

Chicken Infectious Anemia Virus (CIAV) in Broilers and Laying Hens in Sharkia Province, Egypt

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Abstract : CIAV was identified in three broiler flocks from different localities of Sharkia province, Egypt using PCR assay, where DNA extraction from liver, spleen, bone marrow, and thymus of examined chicken yielded positive reaction against primers. The flocks showed clinical signs and lesions suggestive to CIAV infection. The examined birds showed generalized weakness, depression, droopy appearance, pale comb and wattles, stunting, growth retardation, high mortalities, and markedly reduced packed cell volumes (average PCV was 17% - 22%). The necropsy findings were yellow fatty bone marrow, generalized lymphoid atrophy, and enlarged liver and spleen. Hypoplastic bone marrow and lymphocytic depletion were the most consistent histopathological findings. A serologic survey in unvaccinated commercial layer and broiler flocks from different localities of Sharkia province, Egypt was also performed using ELISA assay. Out of 180 tested serum samples of commercial layer flocks, 147 were positive (with percentage of 81.67% & ELISA titers ranging from 1582.74 to 2320.88) and 158 out of 180 tested serum samples of commercial broiler flocks, were positive (with percentage of 87.78% and ELISA titers ranging from 1821.04 to 2803.30). The present serological finding showed that CIAV was widely distributed in Sharkia province, Egypt. [Journal of American Science 2010; 6(9):752-761]. (ISSN: 1545-1003).

Key words: CIAV, PCR assay, PCV, Histopathology, ELISA

1. Introduction:

Chicken infectious anemia virus (CIAV) was first isolated in 1979 in Japan (Yuasa et al., 1979). In fact, it was not a new disease but a newly recognized one. A retrospective serological survey evidenced that CIAV was present in the southeastern United States since at least 1959 (Toro et al., 2006) although it was first isolated in 1989 (Goodwin et al., 1989). Since then, an increasing interest was paid to that virus, as it was found to have a great economic impact on poultry industry in all major chicken-producing countries of the world (McNulty et al., 1988; Jørgensen, 1990; FarKas et al., 1992; Buscaglia et al., 1994; Al-Ankari et al., 1996; Ragland et al., 1998; De Herdt et al., 2001; Owoade et al., 2004; Mahzounieh et al., 2005; Dergham, 2006). The virus is incriminated in a disease of young chickens, characterized by a transient severe destruction of erythrocytic and granulocytic series of the bone marrow cells, resulting in aplastic anemia. It also causes immunosuppression, whether directly by itself as it causes severe depletion of lymphocytes from primary and secondary lymphoid organs, (Yuasa et al., 1979; Taniguchi et al., 1982 & 1983), or indirectly as it participates other immunosuppressive viruses such as infectious bursal disease virus

(IBDV) (Bülow et al., 1986 a; Yuasa et al., 1980), Marek's disease virus (MDV) or reticuloendotheliosis virus (REV) (Bülow et al., 1986 a). In dual infections, anemia was produced even when the chickens were inoculated at two or more weeks of age, or in chickens with maternal antibody (Bülow et al., 1986 a; Yuasa et al., 1980). CIAV is proved to enhance the pathogenicity of a wide range of co-infecting pathogens such as Newcastle disease virus (De Boer et al., 1994), Marek's disease virus (De Boer et al., 1992; Miles et al., 2001), Adenovirus (Bülow et al., 1986, b; Toro et al., 2000), Reovirus (Engström et al., 1988; McNeilly et al., 1995), *Staphylococcus aureus* (Randall et al., 1984; McNamee et al., 1999), *Clostridium perfringens* (Goodwin et al., 1989), *Eimeria tenella* (Ibrahim, 1997), and *Cryptosporidium baileyi* (Hornok et al., 1998). The infected birds showed poor performance and high mortality rate generally between 10-20%, but might reach 60% especially in complicated cases (Gelderblom et al., 1989). This work aimed to detect the presence of CIAV by PCR and its prevalence by using ELISA in commercial broiler and layer flocks.

Materials and methods:

1. Tissue samples:

Thymus loops, bone marrow, bursa of Fabricius, liver, spleen, and intestine samples collected aseptically from freshly dead and sacrificed birds ranged in age from 12 to 35 d representing different breeds and localities in Sharkia province, Egypt and showing clinical signs and lesions suggestive to chicken anemia virus infection were subjected to clinical and postmortem examinations.

