Chicken infectious anaemia virus in Egypt: Molecular diagnosis by PCR and isolation of the virus from infected flocks

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ABSTRACT

In the present study, a chicken infectious anaemia virus (CAV) was isolated and identified from two (clinically and subclinically) infected broiler-breeder flocks aged, 28 and 60 days, respectively. The clinical symptoms of the disease have been observed only in the young flock. However, the chicks in both cases experienced various degrees of characteristic lesions of gangrenous dermatitis (secondary bacterial infection) in the wings and on the back. Low hematocrite values have been recorded in both cases of CAV infection. These findings strongly suggested that CAV was the primary causative agent of the problem. Polymerase chain reaction (PCR) was employed to investigate the presence of CAV-DNA in heparinized blood and a pool of tissue samples collected from the suspected cases of CAV using complementary oligonucleotide primers to region of the CAV DNA genome. These primers generate a 418 bp amplified product, which was sized by agarose gel stained with ethidium bromide after electrophoresis, along with a molecular weight marker. DNA was extracted from heparinized blood and a pool of tissue samples using the standard extraction method and used in the PCR assay. The results revealed that both cases were positive for the presence of CAV-DNA genome. Tissue homogenates that demonstrated positive results for CAV were processed, chloroform treated and inoculated in one day-old SPF chicks for virus isolation. Virus passages in susceptible SPF chicks and cell culture were carried out. Two CAV isolates (H91/2000 and H93/2000) were recovered during the virus isolation trials, demonstrated by PCR, flourescent detection of the viruses in a MDCC-MSB-1 cell line, and histopathological finding of the CAV-induced lesions in the inoculated SPF chicks. The results of the present study demonstrate strong evidence on the isolation and characterization of CAV in clinical and subclinical infections among broiler breeder flocks in Egypt.

Keywords: *Circoviruses, chicken infectious anaemia, polymerase chain reaction, isolation, molecular diagnosis.*

INTRODUCTION

Circoviruses are small, non-enveloped, icosahedral viruses with single stranded DNA genomes (Todd, 2000). The family comprises three members, chicken anaemia virus (CAV), porcine circovirus (PCV) and psittacine beak and feather disease virus (BFDV). CAV is an important avian pathogen worldwide, first isolated in Japan in

1979 (Yuasa *et al.*, 1979). The incidence of CAV infection is high, however, the clinical disease is very uncommon (McNeilly, 1991). The clinical disease occurs in very young chicks which become infected with vertically transmitted virus from breeders (Adiar, 2000). Typical symptoms observed in the clinical disease include weakness, depression, anorexia and stunting (Yuasa *et al.*, 1979). Anaemia in comb and wattles, eyelid and legs with quite pale carcass. The clinical disease is characterised by skin lesions most commonly in wings which are caused by secondary bacterial infections (Engstrom and Luthman, 1984). Affected chicks are generally depressed and mortalities are up to 60%. The gross lesions include anaemia, pale bone marrow, thymic, spleenic and bursal atrophy, in addition to characteristic skin lesions (anaemia-dermatis syndrome). A decreased hematocrite and decreased red and white cells and platelet counts are common findings (Goodwin *et al.*, 1992). Definitive diagnosis of the anaemia-dermatis involves virus isolation or demonstration of virus-specific antigen or virus nucleic acid (Todd, 2000). Since the isolation of the virus in SPF chicks or in cell cultures (MDCC-MSB1 cells) is laborious and time consuming, the virus infection has been diagnosed rapidly by detecting the virus DNA by hybridization or PCR in tissue samples from infected birds (Todd *et al.*, 1992; Tham and Stanislawek, 1992; Yamaguchi *et al.*, 2000). In Egypt, El-Lethi *et al.*, (1990) reported the suspicion of CAV in dressed poultry and serological investigation have been proved the intensive exposure of commercial chicken to CAV (Zaki and El-Sanousi, 1994; Sabry *et al.*, 1998; Amin *et al.*, 1998). The present study describes the detailed data of our recent report on the molecular diagnosis of the CAV for the first time in Egypt (Hussein *et al.*, 2001) and the isolation of the CAV from clinical and subclinical infected broiler-breeder flocks.

