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Full Length Research Paper

Prevalence of equine herpes viruses 1, 2 and 4 in Arabian horse population in Egypt

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The Egyptian Arabian horse is one of the oldest and most popular horse breeds in the world. No previous efforts have been made for investigating the existence and prevalence of equine herpes viruses (EHVs) in this precious horse breed. In this report, ninety three clinical samples were collected from a cohort of Arabian horses located in Cairo, Egypt. Screening of the clinical samples for the presence of EHV antigens by cell-ELISA utilizing a polyclonal antibody pool against EHV-1, 2, and 4 identified 34 (36.56%) positive samples. Virus-specific semi-nested PCR assays were used for typing the positive samples. Three samples were found positive for EHV-1, seventeen for EHV-2, seven for EHV-4, one was a mixed infection of EHV-1 and EHV-4, and six did not produce any amplification signal with all assays. Sequence analysis of the amplified semi-nested PCR products of representative EHV strains further confirmed the virus identity. This study is the first that outlines the involvement of EHV infections in Arabian horse diseases worldwide. Besides, it presents new data about the prevalence of EHVs in the Egyptian horse population.

Key words: Arabian horses, Egypt, equine herpes viruses, prevalence.

INTRODUCTION

Equine herpes viruses (EHVs) are significant causes of serious illness and mortality in domestic horse population worldwide (Slater et al., 2006). Among the five EHVs recognized so far, three are classified as members of the subfamily *alphaherpesvirinae*; EHV-1 (equine abortion virus), EHV-3 (equine coital exanthema virus), and EHV-4 (rhinopneumonitis virus), while EHV-2 and EHV-5 belong to *gammaherpesvirinae* (Slater, 2007).

EHV-1 and EHV-4 are considered the most relevant pathogens both clinically and economically. Both viruses are primarily involved in respiratory tract infections of varying degrees, and establish long-live latent infections in the recovered horses. However, EHV-1 may progress to induce more severe manifestations in the form of abortion, peri-natal mortality and neurological disorders (Allen and Bryans, 1986; Studdert et al., 2003). EHV-2 and EHV-5 are ubiquitous and have been isolated from both healthy and diseased horses' worldwide (Borchers et al., 1997; Nordengrahn et al., 2002). Although EHV-2 was considered as a potential cause of different patho-logical disorders including upper respiratory tract disease, keratoconjunctiviris, fever and enlarged lymph nodes (Browning and Studdert, 1988; Sledge et al., 2006; Leon et al., 2007), its precise clinical impact and tissue tropism remains uncertain. However, some reports suggested that EHV-2 may play a predisposing and/or activating role for equine alphahepesviruses and bacterial pathogens (Welch et al., 1992; Nordengrahn et al., 1996).

A variety of detection methods has been utilized for diagnosis of EHV infections in clinical samples (OIE, 2008). Traditionally, the virus isolation in equine-derived cell culture was the standard diagnostic approach.

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Table 1. Definition of samples used in the study.

Disease form	Animal type/age	Sample type	Sample number		
			2005	2006	Total
Abortion	Mature females/over 2 years	Uterine swabs	6	9	15
	Neonatal foals/0-6 months	Tissues*	1	1	2
	Foeti	Tissues	3	0	3
Respiratory disease syndrome	Mature animals/over 2 years	Nasal swabs	17	0	17
	Growing foals /6 months-2 years	Nasal swabs	19	14	33
	Neonatal foals/0-6 months	Nasal swabs	9	14	23
Total			55	38	93

*Tissues included liver, lung, spleen, kidney and lymph nodes.

Further identification of the positive isolates was routinely achieved by immunofluorescence, immunoperoxidase or ELISA using type-specific monoclonal antibodies (Borchers et al., 1997). During the last two decades, several PCR-based assays were developed for detecting and typing EHVs (Welch et al., 1992; Kirisawa et al., 1993; Dynon et al., 2001; Galosi et al., 2001; Varrasso et al., 2001; Szeredi et al., 2003; Diallo et al., 2006; Elia et al., 2006). The results of such assays correlated well with virus isolation in terms of sensitivity and accuracy, but surpassed it in rapidity, simplicity, time saving and the independence on the presence of infectious virus in the clinical sample (Varrasso et al., 2001).

