j.Egypt.net.med.Assoc 79, no 3. 743 – 759 (2019)

ISOLATION AND MOLECULAR CHARACTERIZATION OF CIRCULATING INFECTIOUS LARYNGOTRACHEITIS (ILT) VIRUS IN EGYPT, 2018

By

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ABSTRACT

Infectious Laryngeotrachitis (ILT) outbreaks lead to severe economic losses in the poultry industry in Egypt. At the present study, the aim was to identify the nature of a circulating ILTV (infectious laryngeotrachitis virus) strain in an outbreak occurred in Fayoum province in spring 2018. A field ILTV strain, namely Fayoum 2018, was isolated in SPF-ECE followed by assessment of its pathogenicity in SPF chickens to calculate intra-tracheal pathogenicity index (ITPI). Thereafter, underwent molecular characterization using PCR of two fragments of ICP4 gene (688 bp and 635 bp sizes). The obtained amplicons were sequenced followed by phylogenetic analysis. The isolated Fayoum 2018 virus conferred high virulence with 0.4 ITPI. Phylogeny revealed complete identity with TCO (tissue culture origin) vaccines and the vaccine-related strains, which were previously isolated from Giza and Sharkia provinces through years 2007 to 2016. Our results indicate that Fayoum 2018 isolate retained virulence either through back-passage from bird to bird in unvaccinated flocks or flocks with un-even immunity to ILT, or retained virulence through genomic recombination with other attenuated ILT vaccine virus.

INTRODUCTION

ILT is caused by *Gallid Herpes virus-1* (*GaHV-1),* a member of the family *Herpesviridae*, subfamily Alphah-erpesvirinae, genus Iltovirus **(Murphy *et al.,* 2000)**. ILT disease remains a serious respiratory disease that causes substantial economic losses in poultry industry worldwide **(Bagust *et al.,* 2000).**

In 1982, the first outbreak of the disease was recorded in Egypt, where severe hemorrhagic tracheitis was observed in layers in Cairo and Giza provinces **(Tantawi *et al*., 1983)**.

Later in, 2011 another outbreak of ILT was recognized in a flock of commercial layers in Behera province investigated by sequencing of thymidine kinase (TK) and glycoprotein G (gG) genes. Histopathological examination of infected chickens revealed epithelialsloughing, development of syncytial cells, heterophilic exudation with presence of eosinophilic intranuclear inclusion bodies. The virus was isolated on chorioallantoic membrane (CAM) of embryonated chicken eggs (ECE), and it induced typical pock lesions after two passages. ILTV outbreaks pursued in poultry farms in Behera province **(Shehata *et al.,*2013)** where referred to chicken embryo origin (CEO) vaccine related strains and Sharkia governorate **(Esraa, 2016)** where attributed to tissue culture origin (TCO) vaccine related strains.

Despite their antigenic homogenicity, ILTV strains naturally vary in virulence from highly-virulent strains that induce high morbidity and mortality to low-virulence strains, which cause mild to unapparent infection. Like alphaherpes viruses in different species of animals, ILTV can establish a carrier state in recovered animals **(Bagust, 1986)**.

In chickens this is characterized by periods of latency interspersed with episodes of intermittent virus shedding detectable by tracheal swabbing of live recovered birds and the shed virus continues to infect naive birds and become more virulent simply by bird-to-bird passage. Back passage of vaccine viruses through inadequately vaccinated flocks with concurrent reversion to virulence remains one of the main sources of ILT outbreaks in Egypt and worldwide **(Abdo *et al.,* 2017; Menendez *et al.,* 2014; Shehata *et al*., 2013; Rodríguez-Avila *et al.,* 2007; Saif *et al.*, 1994; Hughes *et al*., 1991; Hughes *et al.,* 1989).**

In the current study, isolation and molecular characterization of ILTV from Fayoum province was performed to identify the nature of the currently circulating virus strain.

MATERIAL AND METHODS

Clinical samples and Virus strains:

Attempting to isolate and identify the causative agent, exudates and epithelial cells were collected from the trachea of 100 diseased birds which housed in an open system farm of 13000 birds/house capacity at Fayoum province. The birds had no vaccination history with ILT vaccines. Exudates and epithelial cells scrapes were diluted 1/5 in nutrient broth containing 1 million i.u. concentrations of penicillin and 1 gm streptomycin antibiotics, then agitated vigorously. The resulting suspension was centrifuged at 1500 rpm at 4°C for 15 minute followed by inoculation onto chorio-allantoic membrane (CAM) of 12 day old SPF embryonated chicken eggs (SPF-ECE) for virus isolation. Field isolate strain, namely Fayoum 2018, was isolated and titrated on SPF-ECE before molecular characterization using polymerase chain reaction (PCR) and nucleotide sequencing. Live attenuated egg adapted ILT vaccine strain (chicken embryo origin vaccine - CEO) of 4.8 $\log^{10}$ EID_{50}/dose approximate titer was used in heam-agglutination test, pathogenicity test and in PCR.

