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Original Article

Detection of equid herpesviruses among different Arabian horse populations in Egypt

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Abstract

Equid herpesviruses (EHVs) threaten equine health and can cause significant economic losses to the equine industry worldwide. Different equid herpesviruses, EHV-1, EHV-2, EHV-4 and EHV5 are regularly detected among horse populations. In Egypt, monitoring is sporadic but EHV-1 or EHV-4 have been reported to circulate in the horse population. However, there is a lack of reports related to infection and health status of horses, likely due to the absence of regular diagnostic procedures. In the current study, the circulation of four infectious equid herpesviruses (EHV-1, EHV-2, EHV-4 and EHV-5) among different Arabian horse populations and donkeys residing the same farm was monitored. Different samples were collected and DNA was extracted and subjected to quantitative (q)-PCR to detect the four equid herpesviruses using specific primers and probes. Antibody titres against EHV-1 and EHV-4 were tested using virus neutralization test and type-specific ELISA. The results showed that EHV-1, EHV-2, EHV-4 and EHV-5 are endemic and can be a continuous threat for horses in the absence of vaccination programs and frequent virus reactivation. There is an urgent need for introduction of active regular surveillance measures to investigate the presence of different equid herpesviruses, and other equine viral pathogens, in various horse populations around Egypt and to establish a standardized cataloguing of equine health status.

Keywords: alphaherpesviruses, gammaherpesviruses, arabian horses, donkeys, co-infection.

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Introduction

Horses are constantly exposed to equid herpesviruses (EHVs), because the viruses circulate in horse populations in all countries and regions of the world. Among the *Equidae*, nine herpesviruses have been identified to date. The viruses belong to the *Alphaherpesvirinae* subfamily [six viruses: equid herpesviruses type 1 (EHV-1), EHV-3, EHV-4, EHV-6, EHV-8 and EHV-9] or the *Gammaherpesvirinae* subfamily [three viruses; EHV-2, EHV-5 and EHV-7] (Davison *et al*. 2009). Horses are the natural host to EHV-1, EHV-2, EHV-3, EHV-4 and EHV-5, while donkeys are the primary host of EHV-6 (also referred to as asinine herpesvirus 1, AsHV-1), EHV-7 (AsHV-2), and EHV-8 (AsHV-3). EHV-9, the newest member of the equid herpesviruses, was first isolated from an infected Thomson gazelle; however, recent studies suggested that zebras or other perissodactyls such as the rhinoceros could be the definitive host for this virus (Fukushi *et al*. 1997; Abdelgawad *et al*. 2015, 2016).

EHV-1 and its close relative, EHV-4, cause significant economic losses to the equine industry due to clinical illnesses, which are associated with lost time for training and performance (Allen & Bryans 1986; Patel & Heldens 2005). Although both viruses cause respiratory disease, only infection with EHV-1 may

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result in abortion, perinatal mortality and neurological disorders with clinical signs that vary in severity but can result in complete paralysis (Patel & Heldens 2005). Infection of horses with either of the two viruses is clinically and serologically difficult to distinguish because of their high genetic and antigenic similarity. Primary infection with either EHV-1 or EHV-4 occurs mainly via direct horse-to-horse contact. Direct contact with EHV-1-infected aborted fetuses, placental tissues or fomites contaminated by respiratory secretions may serve as a source of infection although the viruses are reported to be relatively short-lived in the environment (Reed & Toribio 2004; Harless & Pusterla 2006). However, it was shown that EHV-1 can remain stable and infectious for over a week and up to 3 weeks in water under different conditions of salinity, pH, temperature and turbidity in controlled *in vitro* experiments (Dayaram *et al*. 2017).

