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RESEARCH

Sequence analysis of Seven Equine Herpes Type 1 Viruses circulating in non-vaccinated Arabian and Foreign horses in Egypt

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ABSTRACT

Background: Equine herpes virus 1 (EHV-1) has a significant economic impact on equine industry. It is a highly contagious pathogen, mainly transmitted by inhalation of aerosols of virus laden respiratory secretions especially with high population during equestrian events. Reactivation of virus in non-symptomatic, latently (silently) infected horses is the main cause of circulation of such virus in horses.
Methods: In the present study, molecular characterization of circulating EHV-1 viruses among horses of equestrian clubs in Egypt was carried out. Sixty-two samples of whole blood with anticoagulant were collected and screened for EHV-1 using nested PCR that amplify the conserved fragment of glycoprotein B gene (ORF 33), followed by sequencing and phylogenetic analysis.
Results: The study revealed that 19% of our samples were positive to EHV-1 and phylogenetic analysis showed that the Egyptian EHV-1 isolates were more than 99% similarity to European abortogenic isolates (EHV-1 strains: Army 183 and Suffolk/48/2013). Indeed, the analysis reports that these viruses are circulating in both Arabian and foreign horses in Egypt.
Conclusion: The nested PCR is an appropriate technique for the detection of EHV-1 present in blood of a latently, or silently infected horses. The application of this nested PCR is considered to be a rapid diagnostic tool for use in veterinary laboratories. Thus it will enhance the detection and characterization of the circulating EHV-1 in Egypt. Control strategy for EHV-1 viruses in vaccinated and non-vaccinated animals needs to be addressed.

Keywords: Nested PCR, equine herpes virus type 1 (EHV-1), glycoprotein B gene (gB, ORF 33), horse, Egypt

BACKGROUND

Equine herpes virus 1 (EHV-1) is one of the most important viral infections in horses which has a major economic and welfare impact on equine industry worldwide (Slater, 2014). EHV-1 infection is characterised by different pathogenic potential with different severities (Patel *et al.,* 1982 and Tearle *et al.,* 2003). It is a major cause of respiratory signs in horses, also causes epidemic and sporadic abortion in mares, mortality of new-born foals and the most fatal one, a neurological disease (Charltons *et al.*, 1976), known as equine herpes virus myeloencephalopathy or [EHM] (Allen et al., 2004).

EHV-1 is a DNA virus belonging to genus varicellovirus, subfamily Alphaherpesvirinae within the family *Herpesviridae* (Davison *et al.*, 2009). The main route for EHV-1 transmission is by inhalation thus, virus exit and entry through the respiratory tract facilitates spread of infection between animals especially during equestrian events (OIE, 2017). The virus is characterised by latent infection posing a critical way that help the virus hiding from the immune system of the host for an undetermined period, then under appropriate conditions, latent virus can revert to an infective state (Roizman & Baines, 1991) Latently infected animals are considered as

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a hidden source of infection to other animals following reactivation. This situation represents a challenge to control transmission of virus because clinical signs are usually not easily detected during the reactivation process. The economic impact of EHV-1 infection on the equine industry is apparent by the great effect on the breeding and competition ability of horses (Slater, 2014). The transport of horses is always restricted when outbreaks are detected in an area. A range of 10 up to 30% of abortions has been observed during EHV-1 outbreaks (abortion storms) and EHM outbreaks (Goehring *et al.,* 2006). Mortality rates of EHV-1 infection may reach up to 40% (Allen *et al.,* 2004 and Lunn *et al.,* 2009).

There is no data available on the current situation of EHV-1 infection in Egyptian horses of equestrian clubs. Therefore the aim of the present study is to molecular characterize the circulating strains of Equine herpes viruses in the Egyptian equestrian clubs.

MATERIALS AND METHODS

Field samples

A total of 62 non-vaccinated Arabian and foreign horses from different equestrian clubs in Egypt (The age ranged between 2 to 22years), with or without respiratory symptoms in the period from August to December 2017 were included in the study. Samples were collected from horses by means of jugular venepuncture using a 10 ml needles on EDTA tubes. These tubes were labelled and transported to the laboratory in an ice tank.

DNA extraction

DNA extraction and purification were carried out by using iNtRON, Patho Gene-spin™ DNA/RNA extraction Kit according to the manufacturer's protocol. Purified DNA recovered in 60 µl elution buffer and stored at -20°C for further testing.

