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Isolation and Molecular Characterization of Infectious Laryngotracheitis Virus from Naturally Infected Layer Chicken Flocks in Egypt

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Abstract: Infectious Laryngotracheitis (ILT) is an acute viral disease affecting the respiratory tract of chickens. The present study was conducted for the isolation and molecular characterization of ILT viruses in layer chickens from commercial farms located in four provinces in the Egyptian Nile Delta. In the embryonated chicken eggs (ECE), virus produced white pock lesions on the chorioallantoic membrane (CAM) after the third egg passage. Histopathological examination of ILTV infected CAM revealed the presence of intranuclear inclusion bodies in the infected cells. The identity of the ILT viruses was confirmed by polymerase chain reaction assay (PCR) amplification of a 647bp fragment of thymidine kinase TK gene with subsequent sequencing and phylogenetic analysis. Sequence analysis of the TK gene of the isolates revealed genetic (99-100%) homology to most of the previously published strains.

Key words: ILTV · Egypt · TK Gene

INTRODUCTION

Poultry industry is one of the main agricultural industries in Egypt as it contributes a large part of the country's supply of animal protein (white meats and eggs) [1].Infectious laryngotracheitis is a highly contagious, upper respiratory disease of chickens that cause severe economic losses in chicken farms due to increased mortality, decreased growth rates and sever reduction in egg production [2]. The disease causes respiratory symptoms in chickens, pheasants, partridges and peafowls. Clinical signs include sneezing, gasping, gurgling, rattling, moist rales, conjunctivitis, extension of the neck and coughing of clotted blood. In addition to decreased egg production and high mortality rates due to asphyxiation [3].

The causative agent is Gallid Herpesvirus-1 (GaHV-1), which is a member of genus Iltovirus, subfamily Alphaherpesvirinae and family Herpesviridae [4]. ILT viral particles are icosahedral in shape with hexagonal nucleocapsids about 80-100 nm in diameter consisting of 162 elongated hollow capsomeres. Envelopes surrounding the nucleocapsids have a diameter of 195- 250 nm. [5]. Viral genome consists of a linear double-stranded DNA molecule 155-kb in overall size. It consists of unique long and short sequences flanked by inverted repeat (IR) and terminal repeat (TR) sequences. Five major envelope glycoproteins (gB, gC, gD, gX, gK) and the unique gp60 have been reported which are the major immunogens of ILTV [6].

In Egypt, infectious laryngotracheitis virus (ILTV) has been recorded for the first time during late 1982 and early 1983 causing serious outbreaks of haemorrhagic tracheitis[7]. The ability of ILTV strains to maintain a state of latency in the chickens, especially within the trigeminal ganglion, is a characteristic feature of GaHV-1 that makes the disease difficult to be controlled [8]. The currently available modified live ILTV vaccines provide good protection but may also induce latent infections and even clinical disease if they spread extensively from bird-to-bird in the field [9]. Antigenically, ILTVstrains are considered to be homogenous, but some ILTV strains may naturally vary in virulence [10]. ILTV strains have been differentiated on the basis of plaque size, morphology in cell cultures and pock size on the chorioallantoic membranes of chicken embryos [11].

Laboratory diagnosis of ILTV can be routinely performed by propagation of the virus in ECE and a variety of avian cell cultures such as chicken embryo liver (CEL), chicken embryo lung, chicken embryo kidney (CEK) and chicken kidney (CK) cell cultures [10]. Molecular techniques, including conventional and

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real-time polymerase chain reaction assays, have been used efficiently in the diagnosis of ILTV [12], but unfortunately the characterization of the type of ILTV that is circulating in the field and/or involved in clinical outbreaks is complicated because of the high antigenic and genetic similarity among the ILT viruses [13]. Sequence analysis of the TK and ICP4gene has been established for determining the virulence of ILTV isolates as well as for differentiation between the attenuated vaccine strains and field isolates [14]. In this study we reported the isolation and molecular characterization of infectious laryngotracheitis viruses isolated from clinical samples collected from layer chickens manifested the typical clinical signs of ILT in Egypt during the period from November 2014 till March 2015.

