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ICP4 GENE FOR DIFFERENTIATION BETWEEN GAHV-1 FIELD SAMPLES AND VACCINAL STRAINS USED IN EGYPT

El-kenawy, A. A.*, Samah, M. M* and Selim, A. A.**

*****Department of virology, Faculty of Veterinary Medicine, Mansoura University, Egypt

** National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, , Giza, Egypt

ABSTRACT

Twenty four pooled trachea and larynx samples were collected from different farms in Dakahlia governorate, Egypt from clinically diseased chickens suspected to be infected with Gallid herpesvirus-1 (GaHV-1). Reference GaHV-1 vaccines (tissue culture origin vaccine and chicken embryo origin) and a field isolate, previously isolated and identified by the authors were included in our study. Polymerase chain reaction (PCR) was applied on these samples, field isolate and two reference GaHV-1 vaccines for detection of GaHV-1 ICP4 gene fragments (ICP4-1 and ICP4-2) using specific primers. Twenty two samples from 24 tested samples also the field isolate and the two vaccinal strains gave positive bands at 688bp of the ICP4-1 gene. On other hand from 24 tested samples 21 samples gave positive bands of 635 bp of ICP4-2 gene fragment together with the field isolate and the two vaccine strains. From bands of PCR positive samples of ICP4-1 and ICP4-2 gene fragments, Eleven and eight samples respectively were selected; the DNA of these samples, the field isolate and the two vaccinal strains were sequenced for two gene fragments then phylogenic analysis was performed. From the phylogenic analysis and identity percentage of both ICP4-1 and ICP4-2 gene fragments we found that 7 field samples were more related to the tissue culture origin vaccine and other 4 field samples and the field isolate were more related to the chicken embryo origin vaccine used in Egypt. These results show that the virus circulating in the field is more related to the tissue culture origin vaccine.

Keywords: Gallid herpesvirus-1 Polymerase chain reaction (PCR), Primers, ICP4

INTRODUCTION

Infectious laryngotracheitis (ILT) is an acute highly contagious upper respiratory disease of chickens **Guy and Garcia (2008)**. ILT disease caused by GaHV-1, a species in the genus Iltovirus of the subfamily

Alpha herpesvirinae, within the family Herpesviridae, in the new order Herpesvirales **Davison *et. al.*(2009**). Domestic chickens and pheasants represent the only natural hosts for GaHV-1 **Fuchs *et. al.* (2007).** The first outbreak of ILT in the world was detected in a farm in Rhode Island in 1923 **May and Tittsler (1925)**. In Egypt the virus was isolated for the first time from Cairo and Giza Governorates from an outbreaks of hemorrhagic tracheitis occurred in layers 4-12 months in age in several farms with rapid spread **Tantawi *et. al.* (1983).**

In Egypt both tissue culture origin and chicken embryo origin vaccines were used for control of GaHV-1. GaHV-1 vaccine can be considered a source of field outbreaks due to return to virulence and broiler unvaccinated birds can be considered a precursor source for slow mutation of either vaccinal strain or field isolates of GaHV-1 **Madbouly *et. al.* (1997).** Phylogenic analysis of two different regions of the ICP4 gene showed differences in nucleotide and amino acid sequences between field isolates and attenuated vaccines. The approach using the sequencing of the two fragments of the ICP4 gene showed to be an efficient and practical procedure to differentiate between field isolates and vaccinal strains of GaHV-1 **Chacón and Ferreira, (2009)**.

Although ILT disease is an economically important problem, few publications described the molecular nature of GaHV-1 in Egypt. **Shehata *et. al.* (2013)** described the molecular characterization of GaHV-1 in Behira and Giza governorates, Egypt, based on sequence analysis of ICP4 gene. **Abdel-Moneim *et. al.* (2014)** determined the sequence of TK and gG genes of GaHV-1isolated from an outbreak in layer flock in Sharkia governorate, Egypt. **El-kenawy *et. al.* (2015)** diagnosed the virus depending on the detection of gE genes using PCR and gC gene using real time PCR directly from field samples without virus isolation.