PCR for amplification of glycoprotein B gene of EHV-1

PCR reactions were employed as previously described by Borchers and slater (Borchers & Slater, 1993). Two PCR reactions applied to amplify fragments of gB gene.

The first PCR was consisted of 35 cycles of amplification using specific pair of primers EHV-I PI (5'TCTACCCCTACGACTCCTTC-3', nucleotide positions 1869-1888) and EHV-I P2 (5'GCTTTCTTTTCCTGCTTTTC-3', nucleotide positions 3749-3768) (Borchers & Slater, 1993) to amplify 1880bp fragment, followed by a second nested PCR reaction to amplify 1280bp fragment using specific pairs of primers EHV-I P3 (5'CTTTAGCGGTGATGTGGAAT-3', nucleotide positions 2329-2348) and EHV-I P4 (5'CCTTTGTTGTTATGGGGTAT-3', nucleotide positions 3604-3623) (Borchers & Slater, 1993). The PCR cycling protocol was as following: an initial denaturation step at 95°C for 5 minutes followed by 35 cycle of 95°C for 2 minutes, 56°C for 20 second.72°C for 2 minutes then a final extension step at 72°C for 10 minutes. Ten µl of the PCR products were visualised in ethedium bromide stained gel of 1% agarose in TAE buffer along with Quick-Load Purple 2-log DNA Ladder (0.1- 10.0 kb) and photographed under UV light.

DNA sequencing and phylogenetic analysis

The PCR products were submitted for sequence analysis to Macrogen Inc., Korea (www.macrogen.com). Sequencing of the PCR products was done using Big DyeTM termination V 3.1 cycle sequencing kit with forward and reverse primers of second PCR. Following the Laboratory Information Management System (LIMS), the obtained data were analysed according to NCBI/BLAST. Sequences alignments were performed using CLUSTAL W 1.4 in Bio Edit-

supported software. Phylogenetic trees were constructed using maximum likelihood method in MEGA 7 software and 1,000 bootstrap replicates. The obtained sequences were submitted to the GenBank to get accession numbers.

RESULTS

The nested PCR was applied to amplify 1.88 kb fragment in the first round and in the second round 1.28 kb fragment of ORF 33 gene (gB) using two sets of primers designated specifically for the target gene (EHV-1 gB). Electrophoresis of the amplified products revealed the presence of specific PCR products at the correct expected size of each round. The resulted fragments were photographed by gel documentation system and the results are shown in (Table 1).Out of 62 horses, 12 were EHV-1 positive by nested PCR (Figure 1). These 12 horses had different ages ranged from 1.5 to 22 years, 4 of them had serous nasal discharge, anorexia and depression. There were four samples revealed very weak bands in the first round while they gave strong bands in the second round. We focused only on the positive samples of the first PCR round. Results confirmed the presence of EHV-1 with clear strong bands of the expected size at 1.28 kb when we employed the positive samples from the first PCR in a second PCR.

Table 1: Summary of the obtained PCR results in both first and second PCR rounds.

Sample	Sex	Age	Breed	Respiratory signs	PCR Result in first round	PCR Result in second round
1	Female	15	Arabian	+ve	++	+++
2	Female	5	Arabian	-ve	+	+++
3	Female	2	Arabian	-ve	+	+++
4	Male	14	Foreign	-ve	+	+++
5	Male	17	Foreign	+ve	++	+++
6	Female	21	Foreign	-ve	+	+++
7	Female	14	Foreign	+ve	++	+++
8	Male	22	Foreign	-ve	+	+++
9	Male	12	Foreign	-ve	+	+++
10	Female	1.5	Foreign	-ve	+	+++
11	Male	17	Foreign	+ve	++	+++
12	Male	20	Arabian	-ve	+	+++

