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Molecular detection and phylogenetic analysis of equine herpesviruses associated with respiratory manifestation in Egyptian horses

Yasmeen S. Rashwan[1], Noura F. Alkhalefa[2], Mohamed A. Nayel[3], Abdel-Hamid I. Bazid[1], Yassien Badr[4], Samy G. Kasem[2], Salah El Balal[5], Momtaz Wasfy[6] and Magdy El- Sayed[6,7]

[1]Virology Department. Faculty of Veterinary Medicine, University of Sadat city, **Egypt.**
[2]Virology Department. Faculty of Veterinary Medicine, Kafrelsheikh University, **Egypt.**
[3]Department of Medicine and Infectious diseases. Faculty of Veterinary Medicine, University of Sadat city**, Egypt.**
[4] Department of Animal Medicine, Faculty of Veterinary Medicine, Damanhur University, El-Beheira**, Egypt.**
[5]Pathology Department, Faculty of Veterinary Medicine, University of Sadat City**, Egypt**
[6] Middle East for Veterinary vaccines, second Industrial Area, El-Salhya El-Gedida, El-sharqia, **Egypt.**
[7]Department of internal medicine and infectious diseases, Faculty of Veterinary Medicine, Cairo University, **Egypt.**

***Correspondence:** mohamed.aboalez@vet.usc.edu.eg **Received: 14 Nov. 2019, Revised: 19 Dec. 2019 Accepted: 20 Dec. 2019 e-Published: 26 Dec. 2019, Reviewed by: Prof. Dr. Magdy ElGabry, Prof. Dr. Hosein abdelaal**

A study for identification, and characterization of most prevalent Equine herpes viruses causing disease in horses was carried out. Seventy-four nasal swabs collected from apparently healthy horses and those showing respiratory symptoms at some animal hospitals in Cairo housing private studs and working animals. Multiplex nested PCR conducted using primers targeting DNA polymerase gene of herpesviruses, then conventional polymerase reaction was employed using type specific primers for Equine herpes virus-1. The results revealed the presence of five Equine herpes viruses out of 74 tested samples (6.75%), three of which were Equine herpes virus-2, with nucleotide sequence identity(96.43 to 100%), one was Asinine herpes virus-5(identity % of 87.43 to 98.9%) and one isolate was EHV-1(99.6%) as compared to reference strains. Although the prevalence of EHVs was relatively low, vaccine development studies may be valuable for protecting thoroughbred and working equids in Egypt.

Keywords: Equine herpes viruses, Multiplex nested PCR, sequence, and Phylogenetic analysis

INTRODUCTION

Equine herpes virus type 1 (EHV-1;) is the serious cause of disease in horses including abortion in pregnant mares ,respiratory and neurological disorders ,it classified under Herpesviridae family, sub-family Alphaherpesvirinae, and Varicellovirus genus (Lunn et al., 2009) . Herpes virus is consist of four essential components including a150-kilobase linear double-stranded DNA genome, a capsid, the tegument, and finally an envelope (Roizman, 1996; Telford et al., 1992,). In alpha herpes viruses the tegument proteins are encoded by at least 15 genes (Mettenleiter, 2002).

Nine equid herpes viruses have been identified; equine herpesvirus-1 (EHV-1 to EHV-5) infect horses, EHV-6 to EHV-8 infects donkeys and formerly known as asinine herpesvirus (AHV-1, AHV-2 and AHV-3 respectively). EHV-9 or gazelle herpes virus (GHV) was isolated from Thomson's gazelles (Crabb and Studdert, 1996; Ostlund, 1993; Taniguchi, et al., 2000).

All herpes viruses have capsid structure consist of 162 capsomers (150 hexons and 12 pentons). A ring in the nucleocapsid formed by twelve portal proteins , by which the viral DNA introduced into the capsid (Newcomb et al., 1989, Baker et al.,1990). The envelope surface presents 11 viral glycoproteins. These eleven glycoproteins (gC-gp13,gB-gp14, , gD-gp18, gE, gG, gH, gl, gK, gl,gM and gN) are conserved in other alpha herpesvirus. EHV-1 encodes an additional glycoprotein, gp2or gp300, an immunomodulatory protein (Sun et al., 1996, Smith et al., 2005).

Glycoproteins are very important in infection progress, including viral adsorption, penetration, and cell-to-cell spread. Infection with EHV-1 occurs through inhalation of infected aerosols with the virus, or through direct contact with infected secretions (respiratory, aborted fetus, fetal membranes, and placental fluid) (Neubauer and Osterrider, 2004).

Latency is an important feature of equine herpes viruses, which provides establishment of the virus infection in the horses and difficulty to eradicate the disease. Animals with latent infection are unapparent sources of infection to other animals following reactivation. This situation represents a challenge to control transmission of virus because clinical signs are usually hard to detect during the reactivation process (Slater, 2014).

Several tests have been used for identification of EHV from clinical samples (OIE, 2017)., Virus isolation using equine cell culture considered the standard diagnostic approach; ELISA, immunofluorescence, and immunoperoxidase using type specific monoclonal antibodies (Borchers, et al., 1997a) routinely achieved identification of the positive isolates. In the last decades, many PCR techniques were applied for EHVs identifying and typing (Welch et al., 1992; Kirisawa et al., 1993; Dynon et al., 2001; Elia, et al., 2006, Galosi et al., 2006;). The results of such techniques correlated well with virus isolation in terms of accuracy and sensitivity, but exceeded them in simplicity, rapidity, time saving and independence on live infectious virus in the clinical samples (Varrasso et al., 2001).

The genome sequence of EHVs plays a significant role in molecular epidemiological studies.