EHV-2 and EHV-5 are slow growing cell-associated gammaherpesviruses. Both viruses are widespread in the equine population worldwide (Borchers *et al*. 2006; Hue *et al*. 2014). EHV-2 has been reported to be associated with upper respiratory tract disease, lymphadenopathy, immunosuppression, keratoconjunctivitis, general malaise and poor performance (Borchers *et al*. 2006). EHV-5 in contrast has been reported to be associated with pulmonary fibrosis in horses (Williams *et al*. 2007). Equine herpesviruses, like all herpesviruses, enter a latent state but reactivation from latency may result in recurring disease, which is accompanied by virus shedding and transmission to other horses (Allen & Bryans 1986; Edington *et al*. 1994; Crabb & Studdert 1995).

Standard diagnostic methods for EHV-1 and related viruses are well-established, including virus isolation and serological assays, particularly virus neutralization tests (VNT) and type-specific ELISA assays (Crabb & Studdert 1993; Crabb *et al*. 1995; Lang *et al*. 2013; OIE, 2018). Quantitative PCR (qPCR) methods offer alternatives to virus isolation and have been proven sensitive and time-effective (Pusterla *et al*. 2005).

Currently there are different inactivated or modified live EHV-1 and EHV-4 vaccines commercially available. However, they cannot completely block virus infection and most vaccines only induce reliable protection against respiratory disease (Heldens *et al*. 2001; Patel & Heldens 2005; Ma *et al*. 2013). Possible protection against neurological disease under experimental conditions (Goodman *et al*. 2006) and abortion under field conditions using EHV-1-based vaccines (Bresgen *et al*. 2012) was reported.

In Egypt, there are more than 1.5 million equids, with horses representing around 40% of the total equine population (Animal Wealth development sector, Egyptian Ministry of Agriculture, 2010 http://www.agr-egypt.gov.e.g/Uploads/Studies/d14ee0cb-c15d-4fb9-9f2d-008f5b7ef873.pdf). These horses include pure-bred registered Egyptian Arabian horses and are mainly used for showcasing, semen collection, and export. Although there are no definitive statistics, horses play an important role in Egyptian economics. This includes the direct economic impact of the horse industry (breeding, employment and education) and the indirect impact (related to horse activities like organization of social events). Currently, there is no accurate or official documentation on the health status of horses or vaccination programs. Recently, one study in Egypt detected EHV-1, EHV-2 and EHV-4 DNA in clinical samples collected from horses from 2005 to 2006 (Amer *et al*. 2011). Furthermore, isolation of EHV-1 from aborted fetuses in Egypt was documented (Soliman *et al*. 2008; Abd El-Hafeiz *et al*. 2010). In general, lack of proper monitoring of equine infectious diseases in Egypt could threaten the stability of the horse industry in Egypt and beyond due to international trade in horses. The aim of the current study was to investigate the circulation of different equid herpesviruses, particularly EHV-1, EHV-4, EHV-2 and EHV-5, in selected Arabian horse and donkey populations in Egypt.

Materials and methods

Sample collection

Samples were collected from Arabian horses (n = 176) and donkeys (n = 16) from different parts of Egypt, including the main provinces of the North

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Egypt (Cairo, Alexandria, Giza, Sharkia, Gharbia and Monufia; Table 1 and Data S1). The Arabian horses were used mainly for show purposes and donkeys were used as work and draft animals. The samples were collected either at horse farms or from horses admitted to horse clinics. Tissue samples and placentae (placenta: n = 23 and tissues from aborted fetuses: n = 17; total cases of abortion = 40) were collected from aborted fetuses and mares immediately after abortion. Thirteen nasal swabs (Egyptian Company for Medical Equipment) were collected from horses suffering from fever and respiratory disorders using sterile swabs. The swabs were placed directly in virus transport medium (serum-free MEM with 1% penicillin streptomycin, 1% gentamicin, and 0.1% fungizone). Cerebrospinal fluids (CSF; n = 4) were collected from stallions suffering from neurological disorders (Table 1 and Data S1). Whole blood collected into tubes with EDTA anticoagulant (n = 135) and without anticoagulant, to obtain serum, (n = 110) was obtained from apparently healthy horses and with no history of previous vaccination. After collection, all samples were packed in coolers with ice packs and transported immediately to the laboratory for analysis.

