VIROLOGICAL STUDIES ON INFECTIOUS LARYNGOTRACHEITIS VIRUS (GALLID HERPESVIRUS-1) IN BIRDS

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ABSTRACT

The study was conducted on a total of 20 samples collected from clinically diseased birds suspected to be infected with infectious laryngotracheitis virus (ILTV). Samples were collected from different farms in Dakahlia and Damietta governorates. These samples include trachea and larynx. Isolation of the virus on chorioallantoic membranes (CAMs) of fertile chicken eggs aged 12 days were carried out. Three to six passages were done to each sample depending on appearance of pock lesions on CAMs. Identification of the virus was carried by agar gel precipitation test (AGPT), indirect immunoflourescence (IF), indirect immunoperoxidase (IP) techniques and histopathological examination. Then confirmation of ILTV diagnosis was done by polymerase chain reaction (PCR). Out of 20 samples inoculated on CAMs, there were 14 showed characteristic pock lesions. Out of 14 isolated samples there were 10 samples (71.4%) tested positive by AGPT, 11 (78.5%) by IF and IP tests. This indicate sensitivity of IF and IP over AGPT in ILTV diagnosis. ILTV DNA was detected in inoculated CAMs by PCR. PCR could be used as a rapid and specific technique to confirm ILTV diagnosis.

INTRODUCTION

Avian infectious laryngotracheitis is a herpes viral respiratory disease of chickens with worldwide distribution, the taxonomy of herpesviruses has been updated by the International Committee on Taxonomy of Viruses (ICTV). The former family Herpesviridae has been split into three families, which have been incorporated into the new order Herpesvirales. The infectious laryngotracheitis disease caused by Gallid herpesvirus-1which classified as a member of order Herpesvirales, family Herpesviridae, subfamily Alphaherpesvirinae, and genus Iltovirus **(Davison et al., 2009)**. The primary natural hosts of infectious laryngotracheitis virus are chickens, but ILTV-like viruses have also been isolated from pheasants and peafowl **(Crawshaw & Boycott, 1982)**. Field ILTV isolates differ in their pathogenicity, but clinical signs of acute respiratory disease are typical and include conjunctivitis, sinusitis, nasal discharge, decrease in egg production and low mortality. In severe cases, dyspneas, expectoration of bloody mucus and high mortality have been reported **(Bagust et al., 2000)**.

Vaccination is an integral part of ILTV control programmes in breeder and layer flocks, but it has been demonstrated that serial bird-to-bird passage of chicken embryo-origin (CEO) vaccine viruses can result in increased

virulence **(Kotiw et al., 1995)**. On the other hand, it appears that vaccination with tissue culture origin (TCO) vaccines carries less risk for reversion to virulence **(Guy et al., 1991)**.

Presumptive diagnosis of ILT can be made reliably in cases of severe acute disease based on high mortality with typical signs of the disease such as expectoration of blood. Otherwise, diagnosis should be based on one or more confirmatory laboratory diagnostic procedures. The most common laboratory diagnostic procedures are virus isolation, histopathology, detection of ILTV antigen in tissues, and detection of ILTV-specific DNA **(Guy and Bagust, 2003)**. This study aimed to isolation of ILTV from clinically diseased birds suspected to be infected with the virus on the chorioallantoic membrane of embryonated chicken eggs (ECEs), identification of ILTV by agar gel precipitation test, immunoperoxidase and immunoflourescent techniques then confirmation of diagnosis by polymerase chain reaction.

MATERIALS AND METHODS

Samples collection

Twenty larynx and trachea samples were collected from chickens (5 samples from layer chickens and 15 samples from broiler chickens) suspected to be infected with ILTV. Samples were collected from different farms in Dakahlia and Damietta governorates. Diseased birds were showing clinical signs of recumbency, lacrimation, mucoid discharge caused the eyelids to become glued together, and swelling of the infraorbital sinuses, nasal discharge, and at each inhalation the head was extended forward and upward, spasmodic coughing caused expulsion of mucus or blood with drop of egg production in layers. The postmortem findings were hemorrhagic conjunctivitis, tracheitis with blood stained mucous and blood clots. In some cases caseous material was observed in trachea (Fig. 1). Samples were collected from freshly dead or euthanized chickens in sterile containers containing phosphate buffed saline (PBS) with antibiotic mixture then transported to laboratory in ice box then stored at -20oC until use. Other samples were also collected from healthy birds and used as control negative.