Sequence analysis of EHV-1 strains revealed that the rate of nucleotide variation was approximately 0.1% (Nugent et al., 2006; Slater et al., 2006). ORF68 gene of EHV-1 has the highest sequence variation rate (2%) and is used a phylogenetic marker to distinguish the virus international collection into six groups

(Nugent et al., 2006). This phylogenetic marker helps to detect the original source of the EHV-1 disease outbreaks, but it does not disclose any relationship between phylogenetic groups and paralytic potential of some strains (Slater et al., 2006). However, the sequence variation in DNA polymerase gene encoded ORF30 provides a means for identifying paralytic viruses. Equine Herpes virus Myeloencephalopathy (EHM) is significantly associated with a single nucleotide polymorphism resulting in an amino acid variation of the EHV-1 DNA polymerase Nugent et al., 2006; Goodman et al., 2007; Van de Walle et al., 2009). EHV-1 encoding aspartic acid (D752) has a higher risk of causing neurological disease than EHV-1 with asparagine (N752) at amino acid position 752, which corresponding to abortion. (Nugent et al., 2006).

In Egypt, antibodies against EHV-2 were detected in sera of Egyptian horses during a survey in 1965, but little efforts were made for virus isolation and/or identification of the circulating EHV strains (Matumoto et al., 1995). EHV-1 was isolated in two separate studies from aborted fetal organs on the CAM of the ECEs (Hassanien et al., 2002), and on a BHK cell line (Warda, 2003). Using immuno diffusion and virus neutralization. However, there is a significant defect in information regarding the most prevalent and circulating EHVs in Egypt. The present study aimed to identify the prevalent types of EHVs using accurate and sensitive tools, including PCR, DNA sequencing and phylogenetic analysis.

MATERIALS AND METHODS

Samples:

Seventy-four nasal swabs were collected from horses, and donkeys, either apparently healthy or showing respiratory manifestations. The animals represented different ages, breeds and sex and were all hosted at animal hospitals in Cairo during the period from May 2017 to November 2017. All specimens were placed in a cold transport medium consist (PBS, pH 7.4), 2 mg/ml streptomycin, 1000 U/ml penicillin, 1000 U/ml fungizone and gentamycin 50 mg/ml. Specimens were transferred to the Laboratories of the Middle East for Veterinary Vaccines (MEVAC) for processing. After repeated cycles of freezing and thawing, the samples clarified by centrifugation at 8000 rpm for 15 min then the collected supernatants stored at - 80 oc until used.

2.2 Virus

A neuropathogenic EHV-1 strain (Ab4p), with a titer of 105 PFU/ml was used as a positive control in PCR reaction. It was kindly provided by Dr. A.J. Davison, Glasgow University, Scotland.

2.3 DNA extraction

DNA extraction was performed using Patho Gene-spinTM DNA/RNA Extraction Kit (Cat. No.17154 from iNtRON) according to the manufacturer's protocol .The purified DNA was stored at -20°C for testing. Positive and negative control DNA extractions were prepared from the Ab4p strain and transport medium and ran in parallel with each sample.

2.4: Multiplex nested PCR

The assay amplifies a short chain (210bp) by using 2 sets of primers, KG1, OFA and ILK in the first cycle, then YGV and IYG in the 2nd cycle. The reaction mixture containing 12.5 µl of Dream taq PCR Master Mix (2x) (Thermo SCIENTIFIC), 0.5 µM (1.25 µl) of each corresponding first round PCR primers, 5 µl of the DNA extract and 6 µl of PCR grade water.

Thermocycling conditions involved initial denaturation at 95°C for 5 min followed by 40 cycles of 94°C for 30 s, 46°C for 30 sand 72°C for 30 s and a final extension step at 72°C for 7 min. For the second round amplification, 5 µl of the first round PCR reaction products were diluted (1:10) then added to a reaction mixture containing 12.5 µl of Dream taq PCR Master Mix (2x), 0.5 µl (1.25 µl) of each corresponding second round PCR primer and 6.5 µl PCR grade water. Target DNA sequences were amplified using the same amplification protocol described for the first round PCR. Amplified products were analyzed on 1.5% agarose gels according to the standard procedures (Sambrook et al., 1989), in comparison with 100 bp DNA ladder (Solis BioDyne).

2.5 Conventional PCR

Identification of extracted viral DNA as EHV-1 was done using type specific primers (ORF15 F and ORF 15 R primers). The reaction mixture contained 12.51JI of Dream taq PCR Master Mix (2x) (Thermo SCIENTIFiC), 0.5! JM (1.25 µl) of PCR primer, 5 µl of the DNA extract and 6.5 µl of PCR grade water. cycling conditions included initial denaturation at 94°C for 5 min followed by 5 cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 30s followed by 30 cycles of 98°C for 10 s, 64oc for 30 s, 72°C for 2 min and a final extension step at 72°C for 7 min. 7 µl of PCR products were analyzed on 1.5% agarose gel electrophoresis containing ethidium bromide stain with a final concentration of 0.5 µg/ml at 95 V for 30 min in TBE buffer, against 100 bp DNA Ladder (Fermentas).

2.6 Nucleotide Sequence and phylogenetic analysis:

PCR products of five selected positive isolates (samples No 10, 12, 29, 33, and 39) were purified from the gel using specific kits (Qiagen Inc. Valencia CA) according to the instructions. The purified PCR products were sequenced using Seqscape ® software for primary analysis of the raw data. A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.4 of Meg Align module of Bio Edit supported software. In order to determine similarities and relationships in amino acid and nucleotide sequence, a phylogenetic tree of the nucleotide and amino acid sequences was constructed using MEGA6 software with 1000 bootstrap replicates

RESULTS

Multiplex nested PCR

Multiplex nested PCR reaction was used to amplify highly conserved motifs in the herpesvirus polymerase gene, 210 bp, by using 2 sets of consensus primers. Gel electrophoresis revealed the presence of specific bands in the expected site. (fig1). Out of 74 collected nasal swabs, 4 samples give positive PCR reactions and were coming from foreign and Arabian horses of different age, ranging from 8 months to 20- years old, with and without history of respiratory manifestations, including nasal discharge, coughing, sneezing, anorexia and depression. (Table 2)

3.2 Conventional PCR:

Conventional PCR using type specific primers for EHV1/4 (ORF 15F and R) was applied to all 74 nasal swab samples, encode ORF15 gene (UL45) which is tegument protein. Only the sample (No12) had successfully amplified the target gene sequence of 705 bp without nonspecific extra bands in all 74 tested nasal swab (0.185%) **(Fig 2).** The sample belonged to a male of foreign breed with a case history of respiratory manifestation. As shown in **Table 2**.