This study aimed to differentiate between GaHV-1 field samples, field isolate and vaccinal strains used in Egypt including CEO and TCO vaccines by polymerase chain reaction, sequencing and phylogenic analysis for ICP4-1 and ICP4-2 gene fragments.

MATERIAL AND METHODS

Clinical samples

This study was conducted on 24 pooled trachea and larynx samples (taking the symbols from **S1** to **S24**), each sample was pooled from five randomly selected birds. These samples were collected from different farms in Dakahlia governorate, Egypt from clinically diseased chickens suspected to be infected with GaHV-1. From 24 samples 5 samples including S2, S4, S5, S14, and S23 were collected from layer chickens and the other 19 samples were collected from broiler chickens. Clinical signs appeared on diseased chickens were recumbency, swelling of the infraorbital sinuses and lacrimation with mucoid discharge from the eye caused the eyelids to become glued together.

Respiratory signs were also observed including; spasmodic coughing caused expectoration of mucus or blood stained in severe cases, at each inhalation the head was extended forward and upward due to difficult respiration and drop of egg production in layers. The postmortem examination showed hemorrhagic conjunctivitis, hemorrhagic tracheitis with blood stained mucous and blood clots in trachea **figure (1),** in some cases caseous material was observed in trachea. Samples were collected from freshly dead chickens in sterile containers containing phosphate buffed saline (PBS) then transported to laboratory in ice box then stored at -20^{o}C until use.

Field isolate

One field isolate of GaHV-1(taking the symbol **I**) was included in this study as the virus was isolated in a previous study from trachea and larynx of chickens showing clinical signs of infectious laryngitracheitis

on the chorioallantoic membrane (CAM) of 12 days old chicken embryos and the GaHV-1 was identified by PCR **(El-kenawy *et. al.* 2011)**

Figure (1): Trachea of chicken suspected to be infected with GaHV-1 showing hemorrhagic tracheitis and blood clot in trachea.

Vaccine strains

1-Tissue culture origin vaccine"LT-IVAX Live Vaccine" (taking the symbol **Vt**), supplied from manufacturer (Schering-Plough, Intervet Inc., Omaha, USA). The vaccine was prepared from ASL L-6 strain **(Gelenczei and Marty, 1964; Elkin, 2012)** which prepared by passage in chicken tissue culture.

2- Chicken embryo origin vaccine "Himmvac ILT Live Vaccine" (taking the symbol **Vc**) obtained from manufacturer (KBNP, INC, Korea) The vaccine was prepared from GaHV-1 IVR-12 strain which propagated in specific free (SPF) chicken embryo.

The vaccines were supplied in lyophilized vials, each vial contains 1000 doses. The vaccines and field isolate used as control positive in PCR, sequencing and phylogenic analysis.

Extraction of GaHV-1 DNA from collected field samples, field isolate and vaccinal strains

Twenty four pooled trachea and larynx samples and CAM containing pock lesions of GaHV-1 field isolate were homogenized with PBS using sterile mortars and pestles. The homogenates were centrifuged for 20 minutes at 3000 r.p.m as described by **Chacón *et. al.* (2007)** and the supernatant fluids were collected while two vaccines were re-suspended in PBS; each vial was re-suspended in 10 ml PBS. DNAs were extracted from field samples, field isolate and two vaccinal strains using a commercial kit; Viral Gene- Spin ™, iNtRON biotechnology INC. Extraction was performed in accordance with kit instructions.

