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Molecular Characterization of Infectious Laryngotracheitis Virus Circulating in Egypt during 2023

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ABSTRACT

Since its discovery in Egypt in 1983, the infectious laryngotracheitis virus (ILTV) has continued to spread, leading to substantial losses for poultry farms. This study aimed to identify and molecularly characterize the ILTV currently in circulation in Egypt in 2023. Fifteen pooled trachea and lung samples were collected from affected vaccinated and non-vaccinated layer farms (Shiver, Lohhman, Brown layer, and Aviagel) aged from 10-30 weeks old spread throughout eight governorates in Egypt. A real-time polymerase chain reaction (PCR) is used to detect ILTV. Ten positive samples representing various governorates were chosen for partial sequencing of the *ICP4* gene, and four positive samples were chosen for sequencing of the *TK, gD, and gG* genes. Using phylogenetic analysis, the *ICP4* clusters into chicken embryo origin vaccine and vaccine-like strains (CEO) and tissue culture origin vaccine and vaccine-like strains (TCO) and wild type. The GAHV-1-Egy-WO5-2023, GAHV-1-Egy-WO7-2023, and GAHV-1-Egy-WO8-2023 strains cluster in the CEO group with the CEO vaccine and vaccine-like strains with 98.9%–100% amino acid identity (A.A.); However, the remaining strains (GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO2-2023, GAHV-1-Egy-WO3-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO6-2023, GAHV-1-Egy-WO9-2023, and GAHV-1-Egy-WO10-2023) were clustered in the TCO group with TCO vaccine strains and vaccine-like strains with 100 % A.A. By analysis of *gD, gG, and Tk* genes, there is no difference between the TCO and CEO groups. Compared with several reference CEO and TCO vaccine strains and vaccine-like strains, some new specific mutations (Q161H and Q182H) were recorded in the *ICP4* in GAHV-1- Egy-WO8-2023. In addition, A34G, and P276L were recorded in the *gD* gene in the GAHV-1-Egy-WO1-2023 and GAHV-1-WO4-2023. Also, the R115I, G126A, and S163I were recorded in the *TK* gene in GAHV-1-Egy-WO8-2023 and A99E in GAHV-1-Egy-WO5-2023, which can affect the virulence and pathogenicity of the virus, which may be due to the reactivation of the vaccine strain by the bird-to-bird transmission or viral recombination. In conclusion, the ILTV outbreaks in poultry farms across several regions of Egypt during 2023 were induced by vaccine strains derived from TCO and CEO. Thus employing innovative vector vaccines and reassessing the current vaccination regimens is necessary.

Keywords: Infectious laryngotracheitis, Diagnosis, isolation, Genetic characterization

INTRODUCTION

Infectious laryngotracheitis virus (ILTV) is an acute respiratory disease affecting chickens all over the world and leading to significant economic losses (Bagust, 2003; Hong et al., 2024; Abebe et al., 2024; Shao et al., 2025). The ILTV is identified as Gallid herpesvirus 1 (GaHV-1) and belongs to the Herpesviridae family and subfamily of Alphaherpesvirinae (Elshafiee et al., 2022). The ILTV causes varying rates of morbidity and mortality of about 90% and 70%, respectively, as previously described by Bagust et al. (2000) and Coppo et al. (2013) based on the circulating strains' pathogenicity and whether they are co-infected with other respiratory pathogens (Menendez et al., 2014). The ILTV genome is 150-155 kilobases of linear double-stranded DNA that codes for inverted internal (IR)

and terminal (TR) repeats, unique long (UL), and unique short (US) repeats. UL and US sections encode 80 open reading frames (ORFs), whereas inverted repeats only contain three genes (*ICP4*, *US10*, and *sORF4/3*), as described by Lee et al. (2011) and Wu et al. (2022). The *ICP4* gene plays a crucial role in distinguishing distinct ILT strains (Creelan et al., 2006, Wang et al., 2021, Ponnusamy et al., 2022).

According to Lee (2011), ILTV encodes thymidine Kinase and 11 glycoproteins (*gL, gM, gH, gB, gC, gK, gG, gJ, gD, gI,* and *gE*) that are encoded by highly conserved ORFs. They have an essential role in the virulence and reproduction of viruses. The Glycoprotein G (*gG*) and the *TK* gene have been linked to ILTV pathogenicity (Han and Kim, 2001), and the *gD* protein functions as a virus-binding receptor to vulnerable cells (Di Giovine et al., 2011). Furthermore, the production of neutralizing antibodies is attributed to the glycoprotein B (*gB)* and glycoprotein D (*gD*) proteins (Rouse and Kaistha, 2006; Devlin et al., 2006; Lazear et al., 2012).

To prevent and control ILTV outbreaks, vaccines with modified live viruses are globally approved and utilized (Groves et al., 2019). Vaccine strains are propagated using either chicken embryos (CEO; chicken embryo origin) or cell culture (TCO; tissue culture origin). It has been seen that an attenuated virus vaccine can revert to virulence during bird-to-bird transmission (Groves et al., 2019; Kardoğan, Sarıçam, 2024). Numerous nations across the world, including the USA (Oldoni and García, 2007), Australia (Blacker et al., 2011), and Canada (Ojkic et al., 2006), have reported that modified live ILTV vaccinations, in particular CEO vaccines, are linked to illness outbreaks (Yi et al., 2024).

The most accurate and popular method for detecting ILTV infection is real-time PCR, which has good sensitivity, specificity, and speed (Davidson et al., 2015; Santander Parra et al., 2018; Asif et al., 2022). Viral isolation and serological assays can also identify an ILT virus infection. However, they are not very sensitive (Creelan et al., 2006; Zhao et al., 2013). Certain gene fragment sequencing appears helpful for defining field isolates and distinguishing vaccine strains from field isolates (Chacón and Ferreira, 2009; Shehata et al., 2013).

ILTV was first detected in Egypt in 1983 (Tantawy et al., 1983). Later, from 2011 to 2021, many field outbreaks were identified in various Egyptian governorates, leading to mortality rates and egg production declines of 20% to 35%, respectively. There was a noticeable sequence similarity between the detected viral strains and the vaccination strains utilized in Egypt. Some field isolates were confirmed to be CEO-like viruses (Shehata et al., 2013), and some detected both TCO-like and CEO-like viruses (Bayoumi et al., 2020; Mossad et al., 2022), and they can result in the creation of natural recombinants with each other (Lee et al., 2012). In field outbreaks, field and ILTV vaccination strains are hard to differentiate (Lee et al., 2012). Therefore, molecularly defining the circulating ILTV strains in affected poultry farms is essential.

Several molecular characterization methods are employed to differentiate the strains causing outbreaks. PCR followed by restriction fragment length polymorphism (PCR-RFLP) was used to effectively distinguish field and vaccine strains (Chang et al., 1997; Kirkpatrick et al., 2006; Hermann et al., 2024). However, this technique's applicability is limited by the substantial amount of DNA required (Chacón and Ferreira, 2009). Specific gene fragment sequencing appears to be useful for differentiating vaccine strains from field isolates and characterizing field isolates.

The present study aimed to detect, identify, and characterize four antigenic and functionally relevant genes (*ICP4, gD, gG,* and *TK*) of the ILT virus strains currently circulating in Egypt in 2023.

MATERIAL AND METHODS

Ethical approval

The study was conducted according to the Animal Health Research Institute guidelines of Benha Provincial Laboratory, Animal Health Research Institute, Agriculture Research Canter, Benha, Egypt**.**

Sample collection

During 2023, the tracheal, larynx, and lung field samples were collected from 50 freshly dead affected layer farms (Shiver, Lohhman, Brown layer, and Aviagel) aged from 10-30 weeks with average sizes (10000-15000) birds. The samples included 37 unvaccinated layer chicken farms and 13 vaccinated layer chicken farms. They were vaccinated with TCO (LT-IVAX, USA) and CEO (Nobilis® ILT, Netherlands), administered at 35 and 95 days of age, respectively. The samples were collected from eight Governorates in Egypt, including eight from El Sharkia, ten from Qalubia, fifteen from Dakahlia, seven from Monofia, three from Gharbia, two from Behira, two from Damietta, and three from Alexandria.

The affected chickens experienced severe respiratory symptoms, such as coughing, sneezing, gasping, expectoration of red exudates from respiratory orifices, difficulty in inspiration, infra-orbital sinus enlargement,

and significant mortality (5-15% mortality). Upon post-mortem inspection, the field instances had congested and hemorrhagic tracheitis with blood clots and caseated material, and in a few cases, this led to a blocked laryngeal lumen. The 10 trachea, larynx, and lungs from each farm were homogenized in sterile phosphate buffer saline PBS and then centrifuged at 3000 rpm at 4ºC for 30 minutes to clarify them.

Screening and detection of infectious Laryngotracheitis virus by real-time PCR

DNA was extracted from the supernatant fluid using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. In conclusion, 10 µl of proteinase K, 200 µl of lysis buffer, and 200 µl of sample solution were incubated at 56 °C for 10 minutes, then 200 µl of 100% ethanol was added. 50 µl of elution buffer was used to elute DNA from the lysate. Real-time PCR used specific primers and probes targeting the *gB* gene (Table 1). Using the Takara kit (Kusatsu, Japan) according to the manufacturer's instructions through the Stratagene Mx3005p Real-Time PCR System.