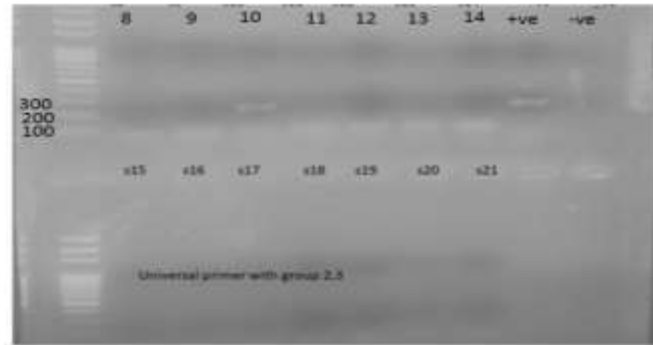


Figure 1: Gel electrophoresis of the amplified products by Multiplex nested-PCR using universal primers. Samples 8 to 21, +ve: positive control, -ve: negative control, L: 100 bp DNA ladder

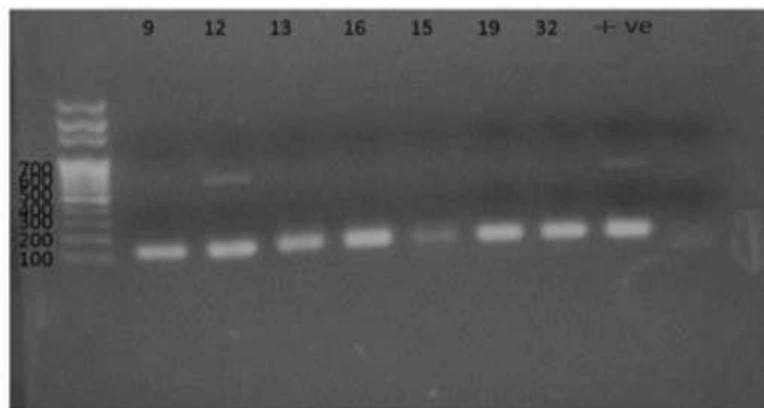


Figure. 2: Agarose gel electrophoresis of the amplified PCR products using ORF 15 primers; samples 12 is positive. +ve: positive control and L: 100 bp DNA ladder

Table (1) Oligonucleotide sequences of the universal and type specific primers used for detection of EHVs

Primer name	type	Sequence (5'-3'_	Annealing Tm.	Product size
KG1	Universal primer	5'-GTC-TTG-CTC-ACC-AGN-TCN-CAN-CCY-TT-3'	46^{0}C	210 bp
DFA		5'-GAY-TTY-GCN-AGY-YTN-TAY-CC-3'		
ILK		5'-TCC-TGG-ACA-AGC-AGC-ARN-YSG CNM-TNAA-3'		
TGV		5'-TGT-ACC-TCG-GTG-TAY-GGN-TTY-CAN-GGN-GT-3'		
IYG		5'-CAC-AGA-GTC-CGT-RTC-NCC-RTA-CAT-3'		
ORF15 F	Type specific primer	5'ACA-AGT-CCG-GAC-TCA-GAT-CTA-TGG-AAG-ATT-ATA-AAT-TAC-TAC-AGC-TGG-AAA-CC3'	55^{0}C in the first five cycles then 64 in 30 cycles	705bp
ORF15 R		5'TAC-CGT-CGA-CTG-CAG-AAT-TCT-TAC-CGG-GGA-AAC-GGT-ATG-ATG-T-3'		

Table (2) Summary of PCR results

Sample No.	breed	age	sex	Respiratory signs	PCR results
10	foreign	8 months	male	Without signs	positive
29	Arabian	2 years	male	Without signs	Positive
33	Arabian	20 years	male	Without signs	positive
39	Arabian	17 years	female	With signs	positive
12	foreign	5 years	male	With signs	positive

3.3 Sequencing and phylogenetic analysis

In order to determine the genetic relatedness of

the obtained viral isolates, a phylogenetic tree was constructed. Using a group of sequences representing some of globally reported genotypes and others from Egypt,.

Partial nucleoid sequence alignment of amplified polymerase gene fragments (ORF 9) of isolates 29, 33, and 39 clustered them with EHV-2 ,as shown in **(Fig 4),** dots (.) represent the identity and alphabets represent the difference in nucleotide sequence **(Fig 3).**

The identity of nucleotide sequences of EHV-2 viruses analyzed in the present study ranged from 96.43 to 100% when compared with each other and with reference strains **(Table 3).** HQ247788.1 Equid herpesvirus 2 strain 276,KY401161.1 and Equid herpesvirus 2 strain G9/92) ,respectively.

Partial nucleoid sequence alignment of identified isolate (sample 10) clusters it with Asinine herpesvirus 5 as shown in **Fig 5, 6 (Table 4).**Partial nucleotides sequence alignment of the amplified fragment of ORF15 (Sample No 12) clustered with EHV-1(**Fig 7 and fig 8)**, with identity percentage of 72.43 % for EHV-4 strain NS80567 and 99.8% for EHV-1 strain Rach and EHV-1strain Ab4 **(Table. 5)**

Table 3: The identity percentage of nucleotide sequences among EHV-2 viruses in the present study compared with each other and with reference strains.

