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Current Genomic Characterization of Circulating Chicken Infectious Anemia Virus in Backyard and Commercial Chicken Flocks in Ismailia and Sharkia Provinces, Egypt

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Abstract

This study aimed to evaluate the occurrence, molecular characterization, partial sequencing, and phylogenetic analysis of the chicken infectious anemia virus (CIAV) circulating in chicken flocks in Ismailia and Sharkia Provinces, Egypt. Tissue pool samples (liver, thymus, spleen, and bone marrow) were collected from commercial and backyard flocks with anemia, uneven growth, and vaccinal failure history. The occurrence of CIAV was 51% (51/100) using specific primers through the polymerase chain reaction test, which was higher in the backyard (26/50) 52% than that in commercial flocks (25/50) 50%. The highest rate of CIAV detection was 77% (13/17) and 75% (9/12) in Saso and Arbor Acer breeds respectively, followed by the Baladi 52% (26/50) and Cobb 27 % (4/15). The histopathological study reflected severe lymphocytic depletions in lymphoid organs with the presence of apoptotic cells and eosinophilic intranuclear inclusion bodies. Partial sequence analysis of six selected field circulating CIAVs showed changes in VP1 at position H 22 Q, VP2 at position A 153 V, T 180 S and VP3 at position R 118 C indicating low affinity of the obtained viruses to grow in the cell line. Some obtained viruses showed mutations in the epitopic site which may develop escape mutation virus from the currently used vaccines. Phylogenetically, the six selected fields CIAVs were classified into two distinct groups. The continuous surveillance activities and epidemiological mapping for CIAV among Egyptian governorates using updating primers are essential to facilitate control program strategies.

KEYWORDS
CIAV, Egypt, Molecular, Phylogenetic, Sequencing.

INTRODUCTION

Chicken infectious anemia virus (CIAV) infection is concerned with several field problems that cause severe immunosuppression in broilers and infected birds develop a chronic or persistent infection with horizontal and vertical transmission of the virus (Maclachlan and Dubovi, 2017). CIAV was first reported in Japan (Yuasa *et al*., 1979), while it was first recorded in Egypt in 1990 (El-Lethi, 1990). Recent researchers have investigated the molecular characterization and turnover sequences of CIAV strains in Egypt (AboElkhair *et al*., 2014; Hussein *et al*., 2016; Abdel-Mawgod *et al*., 2018; Abdelhalim *et al*., 2021). CIAV is a member of the genus Gyrovirus and belongs to Anelloviridae (Adams *et al*., 2016; Rosario *et al*., 2017), and has a single serotype with low genetic variance (Maclachlan and Dubovi, 2017). The CIAV genome contains three partially overlapping open reading frames, called VP1, VP2, and VP3, respectively, with 1350, 651 and 366 nucleotide sequences of different lengths. VP2 and VP3 are protective proteins that stimulate the production of neutralizing antibodies (Koch *et al*., 1995). The main structural protein of the virus is VP1, which has the highest nucleotide diversity and is often used for molecular identification and genetic research of CAIV (Ducatez *et al*., 2006; Craig *et al*., 2009). Detection of CIAV requires sensitive, specific, and rapid molecular techniques (Saini and Dandapat, 2009) since CAIV infection has a high incidence but disease symptoms are uncommon. Clinical signs of CIAV are mainly seen in broiler chicks that get infected vertically by breeders hens (Adair, 2000). The polymerase chain reaction (PCR) assay is a highly sensitive and rapid diagnostic technique for avian pathogens (Cavanagh, 2001). Gene sequencing is a useful tool for studying circulating CIAV variation, and such studies contribute to understanding the molecular epidemiology of the disease and its relationship to other reported viruses and vaccines (Saini and Dandapat, 2009). Recent research has reported that all Egyptian CIAV isolates belong to phylogenetic group different from ancient Egyptian isolates and imported commercial CIAV vaccines (AboElkhair *et al*., 2014; Hussein *et al*., 2016). The goal of the present study was to study the genetic characteristics of CIAV prevalence and its relationship to other Egyptian isolates and vaccine strains.

