Original Article

Molecular characterization of equine herpesvirus-1 and asinine herpesvirus -5 isolated from aborted fetuses of arabian horses

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Abstract

Infections with equine herpesviruses (EHVs) are widespread in equine populations worldwide. Whilst EHV-1 produces well-documented reproductive losses in equids, the role of asinine herpesviruses-5 (AHV-5) to induce abortion is still enigmatic. In the present study, EHV-1 and AHV-5 were detected in aborted fetuses of Arabian horses in Egypt by consensus nested PCR of DNA polymerase gene, with a trial for viral isolation on Rabbit Kidney 13 cell line (RK-13) and identification by indirect immunofluorescence. The partial analysis of DNA polymerase gene sequence revealed that the Egyptian strains EHV-1 and AHV-5 were related to Australian origin EHV-1 838/89 (Accession. no, KF434370) and Austrian strain AHV-5 isolate 1323 (Accession. no, KC825357) by 98.3% and 98.6 % respectively. In conclusion, this was the first detection and isolation of AHV-5 in Egypt, and to our knowledge, this represents the first report of AHV-1 detection from an aborted fetus worldwide.

Keywords: Consensus nested PCR, EHV-1, AHV-5, phylogeny, Egypt

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Introduction

Herpesvirus infections are endemic in equine populations worldwide, including in horses, mules and donkeys. The horse is the natural host of alphaherpesviruses; EHV-1, EHV-3 and EHV-4 and gammaherpesvirus; EHV-2 and EHV-5 (Patel and Heldens, 2005), while the donkey hosts alphaherpesviruses; AHV-1 (EHV-6) and AHV-3 (EHV-8) and gammaherpesviruses; AHV-2 (EHV-7) (Davison *et al*., 2009), in addition to the three unclassified gammaherpesviruses AHV-4, AHV-5 and AHV-6 which have been identified in donkeys (Kleiboeker *et al*., 2002).

In the world, outbreaks of EHVs infection have aroused public interest and EHVs were tagged as an emerging threat. Late abortions in mares with more than 50 % foal losses have been associated with EHV-1 infection (Mumford *et al*., 1987 and Van Maanen *et al*., 2000). In Egypt, EHVs have caused considerable concern in the past years as they are responsible for huge economic loss among Arabian horses (Abd El Hafeiz *et al*., 2010, Amer *et al*., 2011 and Azab *et al*., 2019).

Limited information is accessible on AHVs in horses as the first isolation of AHV-1 from a donkey was from vesicular and erosive lesions of the muzzle of a foal and the external genitalia and udder of its dam (Burrows, 1973). AHV-2 and AHV-3 were isolated from the blood of an apparently healthy donkey and pharyngeal cavity of immunosuppressed donkeys respectively (Browning *et al*., 1988). Moreover, AHV-4, AHV-5, and AHV-6 were associated with interstitial pneumonia in donkeys, characterized clinically with acute and often fatal respiratory disease (Kleiboeker *et al*., 2002). To our knowledge, AHV-5 has not been detected in abortion cases of horses or donkeys until now.

Several PCR assays have been developed for detecting and typing of EHVs in clinical samples (Lawrence *et al*., 1994; Diallo *et al*., 2008 and Wang *et al*., 2007). The results of these assays match well with virus isolation in terms of sensitivity and accuracy but PCR outweighs virus isolation in simplicity, rapidity, time saving and independence from the presence of infectious virus particles in the sample (Varrasso *et al*., 2001). From previous studies, the consensus nested PCR assay has permitted the recognition of all herpesviruses from different subfamilies (VanDevanter *et al*., 1996 and León *et al*., 2008). In the current study, we describe detection of EHV-1 and AHV-5 from aborted fetuses of Arabian horses in Egypt, as well as the genetic characterization of viral isolates obtained from PCR-positive samples.

Materials and Methods

Virological specimens: Twenty aborted fetuses in the third trimester of pregnancy were collected from an Arabian horse stud in Egypt during the years 2014, 2015 and 2016. From each fetus, the spleen, lungs, liver and placenta were collected for laboratory diagnosis. Pooled tissue homogenates 10% (w/v) were prepared in Hank's balanced salt solution minimal essential media (MEM) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B and then centrifuged at 1200 xg for 10 minutes and the supernatant was collected for further analysis (OIE, 2015).