1:	**New_sequence_4EHV39_**	100.00	98.98	99.49	96.43	96.94	98.47	97.96	97.96	97.96	97.96
2:	**New_squence_2EHV29_**	98.98	100.00	99.49	96.94	97.45	97.96	98.47	98.47	98.47	98.47
3:	**New_sequence_3EHV33_**	99.49	99.49	100.00	96.43	96.94	98.47	97.96	97.96	97.96	97.96
4:	**KY628994.1**	96.43	96.94	96.43	100.00	97.45	97.96	98.47	98.47	98.47	98.47
5:	**HQ247791.1**	96.94	97.45	96.94	97.45	100.00	98.47	98.98	98.98	98.98	98.98
6:	**HQ247788.1**	98.47	97.96	98.47	97.96	98.47	100.00	99.49	99.49	99.49	99.49
7:	**KY401161.1**	97.96	98.47	97.96	98.47	98.98	99.49	100.00	100.00	100.00	100.00
8:	**KY407570.1**	97.96	98.47	97.96	98.47	98.98	99.49	100.00	100.00	100.00	100.00
9:	**KY628992.1_**	97.96	98.47	97.96	98.47	98.98	99.49	100.00	100.00	100.00	100.00
10:	**KM924294.1**	97.96	98.47	97.96	98.47	98.98	99.49	100.00	100.00	100.00	100.00

Table 4: Identity percent of sample number 10 (AHV5) compared to other AHV5 and EHV5 sequences based on partial sequences of DNA polymerase gene (ORF9: 191 nucleotides and 63 amino acids).

	AHV5 isolate 1323	**AHV5 strain OVC**	**EHV5 strain 281**	**EHV5 strain BB5-5**	**EHV5 isolate EHV5.2-141**	**EHV5 strain 2-141/67**
Nucleotide Similarity %	98.95	98.43	88.48	88.48	87.43	87.43
Amino acids Similarity %	98.41	96.83	90.48	90.48	90.48	90.48

Table 5: Identity of nucleotide sequences of EHV-1 viruses in the present study when compared with each other and with reference strains.

1:	EHV-4-strain-NS80567	100.00	74.43	72.75	72.75	72.45	72.60	72.60
2:	EHV-9-strain-P19	74.43	100.00	89.19	89.19	88.89	89.04	89.04
3:	EHV-1-strain-Suffolk/110/1994	72.75	89.19	100.00	99.85	99.55	99.70	99.70
4:	EHV-1-strain-V59	72.75	89.19	99.85	100.00	99.70	99.85	99.85
5:	New-sequence-5-_EVH12_	72.45	88.89	99.55	99.70	100.00	99.85	99.85
6:	EHV-1-strain-RacH	72.60	89.04	99.70	99.85	99.85	100.00	100.00
7:	EHV-1-strain-Ab4	72.60	89.04	99.70	99.85	99.85	100.00	100.00