MATERIALS AND METHODS

Ethical approval

The protocol and materials used in the present scientific research were approved by the Scientific Research Ethics Commit-

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tee of the Faculty of Veterinary Medicine, Ismailia Suez University, Egypt, with approval number (2022017).

Sampling and clinical examination

Four hundred and seventy-five broiler chickens located in Ismailia and Sharkia governorates, representing 100 flocks (50 commercial and 50 backyards) with a history of anemia, uneven growth, and vaccinal failure were clinically examined and post-mortem lesions were recorded. Commercial broiler flocks varied according to age (10-90-day), breed and locality. Whereas, all the backyard flocks were from the native Baladi breeds, aged 25-60-days from Ismailia Governorate. One hundred tissue pools (liver, thymus, and spleen) from 3:10 chickens/ each flock) were collected aseptically and stored at -80°C until used and/or fixed in neutral 10% formalin solution until histopathological examination. Preparation of tissue homogenates from frozen samples were performed (Zhou *et al*., 1997).

DNA extraction and detection of CIAV by RT- PCR

Viral DNA was extracted using Gene JET Viral DNA and RNA Purification Kit (Thermo Scientific, Germany, Cat. No. 0821) following the manufacturer's instructions. Two primer pairs were used in a trial assay to select the best primers for CIAV detection. The first oligonucleotide primer-1 (Metabion - Germany) 5'-AAT GAA CGC TCT CCA AGA AG-3' and 5'- AGC GGA TAG TCA TAG TAG AT-3' were used to amplify 582 bp (485 -1066 bp) (Tham and Stanislawek, 1992), while the 2nd oligonucleotide primer-2 (Bio Basic - Canada) 5'- CTA AGA TCT GCA ACT GCG GA-3' and 5'- CCT TGG AAG CGG ATA GTC AT-3' was used for amplification of 420 bp (654 -1073 bp) (Hussein *et al*., 2002). PCR assay was performed using Emerald Amp GT PCR master mix (Takara) with Code No. RR310A kit in a total volume of 25µl containing 12.5µl master mix, 4.5µl of PCR grade water, 2µl for both primers, and 6µl templates. The RT-PCR thermocycler temperature and time-related to the two primers used were an initial cycle of 5 min denaturation at 95°C then 35 cycles for 30 sec at 94˚C, 56˚C for 45 sec and 72˚C for 45 sec and finally one10-min extension cycle at 72˚C. PCR products were inoculated in 1.5% ethidium bromide-stained agarose gel, observed under transilluminator, the PCR products of expected size visualized on DNA Ladder (Qiagen, Gel pilot 100 bp ladder) Cat. No. (239035).

Partial sequencing of CIAV nucleotide and phylogenetic analysis

The PCR fragments were purified by using QIA quick RT-PCR Product extraction kit (Qiagen Inc., Valencia, CA). Six strong and clear positive CIAV bands were selected for partial sequence analysis (3 from commercial broiler flocks and 3 from backyard flocks) (Table 1). The PCR products were submitted to the U.S. Elim Biopharmaceutical Laboratory for sequencing, using PerkinElmer/Applied Biosystems, (Foster City, CA) Cat. No. 4336817, add the prepared Big dye Terminator V3.1 cycle to the purified RT-PCR product. Forward and reverse sequencing was performed using an Applied Biosystems 3130 automatic DNA sequencer (ABI, 3130, USA). Sequence alignment and identity were performed using BioEdit version 7.0.5 (Hall, 1999), MEGA7 software for phylogenetic trees using Neighbor-Joining method and 500-replicate bootstrapping tests (Kumar *et al*., 2016). CIAV reference strains, global strains, Egyptian strains, and vaccine strains used for comparison were from National Center for Biotechnology Information (NCBI) (http://www.ncbi).

Histopathological examination

Histopathological analysis of positive CIAV specimens (liver, thymus, spleen) was performed using 3 µm paraffin sections, stained with hematoxylin and eosin, and then examined microscopically for histopathological changes (Bancroft and Cook, 1994).