DNA extraction: DNA was extracted from the prepared samples using the phenol-chloroform method (Steward and Culley, 2010). Briefly, 400 µl of sample suspension was incubated overnight at 56ºC in 200 µl of DNA extraction buffer (5M guanidinium thiocyanate, 50 mM KCL, 10 mM Tris HCL, 0.5 ml Tween 20, 10 µl Proteinase K 20 mg/ml, and 10% Sodium dodecyl sulfate), then phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) were subsequently used for DNA extraction and purification. Precipitation with absolute ethanol at -20ºC was performed then DNA was pelleted at 10,000 g for 30 mins at 4ºC, rinsed with 70% ethanol, air dried, and suspended in 30 µl of 10 mM Tris HCl–1 mM EDTA (pH 8.0; TE buffer).

Detection of Pan herpesvirus by consensus nested PCR: The assay was employed as described by León *et al*., (2008) with some modifications. Briefly, a nested format was used, in which primary and nested PCRs were performed with degenerate PCR primers targeting a highly conserved region coding for DNA polymerase genes (Table 1).

PCR nuclease free water was used as a negative control and killed virus vaccine (Fluvac Innovator® EHV4/1, Zoetis) as a positive control. The amplified products were analyzed by electrophoresis (1.5% agarose gel) and their size estimated by comparison with molecular weight markers run in parallel.

Table 1 Oligonucleotides degenerate primers and PCR conditions of consensus nested PCR for pan EHVs

Target Gene	Primer name	Role in PCR	Sequence (5`to 3`)	PCR cycle	PCR product
DNA Polymerase Gene	DFA-F	First round PCR	GAYTTYGCNAGYYTNTAYCC	95˚C 5 mins 95˚C 1min 46˚ C 1 min 72˚C 1 min 45 cycles 72˚C 10 mins	215-315 bp
	ILK-F	First round PCR	TCCTGGACAAGCAGCARNYSGCNMTNAA		
	KG1-R	First round PCR	GTCTTGCTCACCAGNTCNACNCCYTT		
	TGV-F	Second round PCR	TGTAACTCGGTGTAYGGNTTYACNGGNGT		
	IYG-R	Second round PCR	CACAGAGTCCGTRTCNCCRTADAT		

F: Forward primer and R: reverse primer

Isolation of EHV: Positive-PCR samples were propagated in monolayers of continuous RK-13 cells grown in 25 cm^2 cell culture flasks according to OIE (2008). The cells were grown in Eagle's minimum essential medium (MEM) with Earl's salts supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 mg/ml streptomycin using standard tissue culture propagation methods. About 0.5 ml of the prepared sample was inoculated then the flask was gently rotated and incubated in 5% Co_2 incubator at 37˚C for 1.5 hours with gentle shaking every 15 minutes.

The inoculum was discarded, and the maintenance medium (MEM + 2 % FCS) added to each flask. Un-inoculated monolayers were used as negative cell controls. Subsequently, all flasks were incubated in 5% Co2 incubator at 37˚C and examined daily by inverted microscope for the presence of EHV specific cytopathic effect (CPE). After that, the inoculated flasks were subjected to three cycles of freezing and thawing and the same sample was passaged for three successive blind passages in the same way.

Identification of viral antigens using indirect immunofluorescence: The inoculated cell culture was tested for the presence of EHV Ag by indirect immunofluorescence using specific reference antiserum against EHV-1 (USDA, APHIS, VS, NVSL), and goat anti porcine IgG conjugated with fluorescent isothiocyanate (KPL, USA) and examined by fluorescent microscope (Gunn, 1992).

Sequencing and phylogenetic analysis: Sequencing was performed on secondary PCR products of the nested PCR. The products were purified by QIAquick® PCR Purification Kit. Sequencing of the viral genomic DNA template was performed according to Sanger dideoxynucleotide-sequencing technology.

Our sequences were aligned with the published sequences on Genbank using Bioedit Sequence Alignment Editor (Version 7.25.0) after making analysis by NCBI/Blast. A phylogenetic tree was performed using the Neighbor-joining method and the reliability of each tree branch was estimated by performing 1,000 bootstrap replicates, in the MEGA 7 software (Kumar *et al*., 2016).

Results

Detection of pan herpesvirus by consensus nested PCR: A band of the expected size (215 – 315 bp) was present in 4/20 samples tested by consensus nested PCR (Fig. 1).

Isolation of EHV: The four pan positive samples produced CPE within 7 days of the first passage and after 4 days in the third passage. The CPE was characterized by rapidly enlarging clusters, cell rounding, cell refractile and partial sheet detachment (Fig. 2).