DNA extraction and PCR

Viral DNA was extracted from collected samples and infected cell cultures using DNA/RNA Virus, Tissue or Blood Mini Kits (Stratec Biomedical, Birkenfeld, Germany) according to the manufacturer's instructions.

PCR reactions were performed using the extracted DNA as a template. Nested PCR amplifications using degenerate PCR primers (Table 2) that specifically amplify a 250-bp fragment of herpesvirus DNA polymerase gene were performed on all samples as described previously (VanDevanter *et al*. 1996). Fourteen randomly selected amplicons were purified and directly sequenced by Sanger sequencing (LGC Genomics) to confirm that the amplified fragments contain herpesvirus DNA. All DNA samples were re-analysed by qPCR with the Applied Biosystems 7500 FAST (ABI, Foster City, CA) using specific primers and probes targeting the highly conserved gB gene (Table 2) (Dynon *et al*. 2001; Pusterla *et al*. 2005; Hussey *et al*. 2006; Dunowska *et al*. 2011). Positive (virus-infected cell culture) and negative (water) controls were included from the beginning of the extraction procedure until the reading of the results.

EHV-specific primers (Table 2) that target various regions of two genes, gB (glycoprotein B; ORF33), and/or POL (DNA polymerase; ORF30), were designed and employed to amplify the corresponding fragments. The amplified products were purified, sequenced by Sanger sequencing (LGC Genomics), and submitted to GenBank (accession numbers: MG732975- MG732978).

Cells

Equine dermal (ED) cells were grown in DMEM medium (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; PAN Biotech) and 100 U ml^{-1} penicillin (Roth, Karlsruhe, Germany) and 100 μg ml^{-1} streptomycin (Alfa Aesar, Kandel, Germany) in a 37°C incubator with 5% CO_2 atmosphere. Cells were grown to confluency in a 100 mm tissue culture dish, washed with phosphate buffer saline (PBS), trypsinized with 0.25% trypsin supplemented with 2.5 μmol L^{-1} EDTA and counted in a Neubauer counting chamber.

Virus neutralization test

Virus neutralization test (VNT) was performed according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2018). Briefly, serum samples were inactivated at 56°C for 30 min. In 96-well plates, serial twofold dilutions of inactivated serum were incubated with 100 plaque forming units (PFU)/100 μl of either EHV-1 or EHV-4 at 37°C. After 1 h, a total of 5×10^5 equine dermal cells (ED) were added and plates were incubated for 2 h at 37°C. The monolayers were overlaid with 1.6% methylcellulose medium and plates were incubated at 37°C for 3 days. The reaction was stopped with 3% formalin and the plaques were stained with Giemsa. EHV-1- and EHV-4-positive horse serum and fetal calf serum were included as positive and negative controls, respectively.

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Table 1. List of samples used in this study

Samples*	Number of samples	History	Sex	Age range/ median (year)	Location
Animals with clinical problems					
Aborted fetus tissues and placenta	40†	Abortion and still birth	F: 40‡	5–12/7	Cairo, Alexandria, Giza, Sharkia, Monufia
Nasal swabs	13	Fever, nasal discharge, and respiratory disease	M: 1 F: 12	2–12/5	Sharkia, Giza, Gharbia
Cerebrospinal fluid	4	Ataxia and hind limb paralysis	M: 4	3–8/4.5	Giza, Alexandria
Total	57				
Apparently healthy animals					
Whole blood	135	No obvious clinical signs	M: 67 F: 68	2–12/7	Gharbia, Giza, Alexandria, Sharkia
Total	192				
• Clinical samples					
• Apparently healthy					
Serology					
Serum	110	No obvious clinical signs	M: 53 F: 57	2–12/7	Gharbia, Giza, Alexandria, Sharkia

*Samples were collected in 2015 and 2016. †Placenta: n = 23; tissues from aborted fetuses: n = 17. ‡Numbers indicate the number of tested animals of each sex.

Antibody titres were expressed as the reciprocal of the highest serum dilution that protected the cells from infection in each well. A reciprocal titre of greater than or equal to 4 was considered positive (Lang et al. 2013). Each test was validated with the positive and negative sera controls.