+++: Strong band **++: Fair band** **+: Weak band**

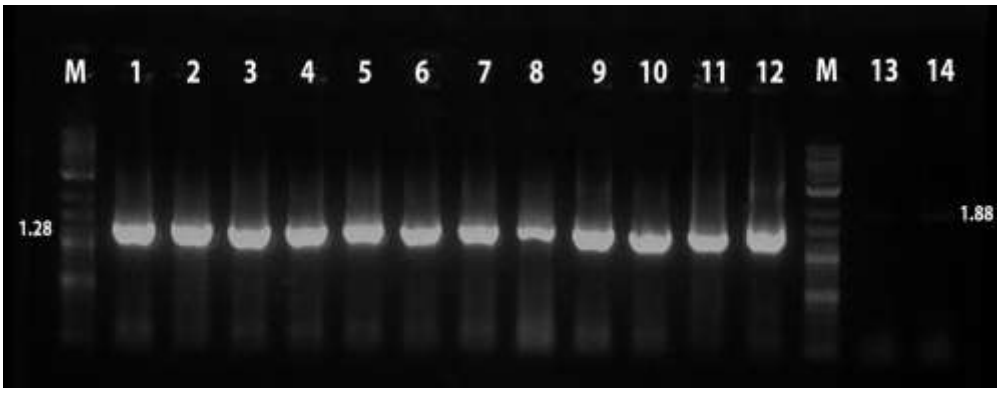

Fig. 1: Ethidium bromide stained 1% agarose gel revealed bands of PCR products at expected size (1.28kb); Lanes 1 to 12 showed positive samples for the second round (at 1.28kb), Lane 13 and 14 showed weak positive samples for the first round (at 1.88kb), Lane M: 0.1-10 kb ladder.

Sequencing of PCR products

The identity of nucleotide sequences among EHV-1 viruses in the present study ranged from 99.1 to 100% when compared with each other (Table 2). While the identity of nucleotide sequences between EHV-1 viruses in the present study and nucleotide sequences of reference strains ranged from 95.7 to 100%.

The deduced amino acid sequences of Egyptian EHV-1 did not exhibit much more variation among each other's. However, one of the detected strains, Egypt/ EHV-1/Cairo/ 3K, has shown different amino acid sequences from other Egyptian strains at positions 547, 564, and 718 (Table 3). Also, mutation was detected in Egypt/ EHV-1/Cairo/ 12L strain at position 694. Whereas we found a difference in amino acid positions 663 and 681 in the strains included in the study compared to reference strains T-965 and Ro-1 strains has shown differences in amino acid composition compared to other EHV-1 strains at positions 663 and 681 (Table 3).

Table 2: Sequence Identity Matrix.