Virus isolation

Three specific pathogen-free (SPF) chicken eggs, ranging in age from ten to twelve days, and 0.2 ml of positive samples were inoculated in the chorioallantoic membrane (CAM). The inoculated eggs were incubated at 37ºC for a maximum of seven days with daily monitoring and investigation of the presence of distinctive pock lesions on the CAMs of dead embryos or even survival eggs in 7 days (Magouz et al., 2018; Wolfrum et al., 2020).

Genetic characterization of *ICP4, gD, gG, and TK* genes

Conventional PCR amplification

Using gene-specific primers, the *ICP4, gD, gG,* and *TK* genes of the isolated ILTV were partially amplified by PCR (Table 2). The PCR was carried out in an ABI thermocycler (ProFlexTM PCR System) using the EmeraldAmp GT PCR MasterMix from Takara, Kusatsu, Japan. Certain PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and examined under an ultraviolet lamp. Their purification procedure used the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Table 1. Real-time primers and probes used for the detection of infectious laryngotracheitis virus

Primers and probe	Sequence	Reference
gB-S	5' CAGTATCTGGCATCGCCTCAT '3	
gB-A	5' CCTGGGAACAGAACCTGAACT '3	(Zhao et al., 2013)
Probe	5' FAM-CTAACCCGTTCGCCGCACTCG-BHQ-1 '3	

Table 2. Primers used for amplification of *ICP4, TK, gD, gG* genes

Primer	Sequence	Weight (BP)	Reference
ICP4-1F	5'ACTGATAGCTTTTCGTACCAGCACG'3	688	(Chacón and Ferreira, 2009)
ICP4-1R	5'CATCGGGACATTCTCCAGGTAGCA'3		
TK1b-F	5'CTTAGCGGAACCTATGCAAG3'	781	The present study
TK-R	5'GAG GCC ATG TGC TGG TAA GT'3		
gD-F	5'ATG CAC CGT CCT CATC'3	1300	(Craig et al., 2017)
gD-R	5'TTA GCT ACG CGC GCAT'3		
gG-F	5'CCT TCT CGT GCC GAT TCA ATATG'3	1480	(Craig et al., 2017)
gG-R	5'AAC CAC ACC TGA TGC TTT TGTAC'3		

Gene sequencing and phylogenetic analysis

Using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and ABI 3500XL Genetic Analyzer (Life Technologies, California, USA) with gene-specific primers, amplicons of ten positive samples representing different governorates and vaccinated and unvaccinated flocks (Table 3) were partially sequenced for *ICP4*. Four of the ten isolates were chosen to be partially sequenced for the *gD, Gg, and TK* genes. The sequence was posted to the GenBank (NCBI) website using the accession number (Table 4). Nucleotide and amino acid sequences of sequenced samples against further strains of ILTV from different groups and vaccine strains obtained from the National Center for Biotechnology Information (NCBI) (Table 5) were aligned using DNASTAR software's MegAlign module and CLUSTAL-W tool (Lasergene version 7.2; DNASTAR, Madison, WI, USA, Burland, 2000). MEGA version 6 generated the phylogenetic tree using 1000 bootstrap repeats and a moderate-strength maximum likelihood approach (Tamura et al., 2013; Kumar et al., 2016). The pairwise amino acid identity percent was calculated using the DNAstar software (DNAStar, Madison, WI, Burland, 2000).

Table 3. The epidemiological data of sequenced isolates of the Infectious Laryngotracheitis virus

Name	Governorates	Breed	Age (day)	Mortality percent (%)	Vaccination
ILT-Egy-WO1-2023	El Sharkia	Shiver	70	5%	Non vaccinated
ILT-Egy-WO2-2023	Dakahlia	Lohhman	100	10%	Non vaccinated
ILT-Egy-WO3-2023	Behira	Brown layer	120	7%	Vaccinated
ILT-Egy-WO4-2023	Monofia	Aviagel	150	8%	Vaccinated
ILT-Egy-WO5-2023	Dakahlia	Lohhman	160	9%	Vaccinated
ILT-Egy-WO6-2023	Qalubia	Shiver	80	12%	Non vaccinated
ILT-Egy-WO7-2023	Gharbia	Lohhman	100	6%	Vaccinated
ILT-Egy-WO8-2023	Monofia	Aviagel	90	11%	Non vaccinated
ILT-Egy-WO9-2023	Domiatt	Lohamn	110	13%	Non vaccinated
ILT-Egy-WO10-2023	Qalubia	Aviagel	180	14%	Non vaccinated

TCO (LT-IVAX, USA) and CEO (Nobilis® ILT, Netherlands) vaccines were administered to the chickens at 35 and 95 days of age in vaccinated flocks.

Table 4. Accession number of sequenced strains of Infectious Laryngotracheitis virus

Name \ Accession number	*ICP4*	*gG*	*gD*	*TK*
ILT-Egy-WO1-2023	OR900664	OR900656	OR900649	OR900660
ILT-Egy-WO2-2023	OR900665	-	-	-
ILT-Egy-WO3-2023	OR900666	-	-	-
ILT-Egy-WO4-2023	OR900667	OR900657	OR900650	OR900662
ILT-Egy-WO5-2023	OR900668	OR900658	OR900651	OR900661
ILT-Egy-WO6-2023	OR900669	-	-	-
ILT-Egy-WO7-2023	OR900670	-	-	-
ILT-Egy-WO8-2023	OR900671	OR900659	OR900652	OR900663
ILT-Egy-WO9-2023	OR900672	-	-	-
ILT-Egy-WO10-2023	OR900673	-	-	-

Table 5. Reference strains provided by the National Center for Biotechnology Information (NCBI)

Name	Country	Virulent/ Vaccine	Accession Number
GAHV1-SA2	Australia	Vaccine	JN596962
GAHV1-TCO-IVAX	USA	Vaccine	JN580312
GAHV1-CEO-TRVAX	USA	Vaccine	JN580313
GAHV1-81658	USA	Virulent	JN542535
GAHV1-USDA-REF	USA	Virulent	JN542534
GAHV1-LTI-IVAX	USA	Vaccine	FJ444832.1
GAHV1-TCO-low passage	USA	Vaccine	JN580315
GAHV1-TCO-high passage	USA	Vaccine	JN580314
GAHV1-TCO vaccine	USA	Vaccine	EU104908.1
GAHV1-40798	Korea	Virulent	MH937566.1
GAHV1-ACC78	Australia	Virulent	JN804826
GAHV1-CEO-high passage	USA	Vaccine	JN580316
GAHV1-V1-99	Australia	Virulent	JX646898
GAHV1-K317	China	Virulent	JX458824
GAHV1-Live attenuated serva (Nobilis)	Australia	Vaccine	HQ630064
GAHV1-CEO-low passage	USA	Vaccine	JN580317
GAHV1-Intervet	Italy	Vaccine	HM230782.1
GAHV1-LT-BLEN	USA	Vaccine	JQ083493
GAHV1-CL9	Australia	Virulent	JN804827
GAHV1-63140	USA	Virulent	JN542536
GAHV1-CEO Vaccine	USA	Vaccine	EU104900.1
GAHV1-Laryngo Vac	USA	Vaccine	JQ083494.2
GAHV1-Poulvac	Italy	Vaccine	KP677882.1

RESULTS

Clinical signs and post-mortem examination

The chicken had red exudates from respiratory orifices, sneezing, coughing, gasping, nasal and ocular discharges, difficulty inhaling, and swelling of the infra-orbital sinus, with a mortality rate of 5-15% (Figure 1).

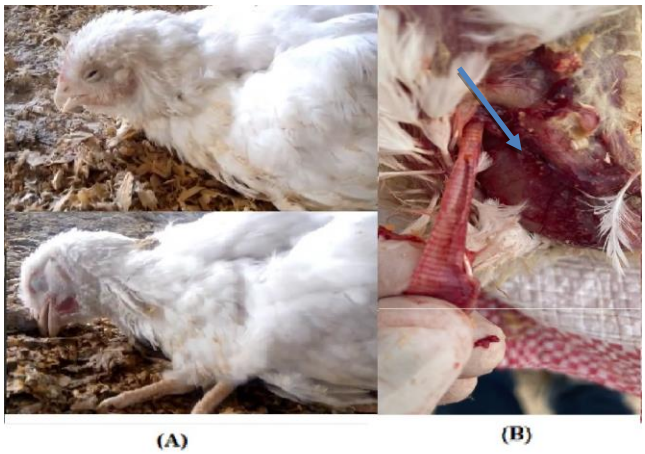


Figure 1. Clinical signs and post-mortem lesions were caused by the infectious laryngitis virus in affected chickens. **A:** Gasping and respiratory signs in affected chickens. **B:** The trachea had Fibrinohaemorrhagic exudate linked to hemorrhage and congestion in the lumen.