2. Serum samples:

A total of 360 serum samples from 27 poultry flocks (14 from commercial layer flocks ranged in age from 16 to 80 w and 13 from commercial broiler flocks ranged in age from 20 to 85 d) representing 5 different localities in Sharkia province, Egypt were collected and stored at (-70°C) until used to study of the seroprevalence of chicken infectious anemia virus (CIAV) in commercial layer and broiler flocks in Sharkia province, Egypt using ELISA assay. There was no history of vaccination against CIAV in any of the tested flocks.

3. Determination of hematocrit values (Packed cell volume: PCV)

Blood samples were collected from heart of chicks suffering from anemia and growth retardation on transferring tubes containing 6% EDTA solution. Blood was then transferred to microhematocrit capillary tube (scientific product, McGraw Park, III). PCVs were determined by using a high speed centrifuge measuring and recording the PCVs (*Duncan and Prasse, 1986*).

4. Detection of CIAV by PCR:

A. Tissues preparation

Collected tissue samples were ground with a mortar and pestle in PBS with addition of antibiotic mixture (1000 I.U. penicillin / ml + 1 mg streptomycin sulphate/ ml) to prepare a 20% tissue homogenate. Three times of repeated freezing and thawing were applied and then the homogenate was centrifuged at 3000 rpm for 20 min. The supernatant was stored at -70°C until used for inoculation (*Zhou et al., 1997*).

B. DNA extraction

DNA was extracted from homogenates of tissue samples (Thymus loops, bone marrow, bursa of Fabricius, liver, and spleen) using commercially available extraction kit (QIAGEN® EZ1 Virus Mini Kit Version 2.0(48), Cat. No. 955134) and automated extractor (QIAGEN® EZ1 (BioRobot), Serial No. 0502f0707) were used under the manufacturer instructions.

C. DNA amplification

The PCR assay was performed in the thermocycler (T3- Thermocycler, Biometra) using:

1-Reddy-Mix™ PCR Master Mix (THERMO Scientific®, Cat. No. AB-0575/LD/A, Lot No. 1404/15).

2-Two primers, CAV A1.1 (5` AAT GAA CGC TCT CCA AGA AG 3`) and CAV A1.2 (5` AGC GGA TAG TCA TAG TAG AT 3`), (MWG-Biotech AG®, Batch No. 523470 & 523471 respectively) were used to amplify a 583 base pair (bp) DNA fragment (*Tham and Stanislawek, 1992*).

3-DNA Ladder of 100 Pb (QIAGEN®, Lot No. 3)

The PCR assay was performed in a final volume of 50 µl containing 25 µl Reddy-Mix™ PCR Master Mix, 18 µl PCR Grade Water, 1 µl of each Primer, and 5 µl template. The amplification was performed under the following conditions: one cycle of initial denaturation step at 95°C for 15m, followed by 30cycles of 95°C for 1m, 56°C for 1m, and 72°C for 1m representing denaturation, annealing, and extension steps respectively. Finally, one cycle of final extension step at 72°C for 5m.

D. Detection of PCR products

The amplified products were analyzed using electrophoresis unit. It was loaded to 2% agarose, stained by ethidium bromide, visualized under ultraviolet light, and photographed by a gel documentation system using Canon Power-Shot® G10 camera and the data was analyzed using computer software (*Sambrook and Russel, 2001*).

5. Histopathological examination of the tissue samples

Samples from thymus, bone marrow, bursa of Fabricius, liver, spleen, and intestine were immediately fixed in 10% buffered neutral formalin solution. Fixed tissues were then trimmed, washed and dehydrated in ascending grades of alcohol, cleaned in xylene, embedded in paraffin, sectioned (4-6 micron thickness), and stained with Hematoxylen and Eosin (H&E) for histopathological examination (*Bancroft et al., 1996*).

6. Detection of CIAV antibodies by ELISA

The test was carried out for detection of antibodies against CIAV studying the prevalence of chicken infectious anemia virus (CIAV) in commercial layer and broiler flocks in Sharkia province. Commercially available chicken anemia virus (CAV) antibody ELISA test kit (ProFLOK® PLUS, Synnbiotics corporation, San Diego, CA, USA; Cat. No. 92127-800-228-4305) was used under the manufacturer instructions.

