RESEARCH ARTICLE

Molecular Characterization of Thymidine Kinase and Glycoprotein G Genes from a Possible Vaccine Induced Infectious Laryngotracheitis Outbreak in Egypt

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A B S T R A C T

Infectious laryngotracheitis virus (ILTV) is a worldwide cause of acute respiratory disease in chickens. In this study, an outbreak of laryngotracheitis in commercial layers flock, Sharkia Province, Egypt in 2011 was investigated by sequencing of thymidine kinase (TK) and glycoprotein G (gG) genes. The chickens showed clinical ILTV infection, histopathological examination revealed epithelial sloughing, development of syncytical 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. The obtained 647 amplicon by PCR confirmed the presence of ILTV genome. Gene sequencing of TK and gG showed high genetic homology between field isolate from commercial layer flock (Sharkia-11) and vaccine strains. To our knowledge, this is the first documented ILTV outbreak attributed to vaccination with live modified ILT vaccine in Sharkia, Egypt based on sequence analysis of TK and gG genes.

To Cite This Article: Ali AMA, SMG Mansour, MHA Mohamed, H Ali and A Shahin, 2014. Molecular characterization of thymidine kinase and glycoprotein G genes from a possible vaccine induced infectious laryngotracheitis outbreak in Egypt. Pak Vet J, 34(3): 381-385.

INTRODUCTION

Infectious laryngotracheitis virus (ILTV) of the subfamily *Alphaherpesvirinae*, family *Herpesviridae* (Davison, 2010) causes an acute respiratory disease in chickens. The disease causes substantial economic losses in poultry industries worldwide (Bagust *et al.,* 2000). The virus contains a linear, double-stranded DNA genome with unique long and short segments flanked by inverted repeats (Johnson *et al.,* 1991).

Similar to other *herpesviruses*, ILTV can set up latent infections within the trigeminal ganglion, inducing latently infected chickens that act as a primary source of ILT outbreaks (Williams *et al.,* 1992). Modified live vaccines are available commercially in two types; tissue culture origin (TCO) and chicken embryo origin (CEO). Major drawbacks of such vaccines are their ability to infect non-vaccinated birds, virulence restoration and induction of latent infection (Guy *et al.,* 1991; Hughes *et al.,* 1991a; Kotiw *et al.,* 1995). Furthermore, the differentiation between the modified live vaccine and field isolates is difficult due to their high antigenic and genomic similarity, and this hinders the control of the disease (Guy and Bagust, 2003).

The disease was recorded for the first time in Egypt in 1982, when severe hemorrhagic tracheitis outbreaks occurred in layers on multiple farms in Cairo and Giza districts (Tantawi *et al.,* 1983). The molecular characterization and recognition of ILTV strains is essential in the epidemiological studies to determine the origin of ILTV strains involved in outbreaks or circulated in the farms. Thymidine kinase (TK) gene has been linked to ILTV virulence (Han and Kim, 2001) while glycoprotein G (gG) reported to play an important role in the pathogenicity of the virus (Devlin *et al.,* 2006).

Sequence analysis of multiple viral genes particularly of the ICP4 and UL47 genes is considered a valuable tool to differentiate ILTV isolates (Creelan *et al.,* 2006; Ojkic *et al.,* 2006). Sequencing of TK and gG genes allowed distinguishing between the vaccine and field isolates (Han and Kim, 2001). To determine the virulence of the virus isolates involved in an outbreak of laryngotracheitis in the Egyptian commercial layers flock reported here, DNA sequencing of TK and gG genes was performed.

MATERIALS AND METHODS

Samples: Samples of larynx, trachea and lungs tissues were collected from chicken for virus propagation, identification and histopathological evaluation. These samples were collected in the summer of 2011 during a major respiratory disease outbreak in a commercial layers farm. Birds exhibited conjunctivitis, depression, gasping, coughing, nasal discharge, expectoration of blood-stained mucous, increased mortality and drop in egg production. Layers were not vaccinated against ILT, yet other hens with unknown history were introduced to the farm occasionally.

Histopathology: Samples were fixed in 10% formalin and processed routinely for histological evaluation. Sections were stained with Hematoxylin and Eosin (HE) and examined under light microscope for any significant findings (Mashkoor *et al.*, 2013).