MATERIALS AND METHODS

Samples Collection: A total of 15field samples including whole tracheas, larynges and lung tissues were collected from clinically diseased commercial layer chicken flocks located in 4 provinces in the Nile Delta, Egypt: Kafrelsheikh, El-Gharbia, El-Behera and El-Dakahlia. The birds exhibited severe respiratory manifestations, drop in egg production and relatively high mortality rates. None of these flocks were vaccinated against ILTV. These samples were collected during the period from November 2014 till March 2015. The samples were immediately transported on ice to the lab and stored at -80°C till used.

Virus Isolation: Organ types were pooled and minced using sterile iced mortar and pestle to prepare 10% suspensions in PBS (pH 7.2) solution containing Penicillin (1000IU/ml) and Streptomycin 100ig/ml). Suspensions were centrifuged at 800xg for 10 min at 10°C.The supernatant fluid of each sample was inoculated into three 13- day old SPF eggs (purchased from Kom Oshim farm for SPF-eggs, El-Fayoum, Egypt) via the chorioallantoic membrane (CAM) route for up to three passages. The eggs were incubated at 37°C for 4-7 days and then examined for the presence of abnormal lesions.

Histopathology: Chorioallantoic membranes (CAM) of 13-day old ECE inoculated with Gallid herpes virus 1 were used for histopathology. The specimens were fixed in 10% formalin and then dehydrated in ascending grading of ethyl alcohol, cleared in xylol then embedded in paraffin. 4μm sections were stained with Hematoxylin and Eosin stain (HE) and examined microscopically for the presence of intranuclear inclusion bodies.

DNA Extraction: DNA was extracted from frozen tissue samples as well as from the lyophilized vaccine (Fowl Laryngeotracheitis. Freeze-dried live attenuated vaccine.LT-IVAX.Shering-Plough Animal Health Corp. Nebraska USA) using Gene Jet^{TM} Genomic DNA purification Kit (Fermentas) as per manufacturer's protocol.The purified DNA was stored at -20 °C for further applications.

PCR Amplification of TK Gene: PCR amplification was performed with a set of oligonucleotide primers that specifically amplify a 647 bp fragment of the TK gene according to [15]. The PCR was performed in 50 μl volumes, in which the reaction mixture consisted of 10 μl of5X Master Mix (Taq/High Yield-Jena Bioscience, Jena, Germany) (5X Conc.of thermostable DNA Polymerase, dATP, dCTP, dGTP, dTTP, (NH4) SO4, MgCl, Tween 20, Noniodet P-40, stabilizers), 1 μl of forward and reverse primers, 5 μl of DNA and 33 μl of PCR grade water. The PCR was performed in (Bio Rad T100 thermal cycler). Cycling protocol consisted of initial denaturation at 96°C for 5 min followed by 40 cycles of denaturation at 95°C /1min, annealing at 53 °C /1min and extension at 70°C/1min with a final extension at 72°C /10 min.DNA extracted from lyophilized vaccine was used as positive control. A negative control containing only PCR master mix, primers and PCR grade water was also included. PCR products were analyzed by2%agarose gel electrophoresis and visualized by ultraviolet light transilluminator after staining with 0.1μg/ml ethidium bromide.A 100bp ladder (GeneON, GmbH, Germany) was used as a molecular weight marker.

Purification and Sequencing of PCR Products: PCR products were purified using Gene Jet PCR purification kits (Fermentas) as per manufacturer's protocol. The purified products were submitted for DNA sequencing in both forward and reverse directions using the same amplification primers. The sequencing reaction was performed in an automated sequencer (Macrogen Inc., Korea ABI 3730XL DNA analyzer).