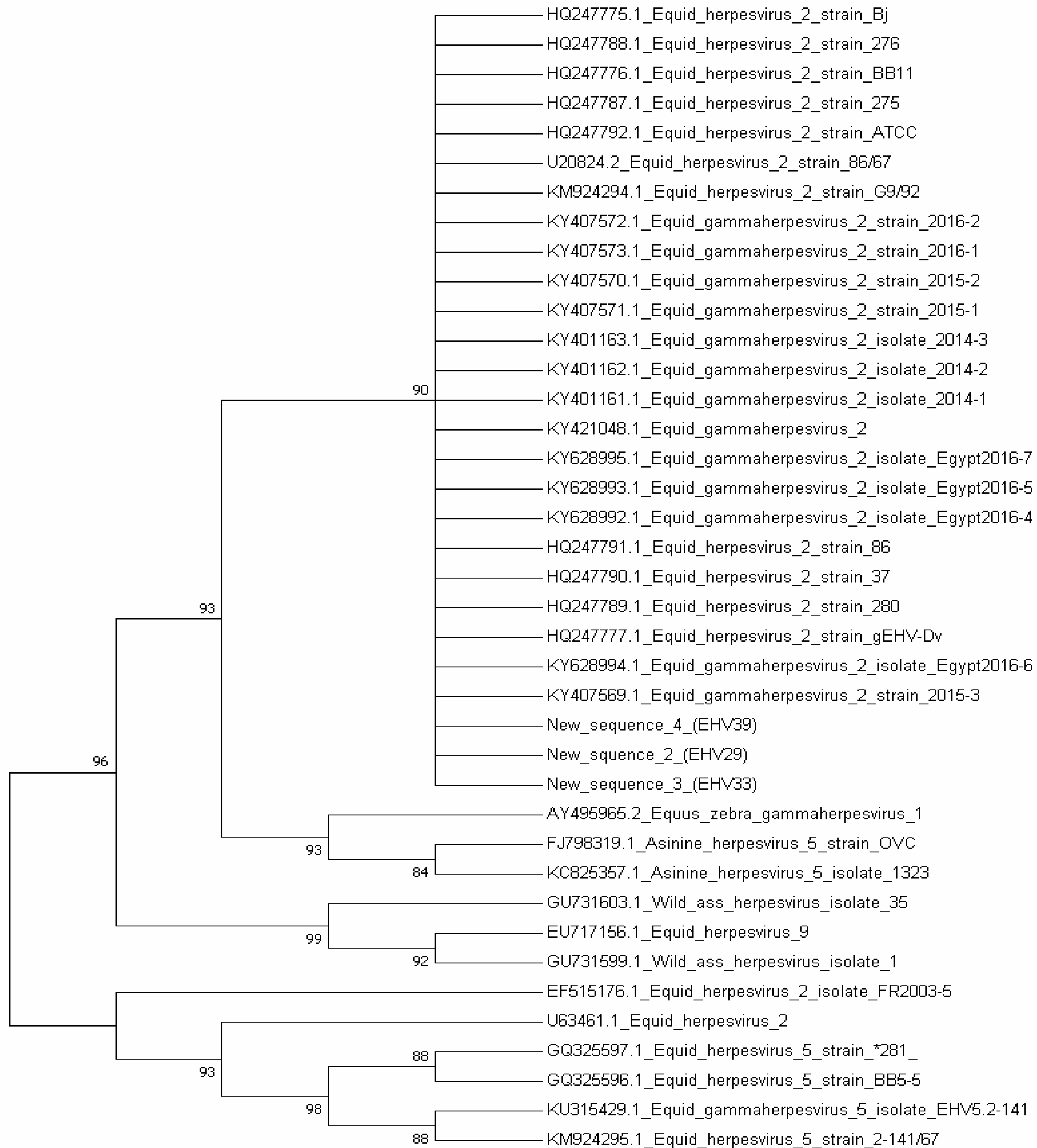
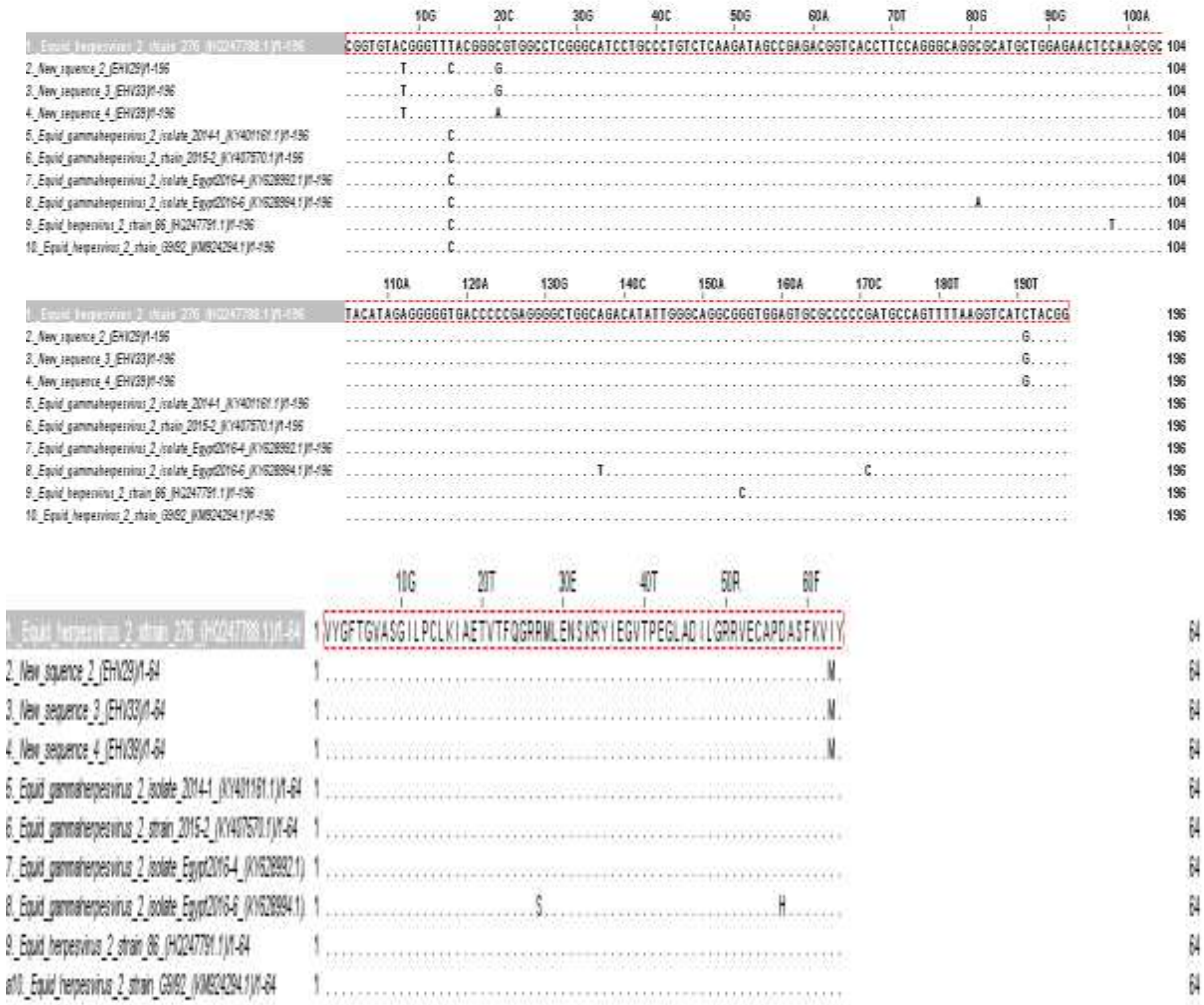


Figure 3: Maximum likelihood phylogenetic tree for amino acid sequences (684 – 747) of the ORF30 of this study isolates (29, 33, 93) and GenBank reference strains.



(Fig 4): Partial nucleotide and amino acid sequence alignment of ORF30 gene of isolates 29, 33, and 39 with the corresponding sequences of EHV-2 strains available in GenBank. Dots indicate site where the sequence is identical to the consensus

