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Isolation and Molecular Characterization of *Equine herpes virus-1* from Horses Localized in Giza Governorate, Egypt

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ABSTRACT

Equine herpesvirus-1 (EHV-1) is endemic in many world areas, including Egypt with significant economic losses in the equine industry through causing a high fatality rate among foals associated with respiratory manifestations and abortion in pregnant mares. The present study conducted to investigate the isolation,, molecular characterization, and phylogeny of the circulating *EHV-1* isolates among native breeds of horses in Giza governorate. A total of 72 samples (aborted foeti, fetal fluids, placenta, vaginal and nasal swabs) were inoculated into Vero cell culture. The DNA was extracted from the infected tissue culture and used for molecular identification by TaqMan real-time PCR (qRT-PCR), DNA amplification by conventional PCR, and DNA sequencing. The results showed successful isolation of 9 out of 72 tested samples (12.5%) after the third passage in Vero cells with a characteristic cytopathic effect for *EHV-1*. The obtained results were confirmed by qRT-PCR. The positive samples were subjected to conventional PCR to amplify 869 bp of the glycoprotein B gene. Sequencing and phylogenetic analysis revealed that *EHV-1* isolates were closely related to each other and those previously isolated from Egypt and the European *EHV-1* strains. In conclusion, the molecular characterization and phylogeny analysis of the circulating *EHV-1* improve the understanding of virus epidemiology in Egypt and implicate good control measures.

Keywords: *Equine, Herpes Virus-1*, Giza governorate, Molecular characterization, Virus isolation.

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The members of *Herpesviridae family* are *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* subfamilies. The *equine herpesvirus1(EHV-1)* belongs to the *genus Varicellovirus, subfamily Alphaherpesvirinae*; it is 200-250 nm in diameter and consists of four main structural components **(Seo *et al.*, 2020).**

The *EHVs* are widely distributed all over the world. Nine types have been identified, including six belonging to the subfamily *Alphaherpesvirinae (EHV-1, 3, 4, 6, 8, and 9)* and three belonging to *Gammaherpesvirinae (EHV-2, 5, and 7). EHV-1 and EHV-4* are the most important types (**Pavulraj *et al.*, 2021**), not only for their widespread but also due to their responsibility for causing different clinical forms of infection and for developing a latent infection status which prevents the disease control **(Ata *et al.*, 2020a,b).** The respiratory form is the common clinical one with severe rhinitis and swollen lymph nodes (**OIE, 2017**). The abortive form is characterized by abortion in pregnant mares usually at the last third of gestation, which could progress into a storm among pregnant mares. The resistance of mares to abortion could be exhibited if the infection occurred early in pregnancy **(Ali *et al.*, 2020).**

The main route for *EHV-1* transmission is by inhalation. Thus, the infected droplets from the respiratory tract or the aborted tissues and fluids facilitate the spread of infection among animals, especially during equestrian events **(OIE, 2018).** Many reports showed the role of donkeys and mules as silent carriers for the disease during outbreaks and vice versa. So, their role should be considered in the epidemiological analysis and outbreak control

strategies **(Pusterla *et al.*, 2012; Rashwan *et al.*, 2019).** Although commercial inactivated or modified live vaccines are available, they cannot provide complete protection against infection and most of them induce reliable protection against respiratory form **(Ma *et al.*, 2013).**

The ability of the virus to establish infection in different types of host tissues, including the respiratory, reproductive, and CNS, and its immune evasion strategies are the main determinant of causing latency. As a result, the virus reactivation and development of an infection occurs when the immunity is suppressed for any reason **(Bueno *et al.*, 2020).**

The detection of antibodies against the virus for the first time in Egypt was observed during a serological survey in 1965 (**Matumoto *et al.*, 1965**). After that the virus becomes endemic and causes significant economic losses even in the vaccinated herds. Several studies discussed the virus situation in Egypt. They concluded that *EHV-1* is an endemic virus and may represent a continuous threat to horses in the absence of vaccination programs and frequent virus reactivation **(Azab *et al.*, 2019; Rashwan *et al.*, 2019; Ali *et al.*, 2020).** Accordingly, this study aimed to update the situation of the EHV-1 in Egypt through molecular characterization, sequencing, and phylogenic analysis of obtained virus isolates from horses in Giza governorate.