Alignment and Phylogenetic Analysis: Phylogenetic tree was constructed via multiple alignments of 647 bp nucleotide sequence. The tree was analyzed using the neighbor –joining (N-J) tree method using the MEGA version 6 software (www.megasoftware.net) and the liability of the internal branches was assessed by 1000 bootstrap replication.

RESULTS

Virus Isolation: The virus was isolated from the larynx and tracheal suspensions collected from the commercial layer flocks. Stunted growth and congestion were observed in the inoculated embryos. Generalized edema and white pocks were formed on the CAMs of eggs inoculated with the viral suspensions on the third egg passage. The membranes appeared cloudy and were slightly thickened compared with the CAM of non-inoculated control eggs (Fig. 1).

Histopathology: The gross examination of the CAMs revealed multiple white opaque pin point foci. Microscopic examination revealed mild congestion, some degenerative changes in some parts of the membrane with infiltration of inflammatory cells in the mesoderm which mostly of lymphocytes and some macrophages, with intranuclear inclusion bodies in the infected cells (Fig. 2).

PCR and Sequencing: PCR was performed for the field samples as well as the lyophilized vaccine (LT-IVAX). Out of fifteen tracheal samples, eleven samples were GaHV-1 TK PCR-positive;with anexpected amplicon size of 647 bp (Fig. 3).

Alignment and Phylogenetic Analysis: Analysis of the nucleotide sequence of the TK gene amplicon of one field isolate confirmed that it was a GaHV-1. It shared 99%homology with the ILTV/LVV13BR strain (GenBank EF552582.1), TCO IVAX strain (GenBank: JN580312.1), USDA strain (GenBankJN542534.1) and the Intervet UL24 strain (GenBank: HM230798.1).Phylogenetic analysis showed the closest relationship between the ILTV isolated in the present study KFS2015 and the Brazilian isolate Laryngovac (GenBank: FJ4448.30.1) (Fig.4).