Pathogenicity test:

Twenty SPF chickens of 3 weeks old were divided into 2 groups, A and B, of 10 birds each, in separate isolators and all had free access to food and water. Group A was inoculated intra-tracheal with 100µl of $10^{3.5}$ EID_{50} of isolated virus. Group B was left as control negative. Birds were scored for clinical signs for 14 days post inoculation**.** Intra-Tracheal pathogenicity Index (ITPI) was calculated as described by **Guy *et al*. (1990)** where clinical signs exhibited by each bird were scored daily for 14 days as follows: O, normal; 1, respiratory signs; 3, death. Indices were determined by dividing the sum of the scores by the total number of chickens and values were expressed as means.

Virus Propagation and Titration:

Virus isolation and propagation was performed following the standard procedure of **OIE (2014)**. Briefly, tissue homogenate of the pooled samples and ILT vaccine strain were inoculated on the CAM of 12 day old SPF-ECEs. Both inoculated and non-inoculated (negative control) eggs were incubated at 37°C and observed by candling for 5 days post inoculation (dpi), the eggs were then opened and CAMs were cut to collect pock lesions areas before pooling and further passage of the virus in ECEs. To titrate the ILT virus, pooled pock lesions were homogenized in sterile PBS as 1/10 (V/V) containing penicillin and streptomycin

at a concentration of 1000 IU and 1000 µg/ml, respectively, then 3 cycles of freezing and thawing were performed before inoculation into 5 SPF-ECEs for each dilution and pock lesions were detected after 5 days post inoculation then titer was calculated using statistical method described by **Reed and Muench (1938).**

Hemagglutination (H.A) test:

Applied on pooled chorioallantoic membranes inoculated with previously mentioned collected samples as described by **Guy *et al.* (1991).**

Extraction of viral DNA:

Virus suspension of isolated virus prepared, as described before, from pock lesions detected on chorio-allantoic membranes of infected SPF chicken eggs, was centrifuged at 4800 x g for 15 minutes at 4°C to obtain a cell free suspension. 400 µl of virus suspension were used to extract viral DNA using PureLink™ viral RNA/DNA mini kit (invetrogen, USA) following manufacturer instructions. Briefly, viral particles in the cell-free prepared sample were lysed using Proteinase K and Lysis Buffer (L22) at 56°C. Ethanol was added to the lysate to a final concentration of 37% and the sample was loaded onto a silica spin column. The viral DNA molecules bound to the silica-based media and impurities such as proteins and nucleases were removed by thorough washing with Wash Buffer. The DNA was then eluted in sterile, RNase free water before preservation at -70°C.

Amplification and sequencing of ICP4 gene fragments and phylogenetic analysis:

Two fragments of the Intra-cellular pathogenicity-4 (ICP4) gene of 688 bp and 635 bp sizes, respectively were amplified in 50 µl volume Polymerase chain reaction (PCR) composed of 5 µl of 10x Dreamt Green buffer, 1 µl 10 mM dNTPs mix, 5 units of Dreamt polymerase (Thermo Fischer scientific, USA), 30 pmole of each primer (ICP4-1F (5'-ACT-GAT-AGC-TTT-TCG-TAC-AGC-ACG-3') and ICP4-1R (5'-CAT-CGG-GAC-ATT-CTC-CAG-GTA-GCA-3') amplify a 688 bp fragment at position 181 to 869; ICP4-2F (5'-CTT-CAG-ACT-CCA-GCT-CAT-CTG-3') and ICP4-2R (5'-AGT-CAT-GCG-TCT-ATG-GCG-TTG-AC-3') amplify a 635 bp fragment at position 3804 to 4440, and nuclease free water to 50 µl volume. PCR conditions were as follows: strand separation at 95°C for 5 min, followed by 35 cycles of 95°C for 15 seconds, 65°C for 30 seconds, and 72°C for 45 seconds. Finally, there was 7 minutes at 72°C for further strand extension. Then, 10µl of the amplified PCR product was separated by electrophoresis on 1.5% agarose gel in parallel with a GeneRuler 1 kilo base DNA ladder ready to use (Thermo scientific, USA) **(Chacon and Ferreira, 2009; Kaya and**

Akan, 2018). Before sequencing, PCR amplicons of ICP4 gene of 688 and 635 bp sizes were purified using QIAquick gel and PCR purification kits (QIAGEN, Germany), and DNA concentration was determined using Qubit 4 fluorometer and DNA high sensitivity (HS) quantification kit (Thermo scientific, USA), thereafter purified amplicons were submitted to GATC company (Germany) for sequencing. The similarity of the sequences was evaluated through alignment and comparison with sequences from GenBank using BLASTn. Homology and phylogenetic analysis were performed using MEGA-X software package.