Peptide-based ELISA

The ELISA test was carried out as described before (Lang et al. 2013) with few modifications. Briefly, 96-well plates were coated with 1 μg ml^{-1} streptavidin (100 μl well^{-1} dissolved in 50 nmol L^{-1} carbonate-biocarbonate buffer; pH 9.6) overnight at 4°C. The wells were washed three times with PBS containing 0.1% Tween 20 (PBST). After coating with 100 μl well^{-1} of the respective biotinylated peptide [EHV-1 E (E1) peptide: KQPQPRLRVKT-PPPVTVP and EHV-4G (G4) peptide: TEGMKN-NPVYSESLMLNV; 2 μg ml^{-1} in 50 nmol L^{-1} carbonate-biocarbonate buffer], the plates were incubated at 37°C for 2 h. The plates were then blocked with 1% goat serum diluted in PBST and incubated for 1 h at 37°C. After washing, serum samples (100 μl well^{-1} in 1:400 dilution) were added and plates were incubated for 1 h at 37°C. Purified goat anti-horse IgG conjugated with horseradish peroxidase (1:20 000; Dianova, Hamburg, Germany) was added and plates were incubated for 1 h. After washing, binding was detected by addition of 100 μl well^{-1} TMB [3,3,5,5-tetramethylbenzidine, dissolved in 42 μg ml^{-1} citric acid, 0.01% H_2O_2 (pH 3.95)]. The reaction was stopped after 10 min with 100 μl ml^{-1} of 1 mol L^{-1} sulphuric acid and the plates were read at a wavelength of 450 nm on a spectrophotometer (TriStar LB 941, Berthold Technologies, Bad Wildbad, Germany). Based on our previous publication (Lang et al. 2013), we have set a cut-off value (OD value = 0.118) above which a sample was considered positive. Samples that produced OD values between 0.118 and 0.100 were considered questionable, and samples with OD values of <0.100 were considered negative. The same cut-off values were used for both EHV-1 and EHV-4 ELISA. Each serum sample was tested for EHV-1 and EHV-4 antibody two independent times. Negative and positive serum controls were included in each plate.

Table 2. Primers and probes used in the study

Product	Primer	Sequence	Fragment	References
All herpesviruses				
Pan herpesvirus	DFA (For)	**G**A**Y**TT**Y**GC**N**A**GYY**T**N**TA**Y**CC*	700 bp	VanDevanter *et al.* (1996)
	ILK (For)	TCCTGGACAAGCAGCA**RNYSGCNM**T**N**AA*		
	KG1 (Rev)	GTCTTGCTCACCAG**N**TC**N**AC**N**CC**Y**TT*		
Pan herpesvirus (nested PCR)	TGV (For)	TGTAACTCGGTGTA**Y**GG**N**TT**Y**AC**N**GG**N**GT*	250 bp	
	IYG (Rev)	CACAGAGTCCGT**R**TC**N**CC**R**TA**D**AT*		
Equid herpesvirus 1 (EHV-1)†				
glycoprotein B (gB)	For	*61722*-CACTTCCATGTCAACGCACT-*61741*	869 bp	Designed for this study
	Rev	*62591*-TCGACTTTCTTCTCGGTCCA-*62572*		
DNA polymerase (POL)	For	*54885*-ACCTCCGGAGGCAAAGTTCA-*54904*	709 bp	
	Rev	*54195*-TTCGCCCCGTTGAGCGACAC-*54214*		
EHV-4†				
gB	For	*61314*-CATGTCTAAAGACTCGACAT-*61333*	1369 bp	Designed for this study
	Rev	*62664*-GATTGGTATTATGGTTTGCG-*62683*		
POL	For	*54660*-CATCACAGTACACTTTTGGG-*54679*	843 bp	
	Rev	*53836*-ACTATAAGCTACTGTGTTTT-*53855*		
EHV-2†				
POL	For	*36400*-GCGCGTGTTGCGCGAGTACT-*36419*	1270 bp	Designed for this study
	Rev	*37651*-GGTGCAGGCACAGCCTGTCT-*37670*		
EHV-5†				
POL	For	*32972*-AAGGGTTTTGAAACAATACA-*32991*	1115 bp	Designed for this study
	Rev	*34068*-GAACTTTCCTTGTTGCCCGA-*34087*		
qPCR				
EHV-1 gB	For	CATACGTCCCTGTCCGACAGAT		Hussey *et al.* (2006)
	Rev	GGTACTCGGCCTTTGACGAA		
	Probe	**6FAM**-TGAGACCGAAGATCTCCTCCACCGA-**BHQ1**		
EHV-4 gB	For	CGCAGAGGATGGAGACTTTTACA		Pusterla *et al.* (2005)
	Rev	CATGACCGTGGGGGTTCAA		
	Probe	**6FAM**-CTGCCCGCCGCCTACTGGATC-**TAMRA**		
EHV-2 gB	For	AGGACTACTACTATGTCAG		Dunowska *et al.* (2011)
	Rev	ATGGTCTCGATGTCAAACAC		
	Probe	**6FAM**-TGACATACCCACCCTACACACCATGA-**BHQ1**		
EHV-5 gB	For	ATGAACCTGACAGATGTGCC		Dynon *et al.* (2001)
	Rev	CACGTTCACTATCACGTCGC		
	Probe	**6FAM**-TCCATCCACGATGGCAGGGA-**BHQ1**		