Seq->	KyA	RacL11	V592	Ab1	Ab4	RacH	Suf/123	Suf/48	Army183	AIV	HVS25A	Mar87	Ro-1	T965	EHV-4gB	NS80567	1A	3K	4B	5S	6K	7S	12L
KyA	ID	0.997	0.998	0.998	0.998	0.998	0.998	0.998	0.998	0.985	0.998	0.986	0.960	0.960	0.852	0.851	0.994	0.994	0.998	0.998	0.997	0.995	0.996
RacL11	0.997	ID	0.998	0.998	0.998	0.998	0.998	0.998	0.998	0.985	0.998	0.986	0.960	0.960	0.852	0.851	0.994	0.994	0.998	0.998	0.997	0.995	0.996
V592	0.998	0.998	ID	1.000	1.000	1.000	1.000	1.000	1.000	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	1.000	0.998	0.996	0.997
Ab1	0.998	0.998	1.000	ID	1.000	1.000	1.000	1.000	1.000	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	1.000	0.998	0.996	0.997
Ab4	0.998	0.998	1.000	1.000	ID	1.000	1.000	1.000	1.000	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	1.000	0.998	0.996	0.997
RacH	0.998	0.998	1.000	1.000	1.000	ID	1.000	1.000	1.000	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	1.000	0.998	0.996	0.997
Suf/123	0.998	0.998	1.000	1.000	1.000	1.000	ID	1.000	1.000	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	1.000	0.998	0.996	0.997
Suf/48	0.998	0.998	1.000	1.000	1.000	1.000	1.000	ID	1.000	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	1.000	0.998	0.996	0.997
Army183	0.998	0.998	1.000	1.000	1.000	1.000	1.000	1.000	ID	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	1.000	0.998	0.996	0.997
AIV	0.985	0.985	0.986	0.986	0.986	0.986	0.986	0.986	0.986	ID	0.986	0.996	0.971	0.971	0.843	0.842	0.982	0.982	0.986	0.986	0.985	0.983	0.984
HVS25A	0.998	0.998	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.986	ID	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	1.000	0.998	0.996	0.997
Mar87	0.986	0.986	0.987	0.987	0.987	0.987	0.987	0.987	0.987	0.996	0.987	ID	0.971	0.971	0.843	0.842	0.983	0.983	0.987	0.987	0.986	0.984	0.985
Ro-1	0.960	0.960	0.961	0.961	0.961	0.961	0.961	0.961	0.961	0.971	0.961	0.971	ID	1.000	0.841	0.840	0.957	0.957	0.961	0.961	0.960	0.958	0.959
T965	0.960	0.960	0.961	0.961	0.961	0.961	0.961	0.961	0.961	0.971	0.961	0.971	1.000	ID	0.841	0.840	0.957	0.957	0.961	0.961	0.960	0.958	0.959
EHV-4gB	0.852	0.852	0.853	0.853	0.853	0.853	0.853	0.853	0.853	0.843	0.853	0.843	0.841	0.841	ID	0.998	0.852	0.849	0.853	0.853	0.852	0.850	0.851
NS80567	0.851	0.851	0.852	0.852	0.852	0.852	0.852	0.852	0.852	0.842	0.852	0.842	0.840	0.840	0.998	ID	0.851	0.848	0.852	0.852	0.851	0.849	0.850
1A	0.994	0.994	0.995	0.995	0.995	0.995	0.995	0.995	0.995	0.982	0.995	0.983	0.957	0.957	0.852	0.851	ID	0.991	0.995	0.995	0.994	0.992	0.993
3K	0.994	0.994	0.995	0.995	0.995	0.995	0.995	0.995	0.995	0.982	0.995	0.983	0.957	0.957	0.849	0.848	0.991	ID	0.995	0.995	0.994	0.992	0.993
4B	0.998	0.998	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	ID	1.000	0.998	0.996	0.997
5S	0.998	0.998	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.986	1.000	0.987	0.961	0.961	0.853	0.852	0.995	0.995	1.000	ID	0.998	0.996	0.997
6K	0.997	0.997	0.998	0.998	0.998	0.998	0.998	0.998	0.998	0.985	0.998	0.986	0.960	0.960	0.852	0.851	0.994	0.994	0.998	0.998	ID	0.995	0.998
7S	0.995	0.995	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.983	0.996	0.984	0.958	0.958	0.850	0.849	0.992	0.992	0.996	0.996	0.995	ID	0.994
12L	0.996	0.996	0.997	0.997	0.997	0.997	0.997	0.997	0.997	0.984	0.997	0.985	0.959	0.959	0.851	0.850	0.993	0.993	0.997	0.997	0.998	0.994	ID

Table 3: The amino acid variation among Egyptian EHV-1 strains compared to reference strains.

Site / Seq	519	530	535	547	553	554	555	557	564	572	648	663	681	694	698	718	730
1A	R	Q	G	K	T	A	G	D	K	T	A	M	N	E	I	F	F
3K	R	Q	G	**E**	T	A	G	D	**E**	T	A	M	N	E	I	**L**	F
4B	R	Q	G	K	T	A	G	D	K	T	A	M	N	E	I	F	F
5S	R	Q	G	K	T	A	G	D	K	T	A	M	N	E	I	F	F
6k	R	Q	G	K	T	A	G	D	K	T	A	M	N	E	I	F	F
7S	R	Q	G	K	T	A	G	D	K	T	A	M	N	E	I	F	F
12L	R	Q	G	K	T	A	G	D	K	T	A	M	N	**K**	I	F	F
T965	R	Q	G	K	T	A	G	D	K	T	A	**V**	**D**	E	I	F	F
Ro-1	R	Q	G	K	T	A	G	D	K	T	A	**V**	**D**	E	I	F	F

Phylogenetic analysis

The sequences of EHV-1 isolates gB gene were submitted to GenBank. **Table 4** shows the designation and the accession numbers of them.

Table 4: EHV-1 isolates designation and their Accession numbers.