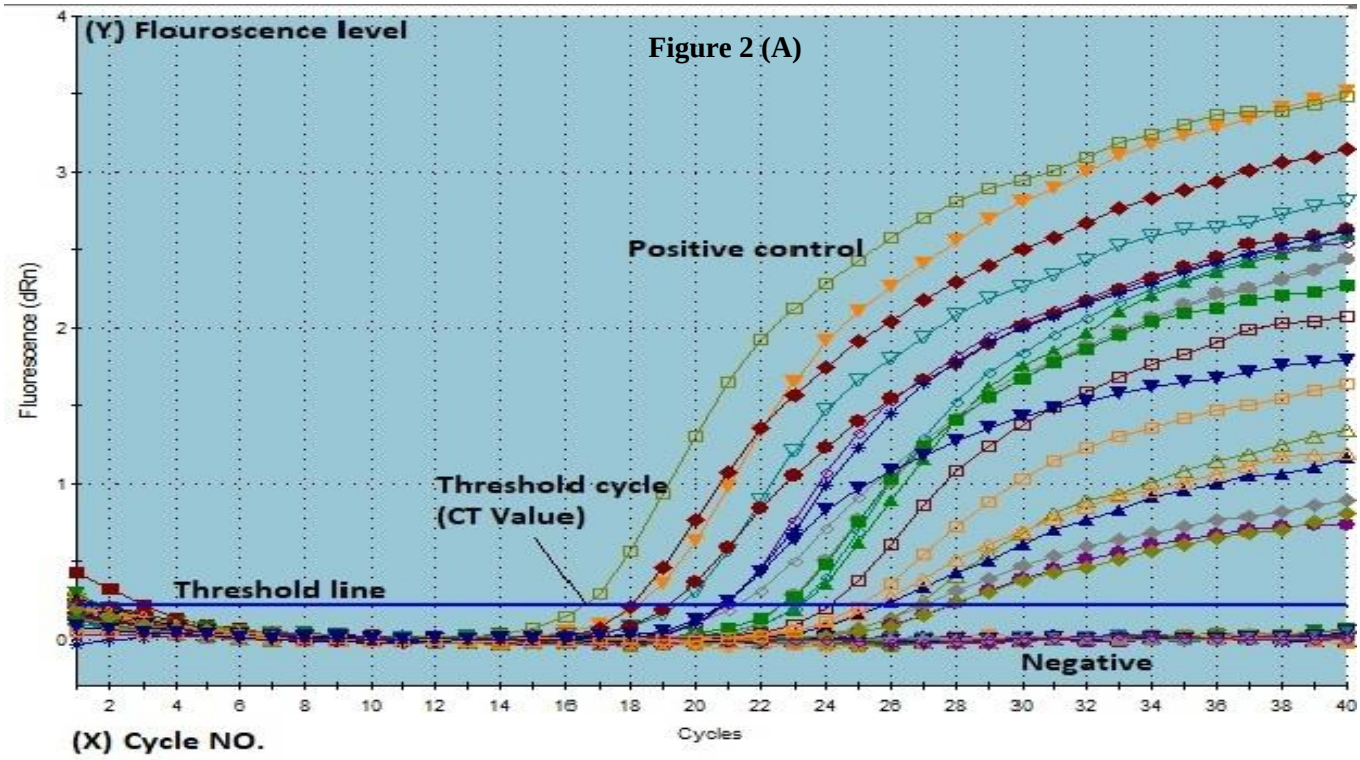
Post-mortem examination of the trachea and larynx indicated hemorrhage and congestion of the mucosa, along with a cast of fibrin-hemorrhagic blood obstructing the lumens. Caseous material was also discovered in the trachea and larynx. The lungs showed areas of congestion and consolidation (Figure 1).

Infectious Laryngitis virus screening by real-time PCR and viral isolation

Real-time PCR for 40 of the 50 samples was positive (5/8 El Sharkia, 8/10 Qalubia, 14/15 Dakahlia, 6/7 Monofia, 2/3 Gharbia, 2/2 Behira, 2/2 Domiatt, and 1/3 Alexandria) (Figure 2). The isolated positive samples on SPF-ECE indicated opaque, raised border pockets-like lesions distinguished by a grey core zone of necrosis and hemorrhagic chorioallantoic membrane. After inoculation, most embryos die 2-7 days later, and they are accompanied by slow development.

Gene amplification by conventional PCR

Ten isolates out of forty positive samples were selected according to different governorates to be amplified by conventional PCR for further sequencing of *ICP4* at 688 bp (Figure 3). Four isolates were partially amplified for *gD*, *gG*, and *TK genes*, gene sequencing at the expected weights 1300, 1480and 781 bp, respectively.



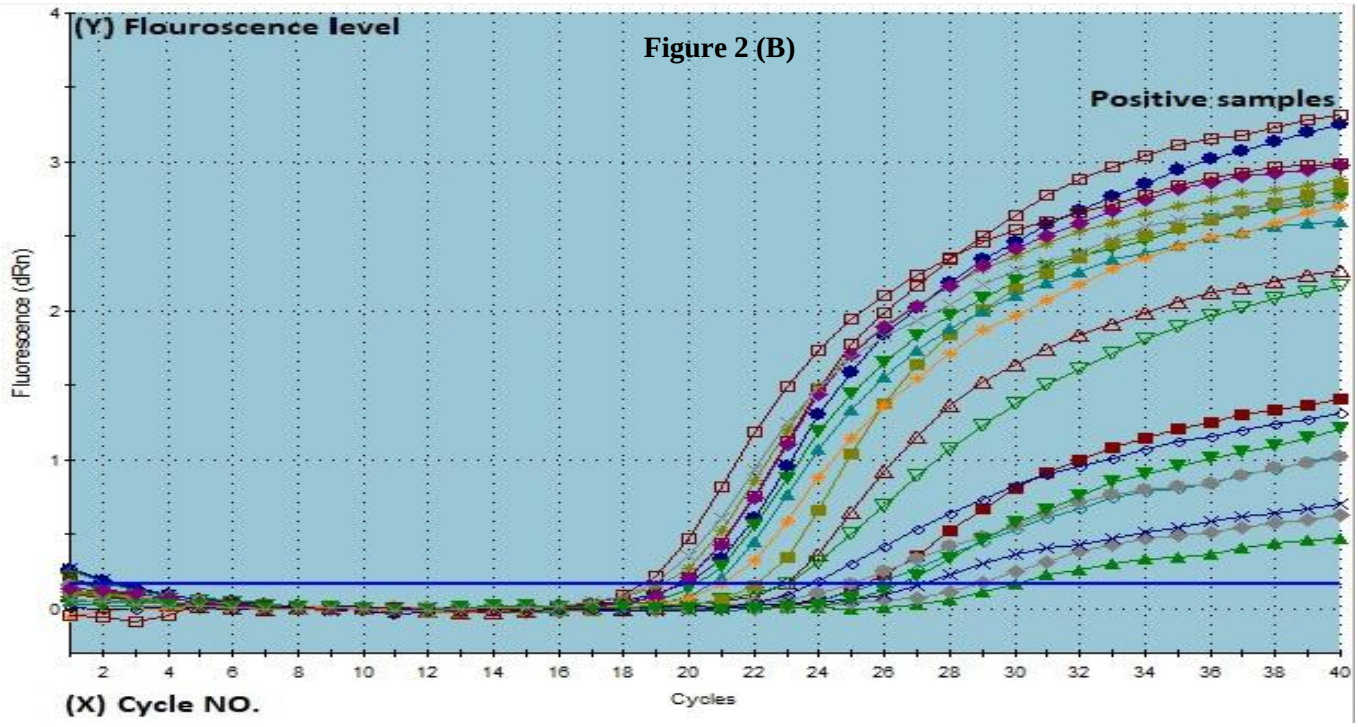


Figure 2. The colored amplification curves of the positive and negative controls, and 40 positive samples, in addition to the curve of 10 negative samples of the infectious laryngitis virus in affected chickens in Egypt (**A** and **B**)

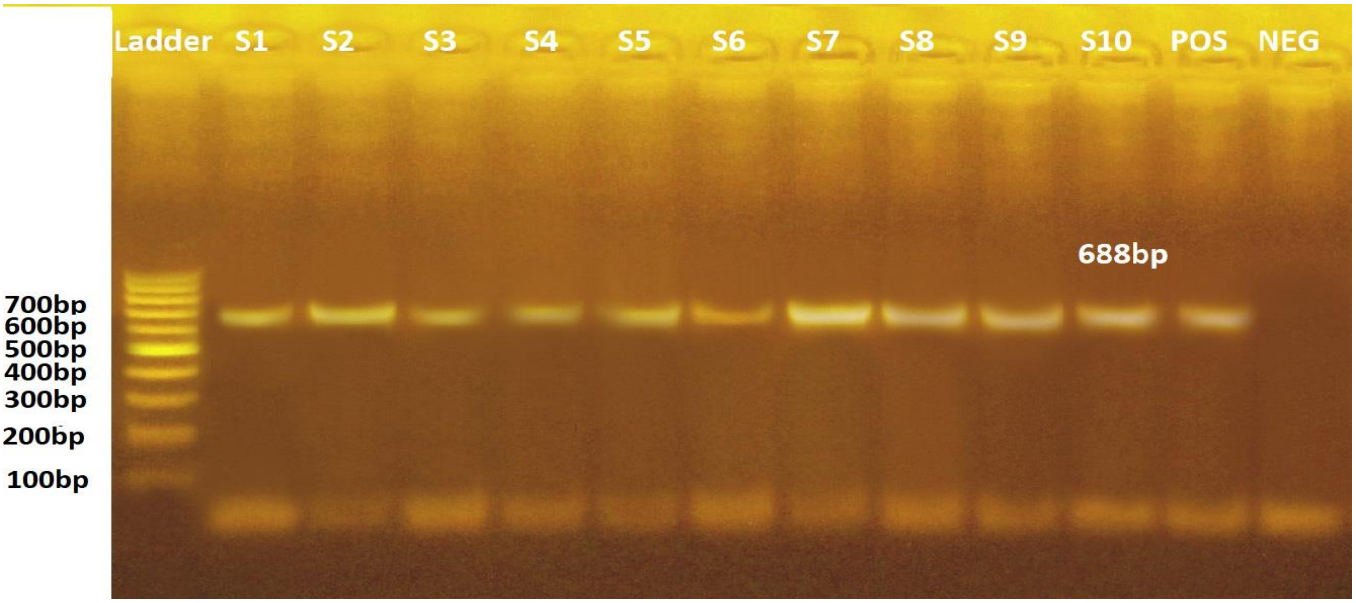


Figure 3. *ICP4* Genes amplification 10 positive samples by conventional PCR of the infectious laryngitis virus in affected chickens in Egypt.