RESULTS

Clinical observations and post-mortem lesions

Commonly observed clinical signs were pallor (eye-led, comb, wattles, beak, and shanks), ruffled feathers, weakness, and uneven growth. Wingtips showed the onset of dermatitis in some cases. Postmortem lesions were seen mainly in lymphoid organs (the size of the thymus, bursa and spleen varies from enlarged to atrophied). The liver and bone marrow were clearly pale, and the muscles, especially the breast muscles, had hemorrhagic lesions (Fig. 1 (a, b, c, d).

Results of RT-PCR for detection of CIAV in examined flocks

Trial for choosing suitable primers

The test was performed on 15 samples of commercial chicken flocks, among which Primer-1 could not detect any CIAV positive samples 0% (0/15), and Primer-2 detected 4 positive CIAV (4/15) of 26.7% (Figure 2).

Detection of CIAV in examined flocks using RT-PCR

Primer-2 used to amplify of DNA extracted from 100 pooled samples showed CIAV positive DNA bands with a corrected size at 420 bp by RT-PCR [50% (25/50) in commercial flocks and 52% (26/50) in backyard flocks]. In commercial broiler flocks, a higher occurrence of CIAV was observed in Sharkia (95%) (19/20) compared to Ismailia (20%) (6/30). While all backyard chicken flocks were from Ismailia. The highest prevalence of CIAV infection in commercial broilers was detected in flocks older than 40 days (9/10) 90%, however, no CIAV infection was detected in flocks aged 0-20 (0/7). The Saso and Arbor Acer breeds had the highest CIAV detection rates, 77% (13/17) and 75% (9/12), respectively, followed by Baladi 52% (26/50) and Cobb 27% (4/15).

Sequencing analysis of primer-1 in comparison to selected six CIAVs

Partial sequencing analysis of the six selected examined CIAVs was compared with the complement primer-1 reverse transcriptase (at position 1047:1066 bp, sequencing ATCTACTATGACTATCCGCT). Three nucleotide changes were detected at positions A-1047/G, T-1050/A, T-1053/G bp in commercial broiler chicken flocks and one at position T-1053/C in backyard (Fig. 3).

Nucleotides and amino acids sequence analysis of CIAV DNA fragment (420 bp)

The nucleotide sequences (654 to 1073 bp) of the six selected field CIAVs were compared with the reference CIAV strain (NC_001427.1). The obtained nucleotide sequences had 20 substitutions at positions T- 677/G, C-710/G, C-727/G, C-759/A, G-778/A, A-832/G, C-837/T, A- 850/G, A-871/C, C-883/A, T-892/G, G-909/C, A-914/G, C-918/G, G-1035/A, G-1038/A,

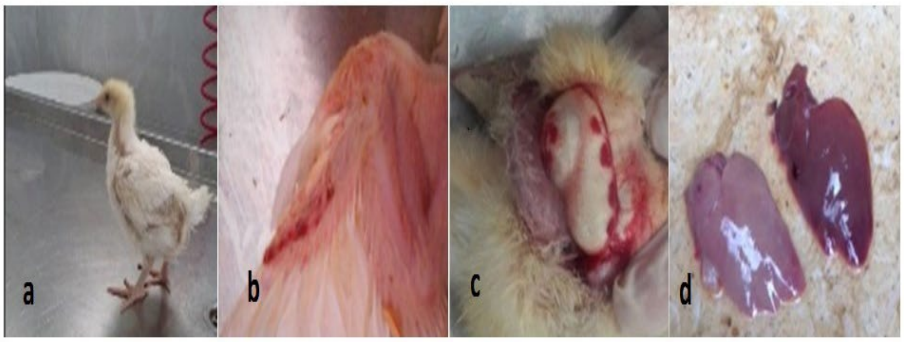

Figure 1. (a, b, c, d): Commercial broiler chicken, showed pale shanks, ruffled feathers, and uneven growth (a): the beginning of wing tip dermatitis (b): dark red rudiment thymus (c): Pale liver was detected in comparison with normal liver lobe (d).