Virus strain

Chicken embryo origin vaccine (Avipro ILT vaccine) obtained from manufacturer Lohmann Animal Health GmbH& Co. KG, Germany) The vaccine was prepared in specific pathogen free (SPF) chicken embryo. It was supplied in lyophilized vials, each vial contains 1000 doses, and each dose contains 103 EID50 ILTV. The vaccine used in preparation of hyperimmune serum against ILTV and as a control positive in different diagnostic techniques.

Virus isolation

Trachea and larynx samples were homogenized using mortar and pestle, treated with antibiotic solution (Penicillin G sodium, 1000000 IU & Streptomycin, 1 gm & Kanamycin, 1 gm & Mycostatin 250-500 IU and Distilled water100 ml). The samples were then centrifuged at 3000 r.p.m for 20 minutes as described by **Chacón et al. (2007)**. The supernatant fluids were collected then 0.2 ml of supernatant fluids was inoculated by dropped membrane method onto the CAM of 12 days old chicken embryos as described by **Xie et**

al. (2010). Inoculated eggs were incubated horizontally with the site of inoculation uppermost at 37°C for six days. Embryos which died within the first 24 h were discarded and considered to be nonspecific deaths. The inoculated eggs were examined through six days for the presence of pock lesions. Three to six passages were done depending on appearance of distributed pock lesions. Avipro ILT vaccine was resuspended in PBS; each vial was resuspended in 10 ml PBS to reach final concentration of 10^5 EID_{50} and inoculated on the CAMs of ECEs as described for field samples. The vaccine used as positive control antigen. CAMs showing distributed pock lesions were collected, part of these CAMs were homogenized and centrifuged. The supernatant fluids were collected and used for detection of the virus by AGPT and confirmation of virus diagnosis by PCR. Part of these membranes were collected in 10 % formalin solution and used for histopathological examination. Another part of these CAMs were frozen in cryostat and used for virus detection by indirect immunoflourescent and indirect immunoperoxidase techniques.

Preparation of hyperimmune serum (HIS) against Avipro ILT vaccine:

hyperimmune serum against Avipro ILT vaccine was prepared according to **Rossi (1971)** as follows; five white Newzealand rabbits, 7 weeks old and about 2.5 Kg each. Four rabbits were given firstly 0.25 ml Avipro ILT vaccine having a titer of 10^5 EID_{50} /ml with equal volume of Freund's complete adjuvant by intradermal injection, secondly intramuscular injection of the same viral dose in Freund's complete adjuvant 2 weeks later, finally three intramuscular injections at weekly intervals thereafter. The rabbits were bled 2 weeks after the last inoculation and hyperimmune serum was separated by centrifugation at 3000 r.p.m for 10 minutes and distributed in eppindorff 1ml each and stored at -20°C till use. One rabbit was kept as a control negative. Serum then tested for presence of immunoglobulins by comparing of total protein count in inoculated rabbits serum and control one.

Histopathological examination:

Histopathological examination was done according to **Crespo et al. (2007)** as follows; CAMs containing distributed pock lesions were fixed in 10% neutral-buffered formalin. Paraffin-embedded tissues were routinely processed, sectioned at 4 µm and stained with haematoxylin and eosin (H&E) then examined by light microscope.