EHV-1 isolate designation	Accession number
Egypt/EHV-1/Cairo-1A	MH289749
Egypt/EHV-1/Cairo-3K	MH289750
Egypt/EHV-1/Cairo-4B	MH289751
Egypt/EHV-1/Cairo-5S	MH289752
Egypt/EHV-1/Cairo-6K	MH289753
Egypt/EHV-1/Cairo-7S	MH289754
Egypt/EHV-1/Cairo-12L	MH289755

Phylogenetic tree was constructed based on the nucleotide sequence alignment of gB gene of the studied isolates together with other references strains obtained from GenBank

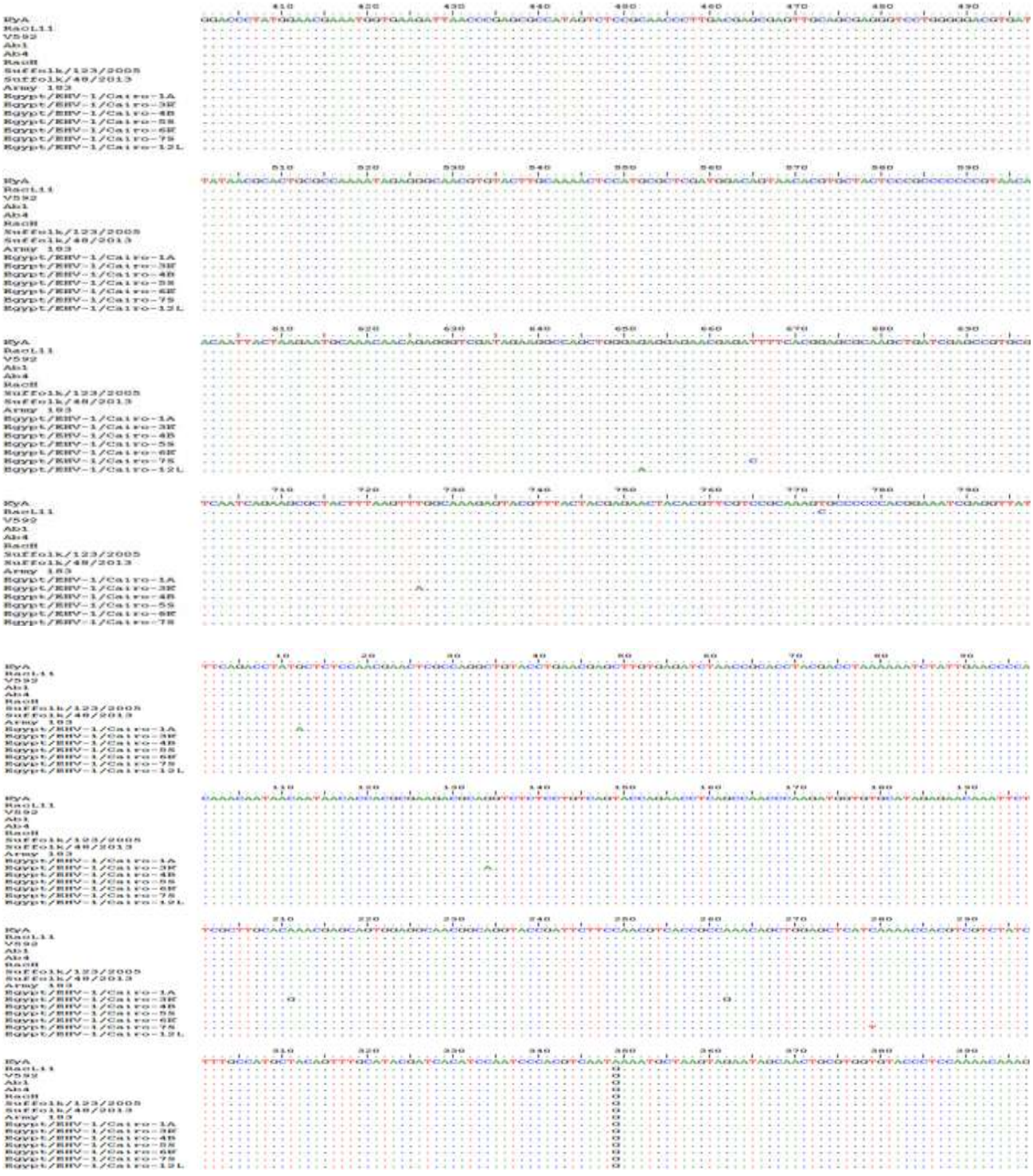

Fig. 2: Partial nucleotide sequences alignment of the amplified fragment of ORF 33 of gB gene of the identified EHV-1 isolates in comparison with other related reference strains from GenBank using Bio-Edit 7.2.6 program. The dot (.) represents identity while single alphabet represents the difference in the nucleotide sequence. [KyA: Vaccinal strain; RacL11, V592, Ab1, Ab4, RacH, Suffolk/123/2005, Suffolk/48/2013, and Army 183: Virulent strains; Egypt/EHV-1/Cairo-1A, 3K, 4B, 5S, 6K, 7S, and 12L: Identified isolates].