Virus isolation: Commercial ECE (10-12 old days) from an ILTV vaccination free flock were used for isolation and propagation of the ILT virus. The samples were inoculated via the chorioallantoic membrane (CAM) and eggs were incubated at 37°C for 5 days. Same procedures were repeated with inoculation of minimum essential medium (MEM) instead of samples as a negative control. Five days post-inoculation, the CAMs were collected, homogenized and passaged four times successively. The presence of plaques on the CAMs indicated ILTV replication. Single plaque was utilized for DNA extraction.

Extraction of viral DNA and PCR amplification: Viral DNA was extracted from CAM plaques using the GeneJET™ DNA purification kit (Fermentas) following the manufacturer's instructions. The DNA was dissolved in 50 µl elution buffer and stored at -20°C. The PCR was performed with a set of ILTV specific primers amplifying a 647-bp fragment as described by Pang *et al.* (2002). The positive ILTV isolates were submitted to molecular characterization.

Sequencing of thymidine kinase and glycoprotein G genes: The DNA from the field isolate was used to amplify a fragment of the TK and gG genes by PCR. Primers and reaction conditions previously described by Han and Kim (2001) were used to amplify 1296 and 1112-bp fragment for TK and gG gene respectively. The products were purified using GeneJET™ Gel Extraction Kit (Fermentas) and sequenced in the forward and reverse directions using the same primers of the amplification (Solgent Co. ltd. Korea).

Phylogenetic analysis: The nucleotide sequences for the TK and gG genes of Sharkia-11 were aligned with the ILTV sequences that have been published and available on GenBank database by the ClustalW method, using the MegAlign module of DNAStar software (Lasergene version 7.2 (DNASTAR, Madison, WI, USA). Multiple sequence alignments of the TK (1089 bp) and gG (879 bp) coding sequences, were used for phlylogenetic analysis. Phylogenetic trees of the aligned sequences were constructed by the Neighbour-Joining method using MEGA software program version 5 (www.megasoftware. net) with 1000 bootstrap replicates.

RESULTS

Pathology: Post-mortem examination revealed mucoid to hemorrhagic tracheitis, laryngitis and diphtheritic bloody mucoid casts along the entire length of the trachea. Microscopically, typical eosinophilic intranuclear inclusion bodies of *herpesvirus* infection were present in the necrotic tracheal epithelium along with syncytia formation (Fig. 1).
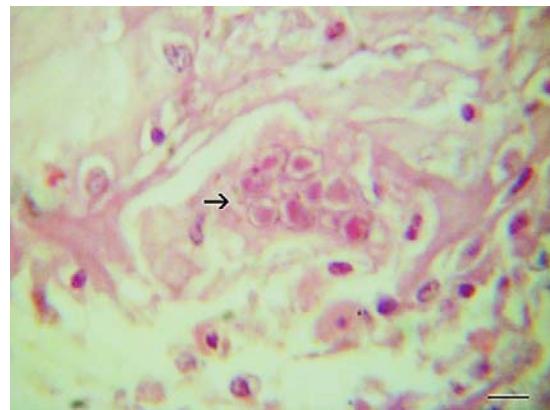

Fig. 1: Trachea (Chicken). Large eosinophilic intranuclear inclusion bodies with syncytia formation (Arrow).H & E. Bar=10µm.

Virus isolation: After five days of incubation, the CAMs of the eggs were examined for pock lesions. The ECEs inoculated with MEM did not show any pock lesions. The virus was isolated from the larynx and tracheal suspension collected from commercial layers flock. Generalized edema and opaque plaques were formed on the CAMs of eggs inoculated with the samples on the second egg passage.

PCR and sequencing: An amplicon size of 647bp was obtained confirming the presence of ILTV. The nucleotide sequences obtained in this study are available in GenBank under accession numbers JX977077 and JX977078.

