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Chicken Infectious Anemia Status in Commercial Broiler Chickens Flocks in Assiut-upper Egypt: Occurrence, Molecular Analysis Using PCR-RFLP and Apoptosis Effect on Affected Tissues

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Abstract: Chicken infectious anemia virus is ubiquitous virus of chickens causing disease in young chickens and immunosuppression in all birds. In the present study, the presence of chicken anemia virus CAV infection using PCR, genetic variability of isolated strains based on restriction of VP1 gene by *Mbo*I and apoptotic changes in the CAV positive broiler chickens in Assiut region, Upper of Egypt, were investigated. The history of problem showed that the clinical features were depression, increased susceptibility to various diseases, vaccination failure and mortalities ranged from 4-21%. Postmortem findings were pale liver, atrophy of thymus and hemorrhages subcutaneously. These findings strongly suggested that CIAV may be the cause of the problem. DNA was extracted from thymus and bursa of fabricius of the collected birds and exposed to PCR assay. A total of 44 Chicken Infectious Anemia Virus (CIAV) DNA was detected genetically from 165 broiler chickens of age up to 7 weeks with a percentage 26.6%, which can be considered high and thus, reflects an attention demanding situation. The data show that restriction endonuclease analysis performed with the 1350-bp PCR product and the *Mbo*I enzyme indicated genetic variations among examined field isolates that two variants of the viruses having differences at genomic level were causing the disease in closely related regions of the province. Twenty five thymuses were collected and processed for DNA extraction. Cellular DNA was subjected to electrophoresis on 1.5% agarose gel and stained with ethidium bromide. Internucleosomal DNA fragmentation was detected in CIAV-infected tissues. These data indicated that infection with CIAV induced apoptosis in lymphoid cells that may affect solely on cellular immunity. Present findings thus highlights the significance of CIAV, therefore, focus should be made on CIAV epidemiology in the Upper Egypt and to further develop and apply reliable diagnostic tools as well as molecular studies so as to advise suitable prevention strategies for this economical important avian pathogen.

Key words: Anemia virus, avian pathogen, poultry industry

INTRODUCTION

The major economic losses for the poultry industry coming from mortality, production losses, condemnation and the cost of preventive medication are due to infectious diseases which caused by viruses, bacteria, fungi and parasites. One of these infectious diseases is Chicken Infectious Anemia (CIA). It was first reported in Japan in 1979 in Specific-Pathogen-Free (SPF) chicks (Yuasa *et al.*, 1979). Chicken infectious anemia is caused by a virus called Chicken Anemia Virus (CAV) and to date there is only one defined serotype from it, but pathogenicity is a little different among CAV isolates (Yuasa and Imai, 1986).

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) for VP1, the major viral structural protein (51.6 kDa); VP2, a novel dual-specificity protein phosphatase (Noteborn *et al.*, 1998) (24 kDa) and VP3, a nonstructural protein (13.6 kDa) named apoptin. VP1 and VP2 are the targets of neutralizing antibodies (Peters *et al.*, 2001).

Infections occurring between 1 and 4 days of age result in clinical symptoms and lesions, observed mainly in 2- to 4-week-old flocks (Todd, 2000; Senthilkumar *et al.*, 2002; Dhama *et al.*, 2002). Outbreaks of the disease are characterized by anemia, thymus atrophy, bone marrow aplasia and hemorrhages (Yuasa *et al.*, 1987; Lucio *et al.*, 1990) and immunosuppression.

In the field, CIAV infection seems to cause few signs of disease, however, dual infections are more serious (McNulty, 1991; Dhama *et al.*, 2002). CIAV infection causes depression of immune response against several vaccine viruses viz. NDV, MDV, ILTV and FPV leading to vaccination failures, vaccination reactions or aggravation of the residual pathogenicity of attenuated vaccine viruses and even could lead to emergence of variant virus (Van den berg, 1996; Todd, 2000; Dhama *et al.*, 2002). The mortality due to CIAV in a flock is generally low but can be high due to secondary complications; therefore the virus seems to play a key role in the etiology of several multifactorial diseases (Pope, 1991; Todd, 2000).