Genetic characterization and phylogenetic analysis

Ten isolates were partially sequenced for the *ICP4* gene, while 4 isolates were selected for *gD, gG, and TK* partial sequencing. To detect similarity levels and analyze gene mutations, a multiple sequence alignment was created using sequenced strains in this study, other strains circulating in Egypt, strains from other countries, chicken embryo vaccine, and tissue culture origin vaccine (Table 5). The ILTV strains were clustered into two categories based on *ICP4* partial sequence-based phylogenetic analyses, including tissue culture origin (TCO) vaccines and vaccine-like strains, and chicken embryo origin (CEO) vaccines and vaccine-like strains. The third group (Wild type), including field isolates from Brazil and the United States, was identified (Figure 4).

The phylogenetic tree of *ICP4* gene (Figure 4) revealed that 7 of Egyptian strains under study (GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO2-2023, GAHV-1-Egy-WO3-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO6-2023, GAHV-1-Egy-WO9-2023 and GAHV-1-Egy-WO10-2023) belonged to the TCO group together with TCO vaccine strains (TCO low passage, TCO high passage, IVAX that used in Egypt) and virulent vaccine like strains from USA (USDA, 81658) and Egypian strains from 2015-2021 (Giza-2, Kaliobia-5, CLEVB-222, CLEVB-221). The remaining three strains in this study (GAHV-1-Egy-WO5-2023, GAHV-1-Egy-WO7-2023, and GAHV-1-Egy-WO8-2023) belong to the CEO group, along with virulent vaccine like strains from Australia (ACC78) and CEO vaccine strains (CEO low passage, CEO high passage, LT Blen, TRVX, and live attenuated serva vaccine that is used in Egypt).

The phylogenetic analysis of the *gD and gG* genes (Figures 5, 6) revealed no difference between the TCO and CEO strains. All strains in this study cluster with the TCO vaccine strains (IVAX), CEO vaccine strains (live attenuated serva, LT Blen, TRVX), and USDA, 81658, ACC78 virulent vaccine-like strains from the USA, Australia, as well as several Egyptian strains (Mansura-2019, Sharkia-2018). However, in the phylogenetic tree of the *gD* gene, the GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 were clustered on a separate branch with Fayoum_2019 and Qalubia_2018.

The thymidine kinase phylogenetic study shows no differences between TCO and CEO strains. All strains in this study were clustered with the TCO vaccine strain (IVAX), CEO vaccine strains (LT Blen, TRVX, live attenuated serva), various Egyptian strains (Sharkia11, Egypt-2015), and virulent vaccine like strains from the USA (USDA, 81658,) and Australia (ACC78) (Figure 7).

The *ICP4* gene of seven strains (GAHV-1-Egy-WO1, GAHV-1-Egy-WO2, GAHV-1-Egy-WO3, GAHV-1-Egy-WO4, GAHV-1-Egy-WO6, ILT-Egy-WO9 and ILT-Egy-WO10) had 100% amino acid identity percent with TCO vaccine strains (IVAX that used in Egypt), virulent vaccine like strains from USA (USDA, 81658) and Egyptian strains (Giza-2, Kaliobia-5, CLEVB-222, CLEVB-221). The other three isolates (GAHV-1-Egy-WO5, GAHV-1-Egy-WO7, and GAHV-1-Egy-WO8) had 98.9%-100% with CEO vaccine strains (live attenuated serva, that were used in Egypt, LT Blen, TRVX) and virulent vaccine like strains from Australia (ACC78) and 96.7%-97.8% with Egyptian strains (Monofia-1-2021 and Kaiobia-3-2021; Figure 8).

The *gD* gene of four strains(GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO5-2023 and GAHV-1-Egy-WO8-2023) had 99.8%-100% A.A. identity percent with -TCO vaccine (IVAX that used in Egypt) and CEO vaccine strains (LT Blen, TRVX, live attenuated serva that used in Egypt) and other virulent vaccine like strains from Australia (USDA, 81658, ACC78). While GAHV-1-Egy-WO5-2023 and GAHV-1-Egy-WO8-2023 had 99.8%-100% relation to many Egyptian strains (Alexandria-2018, Sharkia-2018, Mansura 2019, and Fayum-2019), GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 had 99.7-99.8% relation (Figure 9).

The *gG* gene of all sequenced strains had 100% A.A. identity percent with TCO vaccine strains (USDA, IVAX used in Egypt), CEO vaccine strains (LT Blen, TRVX, live attenuated serva used in Egypt), virulent vaccine like strains from the USA (81658) and Australia (ACC78) and several Egyptian isolates (Sharkia-2018; Mansura- 2019; Fayum-2019; Figure 10).

The *Tk* gene of GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 had 100% A.A. identity percent with TCO vaccine strain (IVAX that used in Egypt), CEO vaccine strains (LT Blen, TRVX, live attenuated serva), virulent vaccine like strains from USA (USDA,18685) and Australia (ACC78) and other Egyptian strains (Sharkia-11, Egypt-2015) but GAHV-1-Egy-WO5-2023 and GAHV-1-Egy-WO8-2023 had 97.2%-99.1% A.A. identity with TCO vaccine strain (IVAX that used in Egypt), CEO vaccine strains (LT Blen, TRVX, live attenuated seva), virulent vaccine like strains from USA (USDA,18685) and Australia (ACC78) and other Egyptian strains (Sharkia-11, Egypt-2015; Figure 11).

Mutation analysis

Comparing several reference CEO and TCO vaccine strains, and virulent vaccine-like strains from the USA, Australia, China, and Egypt between 2015 and 2021, listed in GenBank (Table 5). No mutations were detected in the *ICP4* in the seven strains related to TCO in this study (GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO2-2023, GAHV-1-Egy-WO3-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO6-2023, GAHV-1-Egy-WO9-2023, and GAHV-1-Egy-WO10-2023). All strain-related to CEO related strains in these studies (GAHV-1-Egy-WO5-2023, GAHV-1-Egy-WO7-2023, and GAHV-1-Egy-WO8-2023 had a deletion at 272 to 283 pb and V200M that is characteristic of the CEO vaccine and CEO vaccine-like strain. Also, the GAHV-1-Egy-WO8-2023 had Q161H and Q182H.

The *Gd* genes of GAHV-1-Egy-WO5-2023 and GAHV-1-Egy-WO8-2023 did not have any mutations, and GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 had A34G and P276L. No mutations were detected in the *Gg* gene in all sequenced strains in this study, and it had threonine at positions 67 and 103 was unique to the vaccination strain. The *TK* genes of GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 had no mutations; however, GAHV-1-Egy-WO5-2023 had A99E, and GAHV-1-Egy-WO8-2023 had R115I, G126A, and S163I.