RESULTS

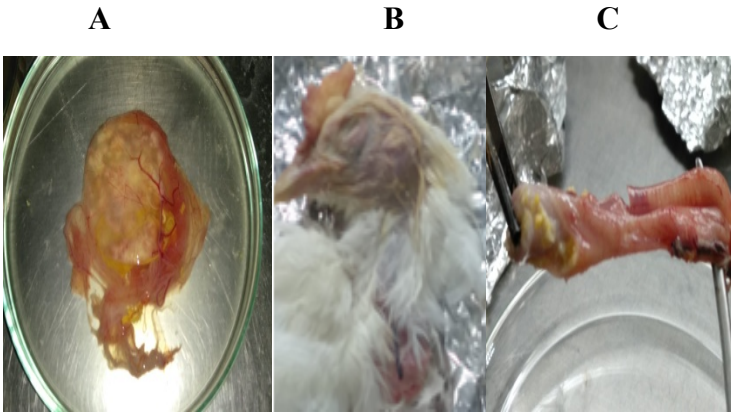

Fig. (1): Isolation of ILTV in embryonated SPF chicken eggs and pathogenicity testing in SPF chickens.

(A). Chorio-allantoic membranes showing pock lesions characteristic for ILTV virus were collected from inoculated eggs.

(B). SPF chickens inoculated by *intra-tracheal route* with homogenates of pock lesions showing swelling of the infra orbital sinuses (almond shaped eyes), and nasal discharge.

(C). Postmortem tracheal hemorrhages and tracheitis of chickens inoculated with chorio-allantoic membranes with pock lesions.

Table (1): Pathogenicity studies of Fayoum 2018 field isolate and CEO vaccine strain in 6 week old SPF chickens.

Virus	Observation for 8 dpi		ITPI*
	Morbidity %	Mortality %	
ILT field isolate	60	40	0.4**
ILT CEO vaccinal strain	0	0	0.0**

*** ITPI: Intra Tracheal Pathogenicity Index**

***ITPI was calculated as described by Guy *et al.* (1991), values are expressed as means.**

The clinical signs exhibited by each bird were scored daily for 14 days as follows: O, normal; 1, respiratory signs; 3, death. Indices were determined by dividing the sum of the scores by the total number of chickens.

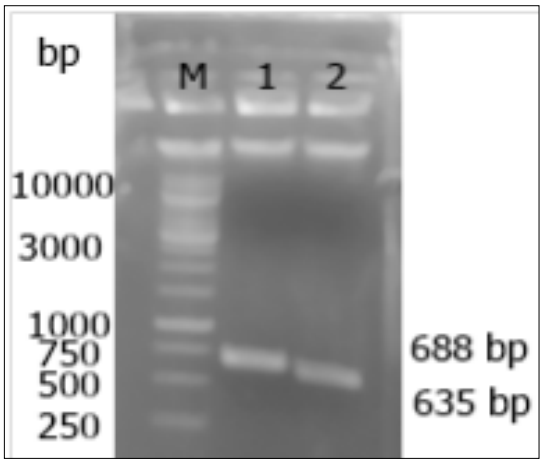

Fig. (2): Amplification of ICP4 gene fragments: Agarose gel electrophoresis of two fragments of ICP4 gene of 688 bp (lane 1) and 635 bp (lane 2) sizes, respectively, when compared to a 10 kb O'GeneRuler DNA ladder (M).

Fig. (3): Evolutionary analysis of amino acids coded by a 635 bp ICP4 gene fragment using Neighbor-joining method. An evolutionary analysis was conducted in MEGA X software by applying Neighbor-Joining method the rooted tree was drawn to scale with branch lengths measured in the number of substitutions per site. This analysis involved 12 amino acids sequences clustered in two main clusters evolved from a root node or a most recent common ancestor. Fyoum 2018 isolate (MN082684) clustered with TCO vaccine strain (EU104908) and Egyptian strains (MH794584, MH794585, MH794586, MH794587, and JX273230) characterized previously as TCO vaccine related. Controversially, Egyptian isolates Behera 2007, 2009, 2010 with gene bank accession numbers JX273231, JX273233, JX273232, respectively, clustered with CEO vaccine strain (EU104900) and originated from a different internal node.