Chickens older than 2-3 weeks of age are also susceptible to infection, but will only develop a subclinical disease that can result in immune suppressive effects such as impaired cytotoxic lymphocyte development, which may have important implications for cell-mediated immunity to a multitude of pathogens (Schat, 2003).

Normally, the virus does not grow in any of the commonly used primary cells and cell lines. Marek's disease virus or avian leucosis virus transformed lymphoblastoid cell lines are susceptible to this virus and the virus usually multiplies with low titre (Natesan *et al.*, 2006). For this reason, different molecular diagnostic methods have been developed for CAV diagnosis, out of which Polymerase Chain Reaction (PCR) seems to be the fastest and most sensitive method for the detection of virus nucleic acid (Noteborn *et al.*, 1992).

Isolation of the virus has been reported in major poultry-producing countries worldwide (Oluwayelu *et al.*, 2005). (The virus was isolated from chickens in Japan, China, Australia, New Zealand, India, Slovenia, Brazil and South Africa (Schat, 2003). Few studies are available about the chicken anemia viruses circulating in chicken population in Egypt and focused esp. in the lower part of Egypt (El-Lethi, 1990; Zaki and El-Sanousi, 1994; Sabry *et al.*, 1998; Amin *et al.*, 1998; Hussein *et al.*, 2001, 2002) and no information was available on the incidence of CAVs circulating in the poultry population in the upper part of Egypt esp. in Assiut province.

Commercial broiler flocks of chickens, 8-41 days of age, in different areas in Assiut province were found to be stunted with poor performance and increased mortality, so the needs was gone to assess the occurrence of Chicken Anemia Virus (CAV) in Assiut province through molecular detection and characterization of CIAV that circulating in the field and to emphasize the importance of CIAV as emerging avian pathogen in the province and help in devising a suitable control strategy against this disease.

MATERIALS AND METHODS

Sample collection: The presence of CAV was investigated by collecting 165 chickens (11 days-6 weeks old) from 15 randomly selected commercial broiler chicken flocks showed clinical sings including depression, increased susceptibility to various diseases, vaccination failure and mortalities ranged from 4-21%. Minimum five birds were collected from an individual farm.

Those farms located in different geographical regions of Asyut province. All flocks had received two or more doses of Newcastle disease and IBD vaccines and no broiler chickens from which samples were taken had received CIA vaccine.

The chicks were then necropsied and gross lesions were recorded. The thymus and spleen from each chick was taken and stored frozen (-20°C) for subsequent DNA extraction after pooling every two organs tissue from each birds.

Preparation of DNA extracts: DNA was extracted from 10 mg of each pooled organ sample (thymus and spleen) as well as from vaccine Thymovac (Lohmann Animal Health, GmbH & Co., Cuxhaven, Germany) as positive control by using DNA extraction QIAamp DNA Mini Kit as the manufacturer's protocol described.

CAV detection by PCR: Specific primers, CAV1)5-GCA GTA GGT ATA CGC AAG GC-3) and CAV2)5-CTG AAC ACC GTT GAT GGT C-3) (Noteborn *et al.*, 1992), amplify a 186-bp region on the highly conserved VP2 coding gene were used for the detection of CAV DNA by PCR, which was carried out in a 25- μ l reaction volume, including 2 μ l of isolated DNA, 12.5 μ l of GoTaq Green Master Mix (Promega, Madison, WI), 1 μ l of each primer (20 pmol) and 8.5 μ l of nuclease-free water.

The cycling condition of PCR reaction consisted of 5 min at 94°C for initial denaturation followed by 35 cycles using the following program: 30 sec at 94°C, 30 sec min at 60°C and 1 min at 72°C for 1 min, followed by final extension for 10 min at 72°C. All PCR assays were carried out in a Techne cyclogene thermal cyler (Perkin Elmer, Foster City, CA). Following amplification, the PCR fragments in 10 μ l of each reaction were resolved in a 1.8% agarose gel in TAE buffer at 100 V for 1 h. The gel was stained in a 0.5 μ g/ml ethidium bromide solution and visualized using an Eagle Eye detection system (Stratagene).