MATERIALS AND METHODS

1. Sampling

From 2018 to 2020, *72* samples were collected; of them, 30 samples (aborted fetuses, fetal fluids, and placenta) were obtained from mares immediately after abortion. While 42 (nasal and vaginal) swabs were collected from the aborted mares and adult horses of different ages with a history of respiratory manifestations. Samples were collected and processed according to **Mahy and Kangr (1996).** The swabs were placed directly into serum-free Minimum Essential Media (MEM) with 1% penicillin-streptomycin, 1% gentamicin, and 0.1% fungizone). After collection, all samples were packed in coolers with ice packs and transported immediately to the laboratory for further processing.

2. Virus isolation

Confluent monolayers of the Vero cell line (70-80%) were inoculated with 10% suspension of the previously prepared samples. After discarding the growth medium, the suspension was left for one hour for adsorption at 37^{o}C and then a maintenance medium was added and incubated at 37^{o} C with 5 % CO_2 with daily examination for the development of cytopathic effect (CPE) of the virus **(Maeda *et al.*, 2007).**

3. DNA extraction

The DNA was extracted from the positive inoculated tissue culture that showed CPE after the third passage using the ID Gene Spin Universal Extraction Kit according to manual instruction (IDvet, France). Meanwhile, according to the instruction manual, the DNA was extracted from the Killed vaccine (Pneumoabort K+ 1b; Fort Dodge Animal Health, Iowa 50501, USA) and used as a positive control in the genetic identification of the isolates.

4. TaqMan real time-PCR for *EHV-1* detection

Based on the glycoprotein B gene, the *EHV-1* MGB F1, the *EHV-1* MGB R1 primers and the TaqMan probe (Table 1) were selected, and the working dilutions were determined to be 0.4µM for the primers and 0.1µM for the probe. The total reaction mixture was 20 µlcontaining (PerfectStar^{TM} II probe qPCR supermix)., 0.4µM of each primer, and 0.1 µM of the probe, and 2 µl of DNA templates. The thermal condition was activating the DNA polymerase at 95 ^{o}C for 10 min followed by 45 cycles consisting of denaturation at 95 ^{o}C for 15 s, primer annealing and extension at 60 ^{o}C for 1 min **(Elia *et al.*, 2006).**

The real-time PCR assay was performed using a Rotor-GeneTM model 3000 from Corbett Research. Data was collected. Fluorescence is generated by specific amplification products and is shown as an exponential curve. Fluorescence is directly proportional to the amount of amplified product. The Ct value is the cycle at which the curve crosses the threshold line. The results were interpreted based on Ct values as follows:

Ct values between 28 and 34were considered positive.

Ct values between 35and 40 were deemed to be suspect and retested again.

While Ct values over 40 and values of zero were considered to be negative **(Elia *et al.*, 2006)**.

Table 1: List of primers and probes used in molecular detection and amplification of the *EHV-1*gB gene in the present study:

Type of Assay	Target gene	Primer name	Nucleotide sequence 5'-3.'	References
TaqMan real time-PCR	Glycoprotein B	EHV1 MGB F1	GCT CTC AGG TTT TAC GAC ATC-	**Elia *et al.*, (2006)**
		EHV1 MGB R1	TTT CAAGGG CCT GGG TAA AG	
		MGB probe FAM	TCA ACG TGG ACA ATA CCG CAG TGA T TA T	
Conventional PCR	Glycoprotein B	The forward primer (tP)	-CACTTCCATGTCAACGCACT	**(Azab *et al.*, 2019)**
		The reverse primer (rP)	TCGACTTTCTTCTCGGTCCA	

5. Conventional PCR for amplification of the glycoprotein B gene

Glycoprotein B gene was amplified using a set of the forward primer and the reverse primer (Table. 1). As a standard protocol and in a final volume of 25 µl PCR reaction mix was applied as 12.5 µl Emerald Amp GT PCR master mix (2x premix), 5.5 µl PCR grade water, 1 µl forward primer (20 pmol), 1 µl Reverse primer (20 pmol), 5 µl template DNA. The amplification cycles were carried out in an exceedingly PT-IOO thermocycler (MJ Research, USA). The cycling condition was optimized to be 95 ˚C for 5 min as an initial denaturation, followed by 35 cycles of 94 ˚C for 30 sec, 55 ˚C for 40 sec and 72 ˚C for 50 sec. A final extension step at 72˚C /10 min was done **(Azab *et al.*, 2019).** The amplified amplicons were electrophoresed in 1.5% agarose gel. The size of the amplified fragments was determined using 100 bp DNA ladder (A) gene, UK). The positive control was DNA of the EHV-I vaccine strain.