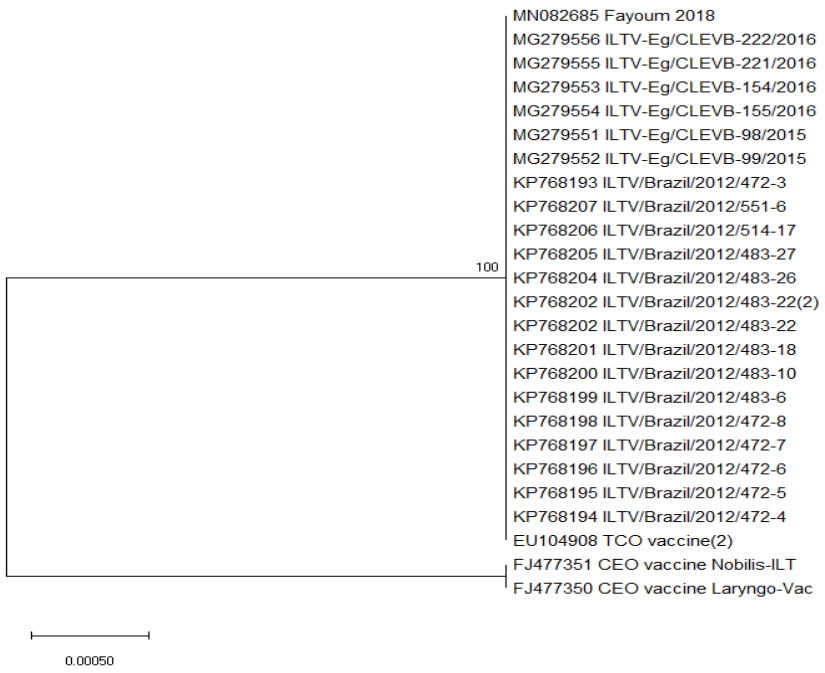

Fig. (4): Amino acids evolutionary analysis of a 688 bp fragment of ICP4 gene. Analysis of 25 amino acids sequences represented in a Neighbor-Joining rooted tree and 1000 boots-trapping replicates using MECAX software package. The tree showed two main clusters originating from the same recent common ancestor. Fayoum 2018 isolate (MN082685), clustered with the TCO vaccine (EU104908) and TCO vaccine related Egyptian isolates(MG279551,MG279552,MG279553, MG279554, MG279555, MG279556) isolated from Sharkeya governorate isolated on years 2015-2016, with 100% confidence of complete identity. At the same context, the same clustered included Brazillian TCO vaccine related strains. In contrast, two CEO vaccines, namely Nobilis-ILT and Laryngo-vac, with gene bank accession numbers FJ477351 and FJ477350, respectively, were clustered separately far away from TCO vaccine and TCO vaccine related strains including Fayoum 2018.