Restriction Endonuclease (RE) analysis: 24 samples were subjected for restriction analysis. The PCR products of 1350 bp were generated by one pair of primers for VP1 gene of CIAV by the following sequence, VP1-F) ATGGCAAGACGAAGTCGACACCG AGAGGC and VP1-R) TCAGGGCTGCGTCCCCCAG TACATGGTGCT) according to the sequence of VP1 gene on the pubmed (GenBank: AJ297678.1). The PCR amplicons that results were purified with the PCR purification kit (Jena Bioscience, Germany). Purified DNAs were digested with *Mbo*I (ER0812), (Fermentas, International Inc) in 20 μ l reaction volumes as per the manufacturer's protocol using specific assay conditions and the buffers supplied. The restriction digests on 2% agarose gel were stained with ethidium bromide and visualized by UV transillumination.

Assessment of apoptosis (Analysis of DNA fragmentation by agarose gel electrophoresis): The thymus and spleen tissue homogenate were used to detect the apoptosis. Tissues DNA was extracted as

described previously. Transfer supernatant to a fresh microcentrifuge tube. Add 1 ml -20°C ethanol. Mix and place in -20°C freezer overnight. Centrifuge sample for 15 min at 15,000 x g, 4°C. Remove and discard supernatant. Add 20 to 30 ul TE buffer and 1 ul RNase. Incubate 1 h at 37°C. Add 1 ul of 20 mg/ml proteinase K and incubate an additional 1 h at 37°C. Add 8 ul of DNA loading buffer. Prepare the cast gel for electrophoresis with 0.9% of Seakem agarose in TBE buffer. Load DNA marker 1 Kb plus DNA ladder (Invitrogen) in the first well and samples in the rest of wells. Run the gel at 70 mA until the bromphenol blue front is 1 to 2 cm from the end of the gel (Zhivotovsky *et al.*, 1995).

RESULTS

Necropsy findings: Postmortem examination revealed pale liver, hemorrhages under the skin and mild to severe atrophy of the thymus and bursa of fabricius were observed in the examined chickens as shown in Fig. 1, 2 and 3.

Detection of CAV-DNA in tissues by PCR: A total of 165 pooled organ samples collected from 165 commercial broiler chickens were tested by PCR assay for the presence of CAV DNA. The extracted genomic DNAs were tested for the presence of CIAV DNA by PCR using CIAV specific primers. As shown in Table 1, the results showed that all flocks of different ages were positive for CAV (Table 1) except flock no. D, G and I were negative. Rates of positive birds among flocks ranged from 20% to 41.2% with a total percentage 26.6%. The size of PCR amplicon was analyzed by comparing them with that of 1 Kb plus DNA ladder (Invitrogen, 10787-018) molecular weight marker using Gene Tools computer software producing the expected amplicons of approximately 387 bp (Fig. 4). The size of the PCR product was the same as that produced using DNA specific to the Cux-1 isolate of CAV Fig. 4.

Restriction analysis: Analysis of the VP1 region of different isolates in comparison with the cux-1 strain revealed differences among the isolated Egyptian strains and between them and cux-1 strain of thymovaccines which assigned the examined strains into three groups. After digestion with *Mbo*I enzyme cux-1 strain showed five cutting sites yielding six products of size 502, 344, 221, 116, 90 and 70 bp (Fig. 5). In contrast restriction profile of PCR amplified VP1 region of different isolates revealed different profiles (Fig. 5). The samples of two farms A and E showed six cutting sites yielding seven products of size 429, 344, 265, 187 and 125 bp, in contrast to isolates detected in farms B, F, G, H and J, the restriction endonuclease profile that