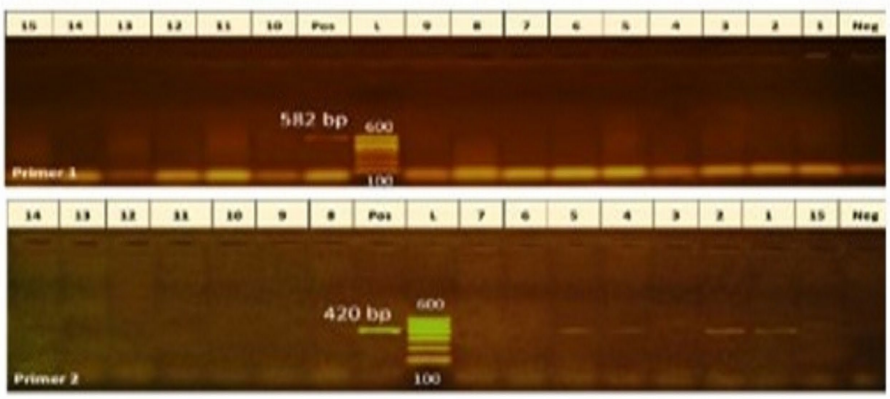

Figure 2. Agarose gel electrophoresis 1.5%, amplified DNA by RT-PCR. Lane L: DNA Molecular Marker, Lane Pos: Positive Control; Lane Neg: Negative control tube.

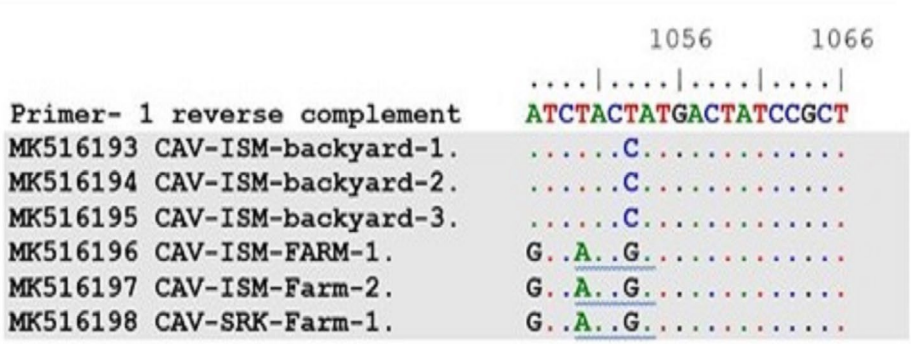

Figure 3. The partial sequence alignment of 6 examined viruses in compared with primer- 1 reverse complement at position 1047:1066 bp.

C-1044/G, A-1047/G, T-1050/A and T-1053/C:G in different CIAVs. The sequenced region was in the overlapping region and gave partial sequences of the three main CIAV proteins VP3, VP2 and VP1. Some nucleotide changes affected different amino acid positions in the six field CIAVs (Table 2). The nucleotide identity of the 3 Ismailia backyard CIAVs was 98.5:99.2 % with each other, while it was 97.3:99.5% with vaccinal strains and 97.3:98.3 % with the commercial CIAVs. The identity of CIAVs in commercial flock with each other was 98:99.2%, while it was 96.9:98.5 % with vaccinal strains.

Phylogenetic Analysis

Phylogenetic trees were constructed from nucleotides and amino acids to assess the genetic relationship among CIAVs. The phylogenetic trees of 420 nucleotides were affected by silent nucleotide mutations detected in the VP1 region at positions (1047, 1050 and 1053) and the six CIAVs were classified into two distinct groups. Group I included three Ismailia backyard CIAVs which found to be closely related to the vaccinal strains, China, USA, Netherland, Vietnam, India, and Taiwan. While group II included commercial broiler CIAVs (2 Ismailia and 1 Sharkia) with Taiwan, Brazil, Chile, Australia, Japan, Malaysia, China, and Argentina (Fig. 4).

While the phylogenetic trees of VP1 and VP2 amino acids were similar to each other. It was classified into two groups. Group I included Sharkia commercial flock CIAVs, vaccinal strains, China, USA, India, Chile, Netherland, Iran, Australia, Brazil, Malaysia, Vietnam, Argentina, and Taiwan. Group II included Ismailia CIAVs (backyard and commercial CIAVs) with USA, China, and Taiwan (Fig. 5). The phylogenetic analysis of VP3 aa was with no significant or clear classification.