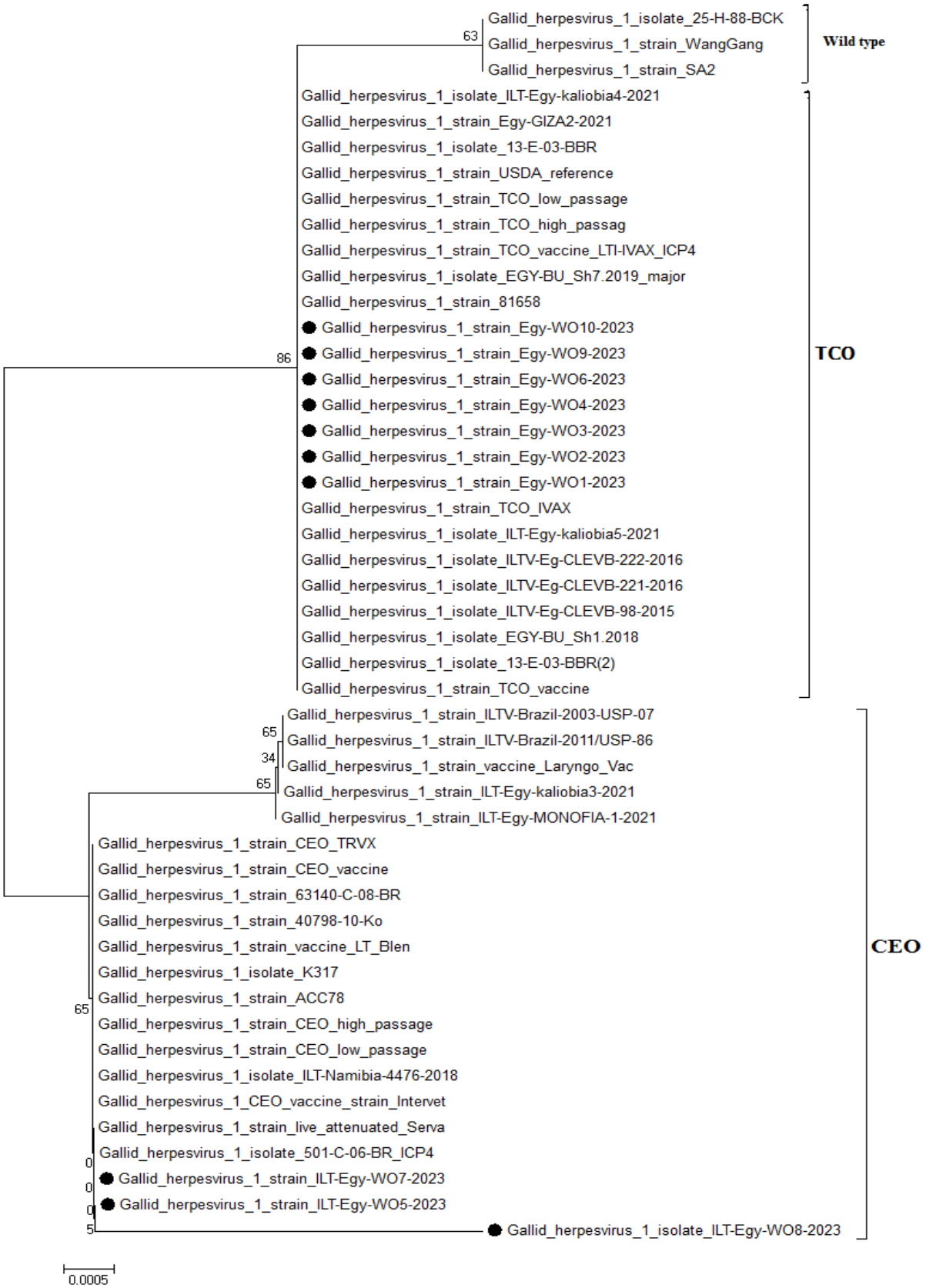


Figure 4. The phylogenetic analysis of the *ICP4* gene of the study strain in comparison to several field and vaccination strains of infectious laryngitis virus (ILTV) using the MEGA 6.0 program's maximum likelihood technique. The strains in this study are indicated by black dots.

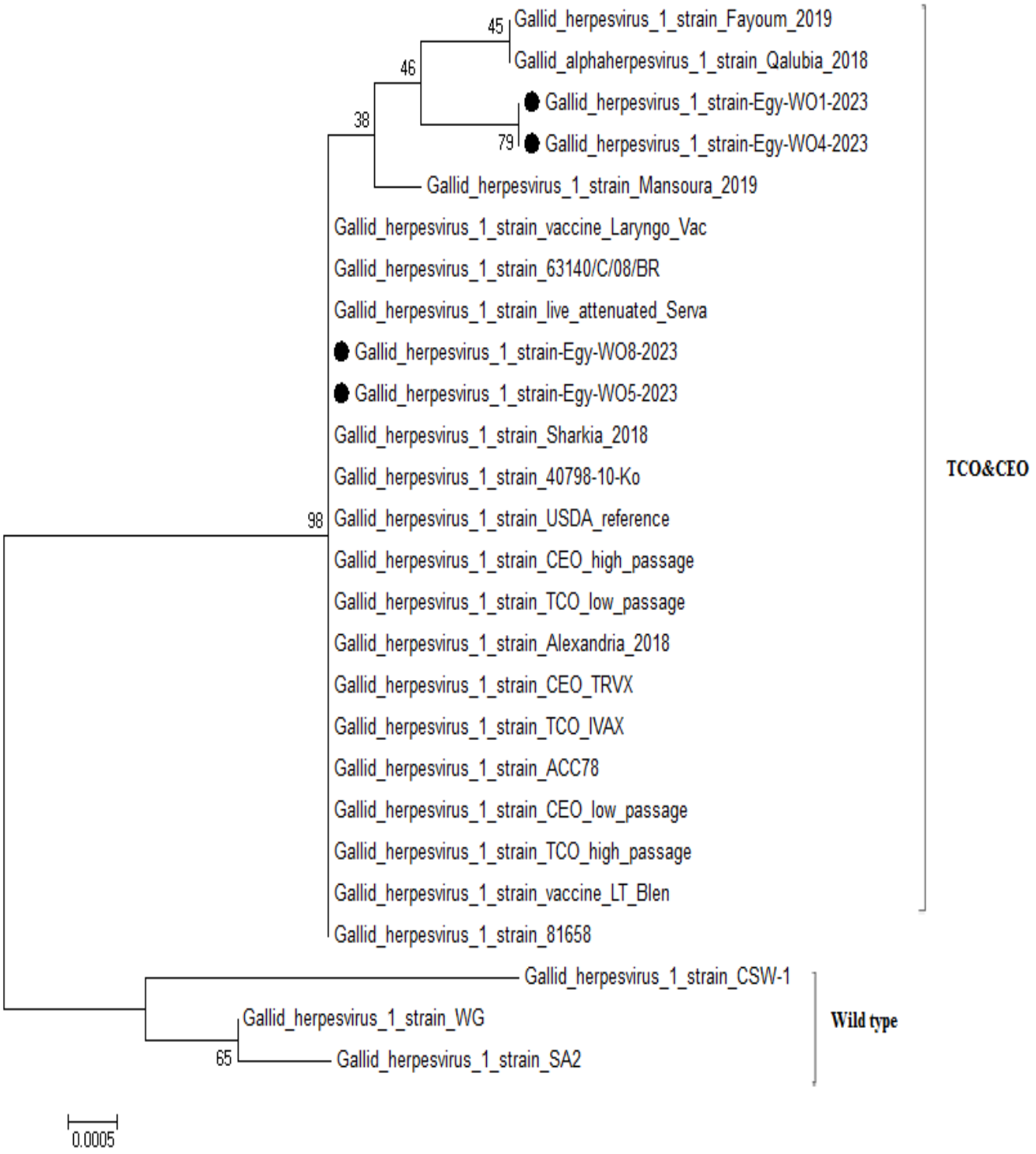


Figure 5. The phylogenetic analysis of the *gD* gene of the study strain in comparison to several field and vaccination strains of infectious laryngitis virus (ILTV) using the MEGA 6.0 program's maximum likelihood technique. The strains in this study are indicated by black dots.

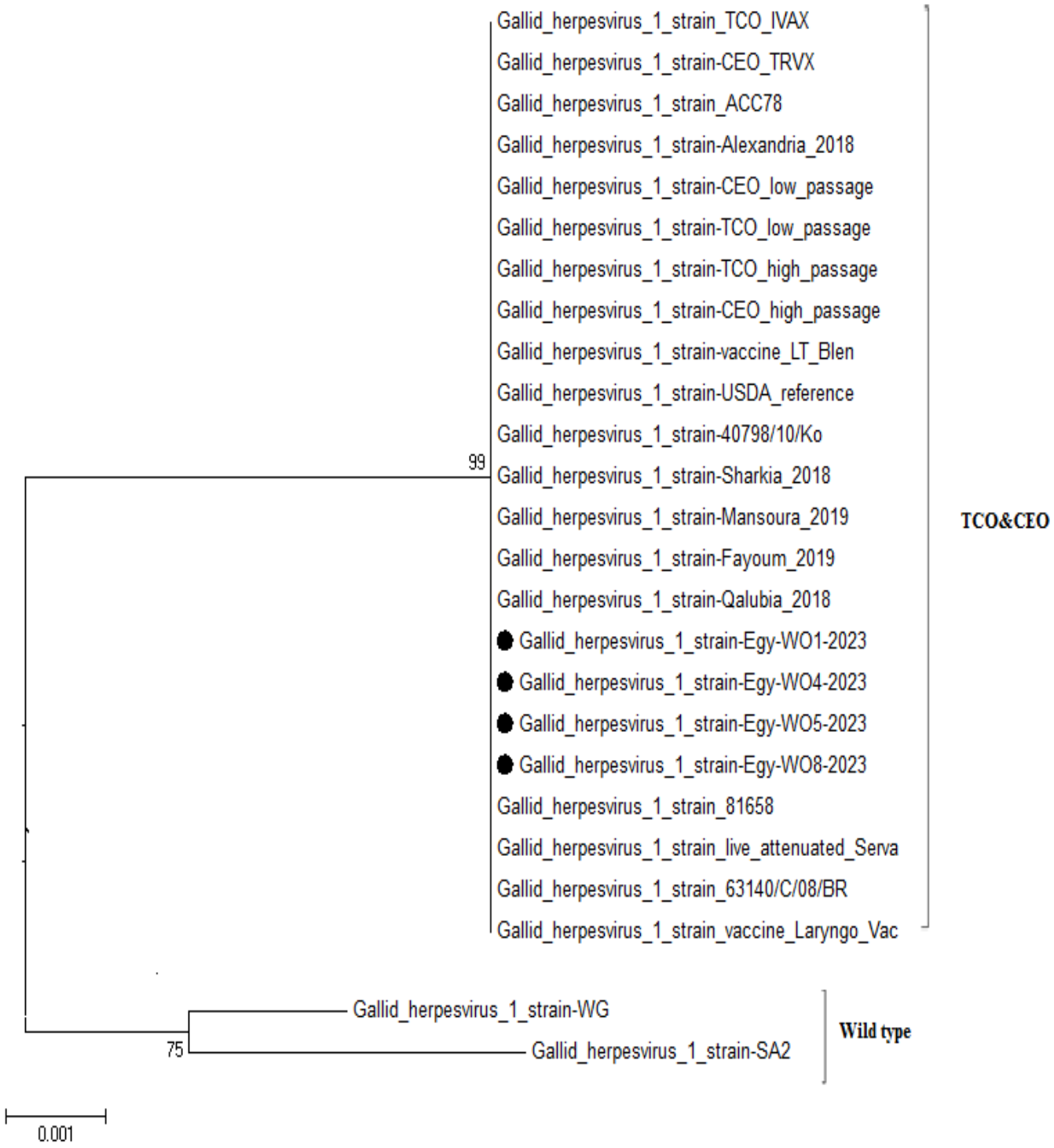


Figure 6. The phylogenetic analysis of the *gG* gene of the study strain in comparison to several field and vaccination strains of infectious laryngitis virus (ILTV) using the MEGA 6.0 program's maximum likelihood technique. The strains in the current study are indicated by black dots.

