



Official Journal Issued by  
Faculty of  
Veterinary Medicine

## Benha Veterinary Medical Journal

Journal homepage: <https://bvmj.journals.ekb.eg/>



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### Original Paper

## Molecular and Virological characterization of local isolate of Equine Herpes Virus -4 among aborted mares from Egypt 2022

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### ARTICLE INFO

#### Keywords

Abortion  
Isolation  
gB gene  
Phylogenetic analysis

Received 14/12/2023

Accepted 31/12/2023

Available On-Line

31/12/2023

### ABSTRACT

This work aimed for detection of EHV-4 in samples from suspected mares and aborted feti from Cairo and Giza, Egypt, 2022 followed by studying its phylogenetic, serological and culture characters on primary RK cells and VERO cell line. Samples were collected, prepared as virus suspension, undergone molecular identification by cPCR using primers specific for gB of EHV-4. Results revealed amplicon size of the amplified DNA product at 508 bp. Sequencing of the partially amplified gB gene and phylogenetic analysis of positive samples showed 100%, 99.8% and 99.6% nucleotide similarity with other EHV-4 strains reported in the GenBank. Virus isolation showed appearance of CPE after 5 dpi at 5th passage and 3dpi at 3rd passage for RK and VERO cells respectively. CPE of EHV-4 isolate characterized by cell rounding, aggregation, clustering and cell lysis. Titration was done on both primary RK cell culture and VERO cell line for all passages, the virus titer increased gradually to reach the highest level in the 5th, and 6th passages in RK cells and 3rd, 4th passages in VERO cells. The growth kinetics of EHV-4 isolate using 0.5 ml virus dose with dilution 10<sup>-1</sup> cell/ml revealed that the MOI of EHV-4 isolates at 4 and 5 dpi and the optimum titer was log<sub>10</sub> 5.5 TCID<sub>50</sub>/ml. Serological identification of the virus isolate showed positive results with IFAT and VNT using specific reference antisera. Finally, the evaluation approved that our EHV-4 strain is more genetically related to the same old strains in Egypt causing the disease.

## 1. INTRODUCTION

Equine Herpes Virus -4 (EHV-4) is one of the pathogens that cause a storm of abortion in the equine in the last trimester of pregnancy 2-4 weeks after infection (Afifi et al., 2017). It causes high mortality and morbidity, which results in high economic losses for horse farms (Vissani et al., 2016). EHV-4 belongs to the family Herpesviridae, subfamily Alphaherpesvirinae (Pellet and Roizman, 2007), which is an enveloped virus and has icosahedral symmetry with double-strand DNA of approximately 120-250 kbp (King et al., 2012). The genome consists of a 76 open reading frame (ORF), which includes unique long UL and unique short US regions that are interrupted by internal small (IRs) and internal large (IRL) inverted repeated sequences and bordered by terminal small (TRs) and terminal large (TRL) inverted repeated sequences (O'Callaghan and Osterrieder, 2008). The structural proteins of the virus are 30; 6 of them form nucleocapsids which are (ORFs 22, 25, 35, 35.5, 42, 43); 12 of them are tegument, which is (ORFs 11, 12, 13, 14, 15, 23, 24, 40, 46, 49, 51, 76). The last 12 of them are glycoproteins, which are gB, gD, gH, gK, gL, gC, gE, gG, gI, gM, gN, and gP300. They are the most

important proteins because they are the surface proteins responsible for pathogenesis, sensitization of immune response, and neutralization of the virus by specific antibodies (Allen et al., 2004).

Based on molecular characterization and subtyping of EHV, it was found that EHV had been classified into five strains, which are EHV-1, 2, 3, 4, and 5 strains. Among these strains, EHV-1, 4 are the most recorded strains in Egypt (Hassanein et al., 2002; Warda, 2003; Abd El Hafeiz et al., 2010; Al-Shammari et al., 2016; Afify et al., 2017).