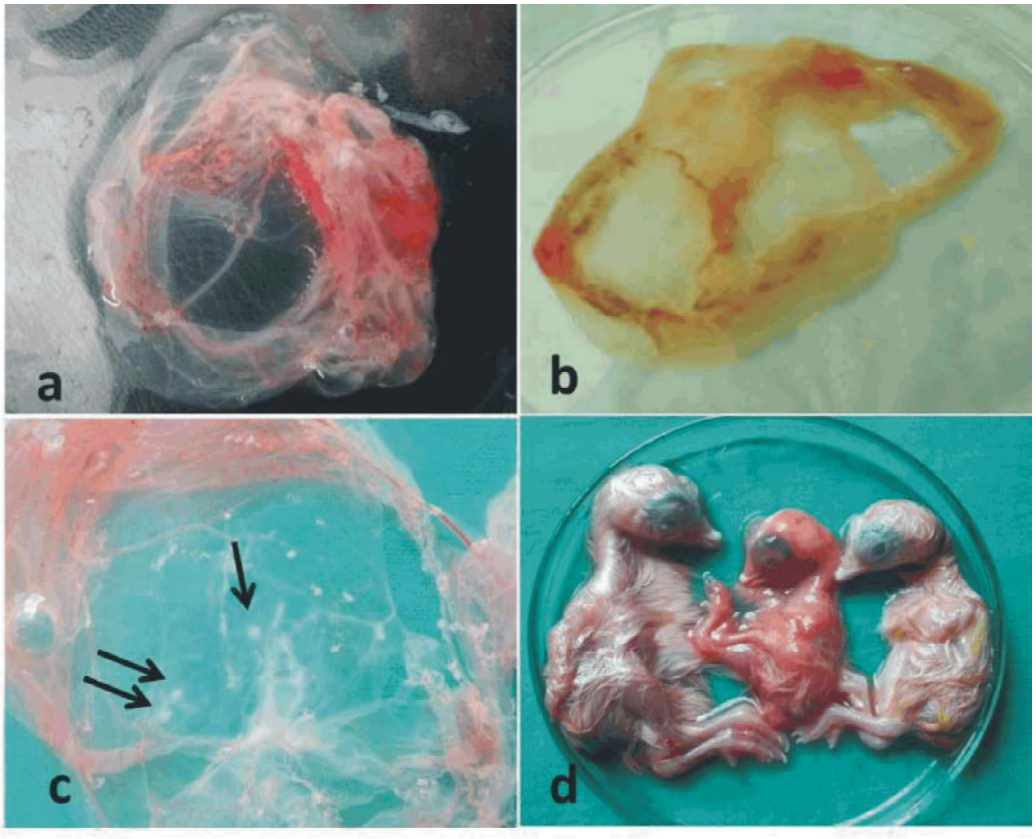

Fig. 1: a.Normal CAM of non-infected embryo. b.CAM of ILTV infected embryo showed thickening and cloudy appearance.c.CAM of ILTV infectedembryo showed white pock lesion after 3rd passage inoculation.d.Normal non infected embryo (left) congested and stunted embryos (right).

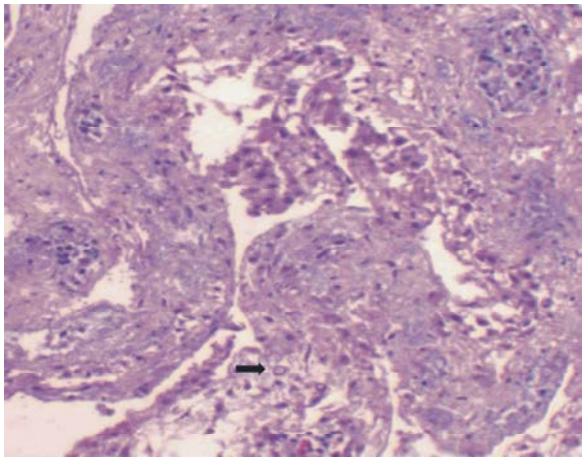

Fig. 2: Histopathology of ILTV infected CAM showing multiple focal lymphocytic infiltrations together withpresence of intranuclear inclusions in the infected cells (arrow). H and E(x20).

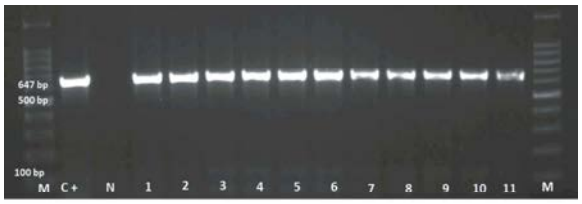

Fig. 3: PCR amplification of 647bp of the TK gene from ILTV infected field samples. Lane M; 100bp DNA size marker.Lane C^{+}; Control positive DNA extracted from ILTV vaccine. Lane N;Negative control. Lanes 1-11 showed positive amplification of TK gene from naturally infected cases.