In Egypt, EHV infections did not receive much concern in literature. Since the early detection of the EHV-2 antibodies in Egyptian horse sera during a serological survey at 1965, no further efforts have been undertaken for isolation and/or identification of the circulating EHV-2 strains (Matumoto et al., 1965). EHV-1 was isolated in two separate occasions from aborted fetal organs on the chorioallantoic membrane of embryonated chicken eggs (Hassanien et al., 2002), and on BHK cell line (Warda, 2003). The later isolates were further identified using virus neutralization and immunodiffusion assays. A significant lack of information regarding the prevalent EHVs circulating among horse population in Egypt still exists and necessitates a series of comprehensive and objective studies.

The Arabian horse is a very unique breed of horses and is considered one of the most highly regarded horses in the world. As a result of its significant economic importance to the horse breeders in Egypt, it was the aim of this report to determine the occurrence of EHV infections in Arabian horses, and to identify the prevalent types using highly sensitive and accurate measures like cell ELISA, semi-nested PCR and DNA sequencing.

MATERIALS AND METHODS

Samples and reference viruses

Ninety-three samples were collected from a cohort of Arabian horses located in Cairo, Egypt, in the years 2005 and 2006 (Table 1). Clinical samples were transported in minimal essential medium (MEM) supplemented with 500 U/ml penicillin and 500 µg/ml streptomycin to the Virology Research laboratory, Faculty of Veterinary Medicine, Cairo University for processing, aliquoting, testing and storage at -80°C. Reference strains of EHV-1 (AIV), EHV-2 (Melissa), and EHV-4 (V01-3-13) were provided by the Institute of Virology, Berlin, Germany (Courtesy of Dr. H. Ludwig), for use as positive controls.

Cell ELISA

Detection of EHV antigens in clinical samples was conducted using cell ELISA as described by Dutta et al. (1983). Briefly, 96-well tissue culture plate was seeded with 100 µl of MEM supplemented with 10% fetal bovine serum (FBS), 0.25 µg/ml amphotericin-B, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, Mo, USA), and containing 2×10^4 equine dermal (ED) cells. The plate was incubated till cell confluence reached 70 to 90%. The clinical samples (100 µl; diluted 1:10 in MEM supplemented with 5x antimicrobials) were used for infection of individual wells. Reference viruses were used as positive controls, while MEM-only was used in negative control wells. After 1 h of incubation at 37°C (adsorption time), the plate was washed twice with MEM and then incubated in MEM containing 1% FBS for 72 h. The culture medium was removed and the cells were fixed using a chilled mixture of acetone, ethanol and methanol in a ratio of 12:5:3 for 10 min. Non-specific binding sites were saturated with 10% blocking buffer (non fat dry milk (NFDM) in PBS, pH 7.4) for 2 h at room temperature. After washing with PBS containing 0.05% Tween-20 (PBS-T), 100 µl of horse serum containing a mixture of polyclonal antibodies to EHV-1, -2 and -4 (kindly provided by H. Ludwig, Institute of Virology, Berlin, Germany, and diluted 1:100 in PBS-T) were added to the individual wells and incubated at 37°C for 1 h. Seco ndary antibody (alkaline phosphatase conjugated antihorse IgG, Sigma); diluted 1:3000 in PBS-T was added to the plate after three successive washing cycles. After incubation for 1 h, another washing repeats by PBS-T was conducted. The color was developed with the addition of para-nitrophenyl phosphate (Sigma) for 20 min. Further color development was stopped by NaOH (1N), and then the plate was read at 405 nm wavelength.

DNA extraction

Total DNA was extracted from 200 µl aliquots of processed samples using GF-1 Blood DNA Extraction Kit (Vivantis, Selangor

Table 2. Oligonucleotide primers for semi-nested PCR.