Alignment and phylogenetic analysis of TK gene: The Sharkia-11 ILTV-TK gene is 1089 bp in length coded 363 amino acids. The sequence of TK gene had 99.5-100% and 98.5-100% homology at the nucleotide and amino acid levels, respectively, with the previously published sequences. Phylogenetic analysis showed that our isolate was closely related to CEO Intervet, TCO IVAX, and USDA strains (Fig. 2). The Egyptian field isolate Sharkia-11 differed from the Swiss field isolate CH04 and Swedish field isolate S04 in one nucleotide at position 540 (T to C), with no change in amino acid. As well, there was another nucleotide substitution with S04 at position 411 (G to A). The deduced amino acid sequence of our isolate was similar to vaccine, 632, 81658 and USDA strains with presence of threonine (ACG) at position 252. In the Swiss field isolate CH95 and in Australian strains (A20 and SA2), the position 252 contained methionine.
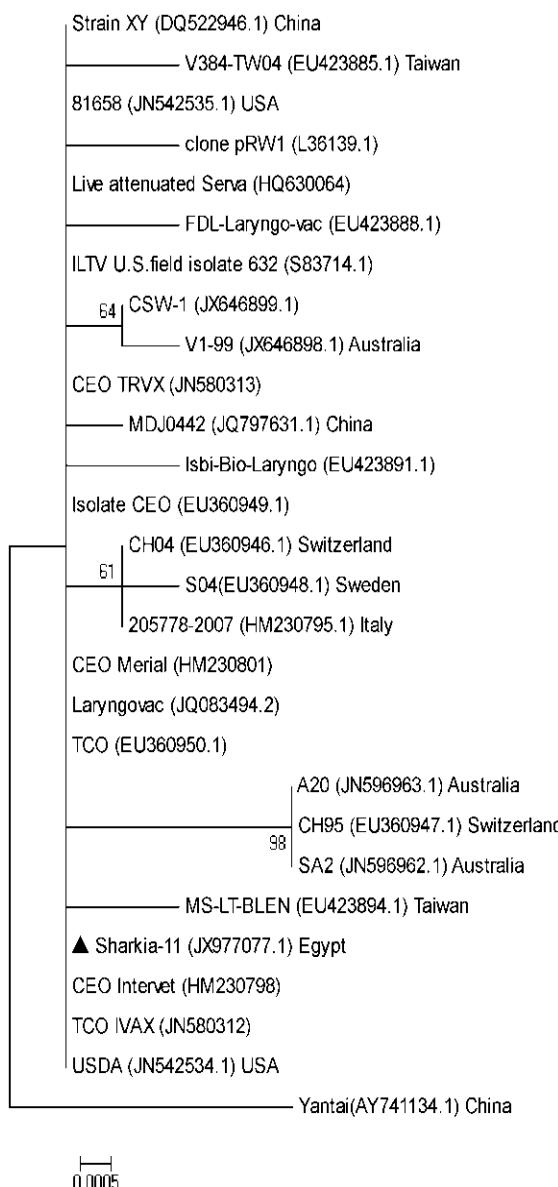
Fig. 2: Phylogenetic analysis of TK gene nucleotide sequences of ILTV isolated from commercial layer flock, Sharkia Province, Egypt and other TK-ILTV sequences available in GenBank. The phylogenetic tree was constructed via multiple alignments of 1089 bp nucleotide sequence. The tree was analyzed by neighbor-joining method using software MEGA 5 (www.megasoftware.net) with bootstrap values calculated for 1000 replicates. The virus isolated in this study is marked with solid triangle.

Alignment and phylogenetic analysis of gG gene: The gG gene nucleotide sequence reported 879 bp in length, coding 292 amino acids. The results of phylogenetic analysis of gG gene were shown in Fig. 3. The comparative sequence analysis showed that the Sharkia-11 isolate shares 98.4-100% and 97.3-100% homology at the nucleotide and amino acid levels, respectively, with previously published sequence of gG gene available in GenBank. The sequence alignment analysis revealed 100% amino acid identity between the isolate Sharkia-11 and vaccine strains (CEO and TCO), as well as with USDA strain (EU104959). On the other hand, the alignment of isolate Sharkia-11 revealed 97.3% amino acid sequence identity to ILTV strains (12/D/02/BCK and 25/H/88/BCK), 97.9 and 98.3% to Anhui-2011-2 and Anhui-2011-1, respectively.

DISCUSSION

Avian respiratory diseases result in severe economic losses for poultry industry due to severity of clinical signs. Infectious laryngeotracheitis virus constitutes an important respiratory pathogen of poultry and continues to have a significant economic impact on the poultry industries worldwide (Bagust *et al.,* 2000). This study presents the detection and molecular characterization of ILTV among layers flocks in Sharkia Province, Egypt.

The clinical observations of the infected farm exhibited great similarity to the clinical picture reported by Hughes *et al.* (1991b). The PM examination revealed diffuse inflammation and petechial hemorrhages of trachea and larynx. Excess mucus was found throughout the respiratory tract and bloody mucoid casts were observed along the entire length of trachea. These gross lesions are consistent with that reported by Guy and Bagust (2003); Moreno *et al.* (2010) and Preis *et al.* (2013).

