Chicken Anaemia Virus in Egyptian Broiler; Clinical Signs and Molecular Characterization

Shahira A. Abdelwahab¹ and Dalia M. H.²
shahira_mohamed@vet.suez.edu.eg - dalia_mansour@vet.suez.edu.eg
¹ Virology Department, Faculty of Veterinary Medicine, Suez Canal University
² Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Suez Canal University

Abstract
A total of 18 flocks, 45 day old broiler from Ismailia, Egypt were tested for chicken anemia virus (CAV) using serological evidence and molecular characterization. Tested birds had history of diarrhea, blue wing, depression, and deaths. Necropsy findings were pale liver, severe atrophy of bursa Fabricius and thymus, pale discoloration of the bone marrow and subcutaneous hemorrhages. The viral DNA of the VP1 gene was detected and sequenced. Nucleic acid alignment showed 100% similarities with many CAV viruses. Antibodies against CAV were detected in 73.9% of the tested samples using commercial ELISA kit. Hemoglobin (Hb) revealed low percentage (4.3% to 8.02%), and packed cell volume (PCV) were measured and showed anemic level (22.5 to 34.2). The data strengthen the concept that CAV may have a negative economic impact on the chicken industry in Ismailia.

Key words: antibodies, chickens, chicken anemia virus, immunosuppression.

Introduction
Chicken anemia virus (CAV) is a worldwide disease of most poultry species causing great economic losses (Chowdhury et al., 2002; Schat, 2009). It transmitted either vertically or horizontally (McNulty, 1991; Sommer and Cardona, 2003). It has been detected in most commercial poultry farms, with a high seroprevalence (Owoade et al., 2004). CAV has Clinical and subclinical forms (Davidson et al., 2004; Jorgensen et al., 1995). Clinical form characterized by severe aplastic anemia, generalized lymphoid atrophy of hematopoietic and lymphoid organs, intramuscular and subcutaneous hemorrhages, retarded growth and high mortality (Sommer and Cardona, 2003). It was enhances susceptibility to other infectious disease agents through reducing response to vaccinations (Adair, 2000; Otaki et al., 1988; Todd, 2000). In older birds the infection is mainly subclinical.

Chicken anemia virus (CAV) is the only member of the genus Gyrovirus
of the family Circoviridae (Niu et al., 2019). CAV is non-enveloped, icosahedral symmetry and measures 22-26 nm in diameter (McNulty et al., 1990; Pope, 1991). The CAV genome is negative-sense, circular, single-stranded DNA (Gelderblom et al., 1989; Noteborn et al., 1991; Todd et al., 1990). The genome encodes for 3 viral proteins (VP1, VP2 and VP3); VP1 is the capsid protein; VP2 is needed for proper folding of VP1 and VP3 (apoptin) induces apoptosis in infected cells (Noteborn, 2004). Diagnosis of CAV infections can be made by detecting infectious virus, virus antigen, virus DNA, or virus-specific antibodies (Chettle et al., 1991; Chettle et al., 1989). Aim of the study was to evaluate seroprevalence of CAV in broilers, determining some pathological indices and hematological parameters, then confirm the serological status by detection and analysis of the viral DNA using PCR and sequencing.

Materials and Methods

1-Chicken
A total of 180 samples were collected from broilers aged 45 days old. Samples were collected from apparently healthy randomly selected from 18 broiler flock in Ismailia, Egypt during winter and summer of 2010. Samples collected from each chicken were whole blood, serum, thymus, spleen, and bursa of fabricius.

2-Whole blood and sera
Whole blood samples were used for determination of packed cell volume (PCV) values and hemoglobin %. Sera were separated and used to detect HI antibody titer against NDV and CAV. Total of 5 randomly selected whole blood samples, one from each of 5 flocks, were used for DNA extraction and genetic analysis. The packed cell volume (PCV) was determined on a blood sample collected from each bird before slaughtering using the microhematocrit capillary tube method (Goodwin et al., 1991).

3-ELISA for CAV antibodies
A commercial ELISA kit was used to detect specific antibodies against CAV (IDEXX Flock Chek CIAV), according to the manufacturer’s instructions; A serum dilution of 1:10 was used. Optical density values were read at 650 nm using a Tecam Sunrise ELISA reader.

4-Nucleic acid extraction
QIAamp DNA Mini Kit (QIAGEN, USA) was used according to the manufacturer’s instructions to extract DNA from 5 randomly selected samples from different farms.