Five amplified PCR products representing the positive ones were selected and purified from the gel and sent for sequencing. The obtained sequence of the different isolates was analysed using the Bioedit software freely available at: https://bioedit.software.informer.com/7.2/.

A comparison with the similar isolates was conducted using the free Basic Local Alignment Search Tool of the National Center for Biotechnology Information https://blast.ncbi.nlm.nih. gov/Blast.cgi to determine the identity percentage. While multiple alignments of the compared sequences were conducted using the Muscle option within MEGA7 software. To have the phylogenetic tree, an analysis of the data using the Maximum-likelihood statistical method **(Kumar *et al.*, 2016).** The confidence level of the tree was evaluated by bootstrapping using 1000 replicates **(Felsenstein, 1985).**

RESULTS

1. Virus isolation

Out of the 72 tested samples, only nine samples showed the CPE which is characterized by cell rounding, granulation of the cytoplasm, cell degeneration, and dispersion in the medium as grapes-like that end with detachment of cells from the culture surface leaving empty spaces after the 3rd passages of inoculation (Fig.1a). In contrast, the non-inoculated cells' negative controls did not show any CPEs (Fig.1&b).

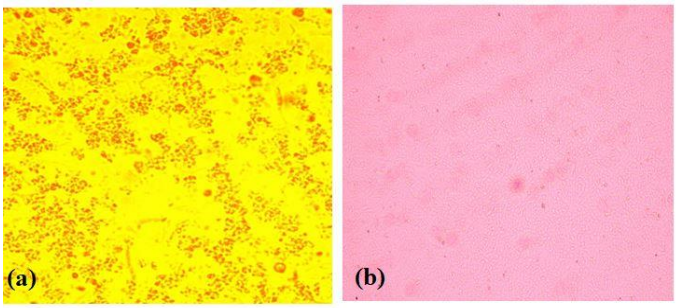

Fig.1: (a) shows the cytopathic effect of EHV-1 isolate on Vero cells in rounding and cell detachment (Mag. 10X). (b): Negative control of non-infected Vero cells.

2. Identification of EHV-1 using TaqMan real-time PCR

Analysis of the real-time PCR revealed successful detection of the *EHV-I*gB in the nine samples as their Ct ranged from 32- 33.5 (Fig. 2). This result confirms the previous virus isolation process.

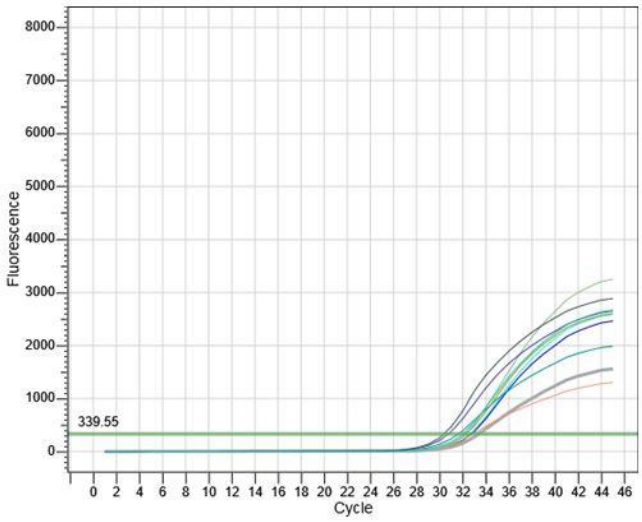

Fig. 2: shows the threshold curve of positive samples of *EHV-1* (their Ct ranged from 32- 33.5).

3. Detection of the glycoprotein B gene

The conventional PCR was applied to amplify an 869 bp fragment of the gB gene. Electrophoresis of the amplified products revealed the presence of the amplified bands at the correct expected size in the tested samples (Fig.3).