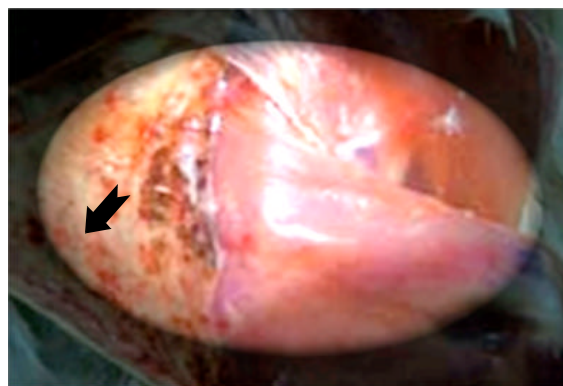


Fig. 1: S/C hemorrhagic of infected chickens with chicken infectious anemia

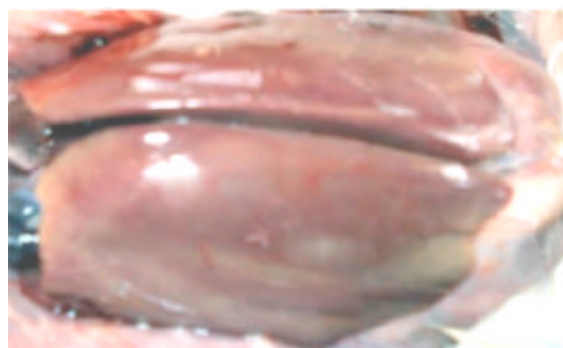


Fig. 2: Pale liver from chicken broilers infected with chicken infectious anemia

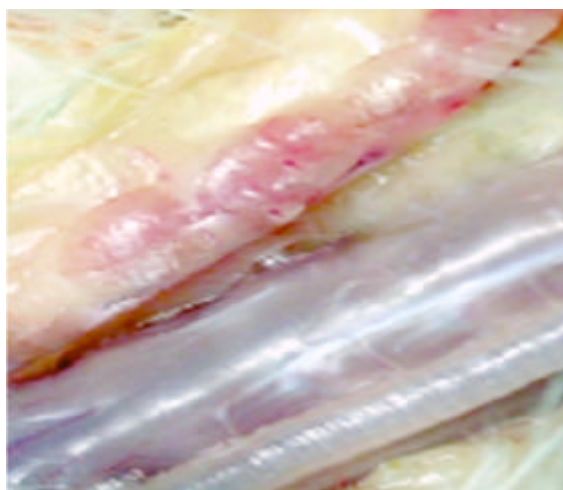


Fig. 3: Atrophied thymus from chicken broilers infected with chicken infectious anemia

obtained was on the following manner; 640, 429 and 281 bp.

Table 1: Frequency of CIAV detection by PCR among examined flocks in different areas in Assiut

Farm ID	Age (Days)	Vaccination To CAV	No. of birds examined	No. positive birds by PCR	Positive (%)
A	14	No	17	7	41.17
B	21	No	32	13	40.6
C	37	No	22	6	27.2
D	15	No	13	0	0
E	23	No	17	5	29.4
F	41	No	10	3	30
G	10	No	9	0	0
H	19	No	20	7	35
I	11	No	13	0	0
J	39	No	7	2	28.57
K	18	No	5	1	20
Total			165	44	26.6

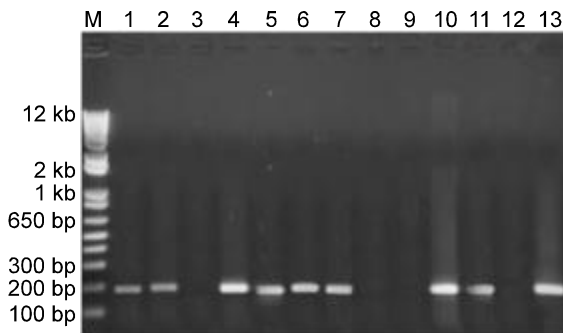


Fig. 4: PCR amplicon for Chicken infectious anemia (CIAV (positive samples Lanes 1, 2, 4-7, 10, 11 and 13. Lanes 3, 8, 9 and 12 are negative samples for CIAV. Lane M = 1 Kb plus DNA ladder) Invitrogen) used as a size marker