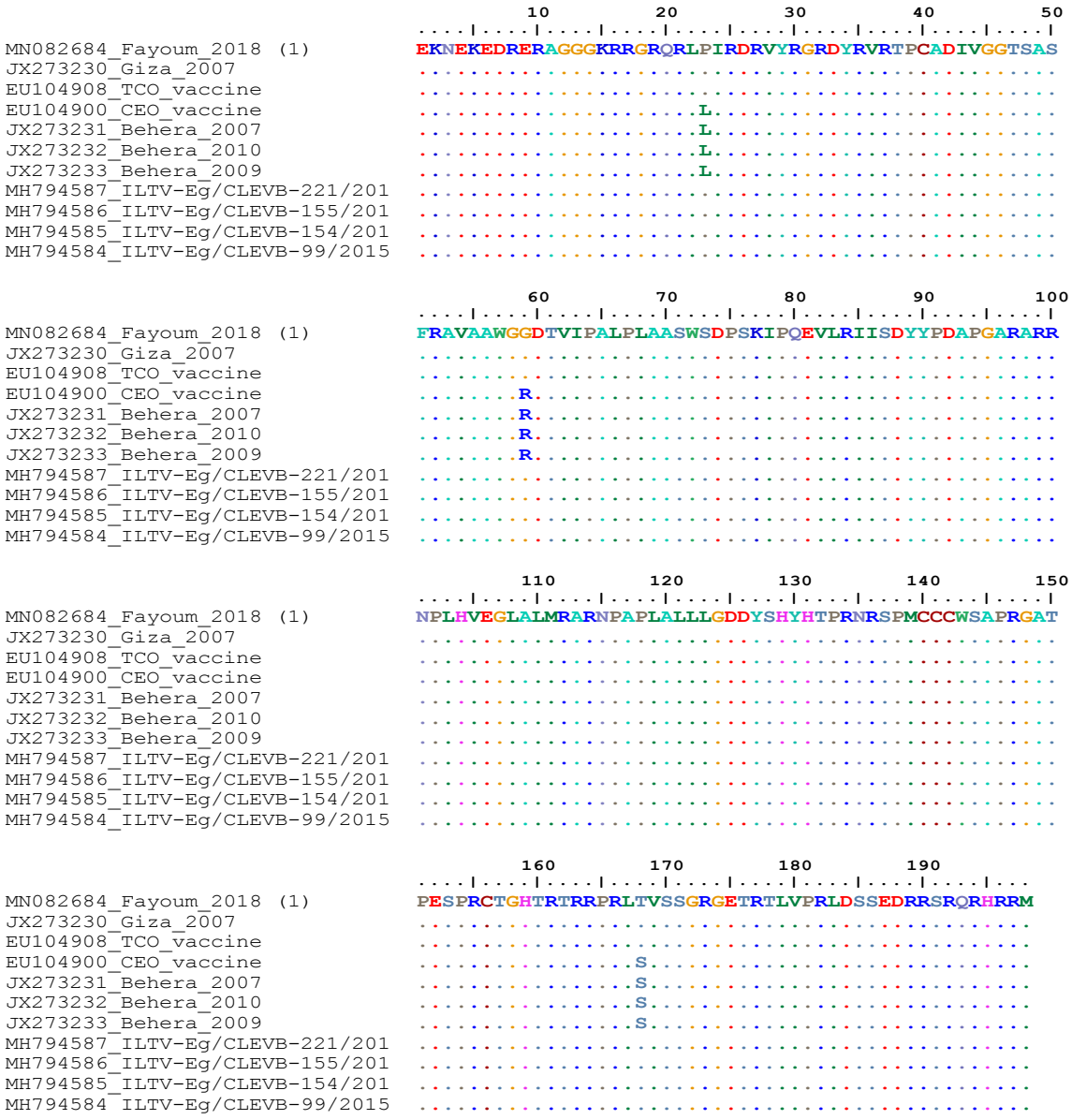

Fig. (5): Deduced amino acids sequence alignment of 635 bp fragment of ILTV ICP4 gene Amino acids sequence alignment revealed complete identity of Fayoum 2018 isolate (GB: MN082684) with TCO vaccine strain (GB: EU104908), and Egyptian TCO vaccine related strains (GB: JX273230, GB: MH794584, GB: MH794585, GB: MH794586, and GB: MH794587). Contrarily, CEO vaccine (GB: EU104900) and Egyptian CEO vaccine related strains (GB: JX273231, JX273232, and JX273233) showed three amino acids mismatches with TCO related strains, namely Lucien (L) instead of Proline (P), Arginine (R) instead of Glycine (G), and Seine (S) instead of Threonine (T).