EHV was reported in Egypt for the first time in 1965 when detecting EHV-2 antibody in Egyptian horse serum (Matsumoto et al., 1965) then EHV-1 was isolated in 2002 (Hassanein et al., 2002) then reported in 2003 (Warda, 2003) then in 2010 (Abd El Hafeiz et al., 2010) and EHV-4 was recently isolated in 2017 (Afify et al., 2017). The first molecular characterization and subtyping of EHV in Egypt was reported in 2011 (Amer et al., 2011). EHV-4 shows a strong antigenic relationship with EHV-1, but there is a minor variation that is only detected by monoclonal antibodies (Allen et al., 2004). The protection of horse farms in Egypt is usually provided with inactivated vaccines (Anonymous, 2015), which are imported from abroad. In the current work,

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molecular detection of EHV-4 in clinically suspected samples collected from vaccinated, non-vaccinated, dead pregnant mares and their aborted fetus and apparently healthy non-pregnant mares from Cairo and Giza governorates, Egypt, during 2022 by conventional PCR targeting gB gene followed by genetic characterization, virus isolation on RK cells and VERO cells, titration and studying growth kinetics of the virus, optimum harvest action time and optimum dose of virus and serological identification by IFAT and VNT.

## 2. MATERIAL AND METHODS:

### 2.1. Ethical approval:

All the procedures in this study were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Benha University, with an Ethical approval number (BUFVTM08-10-22).

### 2.2. EHV-4 strain (KX866264):

A reference isolated strain during outbreak of abortion in Arabian horses Egypt, 2017 (Afify et al.,

2017). It was obtained from the Virology Department at the Animal Health Research Institute, Dokki, Egypt. This virus isolate was adapted to the MDBK cell line and stored at -70 °C. It is still being used as a reference virus for PCR, SNT, and FAT.

### 2.3 Samples:

A hundred samples vary between tissue samples, vaginal and nasal swaps were aseptically collected from Giza and Cairo from June to December 2022 from vaccinated (Pneumabort® vaccine), non-vaccinated pregnant that aborted in the last trimester of pregnancy with congested placenta and some respiratory manifestation in the non-vaccinated mares as rapid and difficult respiration, apparently healthy non-pregnant mares, dead pregnant mares and their aborted fetus. Samples were transmitted to the Lab in an ice box with dry ice prepared as virus suspension and pooled under complete aseptic condition according to OIE, (2015), as shown in Table (1), preserved at -80 °C until being used for isolation at cell culture, serological and molecular identification of EHV-4.

Table (1): Description of the collected samples in the study

Animal	vaccination	type of sample	No. of samples	Pooled sample
pregnant mares	non-vaccinated	placenta	20	S1
	vaccinated	placenta	5	S2
aborted feti	non-vaccinated	lung	20	S3
		spleen	20	S4
	vaccinated	lung	5	S5
		spleen	5	S6
dead pregnant mares	vaccinated	pooled tissue homogenates (placenta lung, spleen, liver)	3	S7, S8, S9
aborted feti from dead mares	vaccinated	pooled tissue homogenates (placenta lung, spleen, liver)	3	S10, S11, S12
apparently healthy non-pregnant mares	non-vaccinated	nasal and vaginal swaps	19	S13:S31

### 2.4. Viral DNA extraction and PCR.

The extraction of viral DNA was carried out from the pooled samples following the instruction of QIAamp DNA Mini Kit (Qiagen, Germany), which provided silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so the total hands-on preparation time is only 20 minutes. The suspected EHV-4 samples were detected by PCR using a set of forward and reverse gB primers (Hafshejani et al., 2015). The primer sequences were EH4 gB forward [5'-TATTGTTCCGCCACTCTTGACG -3'] and reverse [5'-GTAGAATCGGAGGGCGTGAAGC 3']. Midland Certified Reagent Company\_ (Oilgos, USA), supplied the primers for the amplification of 508 bp fragments.

The PCR reaction was performed for gB according to Hafshejani et al. (2015), with initial denaturation at 95 °C for 5 min, then 35 cycles of secondary

denaturation at 94 °C for 30 secs, annealing at 55 °C for 30 secs, extension at 72 °C for 45 sec and final extension at 72 °C for ten min. 5 µl of PCR products were examined by electrophoresis documentation system on a 1.5% agarose gel containing ethidium bromide after amplification (Sambrook et al., 1989).

### 2.5. Sequencing.

The PCR product obtained from the positive sample was purified using the QIA quick gel extraction kit (Qiagen Inc., Valencia, CA). The purified PCR product was then subjected to sequencing in both the forward and reverse directions by an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). The sequencing reaction was performed using the BigDye Terminator V3.1 Cycle Sequencing Kit from Perkin-Elmer/Applied Biosystems, Foster City, CA, with catalogue number 4336817. To verify the sequence identity, a BLAST® analysis (Basic

Local Alignment Search Tool) was conducted. This analysis involved comparing the obtained sequences to the sequences in the National Center for Biotechnology Information (NCBI) database to obtain GenBank accessions for further analysis and identification (Altschul et al., 1990).