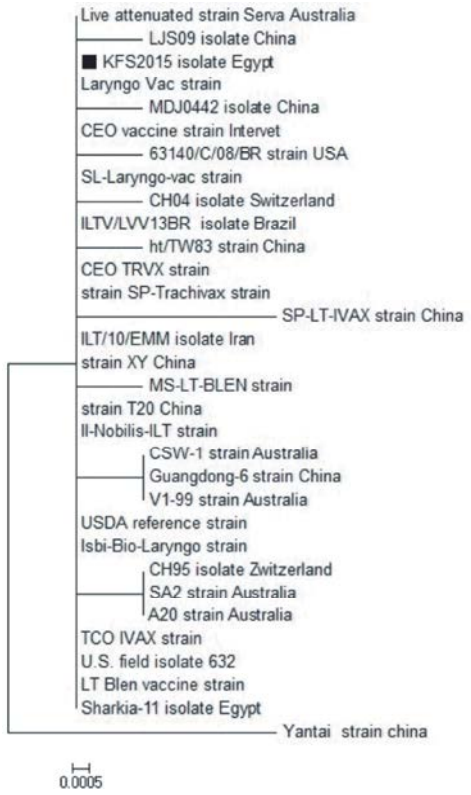

Fig. 4: Phylogenetic analysis of ILTV isolated from Kafrelsheikh province, Egypt,based on TK gene nucleotide sequences of previously published sequences. Phylogenetic tree was constructed via multiple alignments of 647 bp nucleotide sequence and analyzed using N-J analysis with bootstrapping (1000).

DISCUSSION

ILT is a respiratory disease of chickens, pheasant and peafowl caused by Gallid Herpesvirus-1 characterized by severe respiratory manifestations, gasping, sinusitis, conjunctivitis and expectoration of bloody mucus resulting in significant economic impacts on the poultry industries worldwide due to high mortality and/or decreased egg production [16]. In the current study, we described the isolation and molecular characterization of ILT viruses from layer chicken flocks suffering from respiratory distress with relatively high mortality rates. Clinical signs of the examined infected chickens were typically to those previously described [17]. PM examination revealed necrotic tissues and clotted blood along the entire length of the larynx and trachea which is similar to PM signs in previous reports [16]. Virus isolation on CAM of ECE resulted in stunted embryos and mortality within 4-7 days post inoculation in addition to generalized edema and pin point white pock lesions on the CAM which is in accordance with similar studies [10].Histopathological examination of the infected CAM revealed infiltration of lymphocytes and some macrophages in the mesoderm, with intranuclear inclusion bodies in the infected cells which is in consistent with that previously recorded[18].Although virus isolation (VI) technique is regarded as the gold standard among different methods for virus detection, however, as a diagnostic method, VI has some important disadvantages as it is time consuming and samples should be transferred to the laboratory as soon as possible and processed immediately to avoid virus inactivation [19]. PCR assay is the most sensitive and rapid test for the detection of viruses from clinical samples which might appear negative due to inappropriate sampling or loss infectivity during shipment[20]. PCR was performed on all field samples,

eleven samples were TK- PCR positive; with an expected amplicon size of 647 bp. The remaining ILTV negative samples may possibly contain ILT similar viruses such as Avian Influenza virus (AIV), Newcastle Disease virus (NDV) or Infectious Bronchitis virus (IBV). The specificity of the PCR product was confirmed by DNA sequencing of the obtained PCR products. Thus, the PCR described in the present study is a useful tool to confirm the diagnosis of suspected ILT outbreaks as results can be obtained in less than 24 hours, which is an essential point in outbreaks, when fast decisions are required. These results definitely confirm the presence of ILTV in the outbreaks in Egypt.

Vaccination with live-attenuated vaccines has been the principal tool used to control the spread of the disease, two types of live-attenuated vaccines have been widely used, chicken embryo origin (CEO) and tissue culture origin (TCO) vaccines[21].CEO laryngotracheitis vaccine viruses could increase in virulence after bird-to-bird passages causing severe outbreaks in susceptible birds[9].Sequence analysis of the TK gene can be used to differentiate between viruses of low and high virulence as well as between field and vaccinal strains [9].Phylogenetic analysis of the TK gene of our isolate revealed its closest relationship with the Brazilian isolate Laryngovac (GenBank: FJ4448.30.1) while the commonly used ILT vaccine in the area of the current study is the LT-IVAX Shering-Plough, Freeze-dried live attenuated vaccine (Animal Health Corp. Nebraska USA).However, none of the examined flocks were vaccinated. ILT virus strains can move from persistently infected flocks to non-vaccinated birds. In these cases modified live vaccines must to be used, even though they can establish latent infection [6] and so, it is necessary to establish a continuous epidemiological surveillance to determine the incidence and prevalence of the disease, using conventional methods such as isolation in ECE and PCR techniques [6].

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