*Bold letters indicate amino acid residues included in the primers. †Numbers before and after primer sequences represent their positions in the genome.

Statistical analysis

Statistical analyses and graphs of the serology data were performed using GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA). Fisher's exact test was used to compare the frequencies or proportions of EHV-1 and EHV-4 positive samples. Differences were considered statistically significant when the P value was less than 0.05.

Results

Detection of different equid herpesviruses

Using consensus herpesvirus PCR, a 250-bp fragment was amplified in 110/192 (57%) samples. Using virus-specific qPCR, 133/192 (69%) samples were positive for at least one of the four EHVs. The number of samples detected by both assays was $n = 64$ for EHV-1, $n = 5$ for EHV-4, $n = 63$ for EHV-2 and

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n = 37 for EHV-5. The number of samples detected only by virus-specific qPCR was EHV-1 (n = 21), EHV-4 (n = 4), EHV-2 (n = 10) and EHV-5 (n = 8). Four samples were detected only by consensus herpesvirus PCR (Data S1). The 250-bp fragment of 14 amplicons were sequenced and the data revealed 95-100% identity at the nucleotide level to previously reported EHV-1 (n = 5), EHV-4 (n = 1), EHV-2 (n = 7) and EHV-5 (n = 1) DNA polymerase sequences.

Using specific qPCR protocols, we found that the most prevalent virus was EHV-1 (85/192; 44%), followed by EHV-2 (73/192; 38%), EHV-5 (45/192; 23%) and EHV-4 (9/192; 4%). Among the 40 tested samples collected from aborted cases, EHV-1 and EHV-2 were detected in 29/40 (72%) and 16/40 (40%) samples, respectively (Table 3). EHV-4 (4/40; 10%) and EHV-5 (1/40; 2.5%) were also detected. All four herpesviruses (mainly EHV-1 and EHV-5) were detected in donkeys that were present at the same farm as positive horses (Table 3). No herpesviruses were detected in the CSF samples. Co-infections with different herpesviruses were common, especially for EHV-1 and EHV-2 or EHV-2 and EHV-5 (Table 4). Triple co-infections were also detected for all viruses; however, we did not identify coinfection with all four viruses in a single animal (Table 4).

Table 3. qPCR results from different samples

Samples	Total	EHV-1	EHV-4	EHV-2	EHV-5
Clinical problem*	57	38	4	25	6
Apparently healthy	135	47	5	48	39
Total	192	85	9	73	45
Tissue	40	29	4	16	1
Whole blood[†]	135	47	5	48	39
Nasal swabs	13	9	0	9	5
CSF	4	0	0	0	0
Total	192	85	9	73	45
Horses	176	77	8	71	39
Donkeys	16	8	1	2	6
Total	192	85	9	73	45

*Clinical problems included abortion, respiratory disease and nervous disorders (see Table 1 and Data S1). [†]Whole blood was collected from apparently healthy animals.