Polymerase chain reaction for amplification of ICP4-1 and ICP4-2 gene fragments of GaHV-1

The PCR was done according to **Chacón *et. al.* (2009)** with some modifications as follows; the 688bp of the ICP4-1 gene fragment and 635bp of ICP4-2 gene fragment were amplified from 24 field samples**,** the field isolate and the two vaccinal strains using primer sequences **ICP4-1 forward**, 5\-ACTGATAGCTTTTCGTACAGCACG-3\ ,**ICP4-1 Reverse**, 5\- CATCGGGACATTCT CCAGGTAGCA -3\ ,**ICP4-2 forward** 5\- CTTCAGACTCCAGCTCATCTG-3\, and **ICP4-2 Reverse** 5\- AGTCATGC GTCTATGGCGTTGAC -3\. The primers synthesized by Metabion International AG, Lena- Christ- Strasse 44/I, Germany.

PCR was performed with a commercial Kit (Thermo Scientific Dream Taq Green PCR Master Mix) in accordance with kit instructions. The reaction contained the following components, 25 µl Dream Taq Green PCR Master Mix (2X), 0.5µM of each primer (ICP4-1 forward and reverse primers for amplification of ICP4-1gene fragment and ICP4-2 forward and reverse primers for amplification of ICP4-2 gene fragment), 0.5 µg of extracted DNA, then nuclease free water was added to yield a final volume of 50 µl finally the reaction mixture was overlaid with 25 µl of mineral oil as the thermal cycler not contain a heated lead.

The PCR run consisted of an initial denaturation step of 94^{o} C for 3 min followed by 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 64^{o}C for 1 min for ICP4-1 gene fragment amplification and 61^{o}C for 1 min for ICP4-2 gene fragment, and extension at 72^{o}C for 1.5 min. The cycles was followed by final extension step at 72 ^{o}C for 10 mins. In PC , a control tube containing nuclease free water instead of extracted DNA was included as a control negative (no template DNA).

Agarose gel electrophoresis of PCR products

The PCR products were analyzed by agarose gel electrophoresis in 1.5% agarose and stained with 0.5 µg/ml ethidium bromide using Tris-Borate EDTA buffer (89 mM Tris boric acid, 2 mM EDTA).samples were loaded against DNA ladder (Gene Ruler ^{TM} 50 bp DNA ladder, Fermentas Life Science). The gels were run at 5 V/cm for 1 h and examined with an UV transilluminator**.**

Samples that produced the expected band at 688 bp for ICP4-1 gene fragment and 635 bp for ICP4-2 gene fragment were considered positive. These bands were cut from the gel with sterile scalpel and placed in 1.5 ml eppendorf tubes with TBE buffer for DNA extraction from agarose gel.

DNA extraction from agarose gel, DNA sequencing and phylogenic analysis

Eleven field samples **(S1, S2, S3, S6, S9, S10, S12, S14, S15, S16 and S22)** were selected from GaHV-1 ICP4-1 gene positive samples which produced the expected band at 688 bp by PCR, and the DNA from bands of these samples, GaHV-1 field isolate, **Vt** and **Vc** were extracted from gel and submitted to MACROGEN clinical laboratory, Korea for DNA sequencing.

For more discrimination between field samples and vaccine strains, eight field samples **(S1, S2, S3, S9, S10 , S14, S15 and S16)** were selected from 21 GaHV-1 ICP4-2 gene positive samples which

produce the expected band at 635 bp by PCR, and the DNA from bands of these eight samples, Field isolate **(I)**, **Vt** and **Vc** were extracted from gel and submitted to MACROGEN clinical laboratory, Korea for DNA sequencing.

A neighbor-joining distance tree was constructed according to the Kimura-2-parameter model using MEGA6 software (**Tamura *et. al.* 2013**), using ICP4-1 gene fragment sequences from selected eleven GaHV-1 field samples, field isolate and two vaccine strains (Vt and Vc) and ICP4-2 gene fragment sequences from selected eight GaHV-1 field samples, field isolate and two vaccine strains. Sequences of each gene were aligned by Clustal-W method available in the Bioedit software package (**Hall 1999**).