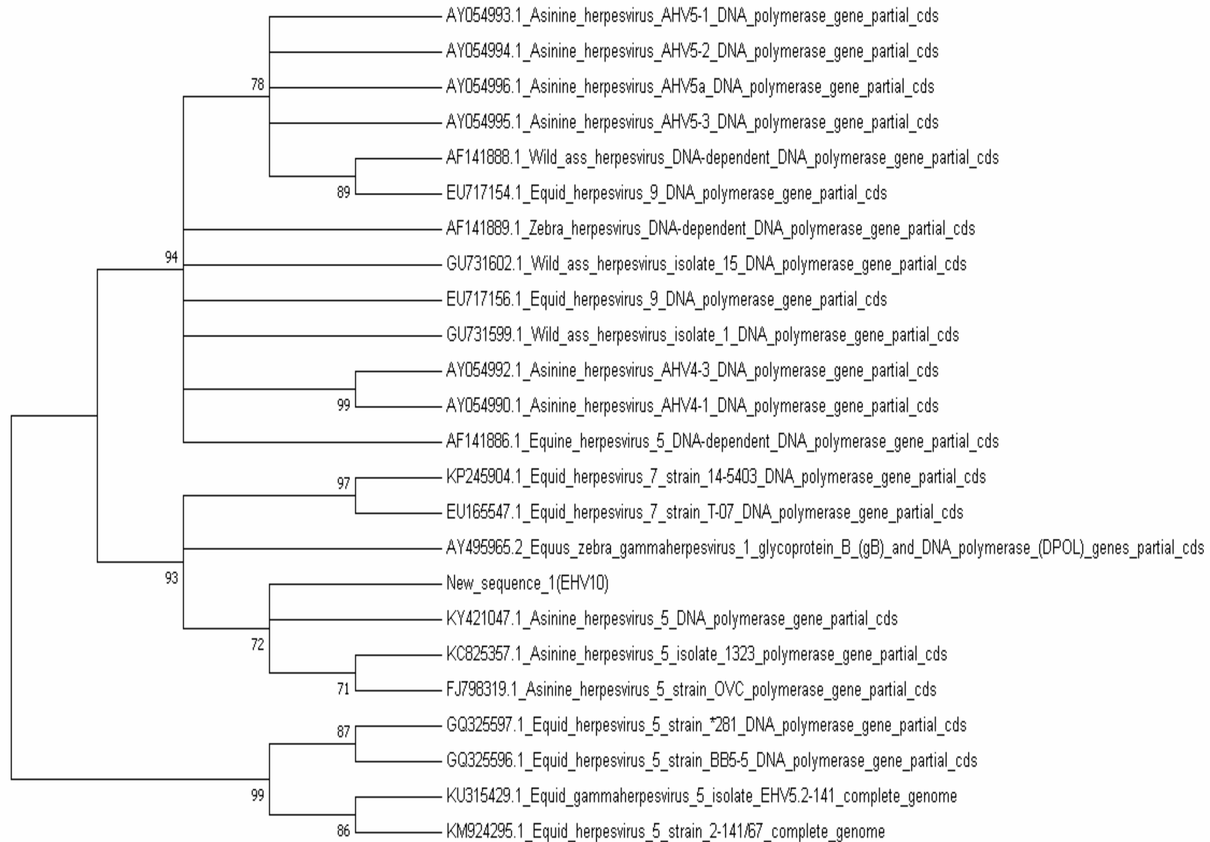


Figure 5: Maximum likelihood phylogenetic tree based on amino acid sequences from 171 to 233 of the ORF30 gene of this study isolate (10) and other reference strains.

Multiple sequence alignment of the partial nucleotide and amino acid sequences of amplified polymerase gene (ORF 30) fragments of isolate (10) in comparison with other related AHV-5 and EHV-5 reference strains from the GeneBank

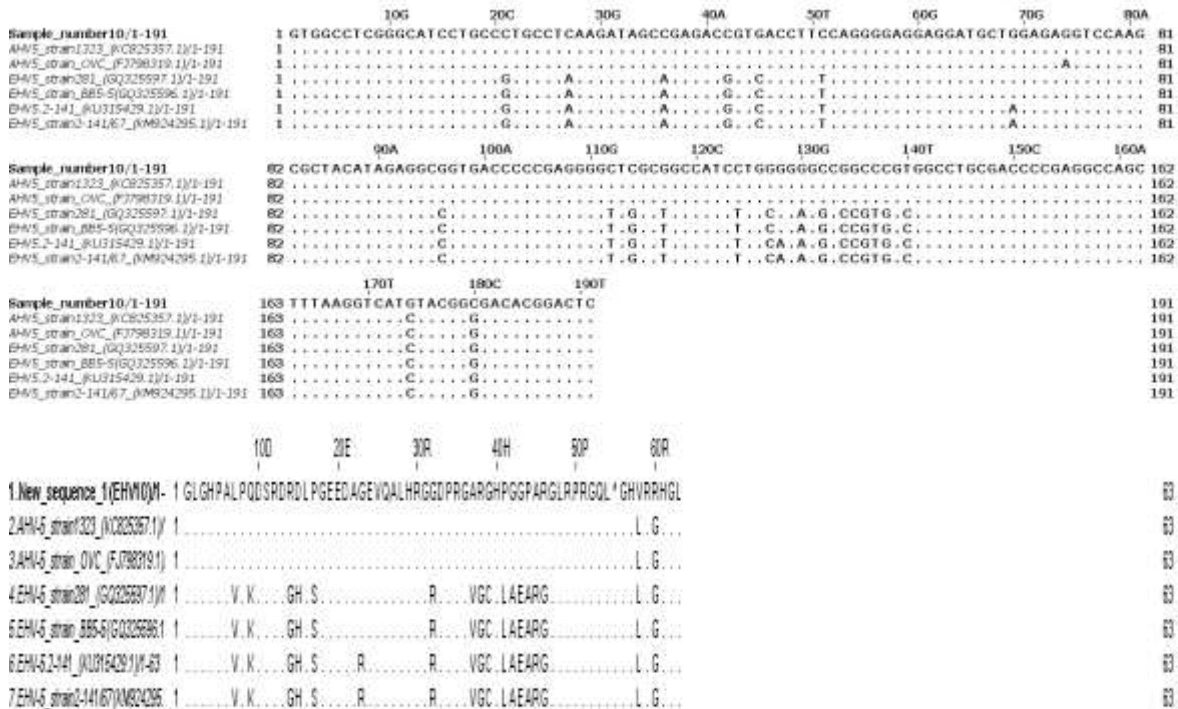


Figure 6 Partial: nucleoid and amino acids sequence alignment of ORF30 of identified isolate (10) in comparison with other AHV-5 and EHV-5 related reference strains from Gene Bank.