Target virus (gene)	Primer name	Role in PCR	Sequence (5`to 3`)	Position	PCR product	Reference
EHV-1 (gH)	EHV1-gH-F	First and second round PCR	AAG AGG AGC ACG TGT TGG AT	72972-72991[a]	First round: 634 bp	Varrasso et al., 2001
	EHV1-gH-R	First round PCR	TTG AAG GAC GAA TAG GAC GC	73586-73605[a]		
	EHV1-gH-RN	Second round PCR	AGT AGG TCA GGC CGA TGC TT	73237-73256[a]	Second round: 285 bp	
EHV-2 (gB)	EHV2-gB-F	First round PCR	CAG TGT CTG CCA AGT TGA TA	33717-33736[b]	First round: 442 bp	Dynon et al., 2001
	EHV2-gB-R	First and second round PCR	ATG GTC TCG ATG TCA AAC AC	34139-34158[b]		
	EHV2-gB-FN	Second round PCR	AGG ACT ACT ACT ATG TCA G	33996-34014[b]	Second round: 163 bp	
EHV-4 (gB)	EHV4-gB-F	First and second round PCR	CTG CTG TCA TTA TGC AGG GA	63539-63558[c]	First round: 507 bp	Varrasso et al., 2001
	EHV4-gB-R	First round PCR	CGT CTT CTC GAA GAC GGG TA	64026-64045[c]		
	EHV4-gB-RN	Second round PCR	CGC TAG TGT CAT CAT CGT CG	63840-63859[c]	Second round: 321 bp	

[a] Nucleotide positions based on the complete genome sequence of Equine herpesvirus 1 strain Ab4, with GenBank accession number AY665713.1; [b]Nucleotide positions based on the complete genome sequence of Equid herpesvirus 2, with GenBank accession number U20824.1; [c] Nucleotide positions based on the complete genome sequence of Equine herpesvirus 4 strain NS80567, with GenBank accession number AF030027.1.

DE, Malaysia) according to the manufacturer's protocol. Purified DNA was recovered in 100 µl elution buffer and stored at -20°C for further testing.

Semi-nested PCR

The primers used for detection of EHV-1, 2 and 4 by semi-nested PCR were previously described by Varrasso et al. (2001) and Dynon et al. (2001) to amplify highly conserved sequences of the virus genome (Table 2). These primers were analyzed by Oligo Design and Analysis Tools (Integrated DNA Technologies, Coralville, USA) and synthesized by Metabion International AG, Martinsried, Germany. First round amplification of each virus was performed in a reaction mixture contained 12.5 µl of 2× ReddyMix^{TM} PCR Master Mix (ABgene, Epsom, Surrey, UK), 0.5 µM (1.25 µl) of each corresponding first round PCR primer, 5 µl of the DNA extract and 5 µl of PCR grade water (Promega, Madison, WI, USA). Thermocycling conditions for the first round amplification were initial denaturation at 95°C for 5 min followed by 35 cycle s of 95°C for 30 s, 60°C (50°C for EHV-2) for 35 s and 7 2°C for 1 min and a final extension step at 72°C for 7 min. For the second round amplification, 2 µl of the first round PCR reaction products were added to a reaction mixture contained 12.5 µl of 2× ReddyMix ^{TM} PCR Master Mix, 0.5 µM (1.25 µl) of each corresponding second round PCR primer and 8 µl PCR grade water. Target DNA sequences were amplified using the same amplification protocol described for the first round PCR. Amplification products (10 µl) were observed in 1.2% agarose gel stained with ethidium bromide, in comparison with 100 bp DNA ladder (ABgene, Epsom, Surrey, UK). Gel images were documented using an image analysis system (IMAGO Compact Imaging System, B&L, USA).

DNA sequencing

The semi-nested PCR products of selected EHV-1, 2 and 4 positive samples were excised from agarose gel and purified by Montáge DNA gel extraction kit (Millipo re, Concord Road Billerica, MA, USA). The nucleotide sequence of the purified fragments was determined using an automated DNA sequencer (Applied Biosystem Inc., Foster City, CA, USA). Sequence result analysis was conducted using BLAST web interface (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Lasergene software, version 3.18 (DNASTAR, Madison, Wis.).

RESULTS

Detection of EHV antigens in clinical samples

The presence of EHV antigens in the obtained clinical samples was first identified by cell ELISA using a polyclonal antibody pool against the most prevalent EHV types 1, 2 and 4. Out of 93 samples, a total of 34 (36.56%) positive samples were recognized. The frequency of detection was extremely similar during the two years of the study period, where it was 20/55 (36.36%) in 2005 and 14/38 (36.84%) in 2006. The ratio of positive samples in the group of aborted mares/foeti was quite higher (12/20; 60%), if it is compared with those suffered from respiratory diseases (22/73; 31.5%).