RESULTS AND DISCUSSION

Polymerase chain reaction for amplification of ICP4-1 and ICP4-2 gene fragment of GaHV-1

Analysis of PCR products of two fragments of ICP4 gene (ICP4-1 and ICP4-2) showed positive amplification of 688bp fragment of ICP4-1 in 22 samples from 24 tested field samples, also field isolate and two vaccine strains gave positive result. On other hand from 24 tested samples, 21 samples gave positive amplification of 635 bp of ICP4-2 gene fragment together with field isolate and two vaccine strains. These results are similar to that obtained by **Chacón and Ferreira, (2009) and Shehata *et. al.* (2013)** who stated that their tested isolates had 688 and 635bp in length for ICP4-1 and ICP4-2 respectively. Moreover **OIE, (2014)** described that ICP4-1 and ICP4-2 gene fragments were used for detection and typing of GaHV-1.

DNA sequencing and phylogenic analysis of the PCR products

The nucleotide sequences of 688 bp of ICP4-1 gene fragment from eleven field samples (S1, S2, S3, S6, S9, S10, S12, S14, S15, S16 and S22), GaHV-1 field isolate (I), Vt and Vc were aligned by MEGA6

software. Phylogenic analysis revealed a close relationship between tissue culture origin vaccine (Vt) and seven field samples (S1, S2, S3, S6, S9, S12 and S22) as they were aligned with Vt in the same clade. But CEO vaccine was more related to the other four field samples (S10, S14, S15 and S16) and GaHV-1 field isolate (I) as they were clustered together in the other clade, so most field samples were more related to Vt than Vc by ICP4-1 gene analysis **figure (4).**

These results are in concurrence with **Chacón and Ferreira, (2009)** who concluded that sequencing of two fragments of ICP4 gene could be used to differentiate between field isolates and vaccine strains of GaHV-1. However our results are in contrast to **Sedeghi *et. al.* (2011)** who found that the phylogenic analysis of tested field isolates, Vt and Vc vaccine revealed that there were no difference between nucleotide sequences between field isolates and Vc.

The identity % between **Vt** and seven field samples aligned with it was 99.7% with both **S2** and **S3**, 99.5% with **S9**, 99.4% with **S12,** 99.2% with **S6,** 98.9% with **S1** and 97.9% with **S22** but the identity % between **Vc** and four field samples aligned with it was 99.5% with both **S15 and S16,** 98.4% with **S10** and 98.2% with **S14**. The identity % between **Vc** and GaHV-1field isolate was 99.4% **Table (1).**

Comparison of the sequenced ICP4-1 gene PCR products of eleven field samples, GaHV-1 field isolate, Vt and Vc demonstrated deletion of a segment of 12 nucleotide from Vc, and four field samples (S10, S14, S15 and S16) leading to absence of four amino acids which permit easy differentiation between CEO, TCO vaccines and field samples depending on ICP4-1 gene analysis. These results were in agreement with the results obtained by **Chacón and Ferreira, (2009)** and **Sedeghi *et. al.* (2011)** who reported absence of four amino acids from of 688 bp fragment of ICP4 gene in CEO strain so these gene fragment could differentiate between CEO, TCO vaccine strains and field strains.

For more discrimination between field samples and vaccine strains, eight field samples (S1, S2, S3, S9, S10 , S14, S15 and S16) were selected from 21 GaHV-1 ICP4-2 gene PCR positive samples and the DNA from bands of these eight samples, Field isolate (I), Vt and Vc were sequenced. Phylogenic analysis of ICP4-2 gene fragment revealed close relationship between tissue culture origin vaccine (Vt) and four field samples (S1, S2, S3 and S9). But Vc was more related to the other four field samples (S10, S14, S15 and S16) and field isolate (I) as they were clustered together in the same clade **figure (5)**.

The identity % between **Vt** and four field samples aligned with it was 100% with both **S1** and **S2**, 97.9% with **S3** and 99.5% with **S9** and the identity % between **Vc** and four field samples and field isolate aligned with it was 100% with **S14** and 99.5% with **I, S10, S15** and **S16**.

