2	>KX171347.1 CAV/Zag1/2015	>KX171350.1 CAV/Zag2/2015	>KX171348.1 CAV/Zag3/2015	>KX171349.1 CAV/Zag4/2015	>KJ955380.1 CAV/Egy-4	>KJ955379.1 CAV/Egy-3	>KJ955378.1 CAV/Egy-2	>KJ955377.1 CAV/Egy-1	>HM460878.1 CAV/EgyptI/Giza2009	>HM460879.1 CAV/EgyptII/Fayoum I	>HM460880.1 CAV/EgyptIII/Fayoum II	>M81223.1 Cux-1M/Germany	>M81223.1 Cux-1N/Germany	>AJ890284.1 CAV/Nobilis P4-vaccine	>D10068.1 CAV/26P4/USA	>AF313470 CAV/Del-Rose/USA	>HQ872043.1 CAV /JS-China 66	>KY888924.1 CAV/140SCH12/Taiwan	>AF311889.1 CAV/98D02826/Alabama USA	>MF770767.1 CAV/LUVAS-CAVT9/India	>LC001785.1 CAV/Fukushima-2/2014/Japan
>MH260568 CAV/Kal.2	ID	39%	37%	40%	39%	38%	37%	38%	38%	86%	86%	86%	38%	38%	37%	37%	38%	39%	40%	39%	99%	99%
>KX171347.1 CAV/Zag1/2015	17%	ID	95%	99%	99%	97%	94%	96%	97%	37%	37%	37%	96%	96%	95%	95%	96%	99%	99%	99%	39%	39%
>KX171350.1 CAV/Zag2/2015	15%	94%	ID	95%	95%	95%	95%	94%	94%	35%	35%	34%	98%	98%	100%	100%	98%	95%	95%	96%	37%	37%
>KX171348.1 CAV/Zag3/2015	17%	100%	94%	ID	99%	97%	94%	96%	97%	37%	37%	37%	96%	96%	95%	95%	96%	99%	99%	99%	40%	40%
>KX171349.1 CAV/Zag4/2015	17%	100%	94%	100%	ID	97%	94%	96%	97%	37%	37%	36%	96%	96%	95%	95%	96%	99%	99%	99%	39%	39%
>KJ955380.1 CAV/Egy-4	17%	99%	93%	99%	99%	ID	95%	98%	100%	37%	37%	37%	94%	94%	95%	95%	94%	97%	97%	98%	38%	38%
>KJ955379.1 CAV/Egy-3	15%	97%	94%	97%	97%	98%	ID	95%	95%	36%	36%	36%	95%	95%	95%	95%	96%	93%	94%	94%	37%	37%
>KJ955378.1 CAV/Egy-2	16%	98%	92%	98%	98%	99%	97%	ID	97%	37%	37%	37%	94%	94%	94%	94%	94%	96%	96%	96%	38%	38%
>KJ955377.1 CAV/Egy-1	17%	98%	92%	98%	98%	99%	97%	98%	ID	37%	37%	37%	94%	94%	94%	94%	94%	96%	97%	97%	38%	38%
>HM460878.1 CAV/EgyptI/Giza2009	86%	17%	15%	17%	17%	17%	15%	16%	17%	ID	100%	99%	36%	36%	35%	35%	36%	37%	37%	37%	86%	86%
>HM460879.1 CAV/EgyptII/Fayoum I	87%	17%	15%	17%	17%	17%	15%	16%	17%	99%	ID	99%	36%	36%	35%	35%	36%	37%	37%	37%	87%	86%
>HM460880.1 CAV/EgyptIII/Fayoum II	86%	17%	15%	17%	17%	17%	15%	16%	17%	97%	98%	ID	36%	36%	34%	34%	35%	36%	37%	36%	87%	87%
>M81223.1 Cux-1M/Germany	15%	96%	97%	96%	96%	95%	96%	94%	94%	15%	15%	15%	ID	100%	98%	98%	98%	95%	96%	96%	38%	38%
>M81223.1 Cux-1N/Germany	15%	96%	97%	96%	96%	95%	96%	94%	94%	15%	15%	15%	100%	ID	98%	98%	98%	95%	96%	96%	38%	38%
>AJ890284.1 CAV/Nobilis P4-vaccine	15%	94%	100%	94%	94%	93%	94%	92%	92%	15%	15%	15%	97%	97%	ID	100%	98%	95%	95%	96%	37%	37%
>D10068.1 CAV/26P4/USA	15%	94%	100%	94%	94%	93%	94%	92%	92%	15%	15%	15%	97%	97%	100%	ID	98%	95%	95%	96%	37%	37%
>AF313470 CAV/Del-Rose/USA	15%	96%	98%	96%	96%	95%	96%	94%	94%	15%	15%	15%	99%	99%	98%	98%	ID	95%	96%	96%	38%	38%
>HQ872043.1 CAV /JS-China 66	17%	99%	93%	99%	99%	98%	96%	97%	97%	17%	17%	17%	95%	95%	93%	93%	95%	ID	98%	98%	39%	39%
>KY888924.1 CAV/140SCH12/Taiwan	17%	100%	94%	100%	100%	99%	97%	98%	98%	17%	17%	17%	96%	96%	94%	94%	96%	99%	ID	99%	39%	39%
>AF311889.1 CAV/98D02826/Alabama USA	17%	99%	93%	99%	99%	98%	96%	97%	97%	17%	17%	17%	95%	95%	93%	93%	95%	98%	99%	ID	39%	39%
>MF770767.1 CAV/LUVAS-CAVT9/India	97%	17%	15%	17%	17%	17%	15%	16%	17%	87%	88%	89%	15%	15%	15%	15%	15%	17%	17%	17%	ID	100%
>LC001785.1 CAV/Fukushima-2/2014/Japan	97%	17%	15%	17%	17%	17%	15%	16%	17%	87%	88%	89%	15%	15%	15%	15%	15%	17%	17%	17%	100%	ID