Agar gel precipitation test:

AGPT was done according to **OIE (2008)** as follows; the gel is made with agarose (1.5%) containing sodium chloride (8%) in distilled water. The ingredients are autoclaved for 15 minutes; 5 ml of the molten agar is poured into a 5 cm diameter Petri dish. When the agar has set, a pattern of wells is punched in the agar, consisting of a central well and six surrounding wells. The hyperimmune serum is put into the central well (25 µl), while the surrounding wells are filled with suspension of prepared CAMs which inoculated with field samples (25 µl each), one well containing Avipro ILT vaccine as a control positive antigen and one well containing supernatant fluid of prepared uninfected CAM of fertile egg as a control negative antigen. Dishes are incubated in incubator at 37°C with humidity, and

examined 24-48 hours later up to 5 days for lines of precipitation.

Indirect immunofluorescent technique:

Indirect immunoflourescent technique according to **OIE (2008)** and **York et al. (1990)** as follows; the infected CAMs of ECEs showing pock lesions and uninfected CAM (as a negative control) were frozen in cryostat chamber and sectioned about 5 µm thick cryostat sections, then picked up on glass slide. Cryostat sections were fixed in acetone for 10 minutes. Rabbit hyperimmune serum against ILTV was applied to these tissues on the slides and the slides were then incubated in incubator at 37°C for 1 hour with humidity. The slides were washed three times (5 minutes each) in a bath of phosphate buffered saline, pH 7.2. Flourescent conjugated goat IgG fraction to rabbit IgG (whole molecule), anti-rabbit flourescene isothiocyanate (FITC) conjugate with 1:200 dilutions was applied on the slide for 30 minutes in dark humidified chamber. The slides were washed as before and then counterstained with 0.01% Evan's blue, mounted with 50% glycerol in PBS, pH 8.6 and examined using a fluorescence microscope.

Indirect immunoperoxidase test (IP):

Indirect immunoperoxidase test was done according to **Guy et al. (1992)** as follows; CAMs showing distributed pock lesions and uninfected CAM (as a negative control) were sectioned with a cryostat, air-dried then fixed in acetone for 10 minutes. Tissue sections were incubated with rabbit HIS against ILTV for 1 hour at room temperature with humidity, then washing of the slides three times (5 minutes each) in a bath of phosphate buffered saline, pH 7.2. Anti-rabbit horseradish peroxidase (HRP) conjugate of dilution 1:200 in PBS was then added to the slides and incubated for 1 hr at room temperature with humidity, then washed with PBS as before. A substrate-chromogen comprised of orthophenyledene diamine (OPD) solution was added and incubated for 30 minutes. Slides were thoroughly washed and examined by ordinary light microscope .

Polymerase chain reaction:

- **DNA extraction:**

DNA extraction was done according to **Han and Kim (2003)** as follows; CAMs of ECEs inoculated with two field samples (field samples were selected as it gave distributed pock lesions on the third passage in ECEs, clear line of precipitation by AGPT and clear results in IF and IP techniques), uninfected CAM (as a negative control) and CAM inoculated with Avipro ILT vaccine (as a control positive) were used to confirm the diagnosis by PCR. These CAMs were homogenized in PBS, 0.1 M, pH 7.4, followed by centrifugation at 3000 rpm for 20 min. The supernatant was used for the extraction of the viral DNA. 100 µl of the supernatant was added to 900µl of Tris-EDTA buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8.0) containing sodium dodecyl sulfate (final concentration, 0.5%) and proteinase K (500 µ g/ml) and incubated at 37°C for at least 2 hr. The suspension was treated with an equal volume of buffer-saturated phenol, phenol: chloroform: isoamylalcohol (25:24:1) and chloroform: isoamylalcohol (24:1). The DNA was precipitated with isopropyl alcohol and 3 M sodium acetate, pH 5.2, at -20°C for 2 hours. DNA was centrifuged at 12,000 r.p.m for 15 min and washed with 70% ethanol and dis-

solved in 20 µl of sterilized distilled water.