Four sequences, Egy-01 (EHV-1 gB sequence; GenBank: MG732975), Egy-02 (EHV-1 POL sequence; GenBank: MG732976), Egy-03 (EHV-2 gB; GenBank: MG732977), and Egy-04 (EHV-5 gB; GenBank: MG732978), were obtained from the isolated DNA and blasted against available EHV-sequences from GenBank.

Detection of EHV-1 and EHV-4 neutralizing antibodies

Of 110 tested serum samples, 59 (54%) and 78 (71%) were seropositive by VNT for EHV-1 and EHV-4 antibodies, respectively (Fig. 1a and Data S1). In horse serum samples (n = 103), 54 (52%) and 75 (73%) tested positive for EHV-1 and EHV-4 antibodies, respectively. In samples collected from donkeys living in the same farm (n = 7), 5 (71%) and 3 (43%) tested positive for EHV-1 and EHV-4 antibodies, respectively (Data S1). The highest neutralizing titre was 128 (Data S1). It is worth mentioning that the horse with this high titre was apparently healthy and positive only for EHV-5 DNA by qPCR. The prevalence of EHV-4 antibodies in the tested serum samples was significantly higher than that of EHV-1 (P = 0.01; Fisher's exact test).

Table 4. Incidence of co-infection as detected by qPCR

Classification	Virus*	No. detected in horses	No. detected in donkeys
Unique detection	EHV-1 only	32	3
	EHV-4 only	0	0
	EHV-2 only	21	0
	EHV-5 only	9	3
Double detection	EHV-1 and EHV-4	4	0
	EHV-1 and EHV-2	24	2
	EHV-1 and EHV-5	4	2
	EHV-2 and EHV-5	15	0
Triple detection	EHV-1, EHV-4, EHV-2	2	0
	EHV-1, EHV-4, EHV-5	2	1
	EHV-1, EHV-2, EHV-5	9	0
Quadrupel detection	EHV-1, EHV-4, EHV-2, EHV-5	0	0

*This includes virus detection in samples collected from clinically affected and apparently healthy animals.

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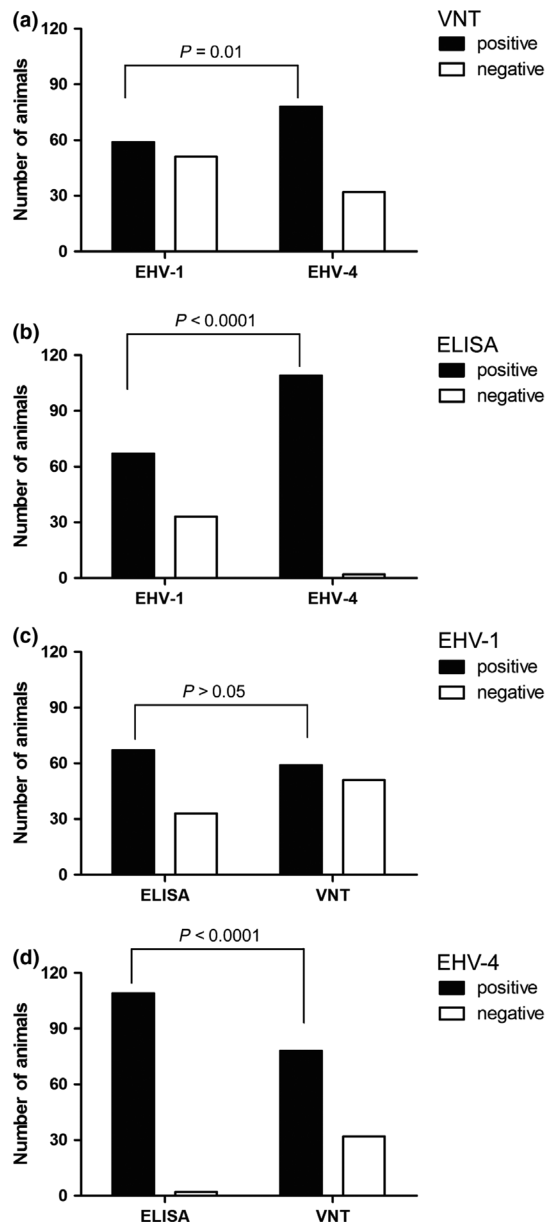