### 2.6. Sequence analysis.

The sequence of the positive EHV-4 sample by PCR was comparatively analyzed with reference sequences through their accession numbers from the Gene bank using the CLUSTAL W multiple sequence alignment program, version 12.1 of Meg Align module of Lasergene DNASTar software Pairwise, designed by Thompson et al., (1994) and also, phylogenetically analyzed using maximum likelihood, neighbor-joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

### 2.7. Isolation of EHV-4:

Primary RK cell culture and VERO cell line were prepared and subcultured according to OIE (2013) using Minimum Essential Medium with Hank's salts and 10% sterile fetal calf serum. EHV-4 was isolated and propagated on the primary RK cells and VERO cell line according to Babiuk et al. (2007), then titrated according to Reed and Muench (1938). The growth kinetics of EHV-4 isolate were studied in VERO cells, according to Trabelsi et al. (2014). Optimization of the virus infectivity titer and harvesting time post inoculation was determined using MOI of the 4th passage in cells at dilution 10<sup>-1</sup>, 10<sup>-2</sup>, and 10<sup>-3</sup> for 2, 4, and 5 dpi.

### 2.8. Serological identification of EHV-4 isolate:

#### 2.8.1. Indirect fluorescence antibody technique (IFAT):

The test was carried out on inoculated VERO cells for identification of EHV-4 isolate according to Gunn (1992) with some modifications on Glass Chamber Slides. EHV-4 reference antiserum as a polyclonal antibody was provided by the virology department, AHRI, Dokki, and Giza. It is used for the identification of EHV-4 at dilution 1/80 in PBS. Rabbit anti-horse IgG conjugated with fluorescence isothiocyanate (SIGMA, kit No.: MFC00163518) was used as a secondary antibody at dilution 1/300 in PBS. Finally, Slides were examined for EHV-4 fluorescence under a fluorescent microscope with a 20 X fluorite lens (OLYMPUS) at the Pathology Research Dept., Animal Health Research Institute, Dokki.

#### 2.8.2. Serological identification of EHV-4 isolate using virus neutralization test (VNT):

It was applied on the virus isolates using the microtiter plate technique according to (OIE, 2018).

## 3. RESULTS

### 3.1 Molecular detection of EHV-4 in suspected pooled samples by conventional PCR (c-PCR):

Samples examined by c-PCR for EHV-4 showed that S1 only was positive for EHV-4 with amplicon size 508 bp as shown in Fig. (1).



Fig. (1): Electrophoretic pattern of c-PCR products based on gB gene of EHV-4 sample on the gel electrophoresis (1.5%). Amplicon size was detected at 508 bp which considered positive results for the EHV-4 in S1. Positive and negative controls are included.

### 3.2. Sequencing and phylogenetic analysis of EHV-4 based on gB gene:

The sequence of gB gene of the sample in this current study, which has Genbank accession number (OP546096), was compared with those of strains available on the GenBank database. The multiple sequence alignment for gB gene partial sequence of EHV-4 sample of this study has shown 100% nucleotide identity with EHV-4 strains RJ4-TR2011 (Acc. No. JN982957) and TR-EHV4-VSA-11 (Acc. No. JX416463) from Turkey 2012, 99.8% nucleotide identity with EHV-4 strains EgeyqH4-19A (Acc. No. MN592995) from Egypt 2020, ER39-67 (Acc. No. KT324748) from Australia 2017 and US4-TR2011 (Acc. No. JN982958) from Turkey 2012 and 99.6% nucleotide identity with EHV-4 strains VRLCU-412-2015 (Acc. No. KP699582) from Egypt 2015 and 157-69 (Acc. No. KT324738) from Australia 2017 as shown in Table. (2).

Also phylogenetic tree showed that EHV-4 strain of our study is clustered with the following EHV-4 strains, RJ4-TR2011 (Acc. No. JN982957), TR-EHV4-VSA-11 (Acc. No. JX416463) from Turkey 2012, EgeyqH4-19A (Acc. No. MN592995) from Egypt 2020, ER39-67 (Acc. No. KT324748) from Australia 2017 and US4-TR2011 (Acc. No. JN982958) from Turkey 2012 and it is located in the same node with the following EHV-4 strains, VRLCU-412-2015 (Acc. No. KP699582) from Egypt 2015 and 157-69 (Acc. No. KT324738) from Australia 2017 as shown in Figure (2).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
2	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT
3	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT
4	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT	TTT

Table (2): Nucleotide identities and divergence of gB gene-sequenced sample of EHV-4 in this study compared to other reference EHV-4 strains retrieved from the GenBank database.