For ILTV diagnosis, histopathology is considered a rapid reliable test (Guy and Bagust, 2003). Microscopically, eosinophilic intranuclear inclusion bodies were seen in the necropsied epithelium and syncytia formed in the trachea. These findings are confirmative to ILTV infection and similar to the lesions that were previously described by Humberd *et al.* (2002). Unfortunately, such confirmatory inclusion bodies are commonly only discerned in early stage of infection due to the considerable respiratory tract epithelial necrosis and sloughing in the subsequent stages (Guy and Bagust, 2003). Thus, the sole reliance on clinical and pathological changes for diagnosis is not possible and other tools should be utilized (Timurkaan *et al.,* 2003).

Virus isolation is still the gold standard method for ILTV diagnosis (Guy and Bagust, 2003). The virus suspension was inoculated in ECE via the CAM, and plaques were observed on CAM after two times passage resulting from necrosis and proliferative tissue reactions. Pock lesions of ILTV isolates from commercial farms of Gazipur District were developed at fourth passage (Islam *et al.,* 2010), contrary to pock lesions in our study which appeared rapidly on CAM at second passage. This could be attributed to the high concentration of the virus and the high virus adaptability to ECE. The findings revealed that the investigated outbreak is induced by ILTV infection with no evidence of co-infection with avian influenza and Newcastle disease viruses as allantoic fluids tested negatively by rapid hemagglutination test. Although virus isolation is a highly sensitive technique, definitive identification of ILTV is required after isolation, and it was accomplished using polymerase chain reaction according to Pang *et al.* (2002).

The modified-live ILT vaccine viruses can induce infection within susceptible birds and are involved in field outbreaks of the disease (Guy *et al.,* 1991). Subsequently, the discrimination of ILTV strains, particularly between outbreak circulating strains and modified-live vaccine viruses is very important in epidemiological studies and the disease control. Many assays have been developed
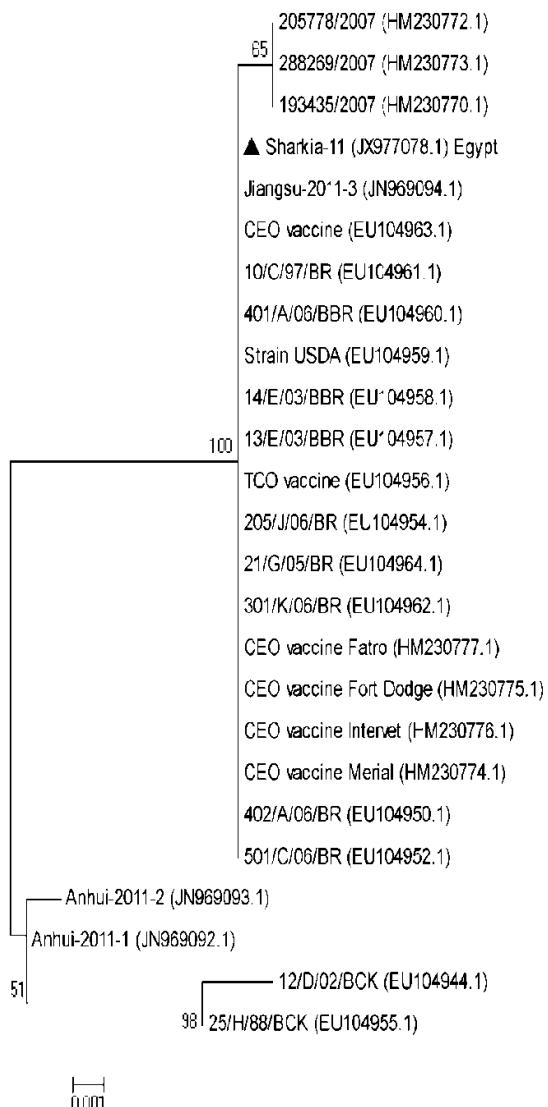
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     ┌ 205778/2007 (HM230772.1)
  65 ├ 288269/2007 (HM230773.1)
     └ 193435/2007 (HM230770.1)
  ▲ Sharkia-11 (JX977078.1) Egypt
  Jiangsu-2011-3 (JN969094.1)
  CEO vaccine (EU104963.1)
  10/C/97/BR (EU104961.1)
  401/A/06/BBR (EU104960.1)
  Strain USDA (EU104959.1)
  14/E/03/BBR (EU104958.1)
  13/E/03/BBR (EU104957.1)
  TCO vaccine (EU104956.1)
  205/J/06/BR (EU104954.1)
  21/G/05/BR (EU104964.1)
  301/K/06/BR (EU104962.1)
  CEO vaccine Fatro (HM230777.1)
  CEO vaccine Fort Dodge (HM230775.1)
  CEO vaccine Intervet (HM230776.1)
  CEO vaccine Merial (HM230774.1)
  402/A/06/BR (EU104950.1)
  501/C/06/BR (EU104952.1)
  Anhui-2011-2 (JN969093.1)
  Anhui-2011-1 (JN969092.1)
  ┌ 12/D/02/BCK (EU104944.1)
  └ 25/H/88/BCK (EU104955.1)
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Fig. 3: Phylogenetic analysis of gG gene nucleotide sequences of ILTV isolated from commercial layer flock, Sharkia Province, Egypt and other gG-ILTV sequences available in GenBank. The phylogenetic tree was constructed via multiple alignments of 879 bp nucleotide sequence. The tree was analyzed by neighbor-joining method using software MEGA 5 (www.megasoftware.net) with bootstrap values calculated for 1000 replicates. The virus isolated in this study is marked with solid triangle.