Results

1. Clinical signs & lesions

Tested broiler flocks for detection of CIAV had clinical signs and lesions suggestive to chicken anemia virus infection. Chicks showed signs of anemia, generalized weakness, depression, droopy appearance, pale comb and wattles, stunting, growth retardation and high mortality rates. The necropsy findings were watery blood, yellow fatty bone marrow, markedly atrophied thymus glands, atrophied bursa of Fabricius, and enlarged liver and spleen. In some chicks of flock No. 1, there were subcutaneous and intramuscular hemorrhages.

2. Hematology

The hematocrit values were markedly reduced. The average PCV was 17% - 22%.

3. Investigation of CIAV by PCR assay

Analysis of DNA extracted from tissues of diseased chicks (thymus, bone marrow, bursa of Fabricius, liver, and spleen) by agarose gel electrophoresis yielded positive PCR product (583 base pairs) as shown in Fig.1

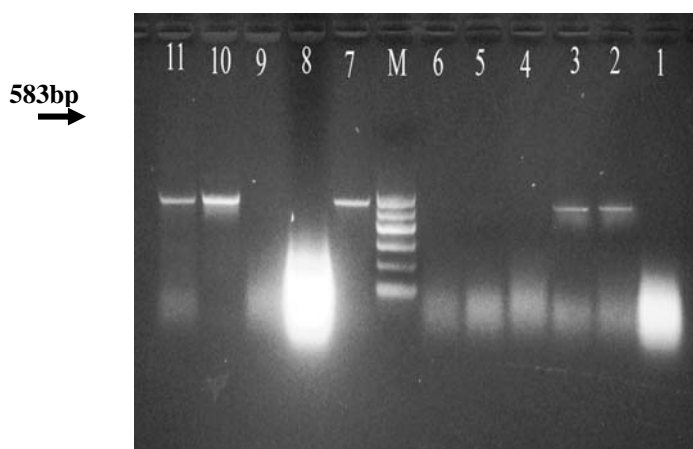


Fig. 1: PCR products (583 bp in size) of amplified CIAV-DNA extracted from tissues of diseased chicks

Lanes 2, 3, 10, and 11 are positive

Lanes 4, 5, 6, 8, and 9 are negative

Lanes 1 and 7 are control negative and positive respectively

M: size marker (100 base pairs ladder)

Table (1): PCR results

Flock No.	Tissues	PCR result	Lane No.
Flock 1	Liver +	+ve	2
	BM +	+ ve	3
Flock 2	Liver +	- ve	9
	BM +	+ ve	11
Flock 3	Liver +	- ve	4
	BM +	- ve	5
	BF	- ve	6
Flock 4	Liver +	- ve	8
	BM +	+ ve	10

4. Histopathology

Histopathological examination of the naturally infected tissues, which were positive to CIAV DNA by PCR assay, showed marked hypoplasia in bone marrow (Fig 2) and generalized lymphocytic and generalized lymphocytic depletion in thymus (Fig 3), spleen (Fig 4), and Peyer's patches (Fig 5). Bursa of Fabricius showed moderate depletion in lymphoid follicles with presence of perifollicular edema (Fig 6). Liver showed cellular swelling and apoptosis with marked apoptotic bodies (Figures 7, 8 and 9).

5. Serological survey by ELISA

Out of 360 serum samples tested for the presence of CIAV specific antibodies, 147 serum samples were positive out of 180 tested samples in commercial layer flocks (with percentage of 81.67 % & ELISA titers ranging from 1582.74 to 2320.88) and 158 serum samples were positive out of 180 tested samples in commercial broiler flocks (with percentage of 87.78 % & ELISA titers ranging from 1821.04 to 2803.30), as shown in Tables 2 and 3.