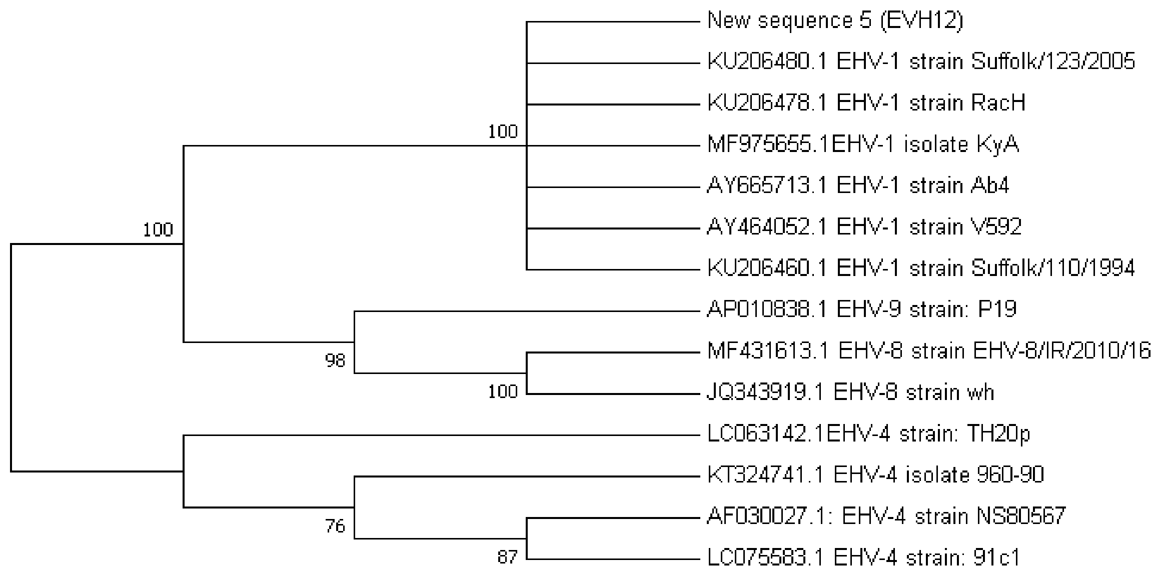


Figure 7: Maximum likelihood phylogenetic tree based on amino acid sequences from 9 to 227 of the ORF15 of this study isolate (12) and other GeneBank EHV reference strains.

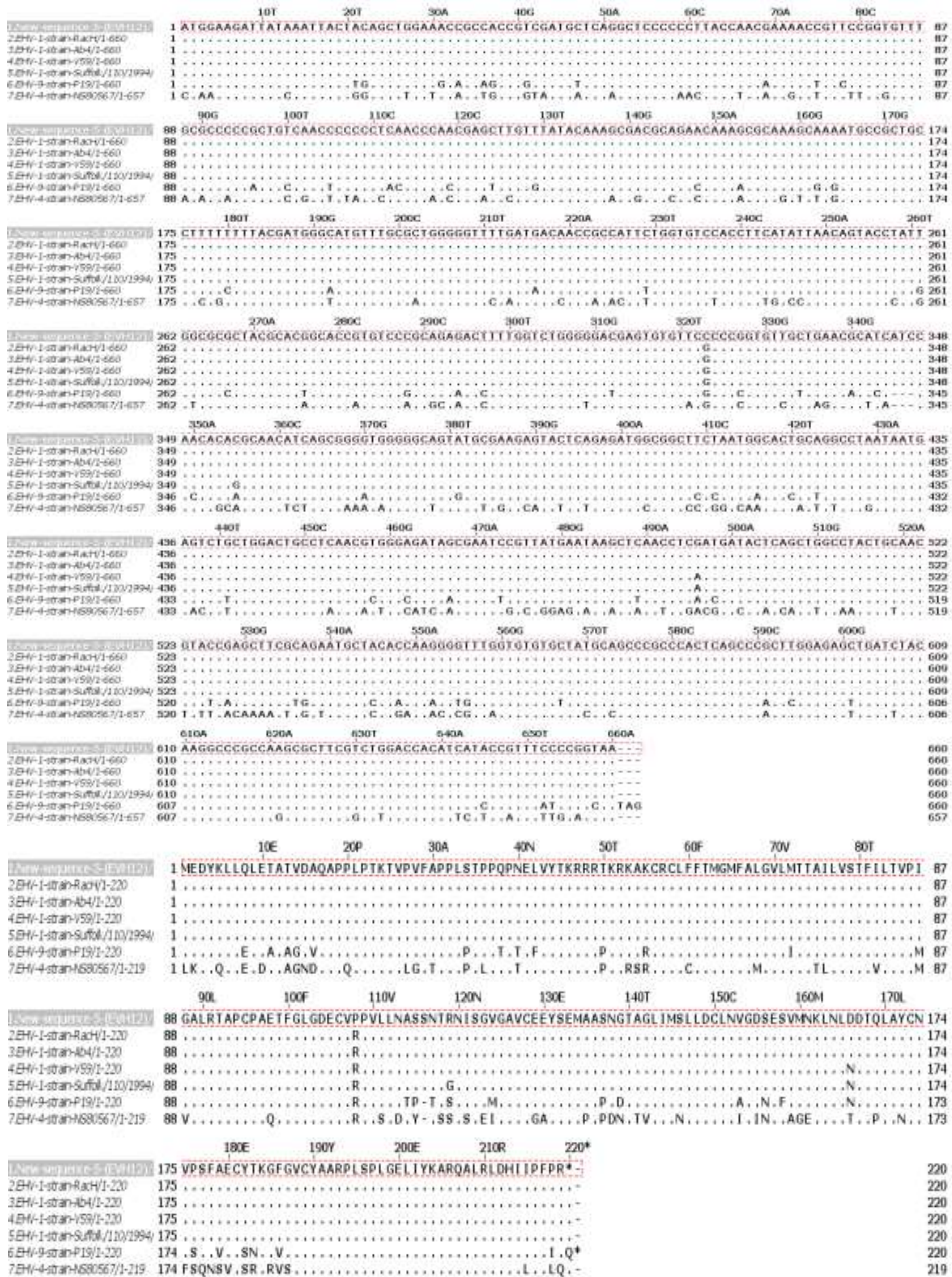


Figure 8: Partial nucleoid and amino acids sequence alignment of ORF15 isolate 12 as compared to related EHV reference strains in the gene bank.