MATERIALS AND METHODS

Field samples

Sera and tissue samples were collected from 2 suspected CAV-infected flocks aged 28 and 60 days. The young flock showed clinical sings including depression and mortalities with skin lesions. The 60 days flock shows only skin lesions with some stunting birds.

Serological investigation

Serum samples collected from both flocks were assayed for the presence of CAV antibodies using commercial ELISA kits (KpL. Laboratories, USA).

Hematology

Blood samples were collected into heparinized tubes. Using the microhematocrit capillary tubes, PCV was determined by centrifugation and measuring the PCV values. Methods of blood collection and PCV determination (Goodwin *et al.*, 1992).

PCR for detection of CAV DNA in heparinized blood and infected tissues

DNA was extracted from heparinized blood and homogenates of tissue samples (liver, thymus, bursa and spleen) using Tripure isolation reagents (Roche Molecular Biochemicals).Two primers (F: 5 CTA AGA TCT GCA ACT GCG GA 3 and R: 5 CCT TGG AAG CGG ATA GTC AT 3) were used to amplify a fragment of 418 bp of CAV DNA genome in the PCR. PCR was carried out as previously described (Hussein, 2001). A total volume of 50 µl PCR reaction containing 5 µl of the extracted DNA and 45 µl of PCR reaction mixture contaioning 1X PCR buffer (10X buffer contains 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 25 mM MgCl2), 0.2 mM of

each primer F and R, 200 µM (each) dATP, dCTP, dGTP and dTTP, 1.25 units of Taq polymerase (GIBCO). Following an initial cycle at 95°C for 3 min, , 50 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec were conducted. The amplification products were analysed by electrophoresis on 2% agarose gels, stained by ethidium bromide, examined under transluminator and photographed by Polaroid Gel Cam camera. The size of the PCR products were confirmed by visualization on an ethidium bromide-stained agarose gel along with a molecular weight marker.

Inoculation of 1-day-old SPF chicks for virus isolation

Tissue samples were prepared according to the method of Zhou *et* al., 1997. Briefly, tissue samples were ground and processed with a mortar and pestle in PBS with addtion of antibiotics to prepare a 20% tissue homogenate. The homogenate was mixed with an equal volume of chloroform for 15 min in a shaker. Three times of repeated freezing and thawing were applied and then the homogenate was centrifuged for 20 min at 3000 rpm. The supernatant was aliqouted and stored at - 70°C till used for chicks inoculation. The primary isolation was carried out by inoculation of 1-day-old SPF chicks intramuscularly in the thigh with 0.2 ml of the supernatant fluids/chick. Two groups of chicks (25/group) and a control group were used for virus isolation. At 10 days post inoculation, the birds were bleeded and liver, thymus loops and spleen were collected and examined for CAV lesions (histopathology as described by Smyth *et al.* (1993) as well as used for preparation of homogenate for passage in another group of chicks. Three passages in chicks were carried out.

Characterization of CAV local isolates

The CAV was characterized in the tissue homogenate collected from infected chicks among the three passages by detecting the virus antigen and the genomic DNA using immunofluorscent assay (Zhou *et al.*, 1997) and PCR, respectively. In addition, histopathology examination was conducted on bursa, thymus and liver collected from inoculated chicks along the three passages.

Inoculation of MDCC-MSB-1 cell line

Recovered and characterized 2 isolates of CAV from the infected SPF chicks were inoculated in MDCC-MSB-1 cell line and the cells were incubated for 36-48 hr, then collected and examined by immunofluorscent for detection of CAV antigen.

RESULTS

Serology

Sera collected from the flock with clinical signs did not demonstrate any titer of antibodies to CAV however the subclinical infected flock demonstrated variable titers ranging from 4066 to 13947 with GMT of 7178.

Hematology

Heparinized blood collected from both flocks showed PCV of low volume with an average of 26% and 29% in clinical and subclinical infected flocks, respectively.

PCR amplification

Analysis of PCR amplification of the extracted DNA from heparinized blood and tissue samples by agarose gel electrophoresis indicated DNA bands of corrected size as expected with a length of 418 bp.