So the results of ICP4-2 gene confirmed the results of ICP4-1 gene and these results are in concurrence with **Chacón and Ferreira, (2009).** They concluded that sequencing of two fragments of ICP4 gene could be used to differentiate between field isolates and vaccine strains of GaHV-1. On the other hand **Shehata *et. al.* (2013)** described that the chicken embryo origin vaccine was responsible for GaHV-1 outbreaks in Egypt.

From the phylogenic analysis and identity percentage of both ICP4-1 and ICP4-2 gene fragments we found that 7 field samples (S1, S2, S3, S6, S9, S12 and S22) were more related to TCO vaccine (Vt) and other 4 field samples (S10, S14, S15 and S16) and the field isolate (I) were more related to Vc used in Egypt. These results show that Vt is more related to the virus circulating in the field. our results are in concurrence with **Chacón and Ferreira, (2009)** who concluded that sequencing of two fragments of ICP4 gene was used to differentiate between field isolates and vaccine strains of GaHV-1. But they are in contrast to

Sedeghi *et. al.* (2011) who found that there was no difference between nucleotide sequences between field isolates and CEO vaccines.

The present study shows that the PCR provide a reliable, sensitive and rapid method for detection of GaHV-1 in field samples, GaHV-1field isolate and the two GaHV-1 vaccinal strains within few hours.Our study also shows that the two GaHV-1 vaccinal strains used for vaccination of birds against the virus are sufficient to induce good protection if there is a good vaccination program. It also showed that the most field samples were related to TCO vaccine which may revert to its virulence after bird to bird passage and from vaccinated to non-vaccinated birds so vaccination should be applied to all broiler and layer birds in the same time to control the disease. More studies are needed for vaccination program evaluation through challenge test. Also field surveillance for GaHV-1 must be routined to monitor the situation of virus evolution.

Table (1): The homology percentage of nucleotide sequences for ICP4-1gene of eleven field samples , GaHV-1 field isolate, TCO vaccine (Vt) and CEO vaccine (Vc).

Percent identity

Percent diversity

	S2	S6	S16	I	Vc	S3	S12	S22	S9	S14	S1	S10	S15	Vt
S2		99.2	98.223	98.223	98.61	99.68	99.18	97.9	99.521	97.556	98.71	99.036	98.388	99.68
S6	0.802		97.895	98.389	97.9	99.52	99.52	98.39	99.197	97.058	99.04	98.877	97.898	99.198
S16	1.777	2.105		99.52	99.500	98.223	98.060	96.890	98.387	98.382	97.895	98.548	99.681	98.221
I	1.777	1.611	0.480		99.358	98.600	98.223	97.395	98.549	98.055	98.227	98.876	99.360	98.551
Vc	1.939	2.103	0.481	0.642		98.06	98.061	96.893	98.389	98.216	97.896	98.387	99.520	98.060
S3	0.320	0.479	1.777	1.447	1.939		99.359	97.895	99.359	97.556	98.876	99.360	98.388	99.680
S12	0.819	0.481	1.940	1.777	1.939	0.641		98.220	99.521	97.226	99.034	98.712	98.063	99.360
S22	2.105	1.614	3.110	2.605	3.107	2.105	1.778		97.730	96.377	97.395	98.222	96.895	97.897
S9	0.479	0.803	1.613	1.451	1.612	0.641	0.479	2.270		97.558	99.035	99.037	98.389	99.520
S14	2.444	2.942	1.618	1.945	1.784	2.444	2.774	3.623	2.442		97.058	98.216	98.384	97.554
S1	1.289	0.963	2.105	1.773	2.104	1.124	0.966	2.605	0.965	2.942		98.553	97.898	98.875
S10	0.964	1.123	1.452	1.124	1.613	0.640	1.288	1.778	0.963	1.784	1.447		98.712	99.360
S15	1.612	2.102	0.319	0.640	0.480	1.612	1.937	3.105	1.611	1.616	2.102	1.288		98.387
Vt	0.320	0.802	1.779	1.449	1.940	0.320	0.640	2.103	0.480	2.446	1.125	0.640	1.613	

Table (2): The homology percentage of nucleotide sequences for ICP4-2gene of GaHV-1 from field isolate (I), eight field samples,TCO vaccine (Vt) and CEO vaccine (Vc).