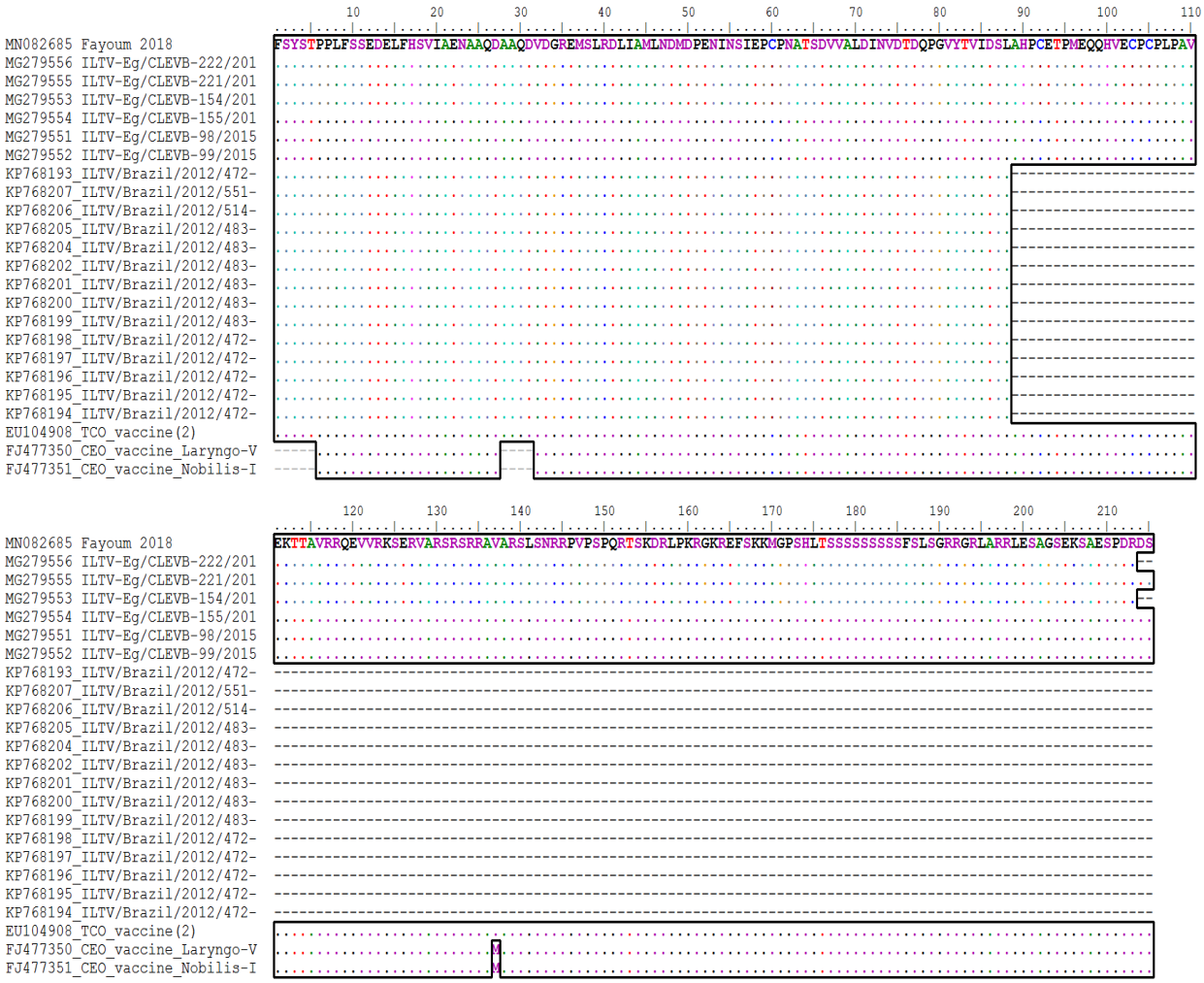

Fig. (6): The deduced amino acids sequence alignment of 688 bp fragment of ILTV ICP4 gene Dots (...) represent identical amino acids (aa) and stretches represents deletion or absence of aa. Identical sequences were outlined with a frame. sequence alignment revealed complete identity of Fayoum 2018 isolate (MN082685) with TCO vaccine (EU104908) and Egyptian TCO vaccine related strains isolated from Sharkeya governorate years 2015 and 2016 (GB accession numbers: MG279551, MG279552, MG279553, MG279554, MG279555, MG279556). Overlapping deduced aa sequence with Brazilian TCO vaccine related strains (GB accession numbers: KP768193, KP768194, KP768195, KP768196, KP768197, KP768198, KP768199 KP768200, KP768201, KP768202, KP768203, KP768204, KP768205, KP768206, KP768207), reveled complete identity. Amino acid mismatches with CEO vaccines Laryngo-Vac (FJ477350) and Nobilis (FJ477351) were observed as a deletion of a four amino acids length stretch deletion [AAQD] and a mutation of valine (V) to Methionine (M).

DISCUSSION

ILT is a respiratory disease of poultry with different degrees of virulence varying from mild, without apparent clinical signs indistinguishable from other mild respiratory diseases, to high virulence with severe tracheal lesions accompanied by difficulty of breathing with extension of the neck gasping, gurgling, rattling, nasal discharges, bloody cough, swelling of head and eyes, conjunctivitis, swelling of the infra-orbital sinuses (almond shape eyes). Post-mortem changes are confined to the upper respiratory tract and are also characteristic, consisting of hemorrhagic tracheitis with blood clots, mucoid rhinitis, and blood-stained mucus along the length of the trachea **(Guy and Bagust, 2003; Ou and Giambrone, 2012**).