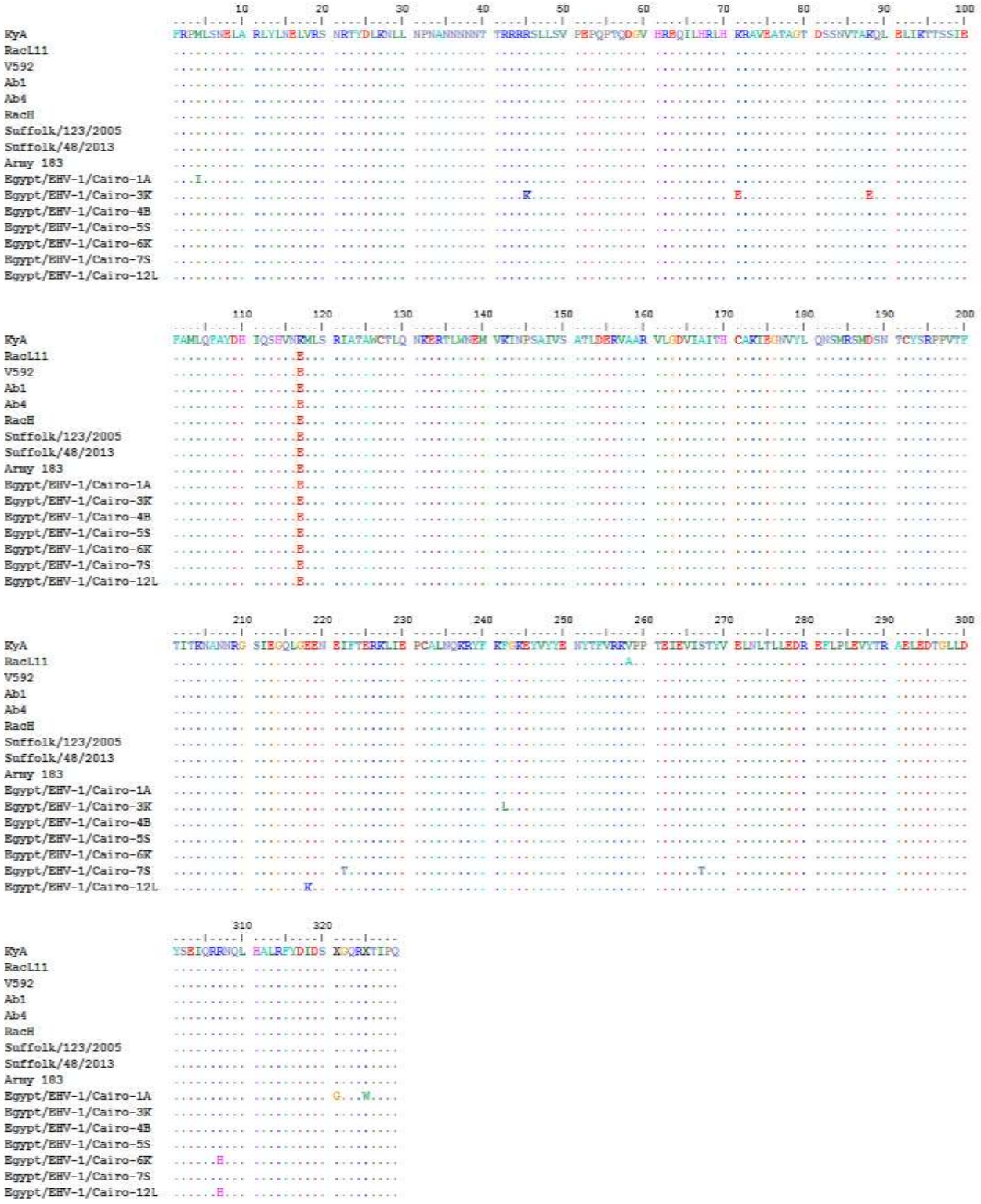

Fig. 3: Partial Amino acids (AAs) sequences alignment of the amplified fragment of ORF 33 of gB gene of the identified EHV-1 isolates in comparison with other related reference strains from GenBank using Bio-Edit 7.2.6 program. The dot (.) represents identity while single alphabet represents the difference in the AA sequence.