Percent identity

Percent diversity

	S9	I	S14	S10	S15	S16	S1	S2	S3	Vc	Vt
S9		98.4	98.43	98.61	98.43	98.43	99.48	99.48	97.53	98.43	99.43
I	1.566		99.48	99.13	99.31	99.31	98.26	98.26	96.28	99.48	98.26
S14	1.566	0.519		99.48	99.48	99.48	98.43	98.43	96.64	100	98.43
S10	1.391	0.866	0.518		99.48	99.482	98.785	98.785	96.998	99.482	98.785
S15	1.568	0.692	0.518	0.518		100.000	98.961	98.961	96.882	99.482	98.961
S16	1.568	0.692	0.518	0.518	0.000		98.961	98.961	96.882	99.482	98.961
S1	0.519	1.740	1.565	1.215	1.039	1.039		100.000	97.895	98.435	100.000
S2	0.519	1.740	1.565	1.215	1.039	1.039	0.000		97.895	98.435	100.000
S3	2.467	3.721	3.361	3.002	3.178	3.178	2.105	2.105		96.640	97.895
Vc	1.566	0.519	0.000	0.518	0.518	0.518	1.565	1.565	3.361		98.435
Vt	0.519	1.740	1.565	1.215	1.039	1.039	0.000	0.000	2.105	1.565	

Figure (2): Agarose gel electrophoresis of conventional PCR products of the ICP4-1 gene (688bp) of GaHV-1. GaHV-1 field isolate **(I)** ,22 field samples, TCO vaccine **(Vt)** and CEO vaccine **(Vc)** give positive results**. S8 and S24** give negative results. In the left DNA marker (**M**) measured by base pair (bP).

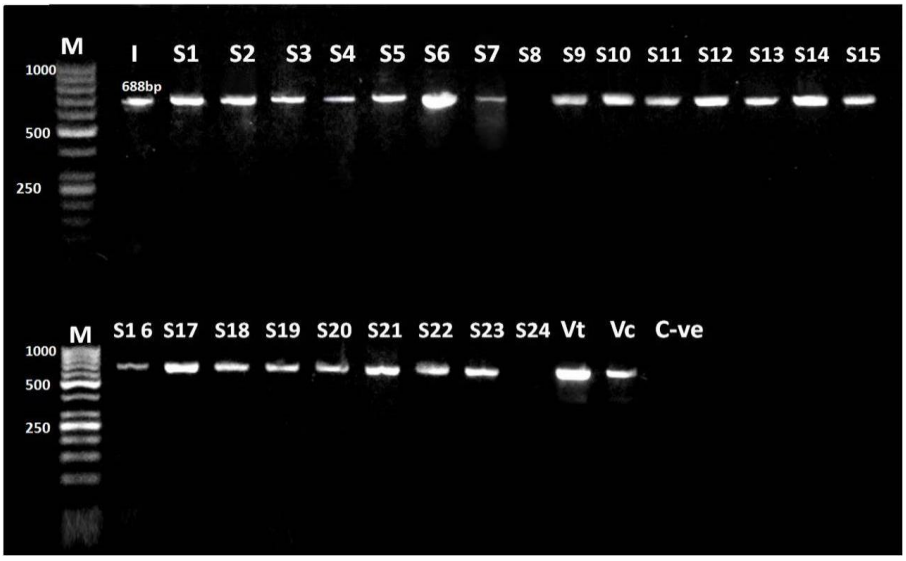


Figure (3):Agarose gel electrophoresis of conventional PCR products of the ICP4-2 gene (635bp) of GaHV-1. GaHV-1 field isolate **(I)** ,21 field samples, TCO vaccine **(Vt)** and CEO vaccine **(Vc)** give positive results**. S11, S20 and S24** give negative results. In the left DNA marker **(M)** measured by base pair (bP).