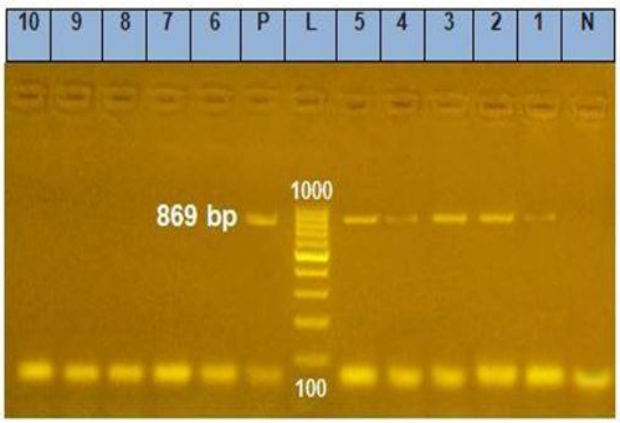

Fig.3: Ethidium bromide stained 1.5% agarose gel revealed bands of PCR products at the expected size (869pb); Lanes 1- 5 showed positive samples submitted for sequencing. Lane P for positive control, Lane N for the negative control, Lane L 100 bp ladder.

4. Sequencing and phylogenetic analysis

Analysis of the amplified gB gene sequences confirmed the detection of EHV-1 isolates. All the sequences were submitted to the Genbank, and the accession numbers are shown in table (2). The sequences of all isolates were the same without differences among them (table 3). Genetic alignment of the obtained sequences with the other sequences retrieved from Genbank revealed high identity with the strains circulated worldwide or the locally identified ones.

Table 2: The designation and the accession numbers of the obtained EHV-1 isolates.

EHV-1isolatedesignation	Source	Accession number
Giza-ARRI-USC-N.EG18	Nasal swab	OL505456
Giza-ARRI-USC-AF.EG19	Aborted foeti	OL505457
Giza-ARRI-USC-VM.EG19	Vaginal swab	OL505458
Giza-ARRI-USC-P.EG19	Placenta	OL505459
Giza-ARRI-USC-FF.EG20	Fetal fluid	OL505460

The detected isolates were 100% identical to the following strains SUFFOLK/91/1994 (accession no: KU206479), RACL11-1950s (accession no: KU206478), Kentucky D (accession no: AB279609), and the Ab4 strain (accession no: AY665713) previously identified in the United Kingdom, and the isolate 438-77 (accession no: KT324734) from Australia. While the identity of 99.9% was recorded with the Suffolk/123/2005 (accession no: KU206480), the Essex/199/2005 (accession no: KU206410) from the United Kingdom. While identity with the strain T616 delta71(accession no: KF644573) and strain 94-137 (accession no: KF644575) from Japan (Fig. 4). In comparison with the previously obtained Egyptian isolates, the identity percentage was 100% for the EH_MG-1 (accession no: OM362231) and the EH_Egy-01 (accession no: MG732975), while 97.5 % was recorded with the Zyat-EH1 (accession no: OM362231).

Table 3: Declares the data of the different isolates used for the phylogram:

Species	Isolate name	Clinical case	Sample type	Accession number	Identity	Country	Reference
EHV-1	SUFFOLK/91/1994	Abortion	Thoroughbred abortion, lung/liver	KU206479	100%	United Kingdom	**(Bryant *et al.*, 2018)**
EHV-1	Racl11 strain	Not available	Not available	X95374	100%	Germany	-----
EHV-1	RACL11 - 1950s	Abortion	Abortion tissue	KU206478	100%	United Kingdom	**(Bryant *et al.*, 2018)**
EHV-1	isolate 438-77	Abortion	Foetus	KT324734	100%	Australia	**(Vaz *et al.*, 2016)**
EHV-1	Kentucky D	-----	-----	AB279609	100%	United Kingdom	**(Ghanem *et al.*, 2007)**
EHV-1	strain Ab4	Paresis	leucocytes of a paraplegic gelding	AY665713	100%	United Kingdom	***(Edington et al., 1986)***
EHV-1	EHV-1	-----	-----	M35145	100%	USA	**(Guo *et al.*, 1990)**
EHV-1				D00401	100%		--
EHV-1	Suffolk/123/2005	Abortion	Thoroughbred abortion, lung/live, /thymus/spleen	KU206480	99.9%	United Kingdom	**(Bryant *et al.*, 2018)**
EHV-1	Essex/199/2005	Abortion	Thoroughbred abortion, lung/live, /thymus/spleen	KU206410	99.9%	United Kingdom	**(Bryant *et al.*, 2018)**
EHV-1	RacL11	Abortion	Aborted foals	AB279606	99.9%	Germany	**(Reczko and Mayr, 1963)**
EHV-1	Hertfordshire/150/2016	Abortion	Thoroughbred abortion tissue	KY852346	99.8%	United Kingdom	**(Bryant *et al.*, 2018)**
EHV-1	Aberdeenshire/84/2013	Abortion	Abortion, tissue	KU206461	99.8%	United Kingdom	**(Bryant *et al.*, 2018)**
EHV-1	Leicestershire/59/1996	Abortion	Thoroughbred abortion tissue	KU206423	99.8%	United Kingdom	**(Bryant *et al.*, 2018)**
EHV-1	strain T-529 10/84	Encephalitis of zebra, onager, and Thomson's gazelle	-----	KF644580	98.4	Japan	**(Guo *et al.*, 2014)**
EHV-1	strain T616 delta71	Encephalitis of zebra, onager, and Thomson's gazelle	-----	KF644573	98.2	Japan	(**Guo *et al.*,** 2014)
EHV-1	strain 94-137	Encephalitis of zebra, onager, and Thomson's gazelle	-----	KF644575	98.2	Japan	**(Guo *et al.*, 2014)**
EHV-1	strain NMKT04	Encephalitis of zebra, onager, and Thomson's gazelle	-----	KF644568	99.8	Japan	***(Guo et al.*, 2014*)***
EHV-1	EHV-1	-----	-----	M36298	99.8	United Kingdom	**(Whalley *et al.*, 1989)**
EHV-1	H3_Allg_92_21/CH/2021	Respiratory signs	Nasal swabs	MZ357402	99.6	Switzerland	**(Kubacki et al., 2021)**
EHV-1	EH_MG-1	Abortion	Aborted feti	OM362231	100	Egypt	----
EHV-1	EH_Egy-01	------	------	MG732975	100	Egypt	**(Azab et al 2019)**
EHV-1	Zyat-EH1	Abortion	placenta	OM362230	97.5	Egypt	-----

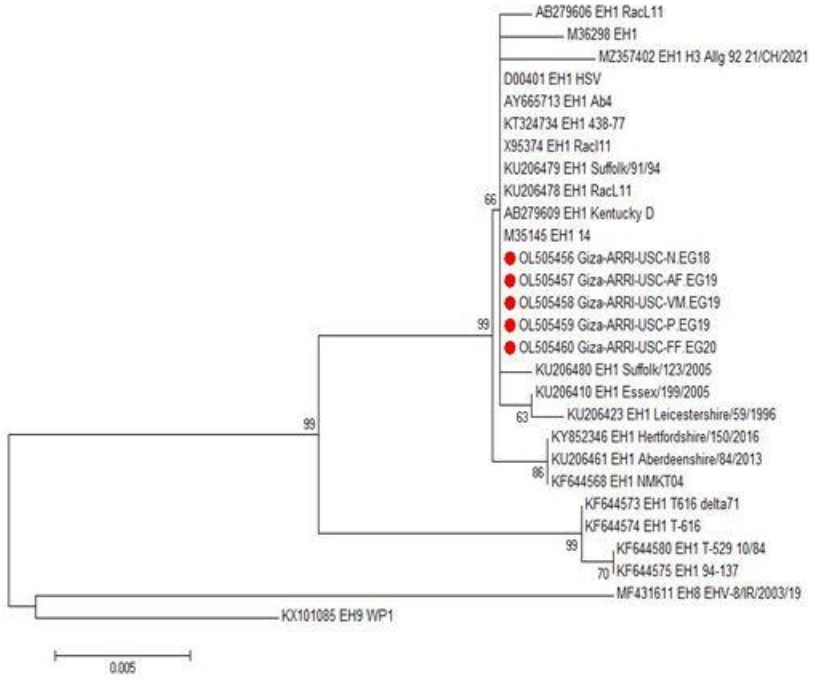

Fig. 4: Phylogenetic tree of the obtained Egyptian *EHV-1* isolates.The evolutionary history was created using the maximum likelihood method based on the Tamura-Nei model. The confidence level of the NJ tree was assessed by bootstrapping using 1000 replicates. The cladogram was carried out using the MEGA7 software. The red circles represented the different sequences obtained in this study, while the black diamond referred to the previously uploaded Egyptian isolates.