PCR typing of EHVs in ELISA-positive samples

Confirmation of the virus existence in cell ELISA

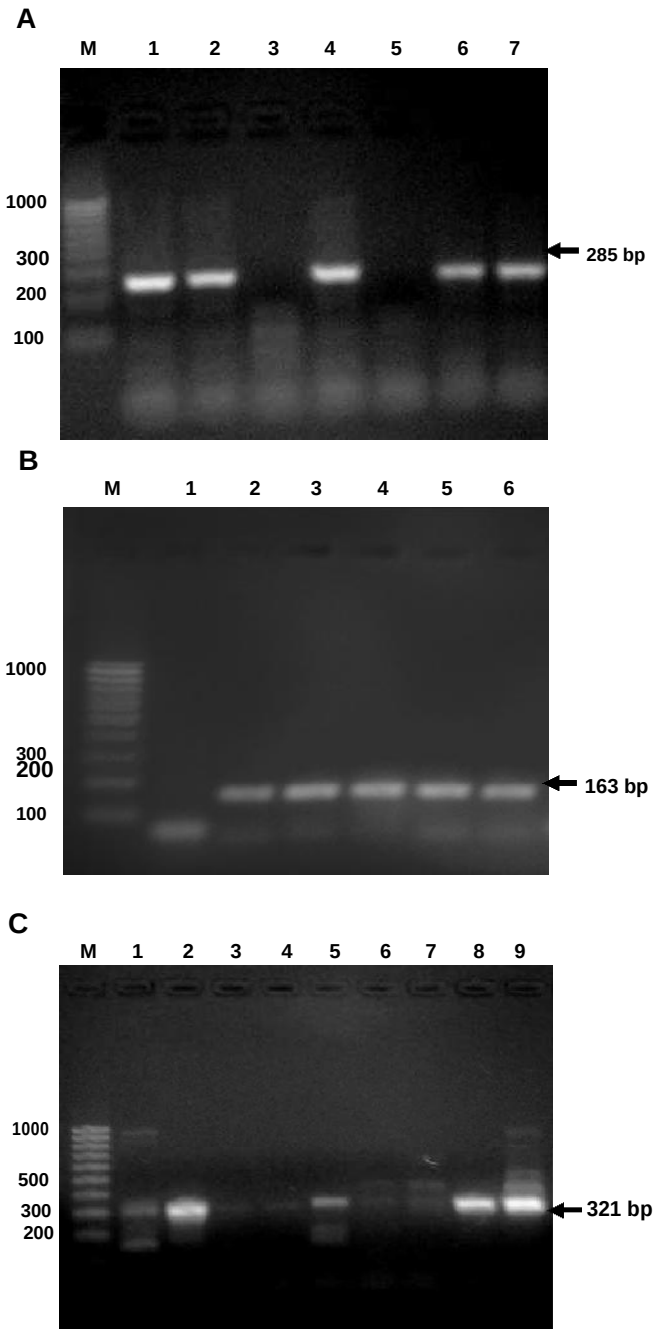

Figure 1. Identification of EHVs in selected clinical samples using semi-nested PCR assays; A) PCR products of EHV-1 positive samples (lanes 1,2,4,6), negative samples (lanes 3,5) and positive control (lane 7); B) PCR products of EHV-2 positive samples (lanes 2-5), negative samples (lane 1) and positive control (lane 6); C)PCR products of EHV-4 high positive samples (lanes 1,2,5,8), low positive samples (lanes 3,4,6,7) and positive control (lane 9). Lane M represents 100 bp DNA molecular weight ladder (ABgene).

positive samples as well as typing of the implicating virus were further executed through separate sets of semi-nested PCR targeting EHV-1, -2 and -4 DNA. While 28 samples produced positive signals of amplification against one or another of the tested viruses, 6 samples were found to be absolutely negative (Figure 1). The positive samples were distributed as follow: 3 EHV-1, 17 EHV-2, 7 EHV-4 and 1 mixed EHV-1/EHV-4. All the EHV-1 positive samples either alone or in combination with EHV-4 were uterine swabs collected in 2006 from mares with recent abortions. EHV-2 was the predominant virus type detected in the nasal swabs (16/19; 84.2%). The frequency of EHV-2 detection in nasal swabs at 2005 and 2006 was considerably relevant (83.3 and 85.7%, respectively). Interestingly, a uterine swab sample (ZH-5U) was only positive for EHV-2. The prevalence of EHV-4 was higher at 2005, where it constituted 6 out of 17 positive samples (35.3%). On the contrary, only one sample (out of 11; 9%) was identified as EHV-4 at 2006. EHV-4 was detected in all kinds of test samples including uterine and nasal swabs as well as tissues of the aborted foeti (Table 3).