Immunohistochemistry Results:

- **Immunoperoxidase:**

The thymus, control negative staining (Fig.3H). The thymus revealed high levels of expression of the CAV antigen (high positive signal) in the nuclei of lymphocytes (Fig.3I), thymocytes and connective tissue (Fig.3J).

- **Immune Fluorescent:**

A negative staining (Fig.3K), the Fluorescent green apple color was seen on organs of SPF infected chicks (with positive real-time PCR sample and stained with fluorescence) showing CAV viral particles inside the nuclei of hematopoeitic cells of stained bone marrow (Fig.3L).

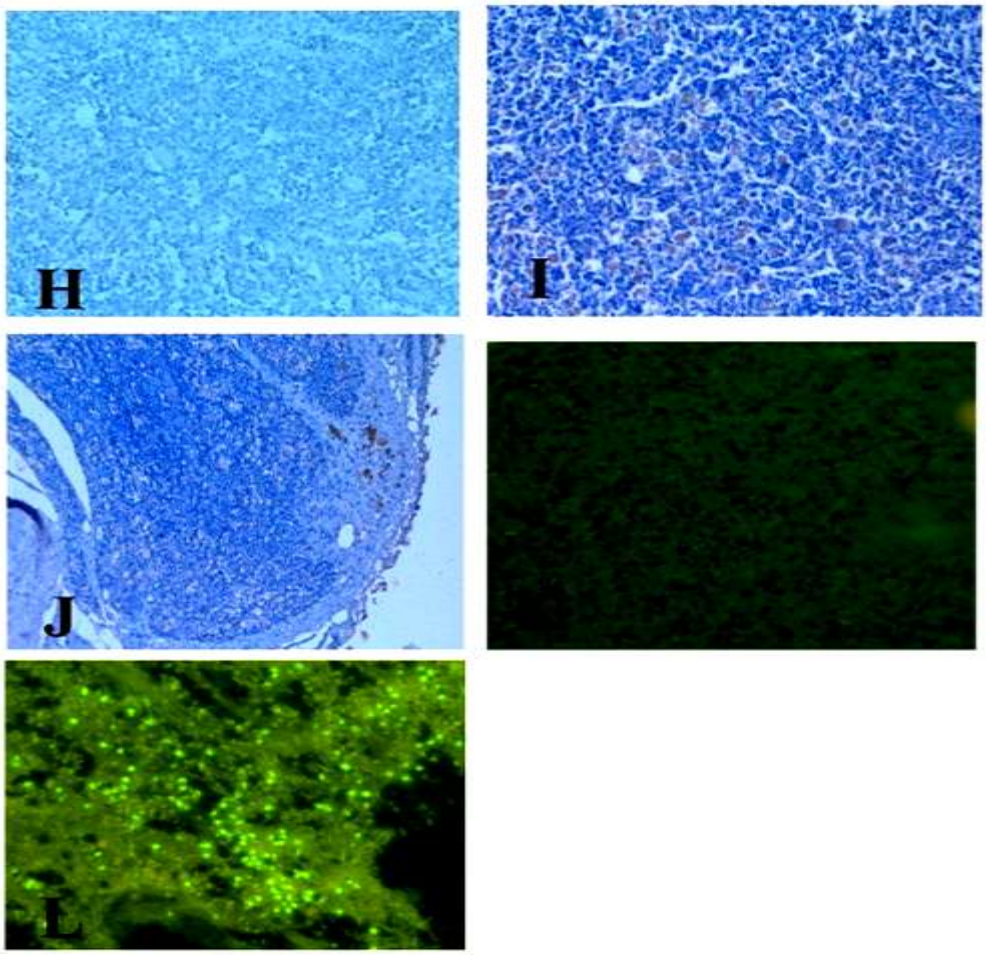

Fig. 3: H: Thymus, control negative staining (X 200). **I:** Positive staining of CAV antigen in the nuclei of lymphocytes (X400). **J**: Positive staining of CAV antigen in the nuclei of thymocytes and connective tissue (X 400). **K:** A negative staining (x 100). **L:** Fluorescent green apple color of CAV viral particles inside the nuclei of hematopoeitic cells (X 400).

DISCUSSION

Chicken Anemia Virus is immunosuppressive viral diseases has become a major cause of mortality and economic losses in poultry industry mainly because of increased susceptibility of second bacterial infections and low response to vaccination. Nowadays chicken infectious anemia is a great threat to poultry industry due to the large number of deaths and immunosuppression it causes. CAV was reported to be spreading among chicken farms in Egypt since the early 1980s when several outbreaks occurred in many breeds (Hussein *et. al.*, 2002). It was confirmed the presence of CAV in both meat and egg type chicken flocks by detection of both CAV antibodies and genome (Hussein *et. al.*, 2002 and Mohamed 2010).

In this study 30 farms from different governorates (Alexandria, Gharbia, Giza and Qalubia) of age 40 to 100-days-old were examined for presence of CAV with real-time PCR, all examined farms were clinically characterized by anorexia, weakness, unthriftiness, weight loss, severe anemia, (paleness of comb, wattle, eyelids and legs) and sudden death, these signs were mentioned before in Japan, China, Australia, New