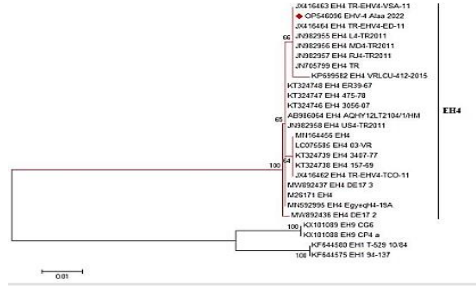


Fig. (2): Phylogenetic tree using maximum likelihood method based on gB gene nucleotide sequences of EHV-4 sample of this study (indicated by red diamond) with other sequences of EHV-4 strains retrieved from the GenBank database.

The scale bar (0.01) means nucleotide changes or substitutions number per nucleotide.

3.3. Virus isolation on cell culture:

Isolation of EHV-4 on primary RK cell cultures showed that specific CPE began to appear at 5th, 4th, 3rd and 3rd dpi for the 3rd, 4th, 5th, and 6th passages, respectively, that were completed at five dpi. CPE was characterized by cell rounding at 48–72 hpi (2-3 days) in the first stage of CPE, cell aggregation forming characteristic grape-like clusters at 96–120 hpi (4-5 days), and finally, the last stage of CPE characterized by necrosis, degeneration, detachment, and cell lysis after five dpi as shown in Fig (3), A, B and C respectively.

Isolation of EHV-4 on VERO cell line showed that specific CPE began to appear at 4th, 3rd, and 3rd dpi for the 2nd, 3rd and 4th passages, respectively that, were completed at 5dpi. CPE as in RK cells as shown in Fig (3), D, E and F, respectively.

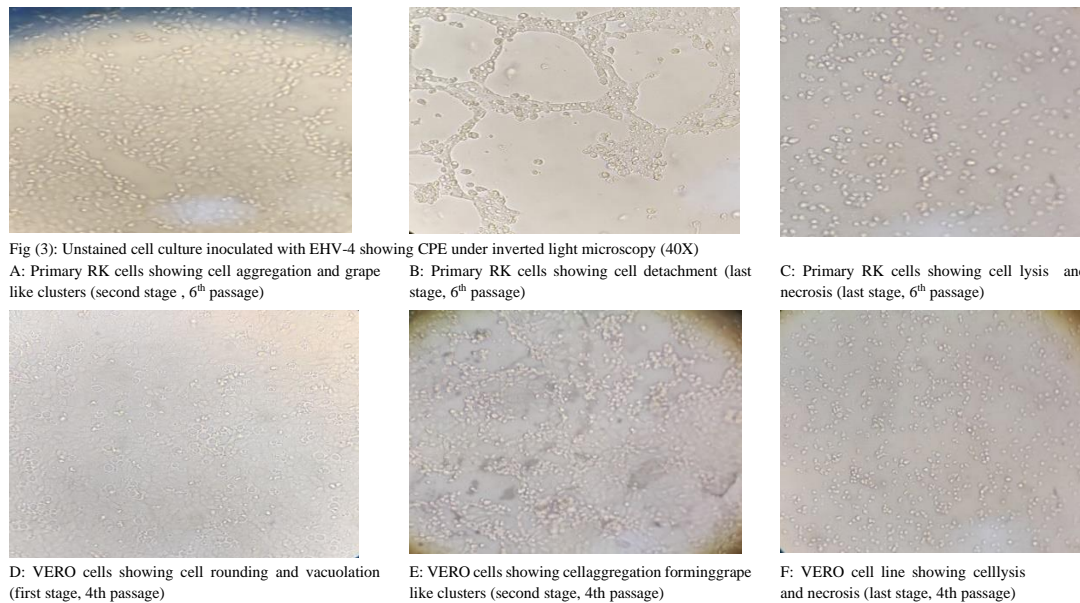


Fig (3): Unstained cell culture inoculated with EHV-4 showing CPE under inverted light microscopy (40X)  
 A: Primary RK cells showing cell aggregation and grape like clusters (second stage, 6th passage)  
 B: Primary RK cells showing cell detachment (last stage, 6th passage)  
 C: Primary RK cells showing cell lysis and necrosis (last stage, 6th passage)  
 D: VERO cells showing cell rounding and vacuolation (first stage, 4th passage)  
 E: VERO cells showing cell aggregation forming grape like clusters (second stage, 4th passage)  
 F: VERO cell line showing cell lysis and necrosis (last stage, 4th passage)