Sequence analysis

The identity of EHV-1, -2 and -4 was further verified by sequence analysis of selected positive samples (ZH-84, ZH-82 and ZH-21, respectively). DNA sequencing targeted the semi-nested PCR products using the corresponding forward primers. The nucleotide sequence data was submitted to the GenBank and have obtained the accession numbers: GU737540 for sample ZH-84, GU737539 for sample ZH-82, and HQ191437 for sample ZH-21. Multiple sequence alignment of each EHV with the respective international strains available at the GenBank, using Megalign program of the Lasergene software, showed a high degree of sequence homology that ranged from 97.15 to 100%. For EHV-1 (ZH-84), two nucleotide mismatches were recognized at positions 73,035 (C/A) and 73,200 (A/T) of the virus genome. Four mismatches were identified at positions 33,901 (C/G), 33,902 (T/C), 33,903 (C/A) and 33,950 (A/G) of the EHV-2 (ZH-82) genome sequence. No sequence heterogeneity was recognized for EHV-4 (ZH-21).

DISCUSSION

Equine herpes viruses have a major economic and welfare impact on all sectors of horse industry worldwide. They are incriminated in several disease forms; including respiratory symptoms, abortion and hind limb paralysis, that greatly affect the breeding and competition ability of horses (Slater, 2007). Although the Arabian horse is considered one of the most proficient and valuable horse breeds in sport racings and tolerance competitions, no data was generated regarding the role of EHV infections in disease status. Moreover, there is a little information about the previous and current situation of EHVs in Egypt in terms of prevalence, circulating types and risk factors.

In this study, an epidemiological screening of EHVs

Table 3. Results of semi-nested PCR for EHV typing.

Strain/sample	Sample type	Collection season	Semi-nested PCR for		
			EHV-1	EHV-2	EHV-4
EHV-1 (AIV)[a]			+	NT	NT
EHV-2 (Melissa)[a]			NT	+	NT
EHV-4 (V01-3-13)[a]			NT	NT	+
ZH-5U	Uterine swab	2005	-	+	-
ZH-5F	Tissues	2005	-	-	+
ZH-6	Nasal swab	2005	-	+	-
ZH-9	Nasal swab	2005	-	+	-
ZH-10	Nasal swab	2005	-	+	-
ZH-11	Nasal swab	2005	-	+	-
ZH-12	Nasal swab	2005	-	-	-
ZH-16	Nasal swab	2005	-	+	-
ZH-18	Nasal swab	2005	-	+	-
ZH-21	Nasal swab	2005	-	-	+
ZH-26	Nasal swab	2005	-	-	+
ZH-27	Nasal swab	2005	-	+	-
ZH-28	Nasal swab	2005	-	+	-
ZH-29	Nasal swab	2005	-	-	-
ZH-33U	Uterine swab	2005	-	-	+
ZH-34	Uterine swab	2005	-	-	-
ZH-36	Uterine swab	2005	-	-	+
ZH-37U	Uterine swab	2005	-	-	+
ZH-38	Nasal swab	2005	-	+	-
ZH-41	Nasal swab	2005	-	+	-
ZH-44	Nasal swab	2006	-	+	-
ZH-45	Nasal swab	2006	-	+	-
ZH-53	Nasal swab	2006	-	-	-
ZH-65	Nasal swab	2006	-	-	+
ZH-72	Nasal swab	2006	-	+	-
ZH-81	Nasal swab	2006	-	+	-
ZH-82	Nasal swab	2006	-	+	-
ZH-83	Nasal swab	2006	-	+	-
ZH-84	Uterine swab	2006	+	-	-
ZH-85	Uterine swab	2006	+	-	-
ZH-86	Uterine swab	2006	-	-	-
ZH-87	Uterine swab	2006	+	-	-
ZH-88	Uterine swab	2006	-	-	-
BD-93	Uterine swab	2006	+	-	+