Histopathological results

The histopathological findings of CIAV-infected flocks were severe lymphocytic depletion in lymphoid organs (thymus and spleen) followed by reticulocyte hypertrophy and proliferation of connective, and adipose tissue. The thymus showed severe lymphatic depletion, particularly in the thymic cortex, which result-

ed in a complete loss of thymus architecture with an indistinct boundary between cortex and medulla. The spleen showed loss of germinal central lymphocytes and disappearance of lymphoid follicles structure with severe lymphocyte depletion, hemorrhage, and inflammatory cells aggregation around the affected area. The liver showed severe hepatocellular necrosis, central vein congestion, sinusoidal dilatation, scattered hepatic and periportal hemorrhages, and focal lymphoid clustering with the liquefied necrotic areas. All examined organ cells contained large eosinophilic intranuclear inclusion bodies (Fig. 6).

DISCUSSION

Chicken infectious anemia virus is an emerging immunosup-

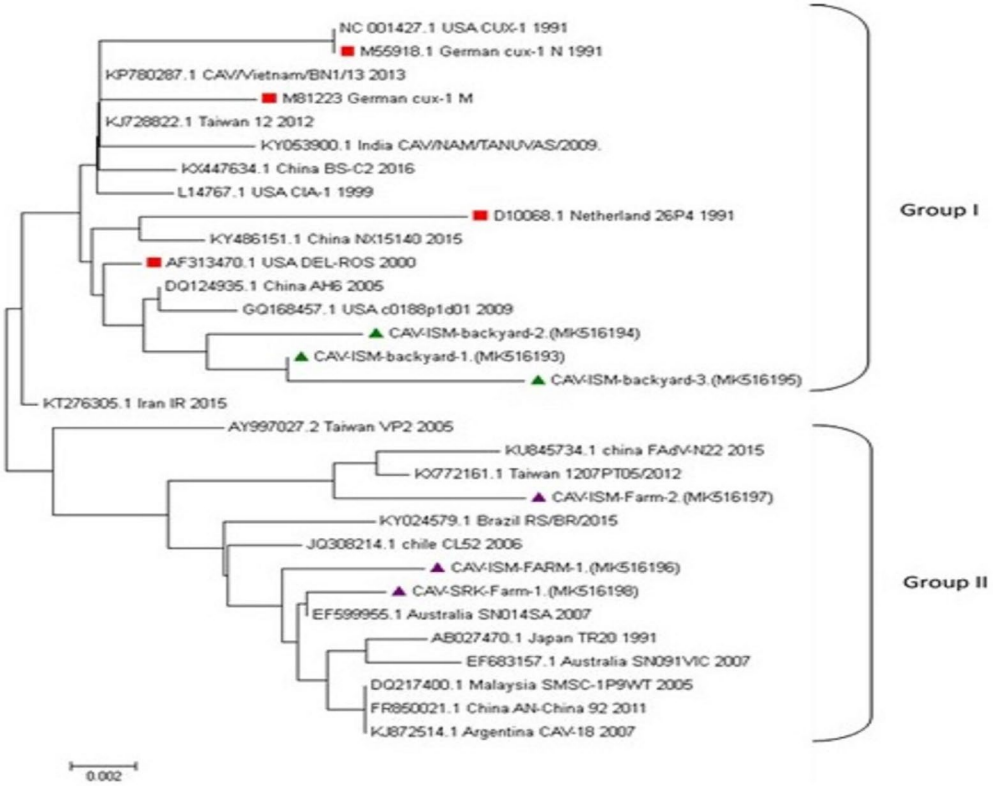

Figure 4. Phylogenetic tree of partial CIAV nucleotides sequence (420 bp) of 6 field viruses in compared with the reference, worldwide and vaccinal strains.
▲ The 6 sequenced isolates ■ vaccinal strains