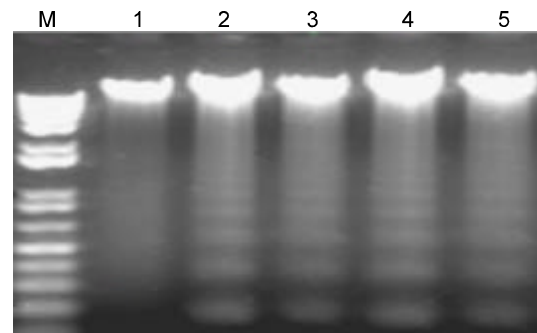


Fig. 6: DNA ladder formation of infected tissues Thymus (lanes 2 and 3) and spleen (lanes 4 and 5) proved to be infected with CIAV, lane 1= control thymus without infection, Lane M = 1 Kb plus DNA ladder) Invitrogen) used as a size marker

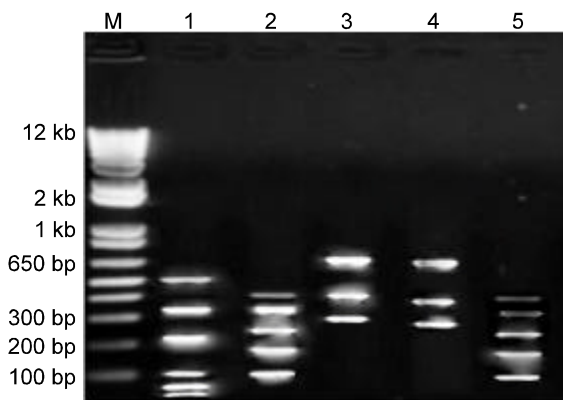


Fig. 5: PCR-RFLP for Differentiation of the isolated Chicken infectious anemia isolated viruses. Restriction fragments generated by digestion with *MboI*; Lane 1 is Cux-1 strain from thymovac vaccine, lane 2 to 5; are field isolated CIAV digested with *MboI*; Lane M 1 kb plus DNA ladder

Apoptosis detection: Apoptosis was detected by DNA ladder assay. A characteristic of apoptosis is the cleavage of DNA of the dying cell at the inter-

nucleosomal regions of genomic DNA extracted from tissues of thymus and spleen. The agarose gel electrophoresis showed nucleosomal laddering pattern yielding multiple bands of 180-200-bp and its multiplication (Fig. 6) that is considered a characteristic feature of apoptosis. This nucleosomal laddering pattern was found in all examined samples of all 15 positive flocks for CIAV by PCR.

DISCUSSION

Through the investigation of different chicken flocks in different farms in Assiut province, gross pathological lesions of pale comb and wattle, subcutaneous hemorrhage and pale liver were suggestive of CIA disease as well as there are no reports available on Chicken Infectious Anemia in Assiut and Upper Egypt, therefore, the need was felt to go for monitoring the incidence of the disease in the state in province to determine the importance of CIA in Assiut and help plan a suitable control strategy against this economically important emerging avian pathogen.

In the present study, the gross lesions showed mainly effect on affected lymphoid organs (thymus and bursa) and some hemorrhages on skin, S/C and muscles as noticed in (Fig. 1). Thymic atrophy (Fig. 3) is the most

consistent lesion of CAV infection especially when chicks develop age resistance and has been reported by (Yuasa *et al.*, 1979; Engstrom and Luthman, 1984; Chettle *et al.*, 1989; Lucio *et al.*, 1990; Toro *et al.*, 2000; Ledesma *et al.*, 2001) in experimental as well as field cases of CIAV infection. Haemorrhagic lesion on skeletal muscle observed by (Yuasa *et al.*, 1979; Chettle *et al.*, 1989) during their investigations.