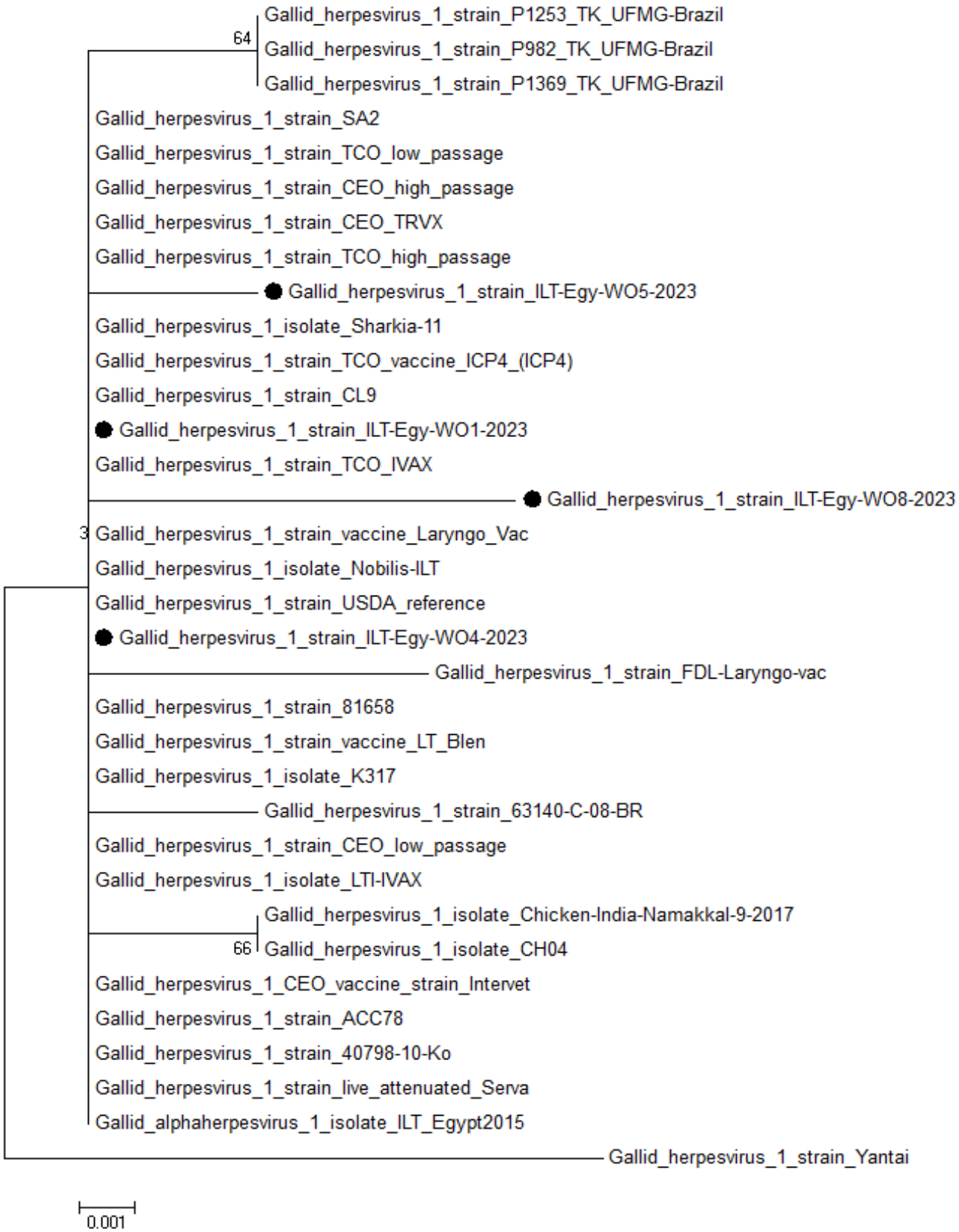


Figure 7. The phylogenetic tree of the *TK* gene of the study strain compared to several field and vaccination strains of infectious laryngitis virus (ILTV) using the MEGA 6.0 program's maximum likelihood technique. The strains in this study are indicated by black dots.

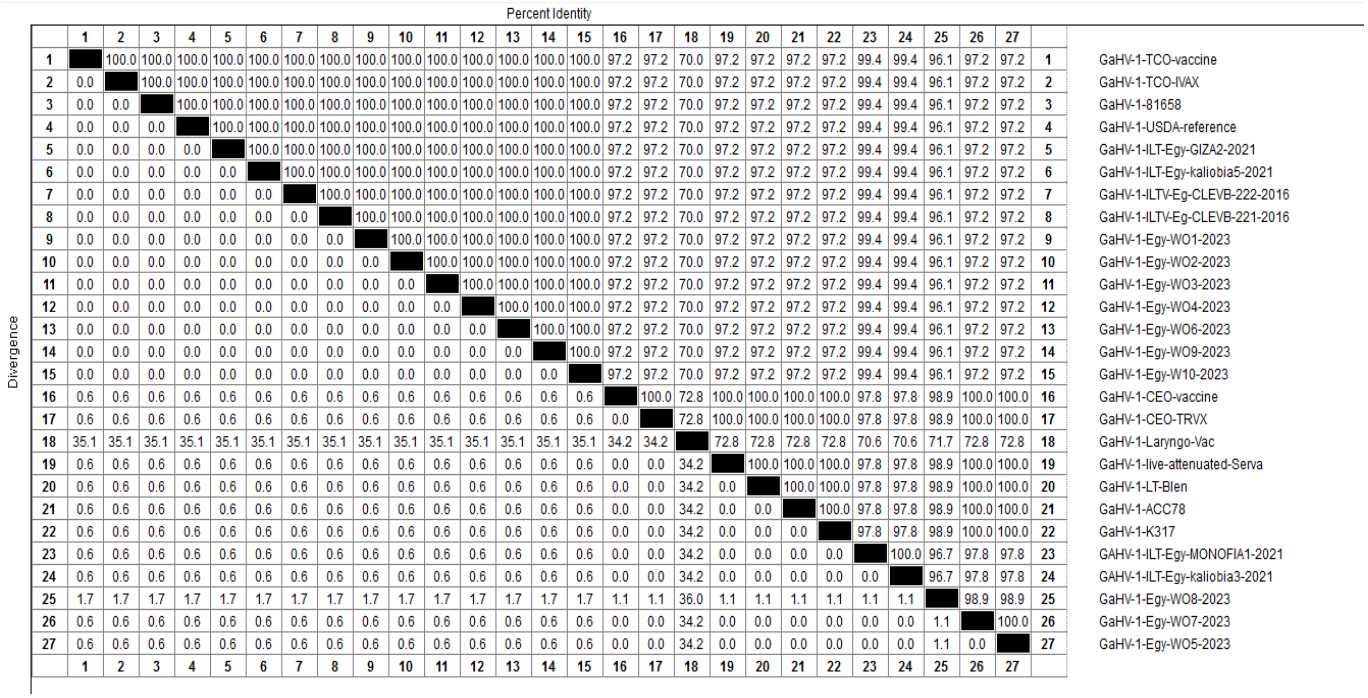


Figure 8. Amino acid identity of the sequenced *ICP4* gene compared to vaccination strains and vaccine-like strains of both CEO and TCO, as well as other Egyptian strains.