DISCUSSION

Egypt is an essential center for raising and marketing the pure breed of Arabian horses. Therefore, the loss of valuable animals has negative consequences for the equine breeding and sports industry **(Al-Shammari *et al.*, 2016).** Although previous reports discussed the epidemiological situation of the *EHV-1* infection in Egypt, continuous searching and studying the circulating strains is important **(Ata *et al.*, 2018b; Ata *et al.*, 2020a,b).**

It was reported that different types of cell lines could be used for the isolation process like rabbit kidney (RK-13), baby hamster kidney (BHK-21), and Madin–Darby bovine kidney (MDBK). Also, cells of equine origin like equine dermal (E-Derm) cells or fetal horse kidney (FHK) cells were superior **(OIE, 2018).** The obtained results declared successful identification of the *EHV-1* in the collected different types of samples after the third passage in Vero cells with a CPE of cell rounding, granulation of the cytoplasm and cell degeneration that end with the detachment of cells from the culture surface. Sometimes, more than 3 passages may be needed, especially for those samples that do not have enough virus concentration to induce CPE **(Abd El-Hafeiz *et al.*, 2010).** These results agreed with **Al-Shammari *et al.*, (2016); Alkhalefa *et al.*, (2018); Azab *et al.*, (2019).**

Although virus isolation on tissue culture is a golden technique, it has many obstacles. Therefore, several molecular techniques were evolved to detect the viral DNA because of its speed, simplicity, and accuracy in different clinical samples **(Azab *et al.*, 2019).**

In our study, molecular identification of the obtained isolates was conducted using TaqMan real-time PCR targeting the EHV-1gB gene which was selected due to its conserved nature among the different EHV-1 isolates **(Elia *et al.*, 2006).** The same results were recorded by **Abd El-Hafeiz *et al.*, (2010).** On the other hand, multiple types of EHVs including EHV-2, EHV-4 and EHV-5, were determined in a previous study based on samples collected from local areas **(Azab *et al.*, 2019).**

It is crucial to clarify that the TaqMan real-time assay is highly sensitive for the diagnosis of EHV-1 as it was able to detect as few as 10 copies of EHV-1 DNA, so it is suitable for the identification of samples with deficient concentrations adding to its simplicity, short time processing, and the absence of post-PCR

processing steps **(Jelocnik *et al.*, 2021).** Moreover, it was used to provide additional insights into the pathogenesis of EHV-1 especially for the latent cases **(Vargas-Bermudez *et al.*, 2018).**

Recently, the importance of the phylogenetic analysis was declared as it did not only help in tracking the virus origin but also explained the causes of some vaccines failure and helped in the control of many pathogens **(Ata *et al.*, 2021; 2018a).**

The DNA of the selected isolates was extracted and subjected to conventional PCR to amplify 869 bp, the conserved fragment of the glycoprotein B gene. The obtained results were consequent with **Azab *et al.*, (2019)** and those of **Meselhy *et al.*, (2019); Yanni *et al.*, (2021),** although they used different primer set for the same gene. It is worth noting that, many genes like gD (US6), gE (US8), and tegument genes, including the ORF11 (UL49), ORF46 (UL16), ORF51 (UL11), and ORF76 (US9) genes could be used for identifying the differences among the isolates **(Ghanem *et al.*, 2007).**

Phylogenetic analysis of the obtained sequences revealed that they were clustered together with other herpesviruses isolated worldwide. These results were congruent with **Hassanien *et al.*, (2020); Meselhy *et al.*, (2019).** The isolated strains of the EHV-1 have 97.5 % to 100%identity with the previously isolated either local or the world-circulated ones, especially those in the United Kingdom, Australia, and Japan. It is worth noting that, the obtained isolates are highly similar to the previously identified ones at the local level. It is expected that, the source of infection could be attributed to contact with diseased cases either locally or during the international races or shows as recorded previously in Swiss horses that attended an international equestrian event in Spain **(Kubacki *et al.*, 2021).**

CONCLUSION

The present data indicated that virus isolation in conjunction with molecular assays like conventional PCR or real-time PCR would lead to a higher virus identification rate of EHV-1. This result is useful for monitoring EHV-1 infection. Moreover, the sequence information for the gB gene from field isolates of EHV-1 would be valuable for developing an effective vaccine against EHV-1 and subsequent phylogenetic analysis provide valuable information about the molecular epidemiology of EHV-1 subtypes prevalent in Egypt.

Declaration of Conflicting Interests

The authors declare that they have no conflict of interest.

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