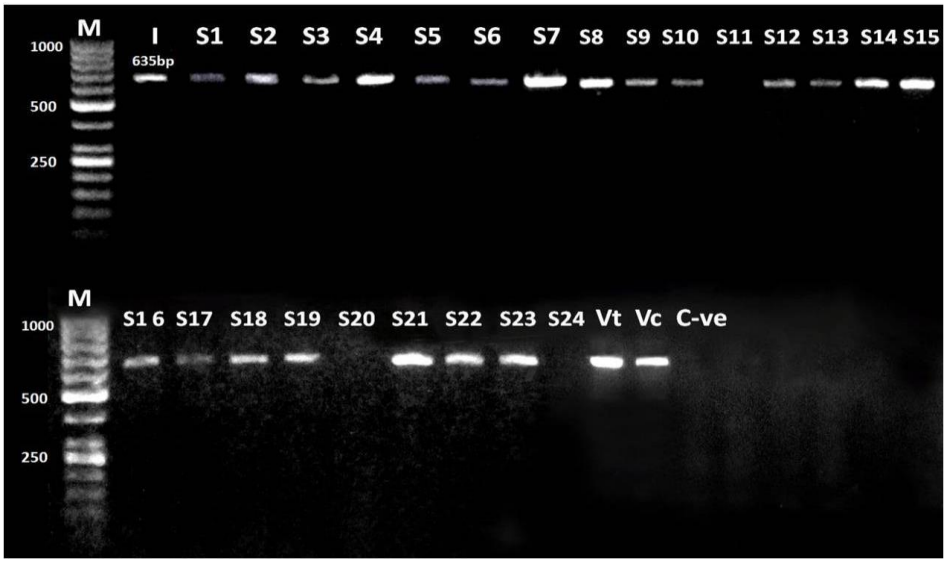


Figure (4): Phylogenic relationship among GaHV-1 following alignment of 688 bp sequences of ICP4-1 gene from eleven field samples, GaHV-1field isolate, TCO vaccine and CEO vaccine (two vaccines were marked with triangle). The tree was analyzed by neighbor-jioning method using MEGA 6 soft ware