NT: not tested; [a] reference strains.

was conducted in one of the largest cohorts of Arabian horses in Egypt. The survey was continued for a period of 2 years (2005 to 2006) utilizing samples from all suspected cases either those showing signs of respiratory disease and/or abortion. As a rapid monitoring of the EHV-positive samples, cell ELISA was performed using ED cell line and a mixed panel of polyclonal antibodies against EHV-1, -2, and -4. Such assay combines the advantages of virus propagation in cell culture with the rapid and specific identification of the viral antigen simultaneously. Moreover, it enables a highly sensitive diagnosis of different EHV types in a large number of samples economically. Typing of the positive samples using semi-nested PCR targeting each of EHV-1, -2 and -4 further confirmed the reliability of cell ELISA for primary screening. However, it identified six samples of false positive results, which may be attributed to the lower specificity of cell ELISA in accordance to the semi-nested PCR (Figure 1, Table 3).

Analysis of the results obtained from cases with

respiratory disease outlines that EHV-2 is the most prevalent type detected, with an overall ratio of 21.9%. This frequency appears relatively constant during the study period. EHV-2 is known to be endemic in all horse populations everywhere, and could be isolated from both healthy and diseased animals (Borchers et al., 1997; Nordengrahn et al., 2002). However, the prevalence rate determined in this study appears low compared to figures in other countries (Reubel et al., 1995; Nordengrahn et al., 2002). The potential impact of EHV-2 in the respire-tory disease symptoms of sampled horses is a matter of debate since its pathogenic role is not clear enough to address such conclusion. Nevertheless, it may be satisfactory to incriminate the virus as a predisposing factor for secondary bacterial infections of possible implication in the disease syndrome (Nordengrahn et al., 1996). Although EHV-1 is endemic in horses worldwide and is responsible for frequent peaks of respiratory disease, it was not identified in any of the test samples during the screening period. On the other hand, EHV-4 was detected in a limited number of samples in both years and in different age groups. These data all together suggest that EHVs, albeit exist, are not the principle cause of respiratory disease in the Egyptian Arabian horses during the study period.

The important role of EHV-1 in equine abortion is well-documented throughout the world (van Maanen. 2002; Smith et al., 2003), whereas the involvement of EHV-4 is less frequent and usually neglected during the routine diagnostic procedures (Ostlund, 1993; O'Keefe et al., 1995). Both viruses can cause lifelong latent infection, with periodic reactivation and spread in the endemic population (Allen and Bryans, 1986). In our report, the two viruses were identified in samples collected from aborted mares and foeti. However, nearly all the samples obtained in 2005 were positive to EHV-4 and all the samples of 2006 were positive to EHV-1 with only one EHV-1/4 mixed sample. Whether this alternative virus circulation constitutes a constant pattern or it is just a result of inadvertent reactivation of latent viruses, this requires extending the screening process on spatial and temporal basis. Moreover, the presence of a mixed uterine sample (BD-93) containing both viruses is a unique observation that were not recorded before except for respiratory samples (Maeda et al., 2004; Ataseven et al., 2009).

More exciting, EHV-2 was detected in a uterine swab sample collected from an aborted mare in 2005 (ZH-5U). Two previous studies reported the identification of few EHV-2 positive samples in tissues collected from aborted foeti and still birth (Galosi et al., 2005; Leon et al., 2007). However, analysis of the tissue samples collected from the aborted foetus of this particular mare (ZH-5F) showed the presence of EHV-4 but not EHV-2. Such observation may incriminate EHV-4 as the primary cause of abortion in such case, while the presence of EHV-2 in the uterine sample of mother can be considered as a potential
sample contamination.

In conclusion, the present report is the first that presents epidemiological data describing the prevalence of EHV-1, -2, and -4 in the Arabian horses worldwide. Moreover, it extends the scanty information regarding the existence of EHVs in the Egyptian horse population. Further objective and comprehensive studies that track EHVs in different horse populations in Egypt need to be addressed to unveil the unclear image, and to justify their precise role in disease state of horses in Egypt.

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