At the current study, isolation and molecular characterization of ILTV were reported, where signs of respiratory disease typical to severe form of ILTV with high mortality rate reaching 50%, were observed in an open system farm of 13000 birds/house capacity at Fayoum province in March 2018. The farm had no vaccination history with ILT vaccines. Pock lesions were detected on dropped CAM of 10-12 days-old SPF-ECE Fig. (1a) as mentioned before for alpha herpes viruses to which ILTV belongs **(Jordan and Chubby,1962).** Assessment of pathogenicity of the virus isolate was performed in SPF chickens by calculating the ITPI in which the clinical signs and mortalities exhibited by each bird were scored daily for 14 days. while negative control birds exhibited 0.0 value for ITPI, Fayoum 2018 isolate showed ITPI value of 0.4 (Table 1). While ITPI values of ILT vaccine strains ranges from 0.0 to 0.14, values of 0.2 to 0.82 were reported for ILT viruses with high virulence **(Guy *et al.,* 1990).** According to these criteria, our local isolate, namely Fayoum 2018, can be characterized as highly pathogenic ILTV strain. This finding comes in accordance with reports of **Shehata *et al.,* (2013)** for the Egyptian CEO ILTV highly pathogenic strains isolated from Behera province on years 2007, 2009, and 2010 which showed ITPI of 0.4 while the low pathogenic TCO isolate Giza 2007 had an ITPI value of 0.0. At the same context, **Esraa (2018)** classified ILTV isolates based on in-vivo pathogenicity test to low and high virulence strains, where the TCO vaccine related strain namely; ILT-155 caused 30% mortalities in SPF chickens and classified as high virulence virus. On the other hand, four TCO vaccine related strains namely; ILT-99, ILT-154, ILT-221, and ILT-222 isolated from field outbreaks and causing

high morbidity and high mortalities in infected poultry flocks. These four strains caused 0% mortalities in SPF chickens and classified as low virulence virus strains.

Esraa (2018) attributed this finding to back passage and circulation of these viruses from bird to bird and from unvaccinated flock to other in the areas of high density poultry populations like Sharkeya, which comes in accordance with our report for isolation of Fayoum 2018 strain from unvaccinated poultry flock at Fayoum province. In parallel to virus back passage in unvaccinated flocks, **Esraa (2018)** mentioned the possibility of bacterial co-infection as a predisposing factor for the high mortality accompanied with the infection of the low virulent TCO ILT-99, ILT-154, ILT-221, and ILT-222. Contrary, negative hemagglutination (HA) results of allantoic fluid of the SPF-ECE inoculated with Fayoum 2018 virus isolate supports the notion that Fayoum 2018 isolate is a highly pathogenic strain causing high mortality rates in the field excluding that a mixed infection with other respiratory disease agents was attributed to high rates of the observed field mortalities. In addition to negative results of H.A.test, no postmortem lesions like pericarditis and/or pneumonia characteristic for bacterial infection was observed. In parallel, postmortem examination of the dyed birds at the pathogenicity test showed almond shape eyes Fig. (1b) due to swollen infra-orbital sinuses and hemorrhagic inflammation with bloody mucous along the trachea Fig. (1c) characteristic for ILTV. Infected cell protein 4 (ICP4) or transcriptional regulator ICP4 is an immediate early (IE) protein of alpha herpes viruses essential for virus gene expression early in infection by controlling host cell RNA polymerase II-mediated transcription. ICP4 is commonly used in epidemiological studies to determine the origin of the circulating viruses in different outbreaks **(Johnson *et al.,* 1995; Chang *et al.,* 1997; Chacon *et al.,* 2010; Chacon *et al.,* 2015). Chacon and Ferreira (2009)** designated two fragments of the ICP4 gene at positions 181 to 869 and 3804 to 4440, to be targeted in PCR followed by sequencing for detection and typing of ILTV. Here, viral nucleic acid was detected in extracts from CAM pocks by PCR targeting the above mentioned two fragments of infected-cell polypeptide-4 (ICP4) gene using the primers designed previously by **Chacon and Ferreira (2009).** PCR generated the expected sizes, 688 bp and 635 bp, for the two amplicons, respectively Fig. (2) characterizing GaHV-1 viruses. To further characterize and elucidate the origin of Fayoum 2018 isolate, obtained fragments were submitted for sequencing. The obtained nucleotide sequences were deposited at gene bank with accession numbers MN082684 and MN082685. Deduced amino acids sequence of the 688 bp fragment Fig. (6) revealed complete identity of Fayoum 2018