SFF inoculation of 1-day-old chicks

Three passages were applied using groups of 1-day-old SPF chicks. In the first passage, lesions associated with CAV infection were observed including enlarged liver in the majority of the chicks with pale muscles, atrophied thymus and variable degrees of coloration in the bone marrow between red and yellow. Most of the chicks in such passage survive till 14 days post inoculation, however, when the tissues collected from such chicks were inoculated for the second passage, mortality of the chicks started at 6 days post inoculation with marked lesions. In the third passage, the chicks became highly affected and mortality increased after one week post inoculation with reproducible lesions. Tissues collected from each passage were examined histopathologically and by PCR. However, tissues collected from the third passage were used for cell culture inoculation.

Histopathology

The histopathology examination of the tissues collected from the inoculated chicks 7 to 10 days after inoculation with the sample preparation showed atrophy in both cortex and medulla of the thymus with degeneration and necrosis of lymphoid cells, atrophy of the lymphoid follicles of bursa with scattered necrotic foci. Liver collected along the three passages showed swollen hepatocytes and simsoidal dilatation with foci of lymphoid aggregation.

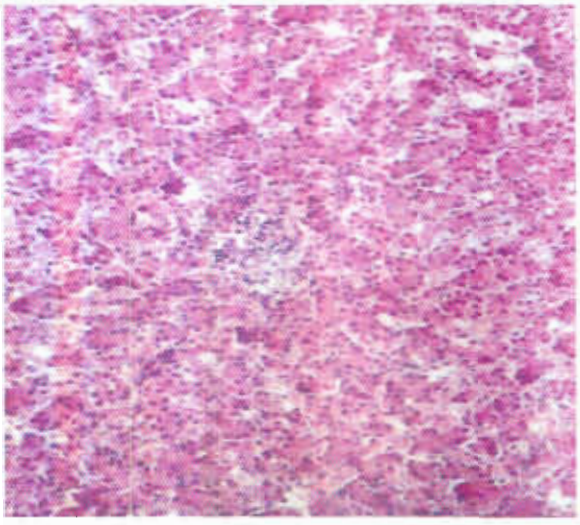

Fig. (1): Photomicrograph showing diffuse, mild-to-moderate hepatocellular swelling with multifocal lymphocytic infiltrates (H&E X100).

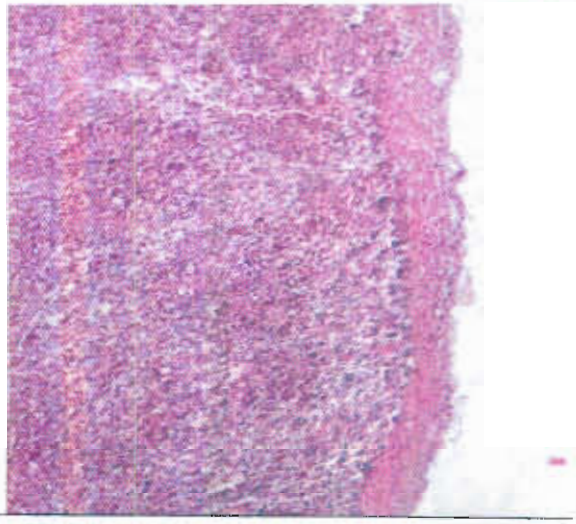

Fig. (2): Thymus with indistinct cortex with moderately depletion of lymphoid follicles (H&E X40).

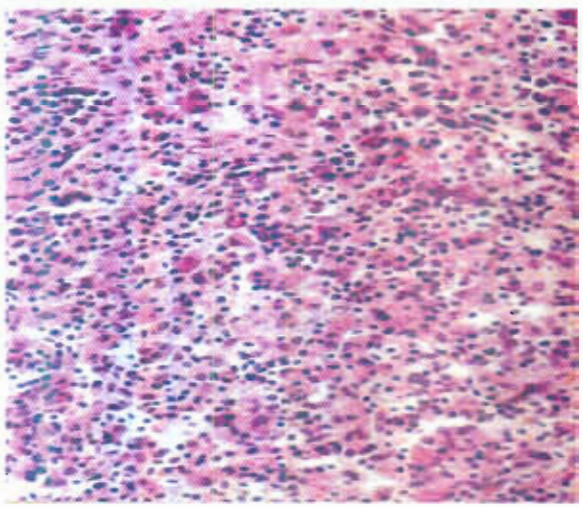

Fig. (3): High magnification of the previous photo (Fig. 2) to show details of Lymphocystolysis (H&E X400).