Identification of viral antigens using indirect immunofluorescence: Three out of 4 viral isolates grown in RK-13 cells emitted perinuclear and intracytoplasmic green fluorescence granules 24 hours post inoculation using reference EHV-1 antiserum. The remaining one was negative by immunofluorescence with EHV-1 antiserum (Fig. 3)

Sequencing and phylogenetic analysis: BLAST analysis of the amplicons obtained from the PCR analysis of two viral isolates (one immunofluorescence positive and one immunofluorescence negative for EHV-1) confirmed that the first one was the most closely related to EHV-1 (designated EHV-1 Egypt 2014) whilst the second one was the most closely related to AHV-5 (designated AHV-5 Egypt 2015). The nucleotide sequences were submitted to the GenBank with accession numbers MF599337 and KY421047 respectively.

Multiple sequence alignments and phylogenetic analysis showed that EHV-1 Egypt 2014 clustered with herpesviruses from other countries, including sequences from Australia (98.3% similarity with KF434373 and KF434370) and France (98.3% similarity with KC924775), whereas AHV-5 Egypt 2015 clustered with AHV-5 from Austria (98.6% similarity with KC825357) and Canada (97.7% similarity with FJ798319). Additionally, the AHV-5 strain had somewhat lower identity 90% or less with other herpesvirus strains (Equine herpesvirus-2, Equine herpesvirus-5, Equine herpesvirus -9, Zebra herpesvirus and Wild ass herpesvirus) from different countries (Fig. 4).

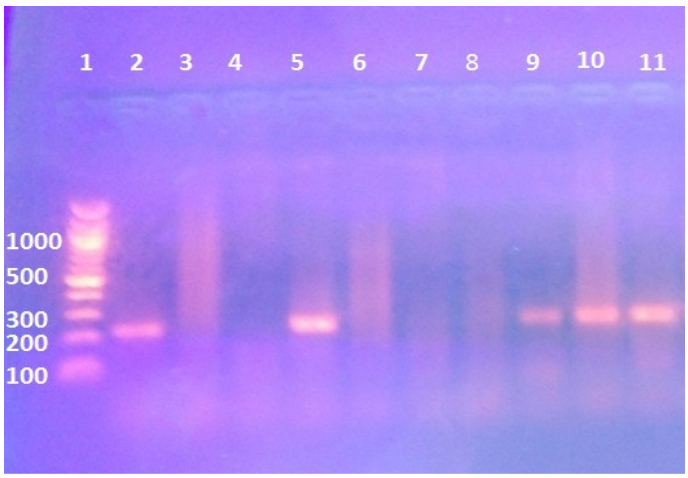

Figure 1 Electrophoresis pattern of consensus nested PCR for pan herpesviruses. Lane 1 (100 bp DNA marker), lane 2 (positive control), lane 3 (negative control), and lane 5, 9, 10 and 11 (positive samples), lane 4, 6, 7, 8 (negative samples).

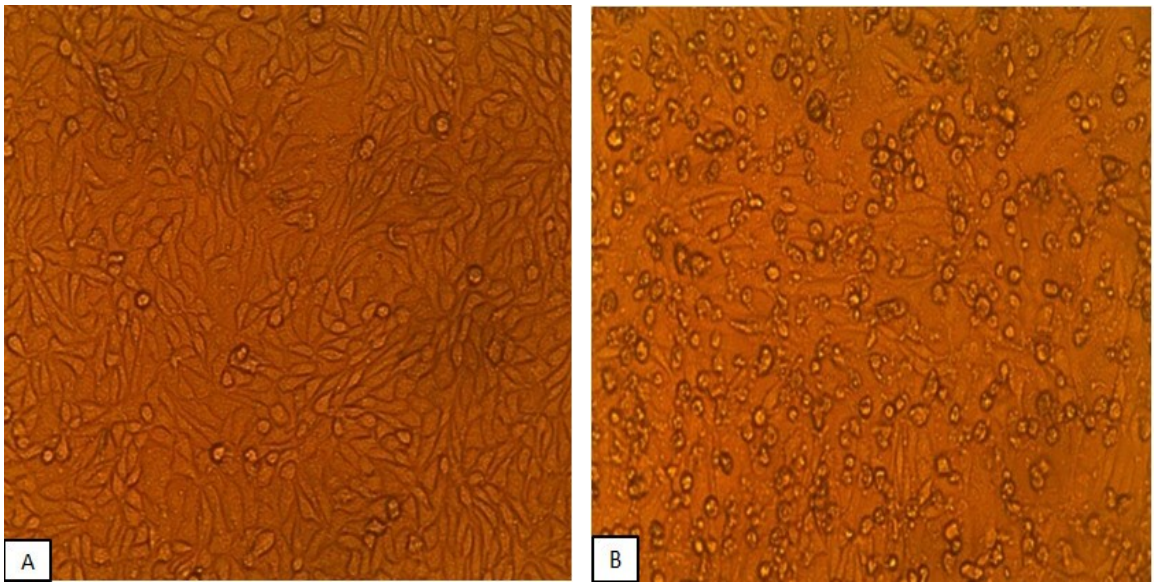

Figure 2 Characteristic CPE of suspected EHV isolates on RK-13 cells showing rapidly enlarging clusters, cell rounding, cell refractile and partially sheet detachment (B) when compared to control non-infected complete sheet of RK-13 cells (A) (low power 10 X).