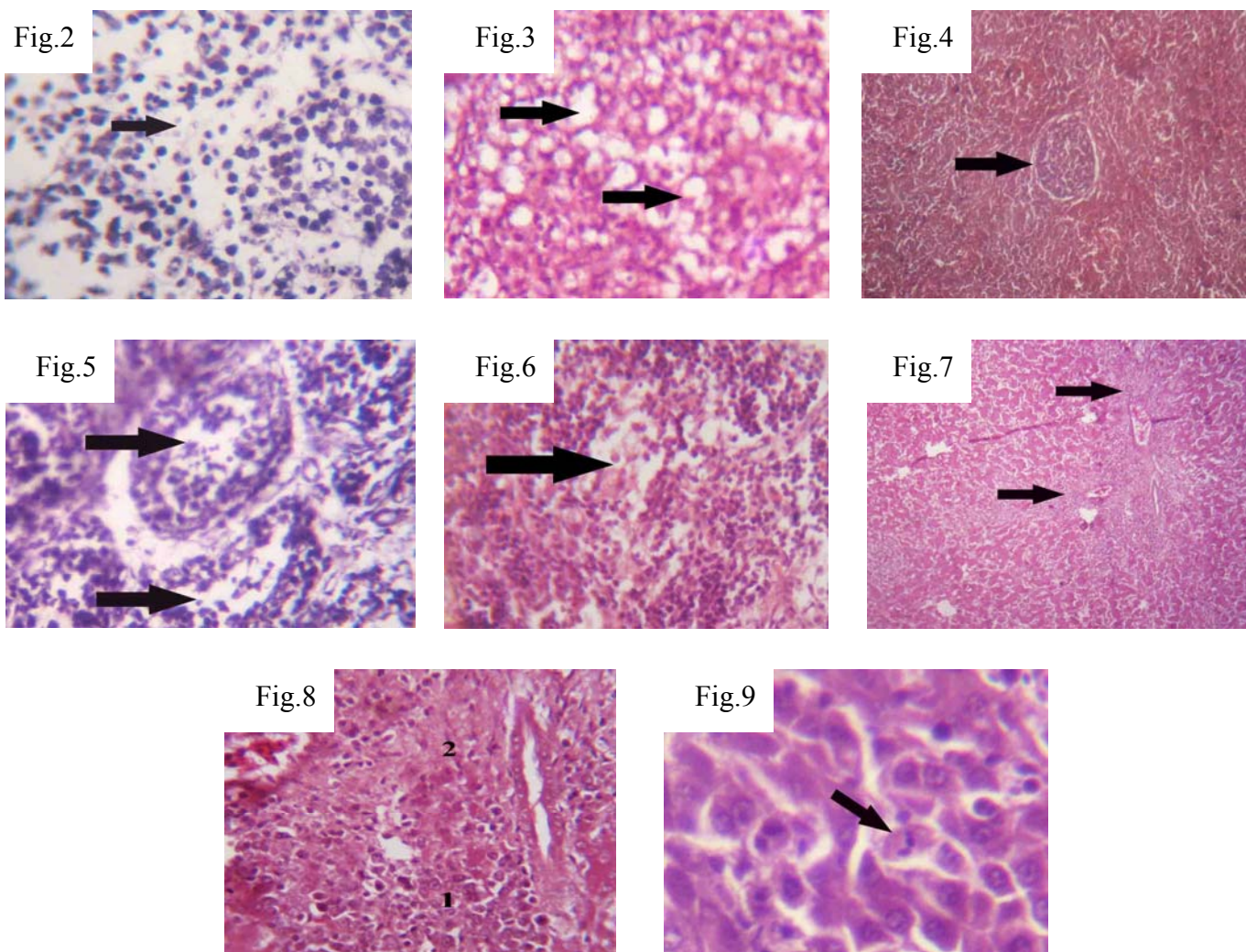


Fig. (2): Bone marrow section showing markedly hypocellular marrow (arrow). H&E., X 1200.

Fig. (3): Thymus showing marked lymphoid depletion (arrows). H&E., X 1200.

Fig. (4): Spleen showing depletion of the white pulps (arrow) and disarrangement of the normal architecture. H&E., X 300.

Fig. (5): Ileum showing depletion of Peyer's patches (arrow). H&E., X 300.

Fig (6): Bursa of Fabricius showing moderate depletion of lymphoid follicles (arrows) with presence of perifollicular edema. H&E., X 1200.

Fig. (7): Liver showing portal aggregation of round cells and proliferation of fibroblasts (arrows). H&E., X 300.

Fig.(8): Liver showing congestion of portal blood vessels with perivascular fibrosis (1) and proliferation of the bile duct (2). H&E., X 1200.

Fig (9): Liver showing hepatocyte apoptosis with marked apoptotic bodies (arrow). H&E., X 1200.