- **Amplification of viral DNA by PCR**

PCR was done according to **Chang et al. (1997)** and **Han et al. (2002)** as follows: A 4.9-kbp fragment of the infected cell protein 4 (ICP4) gene was amplified from isolates using primers as follows: ICP4 forward, 5\ AACCTGTAGAGACAGTACCGTGACCC 3\ and ICP4 Reverse, 3\ CCATTACTACGTGACCTACATTCAGCC 5\. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 0.625 units of Thermo-Start TM Taq DNA polymerase, and 5µl of the extracted target DNA in a total volume of 50 µl The PCR condition for amplification of the 4.9 kbp ICP4 fragment was as following; initial denaturation at 94°C for 1 min followed by 35 cycles of 94°C for 1 min (denaturation), 57°C for 1.5 min (annealing) and 68°C for 4.5 min (extension) then final extension at 68°C for 7 min. The PCR products (12.2 µl) were resolved by electrophoresis with GilPilot 1kb plus ladder (100) (cat no. 239125) in 2% agarose gel (Sigma Chemical Company, USA) and stained with 0.5 mg/ml ethidium bromide using Tris-Borate EDTA buffer (89 mM Tris boric acid, 2 mM EDTA). The gels were run at 90 V/cm for 1 h and examined in an UV transilluminator.

RESULTS AND DISCUSSION

Twenty samples collected from clinically diseased birds suspected to be infected with ILTV were passaged on CAMs of fertile egg aged 12 days. Suspected ILTV could be isolated on CAM in which 14 samples gave characteristic pock lesions in the form of yellowish area with opaque edges (fig. 2) (seven samples after 3rd passage, one sample after 4th passage, 3 after 5th passage and 3 after 6th passage) and the inoculated embryos were dwarfed and died 2-6 days post inoculation. On histopathology, the lesions showing the development of syncytical cells, and eosinophilic intranuclear inclusion bodies (fig. 3). The isolated virus was identified in 14 samples gave clear pock lesions using standard rabbit hyperimmune serum produced against chicken embryo origin vaccine (Avipro ILT vaccine) using different serological tests. Out of 14 samples, 10 (71.4%) tested positive with AGPT and result appear as clear line of precipitin in field samples and control positive well, where control negative well showing no line of precipitin (fig. 4). Eleven samples (78.5%) gave positive results by indirect immunofluorescent technique and the result appear as apple-green fluorescence (fig. 5). Eleven samples (78.5%) gave positive by indirect immunoperoxidase test and the positive results appear as deeply stained dark brown areas (fig. 6).

Analysis of the PCR products obtained from amplification reaction of extracted DNA from inoculated CAMs by agarose gel electrophoresis revealed the positive amplification of 4.9-kbp fragment of the ICP4 gene and (fig. 7) represents the obtained bands.

Table (1): Comparative results of the virus identification using AGPT, IF, and IP test:

positive samples / Total tested samples (percentage)		
AGPT	IF	IP
10/14= (71.4%)	11/14 (78.5%)	11/14= (78.5%)

Avian infectious laryngotracheitis (ILT) is an acute respiratory disease of chickens. Sever epizootic form of ILT are characterized clinically by sever respiratory symptoms such as dyspnea and hemorrhagic expectoration, and are generally associated with high mortality (5-70%). Enzootic infections are associated with mild symptoms as nasal discharge, conjunctivitis, sinusitis, gasping, and decreased egg production or subclinical symptoms **(Mahy and Van Regenmortel, 2010 and OIE, 2008)**.

14 samples gave characteristic pock lesions in the form of yellowish area with opaque edges and the inoculated embryos were dwarfed and died within 2-6 days post inoculation (seven samples gave distributed pock lesions on the CAM after 3rd passage, one sample after 4th passage, 3 after 5th passage and 3 after 6th passage). These results are in concurrence with **Burnet (1934), El-Kenawy et al., (1985), Hidalgo (2003), Chacón et al., (2007)** and **Chacón and Ferreira (2009)** who observed pock lesions on CAMs of inoculated ECEs with death of embryo. On the other hand **Crespo et al., (2007)** and **Sellers et al., (2004)** reported that the ILTV was not easily propagated in ECEs.