During the present study was noticed a wide variation in mortality from 4-21%. This variation in mortality may depends on many factors including synergistic effect of CIAV and other diseases, poor hygiene condition, poor ventilation, low quality feed, etc. One to 50 percent was reported by (Hofstad *et al.*, 1984) and 12% by (Balachandran *et al.*, 1991). Mortality due to CIA alone was reported as high as 60%, but usually it remained between 5-10 (Engstrom and Luthman, 1984). Mortality of 8.83-34.7% in commercial broilers (Chettle *et al.*, 1989) and 20-60% (McNulty, 1991) has also been reported (Jorgensen, 1991) reported 19.2% mortality due to CIA with a range of 8.4-33.9%.

On the other hand, clinical signs of the disease, especially in chicks above 3 weeks anemia, were not seen, but lesions suggestive of CAV such as both lymphoid depletion and atrophy of thymus were recorded. These obtained findings were compatible with that previously mentioned by Ledesma *et al.* (2001) who noticed chicks have infected with virulence strain or high doses of virus after the decay of protective maternal antibodies and the virus has caused the lymphoid lesions without anemia and induction of lesion without clinical signs of the disease was reported previously in inoculation of CAV to specified pathogen-free chicks. Also previous studies were recorded the absence of clinical signs after about 3 wk of age, immunocompetent chickens are resistant to disease, but they can acquire asymptomatic infections (Schat, 2003; Owoade *et al.*, 2004).

The conventional methods for the diagnosis of CIAV are based on the clinical signs and pathological changes. However, more specific methods for the detection of viral antigen from the clinical samples by conventional methods like cell culture, viral marker in tissues, electron microscopy, as well as different serological tests e.g., Virus-Neutralization test, fluorescent antibody test and Enzyme linked immunosorbent assay are available, which have certain limitations and also do not indicate the time of infection (Saini and Dandapat, 2009). The MDCC-MSB1 cell culture can be used for *in vitro* isolation attempts and virus titration (Goryo *et al.*, 1985; Imai *et al.*, 1991; Yuasa, 1983) which are often have certain shortcomings such as highly expensive, time consuming and unable to detect CIAV strains which, failed to replicate in MSB1 or other lymphoblastoid cell lines (Saini and Dandapat, 2009).

PCR, a technique for amplifying a specific DNA fragment *in vitro* is a sensitive and rapid molecular diagnostic technique, being used widely for the diagnosis of a variety of avian pathogens (Cavanagh, 2002; Dhama *et al.*, 2002). It has also been proved to be useful for CAV-DNA detection in field samples (Tham and Stanislawek, 1992; Santeen *et al.*, 2001; Rozypal *et al.*, 1997; Dhama *et al.*, 2002).

The present study demonstrated the presence of CAV genome using one pair of the primer set CAV1 and CAV2 were designed by Noteborn *et al.* (1992) which give 186-bp region on the highly conserved VP2 coding gene amplified products of CIAV DNA as shown in (Fig. 1) from the affected tissue samples. Out of 165 examined birds 44 were positive for CIV by PCR with a total percentage 26.6% and all sampled chicks were not positive. This can be explained with this fact that in field flocks naturally exposed to CIAV, it commonly takes 2-4 weeks until most birds have detected positive (McNulty *et al.*, 1988).

In Parallel with the results in the present study, various other workers detected CIAV DNA by PCR assay from the different tissues, Krapez *et al.* (2003) and Davidson *et al.* (2004) detect CIAV from MSB-I cell line, Chowdhury *et al.* (2002) detected CIAV DNA from 5 field samples out of 125 suspected cases. Miller *et al.* (2003) detected CIAV DNA from embryo and egg shell membrane. Imai *et al.* (1998) detected CIAV DNA from the cell free virus infected MDCC-MSB1 cells, unfixed liver homogenates, formalin fixed liver homogenates and formalin fixed paraffin embedded tissues from experimental or field cases of CIAV infection using PCR assay.

The origin of the CAV present in the studied chickens may be due to vertically infected birds with CAV. Bülow and Schat (1997) reported CIA even in the presence high neutralizing antibodies titers and Imai *et al.* (1999) suggesting that the virus may persist in the presence of antibodies. In addition to the widespread use in chickens of live vaccines produced with CAV-contaminated eggs or cells may have played a role in the dissemination of CAV to chickens due to the previously undetected CAV infection of SPF flocks (Barrios *et al.*, 2009).