Table (2): ELISA results of tested layer flock's sera for CIAV antibodies

Flock No.	Breed	Age (week)	Samples No.	Positive No.	Positive %	Mean Titers	SD
1	Bovans	38	06	04	66.67	1582.74	1110.17
2	Bovans	55	14	12	85.71	2154.49	377.94
3	Bovans	60	15	13	86.67	2119.31	291.13
4	Bovans	65	17	14	82.35	2115.58	433.411
5	Bovans	70	06	05	83.33	2320.88	324.79
6	Bovans	75	14	12	85.71	2031.29	478.17
7	Hisex	22	18	15	83.33	2038.91	357.46
8	Hisex	23	10	9	90.00	2009.54	537.60
9	Hisex	51	10	7	70.00	2067.29	547.94
10	Hy-Line	16	12	12	100.00	2305.3	353.30
11	Hy-Line	33	18	12	66.67	1815.64	596.55
12	Hy-Line	80	14	11	78.57	2062.52	335.56
13	Lohmann	20	16	13	81.25	2053.73	519.58
14	Lohmann	70	10	08	80.00	2054.47	441.53
Total			180	147	81.67	2052.26	478.94

Table (3): ELISA results of tested broiler flock's sera for CIAV antibodies

Flock No.	Breed	Age (day)	Samples No.	Positive No.	Positive %	Mean Titers	SD
1	Cobb	23	10	09	90	2420.12	459.86
2	Cobb	30	20	18	90	2236.17	589.50
3	Cobb	37*	10	10	100	2521.46	215.07
4	Cobb	37*	05	05	100	2507.09	503.35
5	Cobb	40*	10	07	70	2177.29	390.17
6	Cobb	40*	16	13	81.25	2560.70	750.67
7	Hubbard	38	10	08	80	2119.26	514.67
8	Hubbard	42	25	23	92	2319.97	942.48
9	Hubbard	45	12	12	100	2552.03	361.44
10	Saso	60	25	22	88	2525.03	1340.92
11	Saso	70	08	06	75	2803.30	1307.79
12	Saso	78	19	18	94.74	2536.56	581.99
13	Saso	85	10	07	70	1821.04	636.46
Total			180	158	87.78	2392.31	611.11

*Same breed and age but from different areas and farms

4. Discussion:

Chicken infectious anemia (CIA) is a disease of young chickens (Yuasa *et al.*, 1979) characterized by generalized lymphoid atrophy resulting in immunosuppression and severe destruction of erythrocytic and granulocytic series of the bone marrow cells giving rise to aplastic anemia (Yuasa *et al.*, 1979; Taniguchi *et al.*, 1982 & 1983). The mortality rate is generally between 10-20%, but may reach 60% especially in complicated cases (Gelderblom *et al.*, 1989).

In this study, PCR was used for direct diagnosis of chicken infectious anemia virus (CIAV) infection in broiler chicken flocks in Sharkia province of Egypt, while ELISA was used for screening the commercial layer and broiler flocks in Sharkia province of Egypt for the presence of specific antibodies against CIAV.

PCR assay is considered the assay of choice for the detection of CIAV-DNA in chicken tissues, cell cultures, and vaccines. It is proved to be specific and definitely more sensitive than cell culture isolation of the virus; especially that DNA can be extracted from the same tissues as used for virus isolation (Miller *et al.*, 2001; Soiné *et al.*, 1993; Drén *et al.*, 1994). ELISA is the recommended test for routine detection of CIAV antibodies in serum samples and for investigation of the epidemiology of the virus (Todd *et al.*, 1999).

The clinical signs and postmortem lesions go with the previous findings of other researchers (Yuasa *et al.*, 1979; Taniguchi *et al.*, 1982 & 1983; Aly, 2001). In fact, a case of hematocrit value below 27% with yellowish changes in the bone marrow and thymic atrophy may be indicative to chicken anemia virus infection beside other means of diagnosis (Yuasa *et al.*, 1979; Pope, 1991; Ramadan *et al.*, 1998).

PCR assay performed on the extracted DNA from tissues of diseased birds showing clinical signs, PCV values, and lesions suggestive to CIAV infection yielded positive reactions with correct size as primers (583 bp) (Tham and Stanislawek, 1992) indicating that these signs and lesions are due to CIAV infection. In all PCR positive flocks, the thymus and bone marrow samples were positive, this is probably because CIAV targets erythroid and lymphoid progenitor cells in the bone marrow and thymus respectively (Adair, 2000).