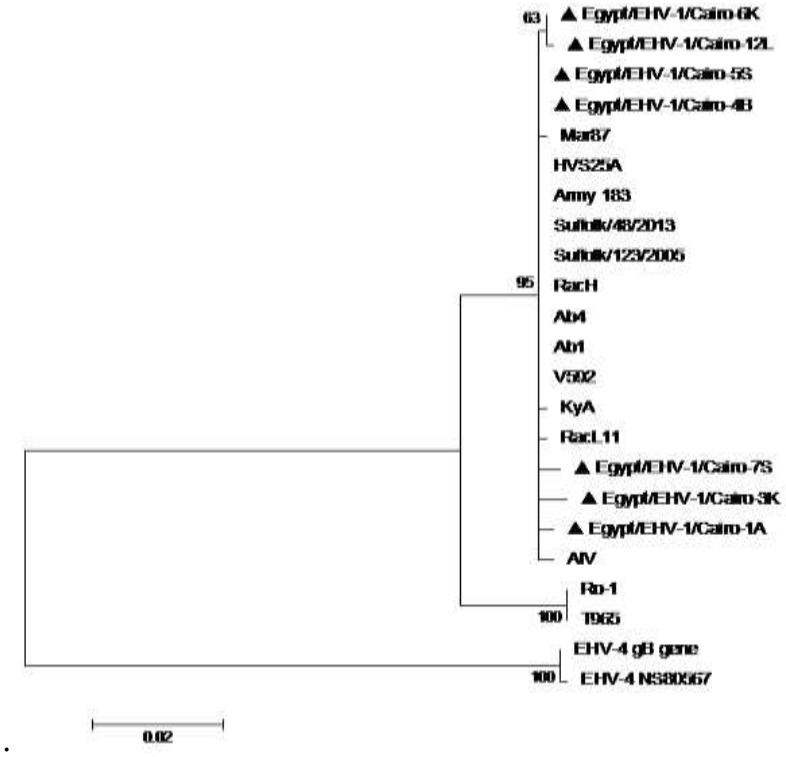

Fig. 4: Phylogenetic tree of Egyptian EHV-1. The Egyptian strains are indicated by black triangles. The evolutionary history was inferred by using the Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences. There were a total of 968 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. [KyA (MF975655); RacL11 (KU206478), V592 (AY464052), Ab1 (KU206468), Ab4 (AY665713), RacH (X95377), Suffolk/123/2005 (KU206480), Suffolk/48/2013 (KU206425), Army 183 (KU206477), AIV (EU087293), HVS25A (D00401), Mar87 (DQ095871), Ro-1 (DQ095872), T965 (DQ095873), EHV-4 gB gene (M26171), and EHV-4 NS80567 (AF030027): References strains; Egypt/EHV-1/Cairo-1A, 3K, 4B, 5S, 6K, 7S, and 12L: Identified isolates].

DISCUSSION

The study included 62 unvaccinated horses with and without respiratory signs in Egypt. EHV-1 was detected molecularly in twelve horses with different ages range (1.5 to 22 years). Four horses showed respiratory signs the remaining 8 horses were positive for EHV-1 despite no respiratory signs were recorded, thus suggesting the existence of latency or reactivation of the virus. EHV-1 respiratory infection is more prevalent in young horses less than 3 years, but older horses are likely to be sub-clinically infected. Interestingly our study revealed that 4 horses with ages ranged from 14 to 17 years showing respiratory signs and were EHV-1 PCR positive, while other two horses with ages less than 3 years were positive EHV-1 PCR despite no clinical respiratory signs were recoded for them. Our findings suggest that the clinical presentation of EHV-1 infection is not entirely depending on age but there are other different factors that

influence the clinical picture of the disease including breed, sex, immune status and stress factors (as transportation, racing and showing events) and other environmental factors (Goehring *et al.,* 2006 and Allen *et al.,* 2008) In our study, no history of neurological signs was recorded for sampled horses.