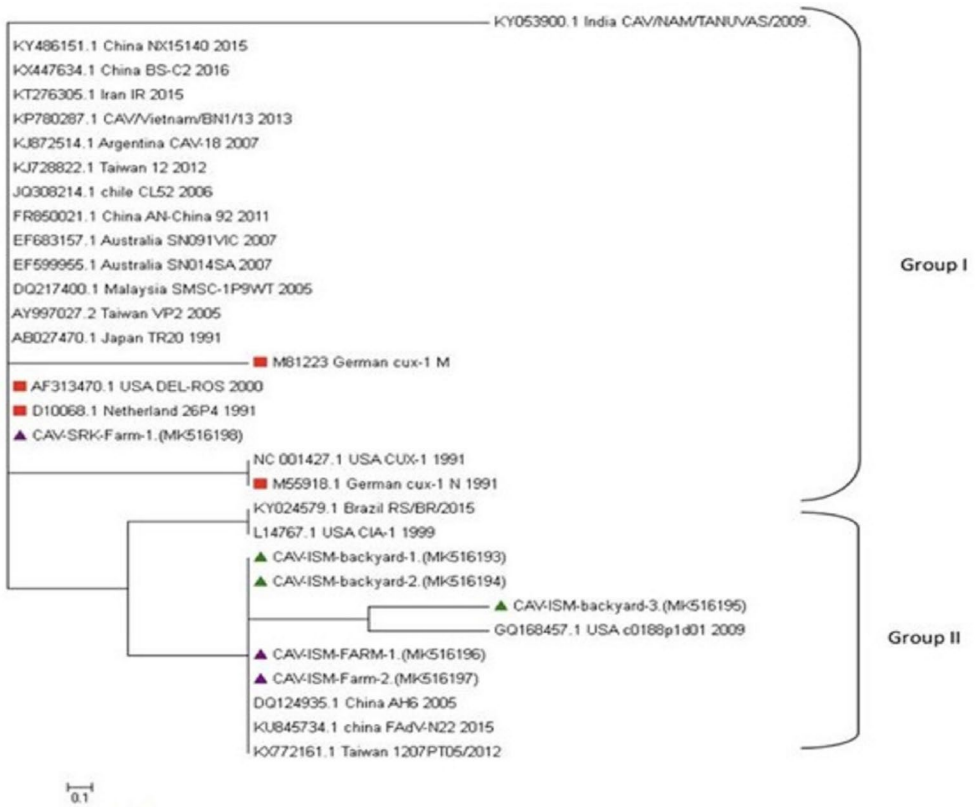

Figure 5. Phylogenetic tree of partial CIAV VP1 aa sequences (73 aa) of 6 field viruses in comparison with the reference, worldwide and vaccinal strains
▲ The 6 sequenced viruses ■ vaccinal strains

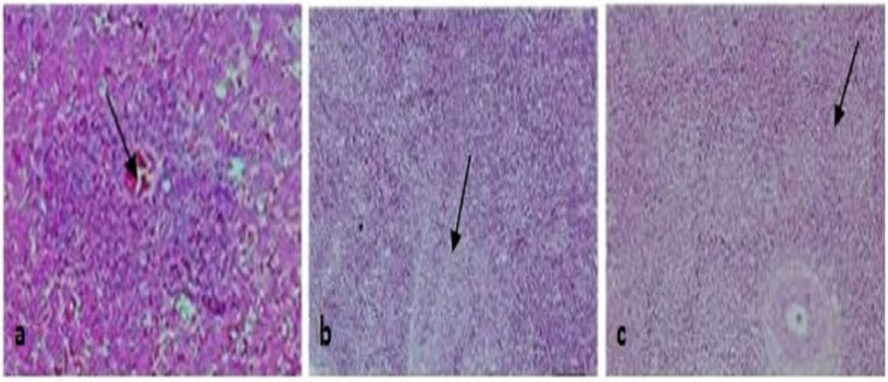

Figure 6. liver showed hepatocytes necrosis, congestion of central vein, sinusoids dilatation, focal lymphocytic aggregation (a): thymus showed severe lymphocytic depletion, especially in the cortex lesion with losses line of demarcation between cortex and medulla (b): spleen showed hemorrhages and eosinophilic intranuclear inclusion bodies by oil immersion lens (c).

pressive disease that poses a serious threat to the poultry industry on a global scale. It targets lymphoid and erythroblast-like progenitor cells, resulting in immunosuppression, reduced efficiency of routine vaccination, frequent outbreaks, and economic losses in commercial poultry farms (Oluwayelu *et al*., 2008; Bhatt *et al*., 2011; Snoeck *et al*., 2012; Nayabian and Mardani, 2013; Giotis *et al*., 2015).