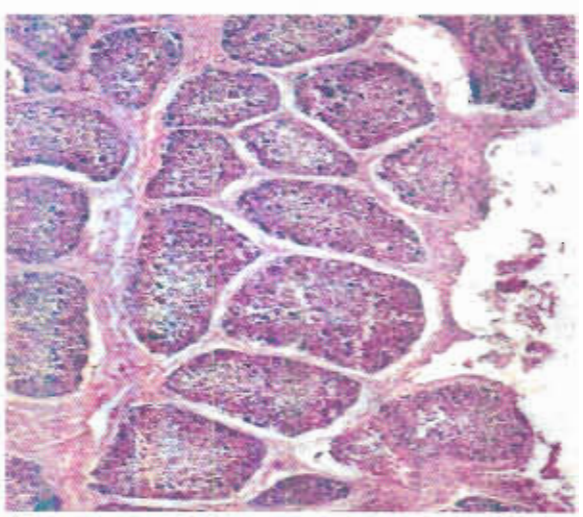

Fig. (4): Lymphoid follicles of bursa of Fabricius crowded and moderately separated by expanded Interstitial (H&E X 100).

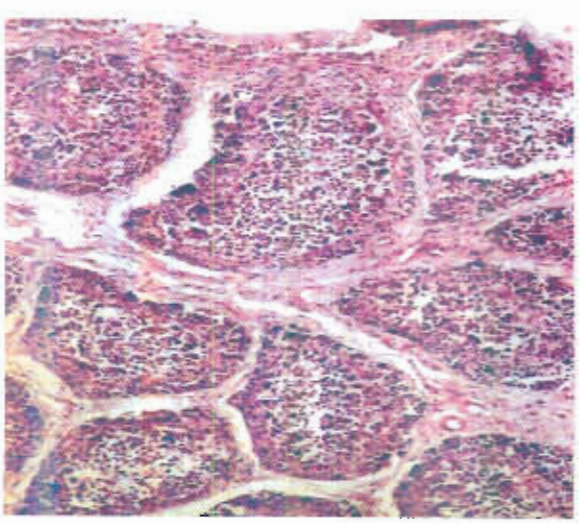

Fig. (5): High magnification of the previous photo (Fig. 4) showing depleted lymphoid follicles and lymphocyolysis (H&E X200).

PCR amplification

Analysis of PCR amplifed DNA extracted from tissue homogenates of organs collected from inoculated SPF chicks by agarose gel electrophoresis, indicated DNA bands of corrected size as expected with a length of 418 bp confirmed the presence of the virus along the passages in chicks.

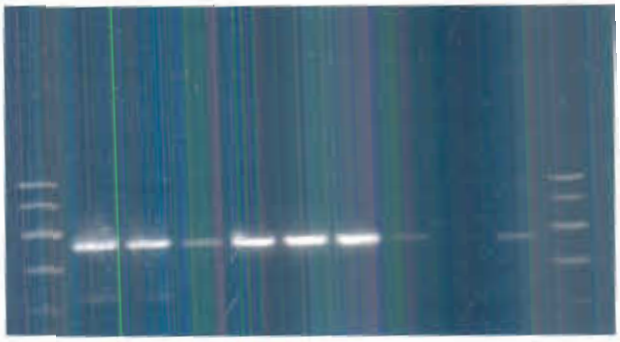

Fig. (6) : The PCR products (418 bp in size) of the amplified CAV DNA genome in liver and blood of clinical and subclinical infected flocks as well as from the inoculated SPF chicks along with DNA molecular weight marker (50, 150, 300, 500, 750 and 1000 bp).

Detection of CAV antigen in inoculated MSB-l cell culture by IFA

Bright staining intranuclear fluorescent was detected in infected cells harvested between 36 and 48 hr post inoculation with the tissue homogenates of the third passage of the isolates in SPF chicks. The cells were enlarged with the characteristic granular fluorescent.