Restriction digestion of the VP1 PCR products obtained from 24 representative farm samples was carried out to confirm the identity of PCR products. The restriction enzyme was selected from the restriction map created using the sequences available in GenBank at <http://www.ncbi.nlm.nih.gov/Genbank/index.html> and NEBcutterV2.0 software available online at <http://tools.neb.com/NEBcutter2/index.php>.

Digestion of VP1 gene PCR amplified product 1350 bp by *Mbo* I revealed three patterns as shown in (Fig. 5), one showing six cutting sites in reference Cux-1 strain, while the others strains having five and two cutting sites, indicating the genetic variation among the field isolates.

So, in the present study the findings indicated that variant viruses having differences at genomic level are causing disease in closely related regions of the district. Hence it is logical to infer from the present findings that viruses causing disease in closely related areas may not be identical. The existence of difference between the isolates can be further analyzed by nucleotide sequencing. However, as reported earlier by (Santeen *et al.*, 2001) RE analysis of the PCR amplified products is a convenient, quick and easy method that can be even used in less equipped diagnostic laboratories. Also, CIAV-PCR and RE analysis can be used to confirm identity of antigenically and pathogenically different isolates.

Present findings revealed difference among the isolated CAV strains at genomic level. These findings can be of importance epidemiologically to distinguish different CAVs circulating in the poultry flocks of the Assiut province. Further antigenic, pathogenic and molecular characterization of these CAV isolates can be useful for devising suitable prevention and control strategies for this emerging avian pathogen.

DNA fragmentation has been suggested to be one of the early irreversible events to trigger cell apoptosis (Duke *et al.*, 1983; Golstein *et al.*, 1991) and DNA ladder assay, which monitors fragmented DNA, is hallmark of apoptosis. In our study, fragmentation of DNA was clearly visible with genomic DNA of tissue from affected birds that broken into low-molecular-weight oligomers made up of multiple of 180 bp as shown in (Fig. 6).

This apoptosis phenomenon has also been described by various other workers in different tissues and cell lines. This was reported in thymocytes and lymphoblastoid cell lines by (Jeurissen *et al.*, 1992), Chicken thymocytes and cultured transformed mononuclear cells by (Noteborn *et al.*, 1994), various human haematological malignant cell lines derived from leukemia and lymphoma and in osteosarcoma cells by (Zhuang *et al.*, 1995), Chicken thymic lymphoma by (Adair, 2000), various lymphoid tissue from CIAV affected birds and CIAV infected MDCC-MSB1 cells by (Chiu *et al.*, 2001) and various transformed cell line by (Oro and Jans, 2004).

So, our results confirm and strength that phenomenon is essential part in the pathogenesis of CIAV infection as noted previously by (Jeurissen *et al.*, 1992) and considered one of the important reasons for atrophy of all lymphoid organs and decrease in WBC and lymphocyte counts.

Since neither vaccination nor other preventive measures are in practice in broiler chicken flocks in Egypt, This study confirmed the widespread occurrence of CAV infection in commercial broiler farms and indicates natural exposure to the virus among the examined flocks. On the basis of these results, it can be assumed that at least a part of lower growth performance and

inadequate response to vaccination observed frequently in broiler chicken farms in the selected regions can be due to CIAV infection.

The results of restriction analysis indicate the circulation of more than one CAV strain among the examined broiler flocks. These findings confirm the emergence of a relatively new disease problem in the Assiut broiler chickens as well as the inability of transferred homologues maternal antibodies to stop the spread of infection.

The present findings highlights the significance of CIAV, therefore, focus should be made on CIAV epidemiology in all provinces and to further develop and apply reliable diagnostic tools as well as molecular studies so as to advise suitable prevention strategies for this economical important avian pathogen and strengthen the necessity for providing adequate immunity of breeder flocks as a means of avoiding the infection in their progeny through vaccination after estimation of cost benefit studies made.

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