The clinically examined chickens showed anemic signs as pallor (eye-led, comb, wattle, peak and shank) as well as ruffled feathers, depression, and uneven growth. Similar variable symptoms were recorded in CIAV infection in chickens (Adair, 2000; Dhama *et al*., 2008; Hussein *et al*., 2016; Swayne DE, 2019; Abdelhalim *et al*., 2021).

Thymic atrophy has been a consistent postmortem lesion among chicken flocks, which may be related to the significant damage caused by CIAV replication in the thymic cortex (Adair, 2000; Ledesma *et al*., 2001; Sharma *et al*., 2014). Spleens of varying sizes from enlarged to small pale colors were observed among the differently affected flocks. These differences may be attributed to the infection stage, infection dose, the virus virulence, and the age of the chicken (Chettle *et al*., 1989; Rimondi *et al*., 2014; Hussein *et al*., 2016). All examined chickens showed obvious pale liver and bone marrow which may be due to the replacement of necrotic tissue with fat cells (Mohamed, 2010) that was proven further by liver histopathological examination which confirmed the presence of severe hepatocyte necrosis and coagulative necrosis; the necrotic areas may be replaced by adipose and connective tissues causing liver paleness. The thymus and spleen are the preferred tissues for CIAV DNA detection (Al-Ebshshy, 2013), Likewise infected liver tissues have high CIAV viral titer and are considered the best for virus isolation (van Santen *et al*., 2004).

A preliminary examination on 15 commercial broiler flocks was conducted to select a suitable primer for CIAV detection. Primer-1 couldn't be able to detect any CIAVs, while primer-2 was able to detect 26.7% positive CIAV in the same 15 commercial broiler flocks. These results disagree with that detected by Hegazy, *et al* (2010) who used primer-1 and detected CIAVs in 44% (4/9) of different Egyptian chicken flocks. Interestingly, when we compared the sequences of reverse primer-1 with the investigated six CIAVs, three silent mutations were discovered at positions A-1047/G, T-1050/A, and T-1053/G bp of the commercial chicken flock viruses that may it causes false-negative results. These mutations appeared near the three endpoints of the reference (primer-1) and may explain the false-negative results obtained (0/15) which were subsequently confirmed when we used another reference primer set (primer-2) for the same samples and it gave positive results. To the best of the authors' knowledge, this is the first record highlighting mutations in the reference primer-1 indicating the need to update and use different combinations of reference primers to avoid false-negative results that might occur.

CIAV detection was recorded as 50% (25/50) in commercial broiler chicken flocks using RT-PCR, while previous studies (Mohamed, 2010; Hussein *et al*., 2016; Abdel-Mawgod *et al*., 2018; Abdelhalim *et al*., 2021) have CIAV in Egyptian commercial chicken flocks by 26.6% (44/165), 65% (26/40), 10.4% (12/115), and 30% (26/86) respectively. At the level of geographical distribution, the prevalence of CIAV in Sharkia was 95% (19/20) and in Ismailia was 20% (6/30), which may be the result of intensive rearing there with the lowest biosecurity distance between farms. Furthermore, the stability and resistance of CIAV to thermal or physico-chemical treatments have complicated its elimination from farms (Miller *et al*., 2003). Detection of CIAV in examined backyard flocks aged 25-60-days using RT-PCR was 52% (26/50). Some internationally published reports demonstrated the high prevalence of CIAV in backyard chickens by RT-PCR, but in the mid-1990s, both industrial and SPF chicken flocks (breeders, broilers, layers) were exposed to vertical infection by CIAV, and which could play a role in the transmitting CIAV to backyard chickens (von Bulow, 1997; Barrios *et al*., 2009).

Gene sequencing technology is a useful tool for studying the variation in CIAVs circulating in the field and understanding the molecular epidemiology of the disease in relation to other reported viruses and vaccinal strains (Saini and Dandapat, 2009). The sequenced region was in an overlapped portion and gave partial sequence for the three main CIAV proteins VP3, VP2, and VP1 and could detect a hypervariable region at the carboxyl terminus of VP3 which affects apoptosis function and replication of the virus in cell culture.