Histopathological findings of this study agree with the statements of other researchers (Goryo *et al.*, 1989; Smyth *et al.*, 1993) and prove that the immunosuppressive effect of the virus may be

attributed directly to the destructive effect of the virus to hematopoietic and lymphopoietic tissues leading to impaired immune response. CIAV infection causes severe defects in splenic T-lymphocyte functions in form of decreased responsiveness to phytohemagglutinin, concanavalin A, and fall in interleukin production (Adair *et al.*, 1991). Macrophage concentration and functions are also severely reduced after in vivo or in vitro exposure to the virus such as interleukin-1 (IL-1) production, Fc receptor expression, phagocytosis, and bactericidal activity (Cloud *et al.*, 1992; McConnell *et al.*, 1993a & b). The adverse effects of the virus on lymphocyte and macrophage functions have substantial negative effects on immune response leading to enhancement of the concurrent infection with other pathogens and vaccination failure (Adair, 2000). In this work, bursa showed moderate depletion in lymphoid follicles. This result agrees with the findings of Sakr and Talaat, (1991) who observed marked depletion of the lymphocytes in the thymus and bursa of Fabricius beside severe hypoplasia in hematopoietic cells in bone marrow. In addition, Hussein *et al.* (2002) reported some bursal changes with various degrees of atrophy in the lymphoid follicles with scattered necrotic foci, which were probably attributed to secondary infections.

Several facts suggest that the incriminated agent in this study is the chicken infectious anemia virus (CIAV) including the observed clinical signs, postmortem lesions, PCR detection of DNA fragments of CIAV genome of correct size as primers used (583 bp), and the histopathological findings which are all indicative to the clinical form of CIAV infection.

The serologic survey performed in this study involved 5 different localities of Sharkia province, Egypt representing different breeds, ages and types of production to express – as much as possible – the field conditions. Bird's age was intended to exceed the age of 3 w to exclude maternally derived immunity that persists for about 3 weeks (McNulty *et al.*, 1988). The overall serological findings of this study proved that CIAV is widely distributed in both commercial layer flocks (with percentage of 81.67% & ELISA titers ranging from 1582.74 to 2320.88) and broiler flocks (with percentage of 87.78% & ELISA titers ranging from 1821.04 to 2803.30) of different ages, breeds, and localities in Sharkia province, Egypt. Such results agree with the earlier findings of the previous surveys conducted in Egypt. Zaki and El-Sanousi (1994) reported an incidence of 70% of CIAV antibodies in serum samples collected from broiler breeder, layer, and day old broiler

flocks. Amin et al., (1998) stated that CIAV antibodies were detected in 97.4% of serum samples collected from 21 native and foreign grandparent, parent, and broiler flocks in 8 provinces. Sabry et al. (1998) used ELISA to test 1916 serum samples from 118 flocks of different breeds and ages in different governorates for the presence of CIAV specific antibodies. Sera showed a high degree of positivity for CIAV antibodies in both imported and locally produced broiler parent, broiler, and layer flocks. Islam, (2003) reported that seroprevalence was 74.6% and 67.3% in commercial broiler and broiler breeder flocks respectively in Sharkia province. Our findings also go with that reported in other countries. The seroprevalence was 85.7% in commercial layer flocks in Afyon region, Turkey (*Kuyucuoglu et al., 2003*); 86% in commercial broiler flocks in Nigeria (*Owoade et al., 2004*); 87.7% in commercial broiler flocks in Shahrekord, Iran (*Mahzounieh et al., 2005*); 82.61% in commercial broiler flocks in Northern Jordan (*Dergham, 2006*); and 67.3% in commercial layer flocks in Khartoum state, Sudan (*Ballal et al., 2005*).

The presence of anti-CIAV antibodies in tested flock's sera indicates that these chickens may be vertically or horizontally infected or even acquired the antibodies passively from their breeders via yolk. 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. (1988). The presence of CIAV antibodies in tested flock's sera with no history of clinical signs or lesions suggestive to CIAV infection or vaccination against the virus certainly indicates that the source of anti-CIAV antibodies detected in tested sera is the horizontally acquired CIAV infection through direct and indirect contact with virus-contaminated dust, water or feed with feces (*Rosenberger, 1991*) specially that the virus shows extreme physical and chemical resistance to inactivation and so persists for long in poultry houses (*Yuasa et al., 1979; Yuasa, 1992*).

It is concluded that chicken infectious anemia virus (CIAV) is widely distributed among both commercial layer and broiler flocks in Sharkia province, Egypt. In addition, the presence of both clinical and subclinical forms of CIAV infection is confirmed using PCR and ELISA respectively. Both clinical and subclinical forms of CIAV infection have destructive effect on lymphoid organs leading to immunosuppression and subsequently vaccination failure, complications with other pathogens, and

great economic losses. The great need for breeders immunization and their monitoring for the presence of CIAV antibodies during rearing period is now clear to avoid vertical transmission of the virus and achieve protection of the offspring by maternal anti-CIAV antibodies.

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8/1/2010