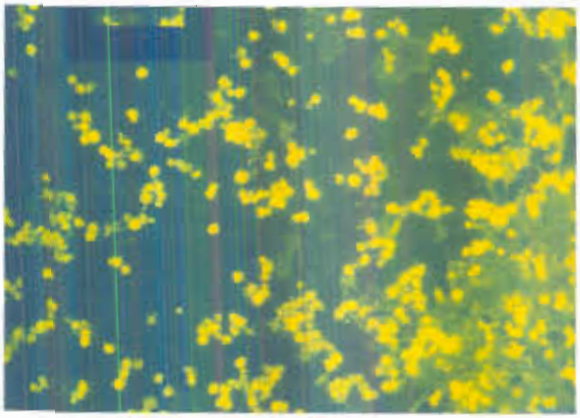

Fig. (7): Intranuclear fluorescence in cells inoculated with the CAV local isolates harvested after 36h post inoculation and stained by IFA using standard anti-CAV antisera.

DISCUSSION

CAV is belived to be spread among chicken in Egypt since the early 1980 s when several outbreaks occurred in many breedrs (Sabry *et al.*, 1998). The presence of CAV antibodies in both meat and egg-type chicken flocks provided strong evidence for the extensive exposure of the chicks to such virus (Zaki and El-Sanousi, 1994; Amin *et al.*, 1998 and Sabry *et al.*, 1998). Hussein *et al.* (2001) previously reported for the first time, the molecular diagnosis of the CAV in clinical and subclinical infected flocks. The present study is reporting of the isolation anc characterization of CAV from such flocks. However, after submission of the previous work (Hussein *et al.*, 2001), another study described the isolation of CAV from broiler

chicken in Egypt has been published (Aly, 2001).

Antibody testing for CAV is an essential part of monitoring the *chicken* flocks for the presence of the virus. However, a link may exist between the genetic background of chicken strain and the development of humoral immune response to CAV and also between the virus strain and host immune responsiveness (Cardona *et al.*, 2000). The presence of an antibody to CAV in chicken indicates that these chicks could have been vertically infected or horizontally infected immediatelly after hatch. However, it is well documented that all seroconversion to CAV after natural infection in SPF chicks occurred at or near sexual maturity (Cardona *et al.*, 2000). Previous observations reported that chicken infected at older ages with CAV showed seroconversion already after 4 to 7 days, whereas in chicks infected at day 1, antibody can be detected only after 2 to 3 weeks (Hoop and Reece, 1991). In the present study, the serology results for both clinical and suclinical chicks were different. Antibodies were not detected in the clinical flock as expected and reported by others (Toro *et al.*, 1997; Cardona *et al.*, 2000). In contrast, sero-positive samples were those of the subclinical flock indicated the CAV infection. Indeed, the presence or absence of CAV-antibody in sera tested in the present study are not indicative for the mode of infection by CAV. Broiler chicks are known to have a higher PCV values than other types of chicken and the PCV values did not change with age (Goodwin *et al.*, 1992). The hematocrite values obtained in the present study clearly demonstrated the presence of anaemia in both flocks. Furthermore, the presence of CAV in such flocks is confirmed in tissues collected from both flocks by PCR. Finding that all tissues examined by PCR are positive, this result directly proves that the CAV is a primary cause of such lower hematocrite values and the clinical symptoms in the young flock. PCR employed in the current study provided its potentiality to rapidly detect the CAV DNA genomic fragment in heparinized blood and organs collected from both flocks. The application of PCR for diagnosis of CAV has been previously described (Todd *et al.*, 1992; Tham and Stanislawek, 1992; Soine *et al.*, 1993; Rozypal *et al.*, 1997; Imai *et al.*, 1998). The gene structure of CAV has been well characterized by sequencing and shown to have high sequence identity among isolates (Noteborn *et al.*, 1991; Frakas *et al.*, 1996). Such finding increased the usefullness of PCR for CAV detection and identification (Yamaguchi *et al.*, 2000). In our previous and present studies, application of PCR for detection of CAV in blood and tissue proved its efficacy and sensitivity. However, it should be noted that in some reactions specially when DNA is extracted from original tissue samples and not the propagated tissue samples, an additional short PCR band was observed. This band was not observed in any of the inoculated SPF which indicates that the band may be non-specific or might be the result of priming at another site in the CAV genome (Rozypal *et al.*, 1997). Inoculation of 1-day old SPF chicks with the CAV-positive tissue samples developed the CAV-associated lesions. The obtained results confirm the diagnosis and success of virus isolation from both flocks. The clinical signs and macroscopic lesions obtained in inoculated SPF chicks among the passages were nearly similar to those reported by others (Toro *et al.*, 1997). Neither clinical nor gross lesions were observed in the control non-inoculated SPF chicks. There have been several reports of the pathologic effect of CAV in diseased chickens (Goryo *et al.*, 1987; Hoop and Reece, 1991 and Smyth *et al.*, 1993). The histopathological lesions detected in the present study in thymus, bursa and liver