Nucleotide's sequence detected 20 changes in different examined CIAVs in comparison with reference strains. The nucleotide change at position C-918/G affects VP1 amino acid at position H-22/Q and VP2 amino acid of the overlapping region at position T- 180/S in Ismailia viruses, that changes made virus poorly replicated in cell culture (Yamaguchi *et al*., 2001; Eltahir *et al*., 2011). Also, there was nucleotide changes at position C-837/T which affect amino acids at VP2 position A-153/V and amino acids of overlapping VP3 region at position R-118/C, which decreased the virus ability to spread in cell culture (Renshaw *et al*., 1996). The change at VP3 position R-118/C considers mutation in the 2nd hypervariable region, affects the NLS2 signal and increases the distribution of VP3 in the cytoplasm than in the nucleus decreasing the nuclear apoptotic bodies formation. There was individual mutation at VP2 amino acid as F-100/V in ISM-backyard-2, that amino acids located at the DSP motif (94–103 aa) which required for virus infection, assembly, cytopathology, virulence, replication, induces apoptosis and maybe play a role in intracellular signaling during viral replication (Todd *et al*., 1990; Peters *et al*., 2002; Cheng *et al*., 2012; Kaffashi *et al*., 2015). Also, a mutation in ISM-backyard-3 at position L-111/V, and in SRK-Farm-1 at position P-127/H; these changes located in VP2 epitope region (111-136 aa) which may develop later escape mutant virus from the currently used vaccines (Koch *et al*., 1995; Noteborn *et al*., 1998; Wang *et al*., 2017).

Phylogenetic nucleotide trees have classified the viruses into two groups. Group-I included three Ismailia backyard viruses, vaccinal strains, China, USA, Netherland, Vietnam, India, and Taiwan.

Group- II included the three commercial broiler farm viruses with Taiwan, Brazil, Chile, Australia, Japan, Malaysia, China, and Argentina, those results matched with previous studies which cluster their isolates (Egyptian commercial broiler CIAV) to phylogenetic groups differed from the vaccinal strain group (AboElkhair *et al*., 2014; Hussein *et al*., 2016). The phylogenetic trees of VP1 aa, and VP2 aa classified the viruses into two groups, Ismailia backyard and commercial farm viruses belonged to group II with China, USA, and Taiwan. While Sharkia commercial farm virus belonged to group I with the vaccinal strains, China, USA, India, Chile, Netherland, Iran, Australia, Brazil, Malaysia, Vietnam, Argentina, and Taiwan. The CIAV strains may differ in their antigenicity due to differences in DNA sequence and protein folding patterns (Koch *et al*., 1995; Scott *et al*., 1999). Therefore, the antigenicity and benefits of using imported live attenuated CIAV vaccines for Egyptian breeder broiler flocks need further studies.

Histopathological examination of the thymus and spleen showed lymphocytic depletion followed by reticular cell hyperplasia, connective, and adipose tissue proliferation. These findings agreed with previous reports (Hegazy *et al*., 2010; Hussein *et al*., 2016). The severe immunosuppressive effect of CIAV on thymic precursor T-lymphocytes leads to vaccination failure and enhanced other pathogens infection (Hu *et al*., 1993; Adair, 2000; Schat, 2003). The liver showed hepatocytes necrosis, congestion of the central vein, sinusoid dilatation and focal lymphocytic aggregation, these findings agreed with previous reports (Chettle *et al*., 1989; Hussein *et al*., 2016). There were large intranuclear eosinophilic inclusion bodies detected in all examined organs (liver hepatocytes, thymus, and spleen lymphocytes), these findings match with previous studies (Smyth *et al*., 1993; Toro *et al*., 1997).

CONCLUSION

CIAV is prevalent in Ismailia and Sharkia governorates but some of the reference primers currently in use could not detected it, so updating the primers set is necessary. Some field viruses in this study have mutations in the epitopic site which may evolve into a fugitive mutated virus from currently used vaccines, thus, further studies are required to recommend an appropriate vaccine program.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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