DISCUSSION

In this study 74 nasal swabs were collected during the period from May to November 2107 from animal hospitals in Cairo. Animals included private studs and working horses with or without respiratory manifestations. Five horses were found positive to EHVs, of which one was positive for EHV-1, three for EHV-2 and one was positive for Asinine herpesvirus-5.

Two out of the five animals that showed positive samples exhibited respiratory manifestations, while the other three animals were asymptomatic, which may be attributed to a latent state of herpes virus infection and difficulty to detect the virus during reactivation process (Slater, 2014). It has been suggested that latency of herpesvirus EHV-2 is ubiquitous and the virus has been isolated from both apparently normal and diseased horses worldwide (Borchers et al., 1997b; Nordengrahn et al.,2002) .The age of infected horses ranged from 8 months to 20 years, in agreement with observation that EHV-1 infection is not restricted to a certain age, but there are other factors involved in the infection, such as sex, breed, stress (during transportation, weaning, pregnancy, racing), immune status, primary infection and reinfection. (Allen et al., 2008).

Although the OIE reported in 2017 that the severity of respiratory symptoms may be associated with age, being more obvious and frequent in the young, the present study showed the two animals aged 5 and 10 years were having clinical manifestation, while the other three aged 8 months, 2 years and 20 years exhibited no respiratory manifestations.

Concerning sex, Goehring et al., 2006 reported a higher infection rate in mares than stallions. In our study, one sample containing EHV-2 was obtained from a mare, while the other 4 samples positive for EHV-1, EHV-2 and AHV-5 were obtained from males. This finding suggests that sex may not play a role in EHV infection in Egyptian horses. Although virus isolation is considered the "gold standard" of virological methods for the detection of EHV infection, it is very strenuous and time consuming, particularly when examining numerous samples. This led us to use molecular methods in the diagnosis of this infection (Carvalho et al., 2000; Milic et al., 2010; Ohta et al., 2011 and Nisavic et al., 2016). Hornyak et al. (2006) compared the sensitivity of PCR with virus isolation for identifying EHV-1 in samples from aborted foals. The results of virus isolation and PCR correlated with each other, although PCR proved to be more sensitive for detecting the virus. Elsewhere, PCR proved sensitive and suitable for screening of latent EHV-1 and EHV-4 infections in horses (Welch et al., 1992; Kirisawa et al., 1993; Borchers et al., 1997a; Allen, 2006, and Pusterla et al., 2012).

Molecular diagnosis using Multiplex nested PCR targeting the highly conserved region of DNA polymerase gene of herpesviruses was described by (Van Denanter et al., 1996). In this work, two PCR cycles were performed with a group of consensus primers to detect motifs of DNA polymerase gene. The primary and secondary PCR were performed with degenerate PCR primers targeting the highly conserved amino acid motifs (Wilks et al., 1989) within the herpes-viral DNA directed DNA polymerase gene (Heringa., et al., 1994 and Ito et al.,1991). The assay successfully amplified a short (215 to 315 bp) region of 21 to 22 different herpes-viral species tested.

Four samples (10, 29, 33, 39) were positive in the consensus primer reaction. Bands appeared in the second PCR cycle indicating that nested PCR was suitable for low viral load samples collected during latency or reactivation of a latent infection. The sensitivity of PCR increased in the second cycle duo to more effective denaturation of smaller amplification products in the second PCR cycle. Borchers et al., 1993 and Edington et al., 1994 mentioned that nested PCR was 100-1000 times more sensitive than standard PCR.

ORF15 encodes the tegument protein that has been identified in both EHV-1 and EHV-4. It was reported that ORF 15 started at the nucleotide 21170 and stopped at 20487 bp and encode 227 amino acids within the total genome of EHV-1, it started at 20698 bp, ended at 20018 bp and encode 226 amino acids of EHV-4 genome. Identity of two strain genomes was found to be more than 69% related (Allen et al., 2004). ORF15 (UL45) is a class II protein responsible for growth in vitro and was declared recently as an important virulence factor of HSV-1 neurotropism..

In this study, ORF15 primer inserted in the standard PCR reaction detected

ORF15 gene of EHV-1 in 74 samples. One sample (#12) showed a specific band size of 705, in agreement with the results of many researchers who reported EHV-1 infection in Egypt (Hassanien et al., 2002, Warda et al., 2003) However, sample number 12 was not reactive with the consensus primer as a herpesvirus, which may be attributed to cross- contamination from the excessive handling of the samples in Multiplex nested PCR reaction (OIE, 2107).

Nucleotide sequencing of positive PCR products was performed in order to complete PCR results and establish phylogenetic relationships between the EHV strains (Milic Nenad et al., 2018). Three samples (29, 33, and 39) were EHV-2, the fourth (10) was AHV-5 and the fifth (12) was EHV-1, as summarized in Table 2. EHV-2 was predominant among the positive samples, with and without respiratory signs. Infection with EHV-2 plays a predisposing role to infection with other respiratory diseases, in a way similar to EHV-1, EHV-4, and bacterial pathogens like Rhodococcus equi (Diallo et al., 2007; Dunowska et al., 2002 and Wang et al.,2007; Amer et al.,2011).

Although all samples in this study were collected from horses, sequencing of sample No.10 showed it as an asinine herpes virus-5 (AHV-5), which means that herpesvirus infection can be transmitted from horses to donkeys during outbreaks and vice versa.

CONCLUSION

The obtained results indicated that the equine herpes virus types 1,2 and 5 were circulating in Egyptian horses and the predominant virus in the positive samples was equine herpes virus-2. Also the study indicated that the equine herpes viruses could be transmitted from donkeys to horses during outbreaks and vice versa.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

Y.R., N.A., Y.B. and A.M. carried out the experiment. A.B. wrote the manuscript with support from N.A., M.N and Y.B. S.K., S.E. and M. W. investigate the findings of this work. M.E. and A.B. conceived the original idea.

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