during last years to differentiate between field and vaccine viruses. DNA sequencing is the ultimate standard of genetic identification, molecular characterization of any virus and in detecting of the pathogenicity determinants (Lee *et al.,* 2013). Produced nucleotide sequences allow the feasible comparison of different virus strains isolated from variable geographic areas (Chacón *et al.,* 2010).

The pathogenicity of herpesviruses is influenced by many genes. Infected cell protein 4 (ICP4) gene help in the gene expression regulation early in infection and frequently used to determine the origin of the strain responsible for the outbreaks (Chacón and Ferreira, 2009). The TK gene is precious to distinguish ILTV vaccine strains from field isolates (Han and Kim, 2001). The gG gene is a pathogenicity factor in ILTV and its deletion from the viral genome causes marked attenuation of the

virus in its natural host (Devlin *et al.,* 2006; Legione *et al.,* 2012).

Although ILT is an economically significant problem, few publications describe the nature of ILTV infections in Egypt. In one article, the authors mentioned the molecular characterization of ILTV in Egypt based on sequence analysis of ICP4 gene (Shehata *et al.,* 2013). In this study, we determined the sequences of TK and gG genes of ILTV isolated from an outbreak of the commercial layer flock. Amino acid sequence analysis revealed that the TK gene of Sharkia-11 Egyptian isolate shared the amino acid threonine at position 252 with ILTV strains either of high virulence, such as strain 632 from the United States (Keeler *et al.,* 1991), or Korean low-virulence field isolates (Han and Kim, 2001). The discrimination of vaccine and field ILT viruses based on sequencing of UL47 and gG genes could serve as a helpful device for future investigations (Ojkic *et al.,* 2006). The alignment analysis of gG gene showed 100% amino acid identity between the isolate Sharkia-11 and vaccine strains (CEO and TCO) as well as with USDA strain (EU104959). The gG gene sequence of Sharkia-11 was compared with that of vaccine and SA-2 strains of ILTV. Stop codon was TAG in Sharkia-11 but TGA in SA-2 strain. The nucleotide sequence of SA-2 was described as 897 bp in size, encoding 298 amino acids. The amino acid sequence analysis of gG gene of Sharkia-11 isolate revealed threonine at position 67 and 103, such amino acid was reported within low-virulence and vaccine strains (Han and Kim, 2001).

The results of the nucleotide and amino acid sequence, suggested that ILTV vaccine strains could be the source of the outbreak occurred in commercial layer flocks in Sharkia, Egypt. These results are in consistency with results reported by Shehata *et al.* (2013) who suggested that CEO-ILT vaccine viruses may become more virulent following bird-to-bird passages causing severe outbreaks in susceptible birds in Egypt. These findings are consistent with the results recorded by Oldoni and García (2007) who illustrated that majority of the commercial poultry ILTV isolates were intimately connected to the vaccine strains. Such outbreaks provoke following the establishment of ILT vaccine viruses replacing the wild circulating virus in the field (Chang *et al.,* 1997).

These results emphasize the significance of using the vaccines based on recombinant DNA together with enforcing biosecurity measures to decrease the risk of ILTV spreading. Further epidemiological investigations including backyard chickens are required to determine their role in ILTV circulation in Egypt.

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