Eosinophillic intranuclear inclusion bodies and syncytial cell formation were found in histopathological examination of ILTV infected CAM cells. These results were similar to those reported previously by **Russell** and **Turner (1983), Timurkan et al., (2003)** and **Portz et al., (2008)** who found syncytial cells and eosinophillic intranuclear inclusion bodies in ILTV infected cells. On the other hand **Sellers et al., (2004)** failed to detect these pathological changes.

The results of AGPT appear as clear line of precipitin, 71.4% of samples tested positive by AGPT. These results were in agreement with **Rialakki (1965), El-Zein et al., (1979), El-Kenawy et al., (1985),** and **El-kady et al., (1997)** who described high sensitivity of AGPT for detection of ILTV but **Bastamy et al., (2007)** mentioned that the results of AGPT showed that the AGPT was less sensitive test for virus identification.

In indirect immunoflourescent test the results appear as apple green fluorescence, 78.5% of tested samples were positive. These results were in agreement with **Madbouly et al., (1997), Humberd et al., (2002)** and **Crespo et al., (2007)** who found an apple-green fluorescence, indicating positivity for ILTV, associated with the epithelial cells of the upper trachea. On the other hand **Wilks** and **Kogan (1979)** mentioned that IF was less sensitive test for detection of the virus.

Immunoperoxidase positive results appear as deeply stained dark brown areas, 78.5% of tested samples were positive. These results were in agreement with **Guy et al., (1992)** and **Abbas** and **Andreasen (1996)** who found that the IP was more sensitive test for ILTV diagnosis.

Figures:

1- Trachea of chicken infected with ILTV showing hemorrhage.
2- Characteristic pock lesions on CAM infected with ILTV from collected samples on the third passage.

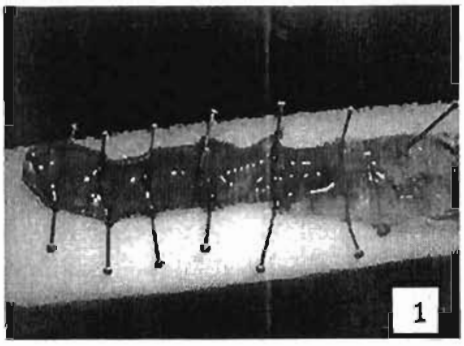
3- AGPT, positive samples produce line of precipitation, A: control positive ILTV, B: control negative (non infected CAM), C, D, E, F: isolated samples on CAMs, S: hyperimmune serum.

4- CAM after indirect If staining. The section show apple green fluorescent color.

5- CAM after indirect IP staining. The section show deeply stained dark brown areas.

6- Section of CAM inoculated with ILTV, showing syncytial cell formation in the CAM cells stained with H & E.