agreed with the previous findings by others and all together confirm the isolation of CAV in SPF chicks. Detection of the genomic CAV/ DNA by PCR in the organs of the inoculated SPF chicks is also a supporting fact of the virus isolation. Atrophy in both thymus and bursa with variable changes in inoculated SPF chicks compared to these non-inoculated control group indicated the effect of CAV in these organs. Also, histopathology changes were observed in liver. Indeed, such changes clearly indicated the isolation of CAV from both flocks and support the implication of CAV in both cases (Clinical and subclinical flocks). This was also confirmed by the detection of the CAV/DNA genomic in the tissues collected from inoculated SPF chicks. The destructive effect of CAV in the organs specially atrophy of lymphoid cells in a wide variety of tissues has been reported Goryo *et al.*, (1989) and Smyth *et al.*, (1993). In the present study, depletion of cortical thymocytes is observed and this strongly supports such effect of CAV, specially as a primary cause of immunodeficiency. This immunodeficiency enhances the concurrent infections and vaccination failure due to the status of immunsuppression. Although, CAV is known to have an effect on both primary and secondary lymphoid organs, the first appearance of CAV in thymic center strongly suggested that the virus selectivity infected immature T cells and hence the primary cause of immunosuppression (Adair, 1996).

Previous studies reported that bursal cells are not susceptibile to CAV infection (Goryo *et al.*, 1989; Smyth *et al.*, 1993) and bursa reaches its immunocompetance and antibody response to the virus are initiated. In the present study, we observed some bursal changes with various degrees of atrophy in the lymphoid follicles with scattered necrotic foci. This observation might be due to secondary infection of these chicks, specially the subclinical flock which showed gangrinous dermatitis or due to CAV itself. It was reported that there is an indirect evidence that CAV infection of older chicks are immunosuppressive includes the observed reduclis in vaccination response to vaccines against MDV, NDV and ILT (Otaki *et al.*, 1988; Cloud *et al.*, 1992; De Boer *et al.*, 1994). Moreover, anenhancement of pathogenicity of a range of co-infecting agents such as MDV, IBD, NDV, Reovirus, Staphylococcus aureus and Cryptosporidia was reported (Todd, 2000). After inoculating of tissue homogenates collected from SPF, inoculated chicks in cell culture, two CAV isolates were recovered from inoculated cells as detected by the characteristic fluoresent in the infected MDCC-MSB-I cell line using immunofluoresent assay. The isolates are designated H91/2000 and H93/2000. The isolated viruses are likelyhood CAV as supported by several facts from the present study including: 1- Histopathology examination of the organs collected from inoculated chicks revealed characteristic CAV-induced lesions. 2- PCR detection of the fragment from DNA genome of CAV in organs collected from inoculated SPF chicks. 3- Resistance of the isolated viruses to chloroform treatment during homogenate preparation. 4- Fluoresent detection of the CAV antigen in inoculated MDCC-MSB-1 cells using standard anti-CAV antiserum.

Finally, the present study reports the isolation and identification of CAV from broiler breeders in Egypt. Further studies on CAV among chickens of layer and meat types need to be addressed before the establishment of a vaccination policy against CAV. Moreover, critical consideration with the nature and immunopathogenesis of CAV infection in relation to other agents have be taken into consideration before the introduction of CAV vaccination program.