3.4. Virus titration on cell culture:

Infectivity titer of EHV-4 isolate on RK cells revealed gradual increase through the successive six passages from 3.5 to 5.5 log<sup>10</sup> TCID<sub>50</sub> /ml as a maximum titer from 3rd till 5th and 6th passages respectively (Fig. 4). Also, infectivity titer of EHV-4 isolate on VERO cell line revealed gradual increase through the successive four passages from 4 to 5 log<sup>10</sup> TCID<sub>50</sub> /ml as a maximum titer from 2nd till 3rd and 4th passages respectively (Fig. 5). These results were compared with the titer of positive EHV-4 control which was six log<sup>10</sup> TCID<sub>50</sub> /ml through the six passages on RK cells and through the four passages

on VERO cell line as well as negative control which gave no titer on both cells.

A selective 4th passage of EHV-4 isolate of this study in VERO cell line was used to follow up the titer of whole infected and determine the optimum titer and the optimum harvesting time with different MOI till 5th dpi. Growth kinetics of 10-fold dilutions of the 4th passage of EHV-4 isolate at 4 and 5 dpi indicate the highest titer of EHV-4 virus at dilution 10<sup>-1</sup>, then decreased at dilutions 10<sup>-2</sup> and 10<sup>-3</sup>. It was revealed that the maximum titer of harvested whole culture virus at 4 and 5 dpi using 0.1 MOI was 5.5 log<sup>10</sup>TCID<sub>50</sub>/ml (table 3).



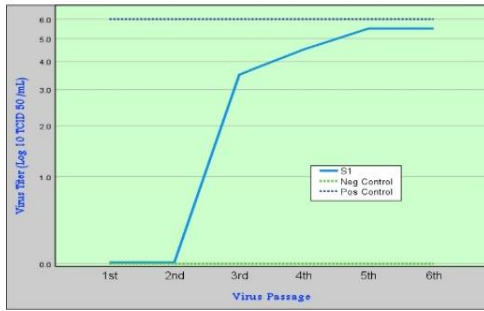


Fig (4): virus titration of S1 (EHV-4 isolate) in primary RK cell culture for 6 passages which indicate that the titer increased gradually from 3<sup>rd</sup> till 6<sup>th</sup> passage.



Fig (5): virus titration of S1 (EHV-4 isolate) in VERO cell line for 4 passages which indicates that the titer increased gradually from 2<sup>nd</sup> till 4<sup>th</sup> passage.

Table (3): titers of the 4<sup>th</sup> VERO passage of EHV-4 isolate in this study at different multiplicity of infection

Day post inoculation	Titration of EHV isolates (log <sub>10</sub> TCID <sub>50</sub> /ml for each virus dilution)		
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>
1	3	2.5	2
2	5	4.5	3.5
3	5	4.5	3.5
4	5.5	5	4
5	5.5	5	4

3.5. Serological identification of EHV-4 isolate:

3.5.1. Indirect fluorescence antibody technique (IFAT):

IFAT was carried out for the detection of specific fluorescence as an identification of EHV-4 isolate on VERO cell line. The test was done on the 4<sup>th</sup> passage of inoculated VERO cells in glass chamber slides. Slides showed specific perinuclear and intracytoplasmic apple green fluorescence as shown in Fig (6) which indicate positive result for EHV-4.

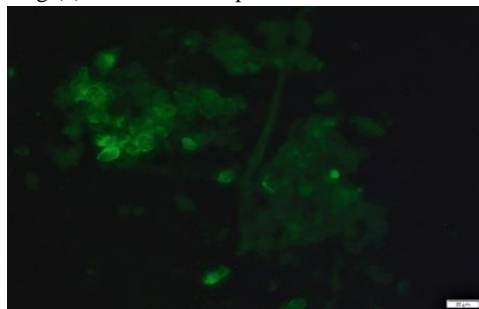


Fig (6): Apple green fluorescence was emerged from 4<sup>th</sup> passage of EHV-4 isolate and detected under fluorescence microscope that confirms the positive result.