5-Partial Amplification of CAV-VP1
A Nested PCR was used for amplification of VP1 fragment of chicken anaemia virus as described previously by (Cardona et al., 2000). The forward and reverse primers were used to amplify 386 bp fragment. The sequence of both primers were shown in table (1).
The nested PCR was done as described by (Cardona et al., 2000). In the first PCR reaction primers O3F and O3R were used, and was programmed as follow; 5 min at 94 °C followed by 35 cycles consisting of 1 min at 94 °C, 2 min at 45 °C and 1 min at 72 °C, followed by 10 min at 72 °C. The nested PCR reaction (second step) was done using primers N3 and N4 using 1 ul from the first reaction. the procedures as described before; an initial denaturation for 5 min at 94 °C, followed by 30 cycles; 2 cycles of 30 sec at 94 °C, 30 sec at 60 °C and 30 sec at 72 °C, followed by 2 cycles at a time with the annealing temperature decreasing by 1 °C down to 56 °C (totaling 10 cycles), and ending with 20 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 30 sec at 72 °C, followed by 10 min at 72 °C.

Sequencing and sequence analysis

Sequencing of the purified PCR products was done at the Molecular Biology Resources Service (UTK) using an ABI prism dye terminator cycle sequencing reaction kit and ABI 373 DNA. The purification was done using ExoSAP-IT PCR Product Cleanup (Affymetrix, Ohio, USA). The sequencing was done using nested second step primer N3 of the first PCR product size 386. The sequence results were analyzed using Mega 6 and the BLAST program in the NCIB website. The trimmed sequence clean part yielded 307 bp for sequence analysis.

Table (1): Nested-PCR primers showing target fragment length and location for each primer pairs on VP1 gene of CIAV

<table>
<thead>
<tr>
<th>primer name</th>
<th>primer position in the genome</th>
<th>Sequence</th>
<th>PCR step</th>
<th>Expected size</th>
</tr>
</thead>
<tbody>
<tr>
<td>O3F</td>
<td>452-471</td>
<td>5’ CAAGTAATTTCAATGAACG 3’</td>
<td>First PCR step</td>
<td>386bp</td>
</tr>
<tr>
<td>O3R</td>
<td>838-819</td>
<td>5’ TTGCCATCCTTACAGTCTTAT 3’</td>
<td>First PCR step</td>
<td>386bp</td>
</tr>
<tr>
<td>N3</td>
<td>489-505</td>
<td>5’ CCACCGGGACCACTCAAC 3’</td>
<td>Second PCR step</td>
<td>209bp</td>
</tr>
<tr>
<td>N4</td>
<td>698-677</td>
<td>5’GGTCCTCAAGTCCGCGACATTC 3’</td>
<td>Second PCR step</td>
<td>209bp</td>
</tr>
</tbody>
</table>
RESULTS

1- Necropsy and hematological finding

Chicken showed depression, sunken eye, conjunctivitis, ruffled feather and pale comb and wattles. Some birds had diarrhea and/or blue discoloration of wings (blue wing disease). Necropsy findings were; atrophied lymphoid organs (bursa, thymus an spleen), and hemorrhages on breast, wings, and thigh muscles. Heart showed petechial haemorrhages with hydro-pericardium. Other findings were noticed as pneumonia, perihepatitis, and air saculitis. Kidneys in some birds were found swollen and pale.

PCV values and hemoglobin; mean values of PCV from different flocks were ranged from 22.5 to 34.2 while mean hemoglobin% values were ranged from 4.3% to 8.02% shown in table (2).

2- ELISA antibody titers against CAV:

Antibody titers against CAV of more than 700 were found in 133 samples which considered positive titers according to the manufacturer’s guidelines. These samples represent a positive percentage of 73.9% of 180 tested samples. While 47 samples had titer less than 1-700 which considered negative according to the manufacturer’s guidelines representing a negative percentage of 26.1% of these samples. The details of positive and negative samples from each flock are shown in figure (1) and table (3).

3- Genetic detection and characterization

All 5 samples used for molecular characterization using nested PCR were positive and produced the target segments (386 bp) of the nucleic acid targeting VP1. Sequence analysis of the obtained PCR products revealed that the full-genome sequence of nucleotides was of 100% sequence identities similar to CAV strains published on the GENBANK; ACCESSION KP899520, VERSION KP899520.

Table (2): PCV, hemoglobin values and percentages of positive in chicken farms in ismailia

<table>
<thead>
<tr>
<th>Flocks</th>
<th>No. of samples (No. of flocks)</th>
<th>% of positive</th>
<th>PCV %</th>
<th>Hemoglobin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>40 (4)</td>
<td>100%</td>
<td>22.5</td>
<td>4.5</td>
</tr>
<tr>
<td>B</td>
<td>40 (4)</td>
<td>90%</td>
<td>25.0</td>
<td>4.3</td>
</tr>
<tr>
<td>C</td>
<td>20 (2)</td>
<td>80%</td>
<td>31.6</td>
<td>5.6</td>
</tr>
<tr>
<td>D</td>
<td>10 (1)</td>
<td>70%</td>
<td>32.0</td>
<td>7.3</td>
</tr>
<tr>
<td>E</td>
<td>60 (6)</td>
<td>50%</td>
<td>34.0</td>
<td>7.2</td>
</tr>
<tr>
<td>F</td>
<td>10 (1)</td>
<td>40%</td>
<td>34.2</td>
<td>8.02</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td>42.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Table (3): ELISA positive values and percentages of Chicken infectious anemia virus in chicken farms in Ismailia:

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of samples (No. of flocks)</th>
<th>ELISA positive</th>
<th>ELISA negative</th>
<th>Titer up to</th>
<th>% of positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>40 (4)</td>
<td>40</td>
<td>0</td>
<td>12301</td>
<td>100%</td>
</tr>
<tr>
<td>B</td>
<td>40 (4)</td>
<td>36</td>
<td>4</td>
<td>7062</td>
<td>90%</td>
</tr>
<tr>
<td>C</td>
<td>20 (2)</td>
<td>16</td>
<td>4</td>
<td>3674</td>
<td>80%</td>
</tr>
<tr>
<td>D</td>
<td>10 (1)</td>
<td>7</td>
<td>3</td>
<td>2228</td>
<td>70%</td>
</tr>
<tr>
<td>E</td>
<td>60 (6)</td>
<td>30</td>
<td>30</td>
<td>5474</td>
<td>50%</td>
</tr>
<tr>
<td>F</td>
<td>10 (1)</td>
<td>4</td>
<td>6</td>
<td>7048</td>
<td>40%</td>
</tr>
<tr>
<td>Normal</td>
<td>180 (18)</td>
<td>133 (73.8%)</td>
<td>47 (26.1%)</td>
<td>Mean (2143)</td>
<td>Mean 71.6%</td>
</tr>
</tbody>
</table>

Figure (1): Titer range of ELISA positive samples in birds

Discussion
In the present study we sought to gain evidence of CAV influence on the health of commercial chicken flocks. The study attempts to contribute to the ongoing debate regarding whether the viral infection causes livability and profitability damages. Several studies indicated the harmful effects of the CIAV clinical and subclinical infection, while others found no significant effects on broiler performance. However, the final effect is the expression of multiple roots, such as management, stresses, genetic background, and additional pathogens (Hagood et al., 2000). Hagood et al., 2000. demonstrated that CIAV was more often detected
in diseased than in healthy flocks, and that finding was associated with thymus and bursal atrophy accompanied by increased mortality and condemnation rates (Pope, 1991). The potential harmful effect of CAV in healthy commercial flocks might be affected by the environment, management factors, and hormonal changes, which may affect the onset of the CIAV replication by activating or affecting cellular transcriptional factors (Li and Cui, 2007).

Necropsy findings and postmortem changes found in examined chickens were indicative for chicken anemia virus infection as atrophied lymphoid organs and hemorrhages on breast, wings, and thigh muscles were found (Brentano et al., 1991; Chettle et al., 1989). Heart and Kidneys lesions in some birds were found to representable to the viral infection.

PCV values and hemoglobin; mean values of PCV from different flocks were ranged from 22.5 to 34.2, this low range of PCV value in comparison to the normal values greater than or equal to 40%-43% supportive that this was caused by the CAV infection (Goodwin and Brown, 1992). While mean hemoglobin% values were ranged from 4.3% to 7.02% and theses values revealed that the chicken experienced anemia (Sommer and Cardona, 2003; Wani et al., 2014).

Serological surveys have shown that CAV infection is widespread in industrial poultry (Schat and Schukken, 2010). Presence of anti-CAV antibodies in tested flock's sera indicates that chickens may be vertically or horizontally infected or even acquired the antibodies passively from their breeders via yolk or even by contaminated vaccines (Stabili et al., 2016). The passively acquired antibodies are unlikely because all tested commercial layer and broiler flock's sera were collected after the age of 3 weeks, the time required for maternal antibodies to decay as mentioned by (McNulty et al., 1989). The presence of CAV antibodies in tested flock's sera with no history of clinical signs or lesions suggestive to CAV infection or vaccination against the virus certainly indicates that the source of anti-CAV antibodies detected in tested sera is the horizontally acquired CAV infection through direct and indirect contact with virus- contaminated dust, water or feed with feces specially that the virus shows extreme physical and chemical resistance to inactivation and so persists for long in poultry houses (Goryo et al., 1985). Although subclinical CAV infection does not produce clinical symptoms of the disease, it is immunosuppressive (McConnell et al., 1993; McNulty et al., 1991).

As detected by, Emikpe et al. (2005) was 66.2% While Oluwayelu & Todd, 2008, and Bulgaria may reflect the differences in the environment conditions, husbandry
practices and samples size also The actual exposure to CAV, most likely from the environment, appears to have occurred at around 28 days of age although the flock may have been susceptible earlier ([Emikpe et al., 2005; Oluwayelu et al., 2008; Todd et al., 2008]).