with TCO vaccine related Egyptian isolates ILT-99, ILT-154, ILT-155, ILT-221, and ILT-222 **(Esraa, 2016).** Similarly, overlapping deduced amino-acid sequence of Fayoum 2018 isolate showed complete identity with the TCO vaccine related Brazilian strains (GB accession numbers: KP768193, KP768194, KP768195, KP768196, KP768197, KP768198, KP768199, KP768200, KP768201, KP768202,KP768203, KP768204, KP768205, KP768206, KP768207) Fig. (6). On the other hand, Fayoum 2018 isolate and TCO vaccine strains are characterized and differentiated from CEO vaccine strains (FJ477350 [Laryngo-vac] and FJ477351 [Nobilis-ILT]) by the absence of a deletion mutation in the 272-283 bp region of ICP4 leading to four amino acids (alanine (A) - alanine (A)-glycine (Q)-aspartate (D) deletion in the 688 bp analyzed fragment Fig. (6), consistent with the data published before **(Chacon and Ferreira, 2009; Couto *et al.,* 2015).** In parallel, CEO vaccine strains analyzed at this study formed a separate branch in phylogentic tree Fig. (4) while Fayoum 2018 isolate clustered with TCO vaccine and vaccine related strains, indicating that Fayoum 2018 is a TCO vaccine related field isolate. Supporting to this notion CEO vaccine strains Laryngo-vac and Nobilis-ILT showed a substitution of valine amino acid with Methionine at ICP4 amino acid position 200 (V200M) Fig. (6).

Sequence analysis of a 635 bp fragment of ICP4 gene at position 3804 - 4440 was reported to discriminate between TCO and CEO origin viruses where CEO strains show three amino acids substitutions in their ICP4 gene to leucine (L), arginine (R), and serine (S) at positions 1302, 1398, and 1447, respectively. On the contrary to the amino acid residues proline (P), glycine (G), and threonine (T) at these particular positions in TCO vaccines **(Chacon *et al.*, 2015; Shehata *et al.,* 2013; Chacon *et al*., 2010).** Sequence analysis of the local field isolate, Fayoum 2018, showed the same substitutions of amino acids, confirming the notion that it is a TCO vaccine related virus Fig. (5). at the same context, phylogenetic tree based on deduced amino acids sequence of the 635 bp fragment Fig. (3) showed clustering of Fyoum 2018 isolate (MN082684) with TCO vaccine strain (EU104908) and Egyptian strains (MH794584, MH794585,MH794586, MH794587,and JX273230) characterized previously as TCO vaccine related **(Shehata *et al.,* 2013; Esraa, 2016).** Controversially, Egyptian isolates Behera 2007, 2009, 2010 with gene bank accession numbers JX273231, JX273233, JX273232, respectively, identified before as a CEO related field isolates **(Shehata *et al.,* 2013)** were clustered with CEO vaccine strain (EU104900) and originated from a different internal node Fig. (3).

Different vaccines against ILTV are used in Egypt including live attenuated TCO and CEO vaccines and like Giza, or live attenuated TCO vaccine strain used to vaccinate neighboring flocks. Latent infection due to virus shedding appears when privilege conditions like flock transportation and re-housing, onset of lay, flock recombinant live vectored vaccines like HVT-ILT and FPX-ILT vaccines. Residual infection and revert to virulence were reported for live attenuated CEO and TCO vaccines strains leading to reemergence of outbreaks even after long time of infection start. This phenomenon was explained by circulation or bird to bird back passage of attenuated vaccine strains or the shed viruses in birds with low levels of immunity against ILT due to uneven vaccination of the flock or infection of unvaccinated susceptible neighboring flocks inducing sever disease. This may be the most possible scenario of our case for Fayoum 2018 isolate which is a possible reverting virus to virulence arose from a TCO vaccine related strain like ILT - Giza 2007 circulating a geographically adjacent area to Fayoum province over crowdedness, or high humidity in rearing house combined by low bio-security measures, occurs, leading to virus spread and continuous contamination of the environment **(Hughes *et al.,* 1989; Williams *et al.,* 1994; Kotiw *et al.,* 1995; Gracia and Riblet, 2001; Neff *et al.,* 2008; Oldoni *et al.,* 2008; Chacon and Ferreira, 2009; Shehata *et al.,* 2013, Couto *et al.,* 2015)**. An alternative scenario for emergence of a TCO vaccine related strain retaining its virulence like Fayoum 2018 isolate, is natural recombination between attenuated ILTV vaccine strains as a mechanism of virus diversity and evolution. Possibility of this scenario directs us to the use of vaccines that have the potency to limit virus replication rather than vaccines limiting clinical signs, as a control measure limiting ILT virus latency and recombination in the infected or vaccinated flocks **(Loncoman *et al.,* 2017; Lee *et al.,* 2012).**

In conclusion,virulent ILTV field strain was isolated from Fayoum province and characterized molecularly as a TCO vaccine related strain. Virulence of Fayoum 2018 isolate was assumed to be retained either by back passaging of an attenuated TCO vaccine strain in susceptible birds in unvaccinated flock, or from genetic recombination of two different TCO vaccine strains, pointing to the need for preparation of recombinant ILTV vaccines that can limit virus replication to control virus latency.

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