3.5.2. Virus Neutralization Test (VNT):

EHV-4 isolate in addition to positive control with reference EHV-4 hyper immune sera at dilution 1/80

were examined by VNT and estimated in 100TCID<sub>50</sub>/ml. It was noticed that the neutralization percentage for EHV-4 isolate was 83% and CPE appeared in only 1 from 6 wells of inoculated microtiter plate. This isolate was examined against positive and negative control.

4. DISCUSSION

EHV-4 infection is the primary cause of economic loss throughout the equine industry worldwide due to their clinical outcomes on the trade of horses for sale, breeding, and competition through it causes a storm of abortion in equine (Vissani et al., 2016). It is well established that EHV-4 has been circulating in Egypt since 2011 (Amer et al., 2011), and our results are consistent with these records.

For viral detection and typing of the strains that are present in Cairo and Giza, cPCR based on gB gene of EHV-4 followed by studying the electrophoretic pattern of product was performed for samples of this study showed positive results(508 bp product size) for EHV-4 strain in one sample(S1). These results agreed with Turan et al., (2012); Azab et al., (2019) and Khattab and Hamdy (2022).

Nucleotide sequencing and alignment were performed for the gB amplicon of our EHV-4 strain and examined for their identity percent when compared with retrieved sequences from the GenBank. The analysis of the nucleotide sequence of our EHV-4 strain which has accession number (OP546096) has shown 100% nucleotide similarity with EHV-4 strains RJ4-TR2011 (Acc. No. JN982957) and TR-EHV4-VSA-11 (Acc. No. JX416463) on the GenBank from Turkey 2012and show 99.8% nucleotide similarity with EHV-4 strains EgyeqH4-19A (Acc. No. MN592995) from Egypt 2020, ER39-67 (Acc. No. KT324748) from Australia 2017 and US4-TR2011 (Acc. No. JN982958) from Turkey 2012and show99.6% with EHV-4 strains VRLCU-412-2015 (Acc. No. KP699582) from Egypt 2015 and 157-69(Acc. No. KT324738) from Australia 2017and this results agreed with Turan et al., (2012) and Khattab and Hamdy (2022).

In our study, EHV-4 sample was cultured on primary RK cell cultures in 6 serial passages that began to give CPE from the 3<sup>rd</sup> passage at five dpi, 4dpi in the 4<sup>th</sup> passage, and three dpi at 5<sup>th</sup> and 6<sup>th</sup> passages, and completed at five dpi. These results also agreed with Damiani et al. (2014), Hassanien (2020), and Stasiak et al. (2020). Isolation on the VERO cell line was done, which began to give CPE in the 2<sup>nd</sup> passage at four dpi and three dpi in the 3<sup>rd</sup> and 4<sup>th</sup> passages and was completed at five dpi. These results agreed with Afify et al. (2017). The inoculated cells showed cell rounding at 48–72 hpi (2-3 days) in the first stage of CPE, the second stage characterized by aggregation forming characteristic grape-like

clusters at 96–120 hpi (4–5 days), and finally, the last stage of CPE characterized by necrosis, degeneration, detachment of cells from the surface of the prescription flasks and cell lysis after five dpi. Then, the titration of the EHV-4 isolate was done on both RK cells and VERO cells. The titers of the virus were determined, revealing that the titers of the EHV-4 isolate in this study increased gradually to reach the highest titer in the 5th and 6th passages on RK cells and the 3rd and 4th passages on VERO cells. Then, the growth kinetics was done to determine the optimum titers of EHV-4 isolate and optimum harvest action time, which was 5.5 log<sup>10</sup>TCID<sub>50</sub>/ml for S1 isolate at 4 and 5 dpi using 0.1 MOI, and the titer decreased with 0.01 and 0.001 MOI. Then, the inoculated cells were identified serologically by IFAT by using a pool of EHV-1 and EHV-4 polyclonal antibodies (reference EHV antisera) as a primary antibody and rabbit anti-horse IgG as a secondary antibody, which revealed perinuclear and intracytoplasmic apple green fluorescence that indicate positive results for EHV-4. These results agreed with Hassanien (2020). Also, virus isolates were identified with VNT, and this result agreed with Lang et al. (2013), Stasiak et al. (2020), and Azab et al. (2019).

## 5. CONCLUSIONS

Depending on our results and the comparison between our isolate of EHV-4 and other reported similar strains in the GenBank revealed that there is no significant change in the gB gene sequence between our isolate and different isolates of the same strains in the world, which does not indicate the possibility of antigenic changes affect the application of vaccine and control of the disease. We found that EHV-4 was identified clearly and present in Egypt.

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