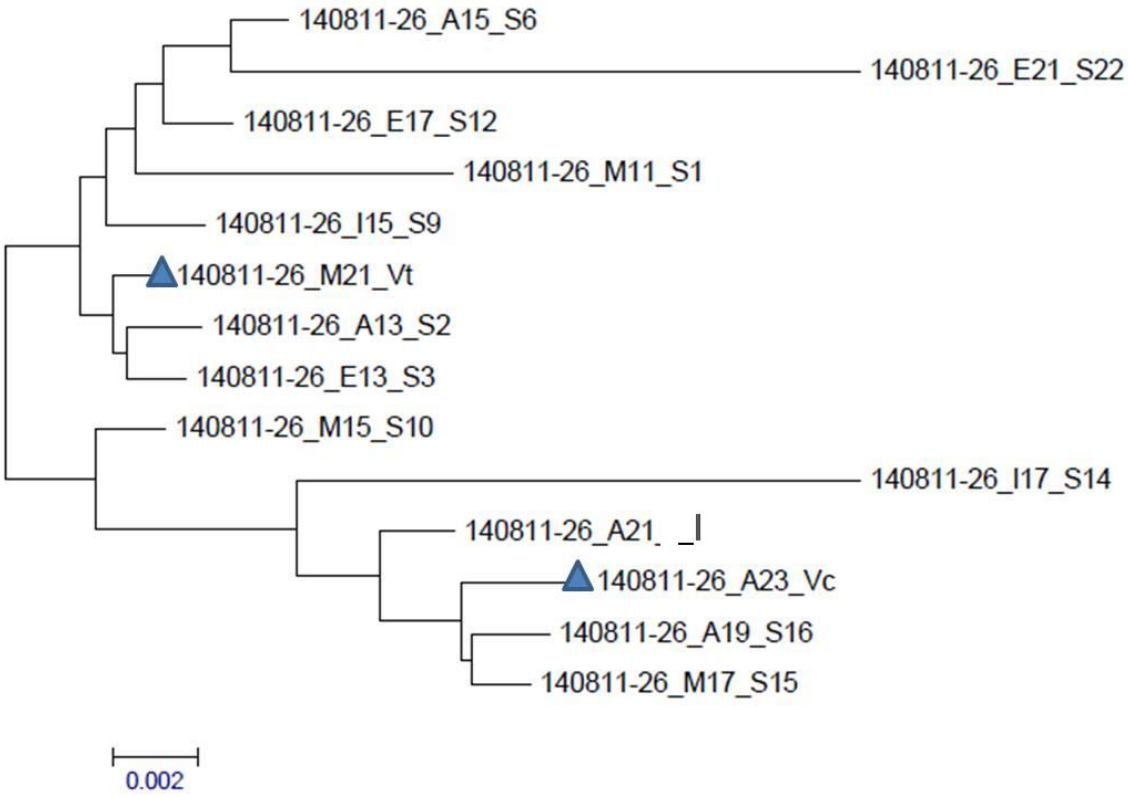
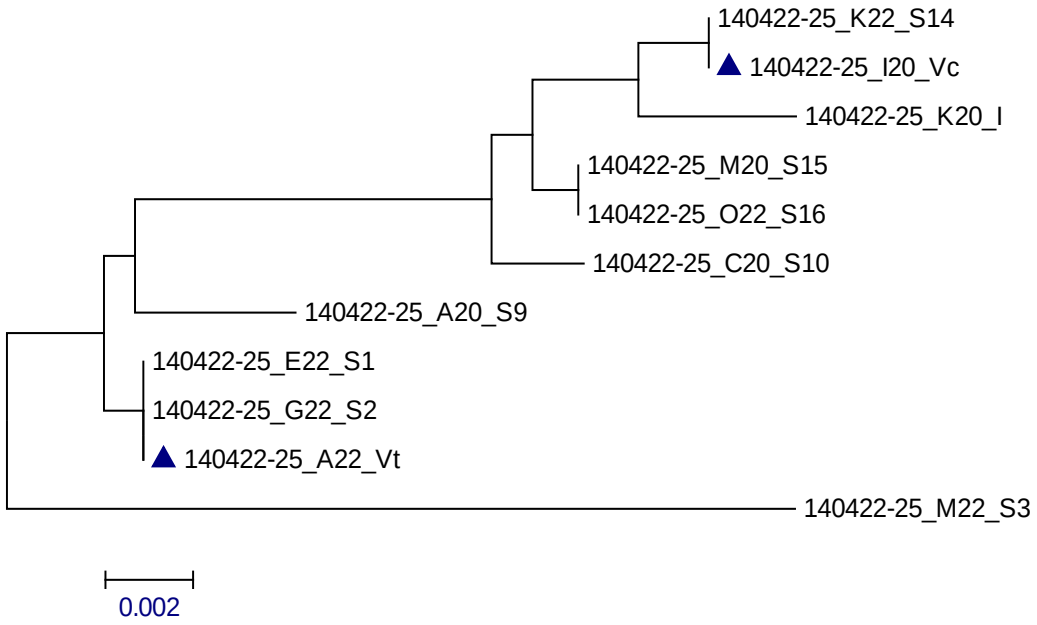


Figure (5): Phylogenic relationship among GaHV-1 following alignment of 635bp sequences of ICP4-2 gene from field isolate, eight field samples, TCO vaccine and CEO vaccine (two vaccines were marked with triangle). The tree was analyzed by neighbor-jioning method using MEGA 6 software.



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