Fig. 1. Detection of EHV-1 and EHV-4 antibodies. Number of animals (n = 110) positive or negative for the presence of specific antibodies either by virus neutralization test (a) or peptide-specific ELISA (b). The prevalence of EHV-4 antibodies was significantly higher in the serum samples tested by virus neutralization test (VNT) or ELISA (Fisher's exact test). Number of animals positive or negative for the presence of EHV-1-specific (c) or EHV-4-specific (d) antibodies using ELISA and VNT. The number of animals tested positive for EHV-4 antibodies by ELISA was significantly (Fisher's exact test) higher than this tested by VNT.

Discrimination between EHV-1- and EHV-4-specific antibodies by peptide-based ELISA

EHV-1 gE (EHV-1_E)- and EHV-4 gG (EHV-4_G)-specific peptides were used to differentiate between EHV-1- and EHV-4-specific antibodies as described before (Lang *et al*. 2013). In total, 67/110 (61%) samples tested positive for EHV-1 antibodies and 109/110 (99%) samples for EHV-4 antibodies, including seven donkeys that were all positive for both EHV-1 and EHV-4 antibodies (Fig. 1b and Data S1). Ten additional samples were classified as questionable for EHV-1 antibodies (Data S1). The data showed that the prevalence of EHV-4 antibodies was significantly higher than that of EHV-1 ($P < 0.0001$; Fisher's exact test).

There were 14/59 (24%) horse serum samples that tested positive for EHV-1 antibodies by VNT, were negative by EHV-1 ELISA (Data S1). In addition, there were 8 and 31 samples that were negative by VNT and tested positive with EHV-1 and EHV-4 ELISA, respectively (Data S1). The data showed that ELISA was significantly more sensitive for EHV-4, but not EHV-1, antibodies (Fig. 1c,d; $P < 0.0001$; Fisher's exact test).

Discussion

The loss of valuable horses and revenue from major horse events has negative consequences for the horse breeding and sports industry. In Egypt, few monitoring studies have been performed but the existence of equine herpesviruses, particularly EHV-1 and EHV-4, in the horse population has been documented (Abd El-Hafeiz *et al*. 2010; Amer *et al*. 2011; Al-Shammari *et al*. 2016; Fararh *et al*. 2016). Egyptian Arabian horses are an important part of the country's economy due to global trade linked to the international reputation of the Egyptian breeding lines. In the current study, we investigated the circulation of equine alpha- and gammaherpesviruses among Arabian horses and donkeys in Egypt.

A total of 192 samples were collected from apparently healthy animals or horses with clinical signs of

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abortion, respiratory disease or neurological disorders. EHV-1 and EHV-2, respectively, were the main viruses detected in clinically affected horses; however, EHV-4 and EHV-5 were also detected to a lesser extent. It is difficult to state whether any of these viruses were responsible for the clinical diseases among horses sampled due to the absence of clear case history and proper diagnosis at the time of disease onset. On the other hand, EHV-2, EHV-1 and EHV-5, respectively, were mostly detected in the apparently healthy horses. Interestingly, all viruses, but predominantly EHV-1 and EHV-5, were detected in the tested donkey samples. Our data as well as previous studies clearly indicate that these equine herpesviruses are circulating in equid populations in Egypt (Abd El-Hafeiz *et al*. 2010; Amer *et al*. 2011; Al-Shammari *et al*. 2016; Mohamed *et al*. 2017). The frequency of detection of each virus among equids varied between studies. This can be explained by the age, health status and breed of horses as well as geographical variability and environmental factors. Detection of EHV in donkeys (in this study) and mules (Mohamed *et al*. 2017) is interesting but it is not clear if the donkeys contracted the infection from horses or whether they can play a role in further spreading of the infection.