The molecular diagnosis has been done using nested PCR targeting the EHV-1 gB described by Borchers and Slater, 1993. Two consecutive PCRs using gB specific primers were applied to detect gB gene in the collected whole blood samples. Some samples gave very weak bands in the first round but they gave strong bands in the second round giving a strong evidence for nested PCR sensitivity especially in case of low viral loads that usually attributed to latency or subclinical infection (reactivation from latency). The sensitivity of the PCR was increased in the second round. The nested PCR for EHV-1is considered 100-1000 times more sensitive than the standard PCR due to the more effective denaturation of the smaller amplification products added to the second PCR reaction (Borchers and Slater, 1993).

Gel electrophoresis of the nested PCR products revealed positive bands in first and second rounds for samples of clinically diseased horses while samples from horses without clinical respiratory signs revealed very weak band in the first round but they resulted in a strong band in the second run (Table 1). This increase the suggestion of latent or subclinical infection depending on findings of Baxi and his co-worker who had found that samples from latently infected mice had given a negative result in the first round while they gave very clear bands in the second round. (Baxi *et al.,* 1996).

In the current study, the gB gene was targeted for detection and investigation of nucleotide variation in Egyptian EHV-1 detected by nested PCR. In other studies, genes coding for glycoprotein D was targeted (Abd El-Hamid *et al.,* 2015).

The identity of nucleotide sequences among EHV-1 viruses in the present study and between reference strains was carried out and shown in Table 2. A minor difference in identity of nucleotide sequence was revealed among viruses employed in this study where it ranged from 99.1 to 100% when compared with each other (Table 2). While the identity of nucleotide sequences between EHV-1 viruses in the present study and nucleotide sequences of reference strains was 95.7 up to 100%. Thus indicate that there are some mutations in nucleotide sequences which consequently translated to change in some amino acids and that was clear in Egypt/ EHV-1/Cairo/ 3K, one of our viruses included in the study It was reported that a single nucleotide polymorphism (SNP) in ORF30 could be associated with neuropathogenesis of the strains (Turan *et al.,* 2012). The variation of amino acid sequences among Egyptian EHV-1 isolates included in the study and in comparison to reference strains revealed that there is a little difference. One of the detected strains, Egypt/ EHV-1/Cairo/ 3K, has shown different amino acid sequences from other Egyptian strains at positions 547, 564, and 718 (Table 3). Also, mutation was detected in Egypt/ EHV-1/Cairo/ 12L strain at position 694. Whereas we found a difference in amino acid positions 663 and 681 in the strains included in the study compared to reference strains T-965 and Ro-1 (Table 3). The effect of these amino acid substitutions on the pathogenesis of the strains should be further investigated.

The resulting phylogenetic tree has shown that the Egyptian EHV-1 strains were homogenous and grouped in one cluster with two subclusters (Figure 4). They were different from equine herpesvirus-1 isolated from Grevy's zebra (T-965) and a blackbuck (*Antelopa cervicapra*) (Ro-1) which formed a distinct cluster within equine herpesvirus-1(Figure 4). Although there were previous reports (Hassanin *et al.,* 2002; Saleh *et al.,* 2009; kalad *et al.,* 2013; Abd El Hamid *et al.,* 2015; Mohamed *et al.,* 2017) studied EHV-1 infection in Egypt, they did not target the same gene or the same PCR product we used in our study (gB protein) except Mohamed *et al.,* 2017 who targeted the same gene but they didn't include any data about

sequencing. So, we couldn't find any Egyptian isolates sequences in order to compare and include in our phylogenetic and genetic analysis. The sequence analysis based on the gB gene of the Egyptian EHV-1 isolates indicates that predominantly European EHV-1 strains are circulating in Egypt. Our isolates resemble Army 183 and Suffolk/48/2013 strains which were isolated from abortion cases (Bryant *et al*., 2018). This result is expected to be highly useful for monitoring of EHV-1 infection. Moreover, the sequence information for the gB gene from field isolates of EHV-1 would be valuable for the development of an effective vaccine against EHV-1.

CONCLUSION

In conclusion, we demonstrated that the whole blood sample is a good choice for detection of EHV-1 latency and nested PCR is an appropriate technique for the detection of EHV-1 present in blood of a latently, or silently infected horses. The application of this nested PCR is considered to be a rapid diagnostic tool for use in veterinary laboratories. Thus it will enhance the detection and characterization of the circulating EHV-1 in Egypt. The control measures EHV-1 must include official screening or monitoring. Control of movement inside the country must be conducted to prevent spread of EHV-1 through carriers.

AUTHOR DETAILS

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