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الملخص العربي

فيروس الأنيميا المعدية للدجاج في مصر : عزل الفيروس من القطعان المصابه و تشخيص المرض بإستخدام البيولوجيا الجزيئية عن طريق تفاعل البلمره المتسلسل

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[1] قسم الفيروسات-كلية الطب البيطرى – جامعة القاهرة – جيزة – مصر. [2] إيجيتك – دقي – جيزة – مصر
[3] المعامل المركزية للرقابة الصحية علي المستحضرات البيطرية – العباسية – القاهرة – مصر

من خلال هذه الدراسة تم عزل و تصنيف فيروس أنيميا الدجاج المعدي من حالات مرضية و أخري لم تظهر عليها الأعراض المميزة من قطيعين من دجاج أمهات التسمين و التي تبلغ أعمارها ٢٨ يوما في القطيع الأول و ٦٠ يوما في القطيع الثاني. و علي الرغم من ملاحظة مراحل متدرجة من إلتهابات الجلد الغرغرينية (نتيجة عدوي بكتيرية ثانوية) في الأجنحة و علي الظهر في كلأ القطيعين، إلا أن الأعراض الإكلينيكية المميزة للإصابة بفيروس أنيميا الدجاج المعدي لوحظت فقط في الأعمار الصغيرة فقط من الدجاج. و قد أثبتت نتائج تحليل عينات الدم المأخوذة من الحالات المختبرة أن جميعها كانت منخفضة علي إختلاف الحالة الإكلينيكية للدواجن المصابة. هذه القراءات المعملية التشخيصية ترجح كلها إحتمال أصابة القطعان المختبرة بفيروس أنيميا الدجاج المعدي كمسبب أول للأعراض المرضية الملاحظة في القطعان المختبرة. وبإستخدام تفاعل البلمرة المتسلسل للكشف عن وجود الحمض النووي (الدنا) لفيروس أنيميا الدجاج المعدي في عينات دم غير متجلط و أيضا أنسجة مجمعة من كل الحالات تحت التشخيص، فقد تم عزل الحمض النووي للفيروس من هذه العينات وفقا للطرق القياسية، لذلك تم إستخدام زوج من البوادئ المكمل لتتابع خاص بالحمض النووي (الدنا) للفيروس و قد تم التأكد من إيجابية تفاعل البلمرة المتسلسل و خصوصية نواتجه من تمرير النواتج في جل مصبوغ بمادة بروميد الإيثيديم خلال تيار متردد للفصل الكهربائي للحمض النووي تبعا لطوله و مقارنة هذه النواتج لتدريج مكون من قطع معلومة الطول من الحمض النووي. و قد كانت جميع الحالات إيجابية من حيث نواتج إختبار البلمرة المتسلسل.و بالنسبة لعينات الأنسجة المطحونة التي أظهرت نتائج إيجابية في تفاعل البلمرة المتسلسل فقد تمت معالجتها بالكلوروفورم ثم حقنت في كتاكيت خالية من الأجسام المناعية و المسببات المرضية عند عمر يوم من الفقس لعزل فيروس أنيميا الدجاج المعدي و أيضا محاولة العزل علي خلايا الزرع النسيجي (MDCC-MSB-1). و قد تم عزل عترتين من فيروس أنيميا الدجاج المعدي هما H93/2000 , H91/2000 من العينات المحقونة و هو ما تطابق مع ما أظهرته الإختبارات المتعددة التي طبقت علي العينات موضع الدراسة و هي:إختبار البلمرة التسلسلي، إختبار الفلورسنتي المناعي علي خلايا الزرع النسيجي MDCC-MSB-1 المحقونة بالعينات ، الإختبارات الهستوباثولوجية علي التغيرات المرضية الناتجة عن نمو فيروس أنيميا الدجاج المعدي في الكتاكيت الخالية من الأجسام المناعية و المسببات المرضية و المحقونة عند عمر يوم من الفقس. مما لا شك فية أن النتائج التي تم الحصول عليها من الدراسة الحالية أثبتت وجود فيروس أنيميا الدجاج المعدي في قطعان أمهات دجاج التسمين في مصر حيث أمكن عزل و تشخيص الفيروس من الحالات المرضية و أيضا تلك التي لم يظهر عليها الأعراض بعد بطرق متعددة.