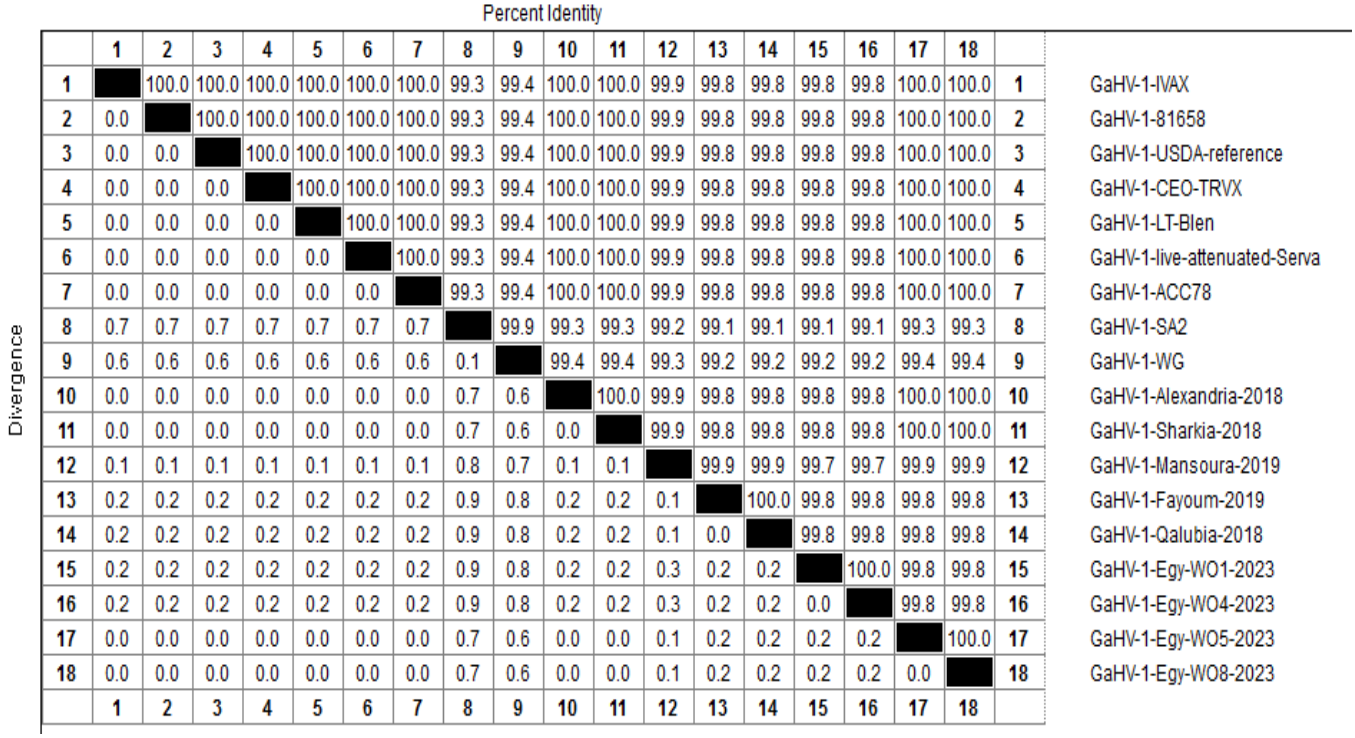


Figure 9. Amino acid identity of the sequenced *gD* gene compared to vaccination strains and vaccine-like strains of both CEO and TCO, as well as other Egyptian strains.

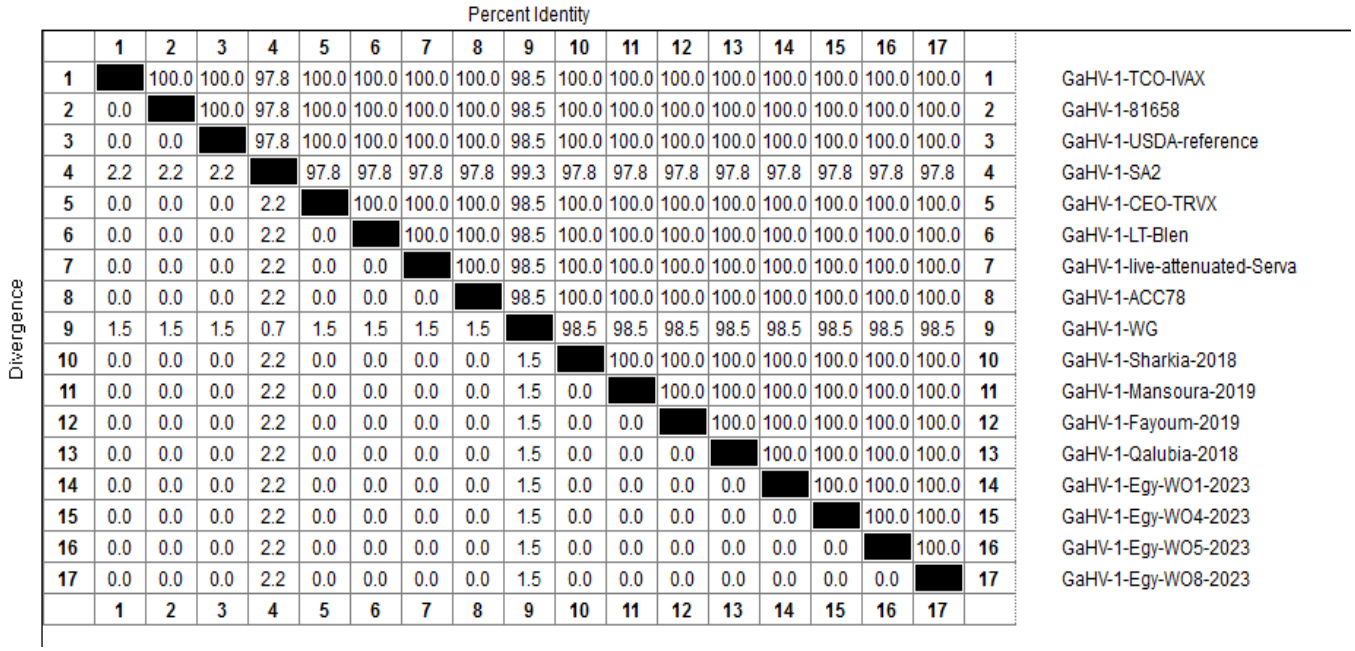


Figure 10. Amino acid identity of the sequenced *gG* gene compared to vaccination strains and vaccine-like strain of both CEO and TCO, as well as other Egyptian strains.

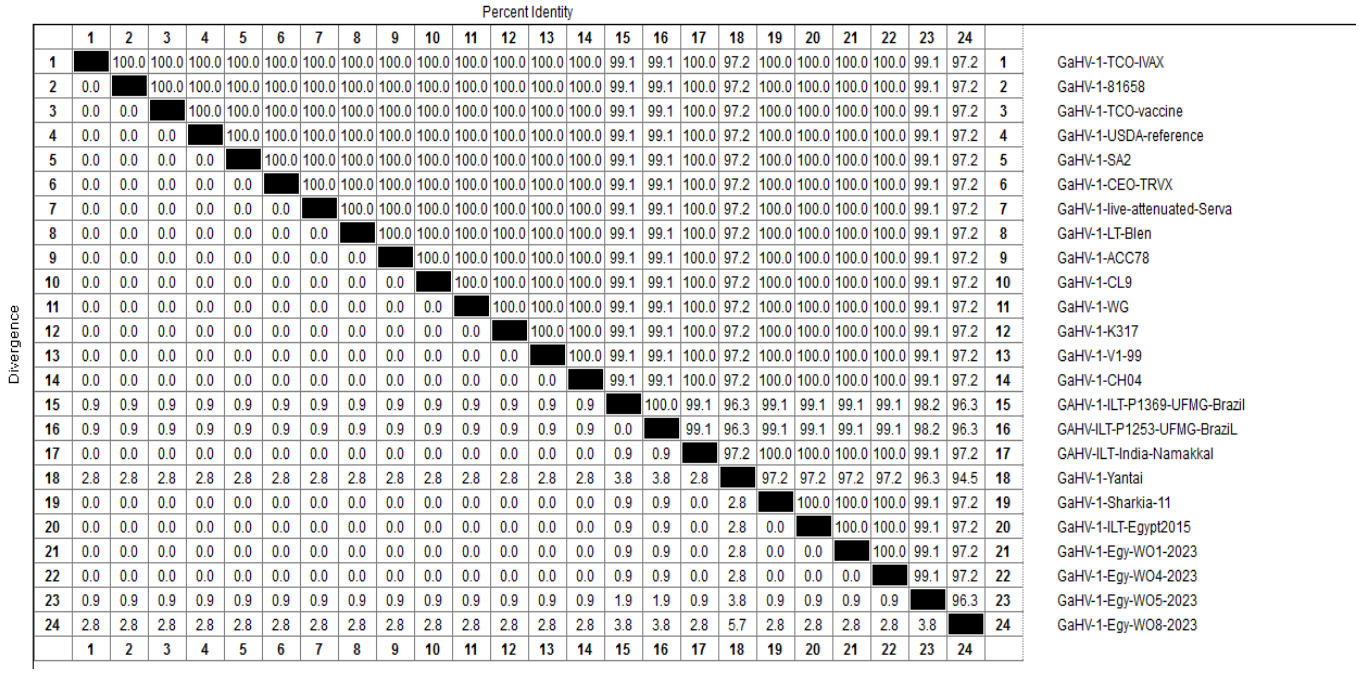


Figure 11. Amino acid identity of the sequenced *TK* gene compared to vaccination strains and vaccine-like strains of both CEO and TCO, as well as other Egyptian strains.

DISCUSSION

The ILTV has been identified in Egypt for the first time in 1983 (Tantawi et al., 1983), and outbreaks have continued until the most recent outbreaks in layer and broiler farms in 2021 (Ali et al., 2014; Bayoumi et al., 2020; Ibrahim et al., 2021; Mossad et al., 2022). ILTV still spreads throughout Egypt, causing outbreaks and significant losses in chicken farms (Bayoumi et al., 2020; Ibrahim et al., 2021; Mossad et al., 2022).

This study attempted to determine the detection and identification of the infectious laryngotrachitis virus that

was an outbreak in Egypt during 2023 and the genetic characterization of four antigenic and functional genes (*ICP4, gD, gG, and TK*).