Zealand, India, Slovenia, Brazil, Nigeria and South Africa Kuscu and Gurel, 2008. The clinical signs of CAV infection, especially in chicks above 3 weeks were not seen, but the lesions which suggestive to be CAV such as both lymphoid depletion and atrophy of thymus were recorded. These were compatible with that previously

mentioned by Ledesma *et al.,* (2001) who recorded that, the chicks infected with virulence strain or high doses of virus after the maternal antibodies were decline usually suffering from lymphoid lesions without anemia. Moreover, the previous studies find the absence of clinical signs after about 3 weeks of age and the immunocompetent chickens were resistant to disease, but they can gain asymptomatic infections (Schat, 2003).

The histopathological examination of the positive farm showing generalized lymphoid aplasia in thymus, spleen and bone marrow; these findings were as similar as described by (Dhama *et al.,* 2002; Smyth *et al.,* 1993). The severe depletion of hematopoietic cells in bone marrow replaced by adipocytes and this is due to the destruction of hemocytoblasts in bone marrow leading to severe depletion of myeloid and erethroid cells producing anemia, theses were agreement with Adair, 2000. Spleen exhibited depletion of lymphocytes with focal coagulative necrosis, in addition to congested blood vessels due to the infection of mature lymphocytes with CAV. The recorded lymphoid aplasia could be bravely described by the strong immunosuppressive effect of CAV which induces marked destruction both to bone marrow stem cell and precursor T-lymphocytes in thymus (Goryo *et al.,* 1989; Smyth *et al.,* 1993). Focal starry sky depletion in cortex of thymus with cellular necrotic foci and severe hemorrhage in the medulla. Moreover, widening of few hussal's corpuscles forming cysts containing necrotic debris and granulocytes. Thymic lesions confirmed with the results of (Adair, 2000) as he proved that the destruction of T lymphocytes progenitor cells in thymus results in depletion of helper and cytotoxic cells inducing severe atrophy and depletion of lymphocytes.