Comingling of birds from different generations in the same flock, a common vertical transmission, which is an important way of viral spread at the conditions of industrial poultry farming due to the ability of CAV to persist in the ovaries of laying hens even at a presence of circulating antibodies ([Brentano et al., 2005; Hoop et al., 1992]), also may play a role in CAV epidemiology. Our study detected CAV seroreagents in all flocks surveyed. These results are indicative for the high rate of environmental contamination and reflect the possibility for easy virus transmission between farms by environmental factors.

Seropositivity to CAV does not necessarily relate to overt disease, it indicates that birds have undergone at least subclinical infection with possible consequent immunosuppressive effects such as increased predisposition to other viral or bacterial infections or suboptimal response to vaccination. Thus, considering its widespread occurrence, CAV infection must be taken into account as a risk factor related to the epizootological picture of backyard chicken flocks.

The current study used the primers coding for the casid viral protein VP1. The circulating chicken infectious anemia virus has the viral capsid protein VP1 (51KDa) which is relatively conserved compared to the other two viral nonstructural proteins VP2 (24KDa) and viral apotin protein VP3 (13KDa) which are highly stable and highly conservative, the neutralizing antibody are produced against VP1 and VP2 ([Koch et al., 1995]).

The obtained nested-PCR product was sequenced, the nucleotide identity was 100% to most CAV strains when compared by aligning. This result is in agreement with studies performed in different placed worldwide ([AboElkhair et al., 2014; Erfan et al., 2018; Hailemariam et al., 2008; Ou et al., 2018]). The Vietnamese CAV strains were closely related to the Chinese, Taiwanese, and USA strains ([Van Dong et al., 2019]).

While the deduced amino acid sequence of the portion of VP1 determined differed from that of most Alabama CAV sequences previously determined at five positions and was different from all other CAV VP1 deduced amino acid sequences available, with the exception of Alabama 98-7370, in at least one position

**Conclusion**

Chicken infectious anemia virus infection is a worldwide disease with clinical and subclinical forms and it has been described in most countries
where chickens are raised commercially. The infection have a high economic losses due to destructive effect on lymphoid organs leading to immunosuppression and subsequently vaccination failure and complications with other infections. This study revealed a high CAV infection in Ismailia governorate clinically and serological testing showed 68% positive by ELISA test and genetic analysis revealed that the circulating virus in positive sampled are 100% similar to the worldwide strains. Further investigations are necessary to evaluate the economic losses caused by CAV and the cost-benefit of vaccination.

References
Serological evidence of chicken anaemia virus infection in Nigerian indigenous chickens. The Onderstepoort journal of veterinary research 72, 101-103.


فيروس أنيميا الدجاج المعدي من الأمراض الفيروسية العالمية و التي تتسبب في خسائر اقتصادية كبيرة في صناعة الدواجن حيث أنها تسبب تلف وضمور في الأعضاء الليمفاوية و التي بدورها مسؤولة عن المناعة في الطيور مما يؤدي إلى الوفيات بين الطيور المصابة لضعف المناعة و يجعلها عرضة للإصابة بالعدوى بأمراض أخرى و يحدث فشل الاستجابة لتطعيمات ضد الأمراض.

في هذه الدراسة تم تجميع عدد 180 عينة من 18 مزرعة مختلفة من محافظة الإسماعيلية لدراسة الفيروس. و قد تم فحص الحالات حقيقة و تم تسجيل الأعراض و التي تتفق مع أعراض مرض الأنيميا الفيروسي المعدي و منها حالة اعياء عامة و أصفر للجلد و شحوب للعرف بالإضافة إلى وجود حالات من الأسهال و تلون الجناح باللون الأزرق. و تم تجميع عينات الدم و الأعضاء الليمفاوية لعمل فحص الفيروس و العينات الدموية في الدم و نسبتهما في الدم و العينات الليمفاوية تراوحت نسبة الهيموجلوبين بين 4.4-9.8٪ و هي أقل من النسبة الطبيعية (10٪). و بالفحص السيرولوجي باستخدام اختبار الاليزا كانت نسبة العينات الإيجابية 73.9٪.

تم اختبار بعض العينات الإيجابية باختبار انزيم البلمرة المتسلسل و عمل التسلسل للقواعد النيتروجينية وقد وجدت متطابقة 100٪ مع فيروسات الأنيميا المعدية المنشورة على بنك الجينات و تم تسجيل الفيروس أيضا على بنك الجينات العلمي.

قد يكون انتشار الاصابة بهذا الفيروس نتيجة عوامل بيئية أو ان يكون الفيروس انتقل عمليا من الأمهات و بعض الدراسات ترجع انتشار الفيروس لتوث بعض التطعيمات به و لذلك يجب عمل دراسات إضافية للمواقف على أسباب انتشار الفيروس و كيفية القضاء عليه.