Co-infections with up to three viruses were detected in both clinically infected and apparently healthy horses and donkeys. This dual infection was reported before in horses but also in wild equids (Amer *et al*. 2011; Back *et al*. 2015; Abdelgawad *et al*. 2016; Laabassi *et al*. 2017; Negussie *et al*. 2017). However, their potential synergistic effect on the disease outcome remains to be elucidated. The detection of EHV-1, associated with other EHVs in donkeys, provides hallmarks on the potential source of infection. Furthermore, donkeys in Egypt are always stressed due to the assigned hard work, which might increase the probability of EHV shedding after reactivation.

It was clear that both EHV-2- and EHV-5 are circulating among apparently healthy horses (Table 3). This high prevalence may increase the risk of other possible infections due to compromising horse immunity (Nordengrahn *et al*. 1996; Negussie *et al*. 2017). Further studies are needed to better understand the clinical outcomes of EHV-2 and EHV-5. The role of EHV-4 in disease outcome cannot be ideally assessed due to the low number of detected positive samples.

Serological analysis was conducted for all collected serum samples. Although VNT is known to be an efficient and robust test, it cannot accurately discriminate between antigenically similar viruses, like EHV-1 and EHV-4 (Crabb *et al*. 1995; Lang *et al*. 2013); this was further confirmed in our current data. We found that 14 of 59 horse serum samples, which tested positive for EHV-1 antibodies by VNT, were negative by EHV-1 ELISA but positive by EHV-4 ELISA. This result indicates that the detected EHV-1 neutralizing antibodies were a result of cross-reactivity with EHV-4. In addition, there was a clear difference in sensitivity when comparing VNT with ELISA (Fig. 1c,d). In general, our data clearly support the previous data indicating that peptide-ELISA is considered a specific and sensitive serological test for detection of EHV-1 and EHV-4 seroprevalence (Lang *et al*. 2013; Damiani *et al*. 2014). VNT and specific peptide-based ELISA demonstrated that the prevalence of EHV-4 antibodies among sampled horses was significantly higher than that of EHV-1. These results strongly suggest that EHV-4 is omnipresent in horse population in Egypt and that most of the horses probably contracted the infection during their early life.

Equid herpesviruses EHV-1, EHV-4, EHV-2 and EHV-5 circulate among various Arabian horse populations in Egypt. In the present study, all viruses were detected in apparently healthy and clinically ill equids. Although Arabian horses are considered one of the most proficient and valuable breeds used in breeding, show competitions and exportation, no regular monitoring programs are performed to track the health status of these animals, including EHV infections. In addition, vaccination programs against equid herpesviruses, that might reduce the risk of infection, are not routinely performed by veterinary practitioners in Egypt. In the current study, we shed light on the frequency of detection of various equine herpesviruses among sampled population(s) of Arabian horses in Egypt. These data will help to guide efficient diagnosis and preventive measures against

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these viruses through establishing regular testing (molecular and serological) and vaccination programs.

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Conflict of interest

The authors declare that they have no conflict of interest.

Contributions

Walid Azab and Sameh Bedair have contributed equally to this work by designing and carrying out the experiments, analyssing and interpreting the data. Azza Abdelgawad did peptide-specific ELISA, data analysis and helped with manuscript writing. Kathrin Eschke helped with PCR, sequencing and data analysis. Gemelat K. Farag helped with sample preparation and data analysis. Ali Abdel-Rahiem, Alex D. Greenwood, Nikolaus Osterrieder and Ahmed A. H. Ali contributed to the drafting of the manuscript, discussion and writing the manuscript.

Ethical approval

Sample collection was done according to the Ministry of Health and Population (General Administration of Medical Licenses; approval number 36490).

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Detailed descriptions of collected samples and presentation of molecular and serological results of each tested sample.

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