PCR is the most advantage of adequate, faster and sensitive detection of more fastidious viral pathogens that might require several days and sequential passages in culturing for virus isolation and provide the faster diagnosis of viruses that's usually to be isolated in vitro cell culture (Cavanagh, 2002 and Dhama *et al.,* 2002). The 30 farms which were examined by real-time PCR, one farm is positive with C.T. reading =26. This sample is of age 100-days-old of ISA type from Qalubia governorate, our results was confirmed with Adair (2000) who's said that, the complete protection against CAV induced disease was grant by maternal antibodies which confirmed by many studies that are showed CAV infection and development of specific antibodies in chickens of eight and 12 weeks age as maternal antibodies completely disappear at 2-3 weeks of age so such infections are mostly due to horizontal transmission.

Sequence of the VP1 gene is commonly used to determine the relationship of different CAV isolates due to the fact that most of the amino acid substitutions between isolates lie in VP1 gene and more specifically in the N-terminal half of VP1 gene (Craig *et al.,* 2009 and Hailemariam *et al.,* 2008). PCR assay performed on the extracted DNA from infected tissues of SPF, giving positive reactions with DNA fragment at 418 bp (Hussein *et al.,* 2002). This was sequenced and submitted to the GenBank under accession number MH260568 for the partial sequenced to VP1 gene fragments.

The strategies to control immunosuppression are mostly depending on vaccination programs for breeders and their progeny. The most suitable way to minimize the prevalence of this disease is to use the best progress vaccines available, correctly apply them and ensure the good level of nutrition to get the highest possible response to these vaccines (Cloud *et al.,* 1992). Currently, characterize circulating CAVs in Egypt is so important to improve methods of virus control and to knew the relationship of circulating CAV with vaccine strains and other CAV strains. Now a large number of isolates have been fully or partially sequenced as we done on this work, partially sequenced VP1gene revealed that ours strain according to phylogenic tree was markedly varied from a commercial CAV vaccine (Nobilis® CAIV P4 and Cux-1N/Germany) which used as vaccine in our filed. Also according to phylogenic tree, they were present in two clusters our strain with Egypt I/ Giza2009, Egypt II/Fayoum I and Egypt III/ Fayoum II, India

and Japan in one and the other cluster include old CAV isolates in 1990s Egy-1, 2, 3 and 4 by Abo Elkhair *et al.,* 2014 and 5 vaccinal strains (Cux-1M/Germany, Cux-1N/Germany, CAV/Nobilis®P4, CAV/26P4/USA and CAV/Del-Rose/USA,).

The Immunoperoxidase staining techniques are most sensitive in detecting infected cells and the stained tissue sections can be re-examined several times and stored for a long time. Also the identification of cells is possible due to the counterstaining with haematoxylin. This is because the methods consist of two-step antibody reaction results of thymus revealed high level of expressed CAV antigen-specific staining (high positive signal) in the nuclei of lymphocytes, thymocytes and connective tissue this is agreement with (Hoop and Reece, 1991).

The indirect immunofluorescence staining techniques detected the infected cells in bone marrow. The Fluorescent green apple color was seen on organs of SPF infected chicks with positive real-time PCR sample and stained with fluorescence. These observations matched with the histological lesions in the same tissues and correspond with other reports of light microscopic changes due to infection with CAV (Yuasa *et al.,* 1979 and Goryo *et al.,* 1989). Our observations for immunohistochemically examination are the largest amounts of CAV antigen were detected in the thymus, spleen and bone marrow; these were matched with Smyth *et al.,* 1993.

CONCLUSION

The obtained results indicate that our circulating strain has great differences from vaccinal strains which used in the field, so we need periodic characterization of circulating CAVs in Egypt to improve methods of virus control and to usually determine the relationship of circulating CAV with vaccine strains and other CAV strains. The most obvious thing in this study, that's we need to make our vaccine from our strain which circulating in the field with periodic updating.

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