In this study, rapid detection of ILTV-suspected layer farms from 8 governorates suffered from respiratory signs such as Dysnea, gasping, coughing, red exudates from respiratory orifices, nasal discharge, and swelling of the infraorbital sinus and moderate mortalities (5-15%) as previously detected (Hughes et al., 1991; Ali et al., 2014; Bayoumi et al., 2020). Upon post-mortem examination, the suspected chicken had hemorrhagic tracheitis with caseated material and blood clots that may have led to a blocked laryngeal lumen, as previously recorded (Preis et al., 2013; Bayoumi et al., 2020).

The symptoms and post-mortem are not sufficient to diagnose ILTV, and the diagnosis was confirmed by a rapid molecular technique (real-time-PCR) (Pang et al., 2002) due to its rapid sensitivity and reproducibility (Mackay et al., 2002). In the current study, 40 out of 50 samples (80%) were positive for ILTV by real-time PCR in eight governorates (5/8 El Sharkia, 8/10 Qalubia, 14/15 Dakahlia, 6/7 Monofia, 2/3 Gharbia, 2/2 Behira, 2/2 Domiatt, and 1/3 Alexandria), in vaccinated and unvaccinated layer farms in Egypt during 2023.

To describe the genetic development of the ILT virus strains that are presently circulating in Egypt, partial sequencing and phylogenetic analysis of four genes (*ICP4, gD, gG, and TK)* were used. to determine whether an outbreak in Egyptian fields was caused by a wild field strain or a circulating vaccine strain. This was in line with earlier research that showed these genes were commonly used to differentiate between field and vaccine strains and identify the strains' virulence (Shehata et al., 2013; Craig et al., 2017; Bayoumi et al., 2020).

As *ICP4* is a critical viral protein that is typically employed in epidemiologic research to classify circulating virus strains and contribute to the regulation of gene expression of the virus (Shehata et al., 2013) additionally, the *ICP4* gene's nucleotide sequence was able to differentiate between vaccine strains and field isolates (Chacón and Ferreira, 2009). The Phylogenetic tree of the *ICP4* gene was split into two categories: TCO and CEO vaccine and vaccine-like strains, as previously reported (Oldoni and García, 2007; Couto et al., 2015; Bayoumi et al., 2020).

According to sequence and phylogenetic analysis of *ICP4* gene of the strains under study, 7 out of 10 Egyptian strains were clustered in TCO group with TCO vaccine strain (IVAX used in Egypt, TCO low passage, TCO high passage) and virulent vaccine like strains from USA (USDA, 81658) as well as Egyptian strains during 2015-2021 with 100% A.A. identity as previously described (Nagy et al., 2020; Ibrahim et al., 2021; Mossad et al, 2022). The remaining three strains in this study were grouped with the CEO vaccine strains (TRVX, live attenuated serva used in Egypt, CEO low passage, CEO high passage) and virulent vaccine like strains from Australia (ACC78), which had 98.9%-100% amino acid identity as recorded by Shehata et al. (2013), Bayoumi et al. (2020), and Mossad et al. (2022). However, this strain differed from the Egyptian strain that was recently circulated in Egypt in 2021, which had a 96.7%-97.8% A.A. identity. As a result of sequenced strains in the present study, the CEO and TCO-like virus strains were found and circulated in Egypt during 2023, causing severe outbreaks, most of which were TCO-like virus strains (70%). However, compared to TCO reverting, it has been demonstrated that chicken embryo origin reverting causes more serious respiratory illnesses and increased mortality (García, 2017).

According to earlier research by Chacón and Ferreira (2009), the CEO vaccine-like strains are identified by deletions in the 272–283 bp and V200M in the *ICP4* gene. These deletions were recorded in GAHV-1-Eg-WO5, WO7, and WO8 that cluster with CEO vaccine and vaccine like strains. Furthermore, the findings were consistent with earlier research conducted in Egypt by Nagy et al. (2020) and Mossad et al. (2022). Additionally, two novel A.A. mutations (Q161H and Q182H) unique to GAHV-1-Eg-WO8-2023 were discovered in this work. These mutations may impair the protein's structure and configuration, impacting its biological function (Sotomayor-Vivas et al., 2022). We need further research to study the impact of these mutations on the virulence of the virus.

The envelope Glycoproteins genes (G), such as *gG*, *gJ*, and *gD*, have been shown in previous studies to be crucial for the pathogenicity and antigenicity of ILTV in chickens. Additionally, field and vaccine strains can be differentiated from one another by using specific nucleotide mutations in the gG gene, such as 316, which serve as markers (Helferich et al., 2007; Craig et al., 2017). Additionally, Han and Kim (2001) found that the threonine at positions 67 and 103 was unique to vaccination strains. Previously, vaccination strains were distinguished from some Argentinean isolates using the nucleotide mutation at 163 in the gD gene (Craig et al., 2017).

According to *Gd, Gg* phylogenetic analysis, there is no difference in phylogenetic analysis between the CEO

group and TCO group as previously recorded (Ali et al., 2014; Bayoumi et al., 2020). The strains in this study showed 99.7%, 100%, and 100% amino acid sequence identity with the TCO vaccine strain, IVAX, and CEO vaccine strains TRVX, live attenuated serva, virulent vaccine like strains from the USA and Australis (USDA, 81658, ACC78), and Egyptian strains in 2018-2019 respectively. Additionally, there was no variation between the Egyptian and vaccine strains in the nucleotide locations at position 163 of the gD gene and 316 of the Gg gene. These findings are in line with Ali et al. (2014) and Bayoumi et al. (2020), and they had threonine in the gG gene at 67 and 103 as vaccine and low virulent strain as previously detected (Ali et al., 2014). The GAHV-1-Eg-WO1 and GAHV-1-Eg-WO4 had new mutations at A34G and P276L in the gD gene that clustered them in the new branch that may affect the viral virulence (Huang et al., 1997; Bayoumi et al., 2020; Wu et al., 2022). Further studies need to determine their effectiveness on the virulence of the virus.

According to Han and Kim (2001), the *TK* gene is crucial for distinguishing between field isolates and ILTV vaccine strains and has been linked to ILTV pathogenicity and virulence. Different levels of pathogenicity have been associated with single-nucleotide polymorphisms in the TK gene. Furthermore, several Tk gene mutations were obtained to demonstrate viral attenuation for vaccine production. According to the current study's phylogenetically examined and sequenced TK gene from four isolates, there were no variations between the CEO and TCO groups. the strains showed 97.2% to 100% with CEO vaccine strain (LT Blen, TRVX, live attenuated seva, used in Egypt) and the TCO vaccine strain (IVAX, used in Egypt) along with virulent vaccine like strains from the USA, and Australia (USDA, 81658, ACC78) and Egyptian strains. According to the mutational investigation of the *Tk* gene, the GAHV-1-Eg-WO5 had A99E and GAHV-1-Eg-WO8 had R115I, G126A, and S163I that may have an impact on the virus pathogenicity (Han and Kim 2001; Santander-Parra et al., 2022). More research will be required to ascertain the efficacy of the pathogenicity of the virus.

The molecular characterization and phylogenetic findings of four genes (*ICP4, gD, gG, and Tk*) in strains isolated during 2023 indicated that outbreaks in Egyptian commercial broiler chicken flocks were related to ILTV vaccine strains. These findings align with other research on ILTV in Egyptian layer farms (Shehata et al., 2013; Ali et al., 2014; Bayoumi et al., 2020), which postulated that the CEO and TCO vaccination viruses would become more virulent following bird-to-bird transmissions, resulting in devastating epidemics among Egypt's susceptible chickens. Alternatively, the attenuated vaccine might have reactivated and caused outbreaks in susceptible chickens as a result of chicken-to-chicken transmission or the reactivation of latent infection brought on by stress, poor hygiene, and the spread of other pathogens. These results align with those obtained by Oldoni and García (2007), who demonstrated that the vaccination strains were closely linked to most commercial chicken ILTV isolates. According to Chang et al. (1997), outbreaks of ILTV occur when ILT vaccine viruses take the place of naturally occurring viruses circulating in the field. Additionally, it may be recombination that occurred between several attenuated vaccination strains, resulting in virulent recombinant viruses, which became the dominant strains in commercial poultry flocks as recorded by Lee et al. (2012). We recommend performing a full genome sequence of ILTV to detect any recombination occurrence.

CONCLUSION

According to molecular characterization of *ICP4, gG, Gd,* and *TK* genes, the ILTV outbreaks in poultry farms across several regions of Egypt during 2023 might be induced by vaccine strains derived from TCO and CEO, with some acquired mutations that may affect the virulence of the virus. It could be caused by reactivation of a viral vaccine strain from chicken to chicken transmission, or revival of a latent infection, or viral recombination of modified attenuated vaccines. Therefore, to stop ILT outbreaks in the future, there is a need to reevaluate current vaccination programs and use novel vector vaccines.

DECLARATIONS

Authors' contributions

Sabry Omar contributed to collecting samples. Ahmed Abdelhalim and Zienab Mossad performed PCR for TK, gG, gD, and ICP4. Nahed Yehia and Wessam Hassan made the sequence and phylogenetic analyses. Nahed Yehia and Wessam Hassan wrote the manuscript draft and revised it before submission. All authors checked and confirmed all data and the last draft of the manuscript before submission to the journal.

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Ethical considerations

The final version of this study has been reviewed by all authors and submitted for the first time to this publication.

Availability of data and materials

The study's original contributions are contained in the article and supplemental materials. Data is available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

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