7- PCR products of the ICP4 gene (4900 bp in the left) of ILTV DNA extracted from inoculated CAMs of ECEs in stained agarose gel electrophoresis, along with GIBPilot 1kb plus ladder (100) (M). Lane 1 : positive control sample (Avipro ILT vaccine), Lane 2 : negative control sample Lane 3 & 5 : The amplified DNA products prepared from CAMs. In the right DNA marker measured by base pair (bP).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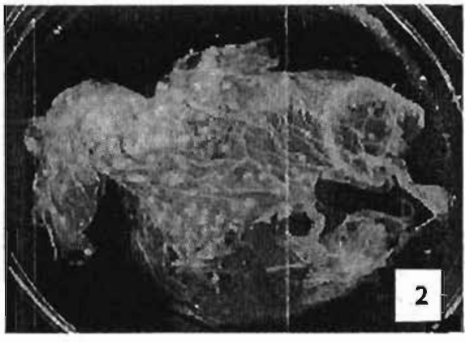
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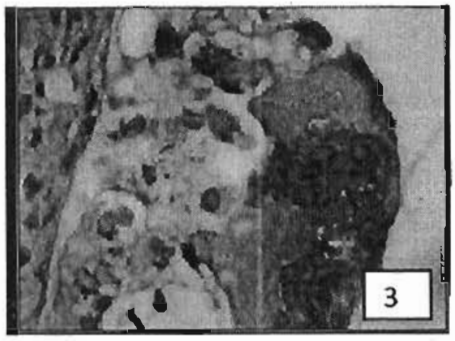
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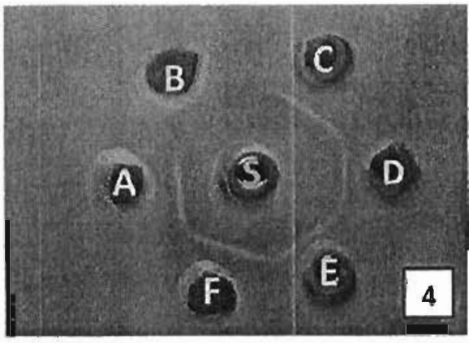
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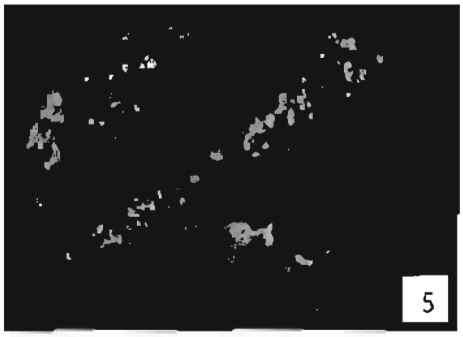
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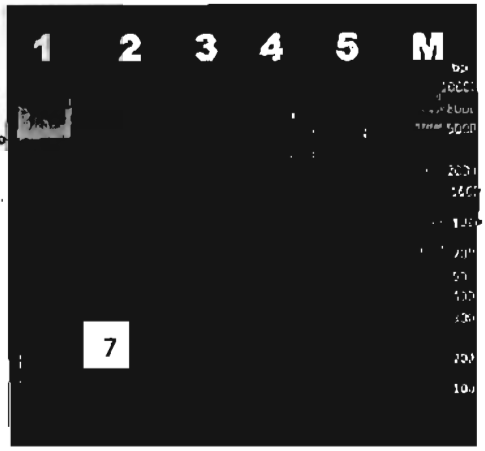
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الملخص العربى

دراسات فيرولجية على فيروس إلتهاب الحنجرة والقصبة الهوائية المعدى (جاليد هريس فيرس- ١) في الطيور

على على ابراهيم القناوى* سماح مسعد مسعد*

على عبد الرشيد على سلامة**

قسم الفيروسات - كلية الطب البيطرى - جامعة المنصورة*

قسم الفيروسات - كلية الطب البيطرى - جامعة الزقازيق**

تمت هذه الدراسة علي ٢٠ عينة تم تجميعها من طيور يشتبه انها مصابة بمرض إلتهاب الحنجرة والقصبة الهوائية المعدى وتشتمل هذه العينات على الحنجرة والقصبة الهوائية .

وقد تم عزل الفيروس على الغشاء اللقانقى المشيمى لبيض دجاج عمر ١٢ يوم وقد تم تمرير العينات من ثلاث إلي ست تمريرات على حسب ظهور بثرات متفرقة على الغشاء.

تم تعريف الفيروس باستخدام تفاعل الترسيب في الاجار و اختبار الفلورسنتى المشع الغير مباشر واختبار الأمينوبيروكسيديز الغير مباشر و الفحص الهستولوجى وتم تأكيد التشخيص باستخدام تفاعل البلمرة المتسلسل.

تم عزل ١٤ عينة على الغشاء اللقانقى المشيمى حيث أعطت ١٠ عينات منها نتيجة إيجابية فى تفاعل الترسيب فى الاجار (بنسبة ٧١,٤٪) و١١ عينة في تفاعل الفلورسنتى المشع الغير مباشر والأمينوبيروكسيديز الغير مباشر بنسبة (٧٨,٥٪) وتوضح هذه النتائج أن اختبار الفلورسنتى المشع الغير مباشر و الأمينوبيروكسيديز الغير مباشر أكثر حساسية من اختيبار الترسيب في الاجار.

وقد تم تحديد ICP4 جين الخاص بالفيروس باستخدام تفاعل البلمرة المتسلسل لبعض المعزولات.