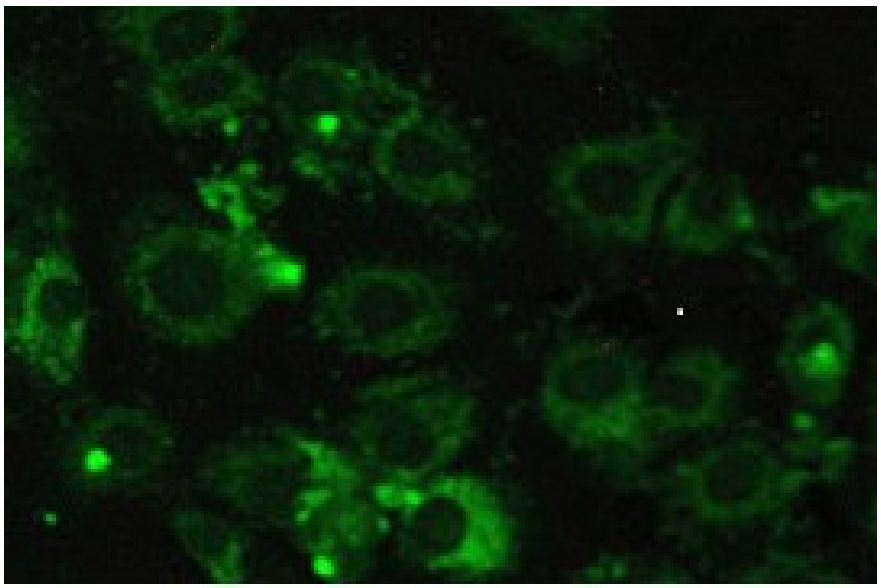

Figure 3 Stained infected RK-13 cells with fluorescein isothiocyanate after 24 hours post inoculation. Notice perinuclear and intracytoplasmic apple green fluorescence emission as a positive result for EHV isolates (Magnification power, 400X).

Discussion

The data presented in the current study reports the first isolation of AHV-5 from a third trimester-aborted equine fetus in Egypt, however, AHV-5 has been previously reported in some countries in the world including; Canada (Vengust *et al*., 2008), France (Fortier *et al*., 2009), Sweden ((Back *et al*., 2012), USA (Kleiboeker *et al*., 2002 and 2004, De Witte *et al*., 2012) and Austria (Rushton *et al*., 2014).

The clinical implications of AHV-5 in either the donkey or the horse have not been fully established. The virus was detected from donkeys with fatal interstitial pneumonia (Kleiboeker *et al*., 2002), neurological disease (Vengust *et al*., 2008) and also from clinically normal donkeys (Browning *et al*., 1998). Similarly, AHV-5 was detected from horses with pyogranulomatous pneumonia and multinodular pulmonary fibrosis (Schwarz *et al*., 2013) but also from healthy horses (De Witte *et al*., 2012 and Fortier *et al*., 2009). While detection of AHV-5 from a single abortion case does not imply causation, it does suggest that the clinical implications of infection with this virus need further investigation.

The AHV-5 sequence obtained from the aborted equine fetus was genetically similar to AHV-5 sequences from donkeys and horses in other countries. In fact, limited sequence data is available on AHV-5 and the longest nucleotide sequence published in Genbank consists of only 881 nucleotides. Due to the high genomic heterogeneity of equid gammaherpesviruses, mistakes in AHV-5 classification are possible. It has been suggested that AHV-5 is a variant of EHV-5 or EHV-2 (Rushton *et al*., 2014). Therefore, AHV-5 has been given its discrete nomenclature based on differences in the given short genetic sequence (Bell *et al*., 2008; Fortier *et al*., 2009). Until now, we cannot determine the exact natural host

of AHV-5 as the virus has been detected in both donkeys and horses (with or without a history of previous contact with donkeys) (De Witte *et al*., 2012 and Rushton *et al*., 2014).

Detection of EHV-1 from four of the aborted fetuses was expected as the role of EHV-1 in equine abortion is well documented through the world (van Maanen (2000), Léon *et al*., (2008)). Furthermore, In Egypt, Abd El Hafeiz *et al*., (2010), Amer *et al*., (2011) and Azab *et al*., (2019) also indicated that EHV-1 is the most predominant viral cause of abortion in mares and results in major financial loss to the horse-breeding industry.

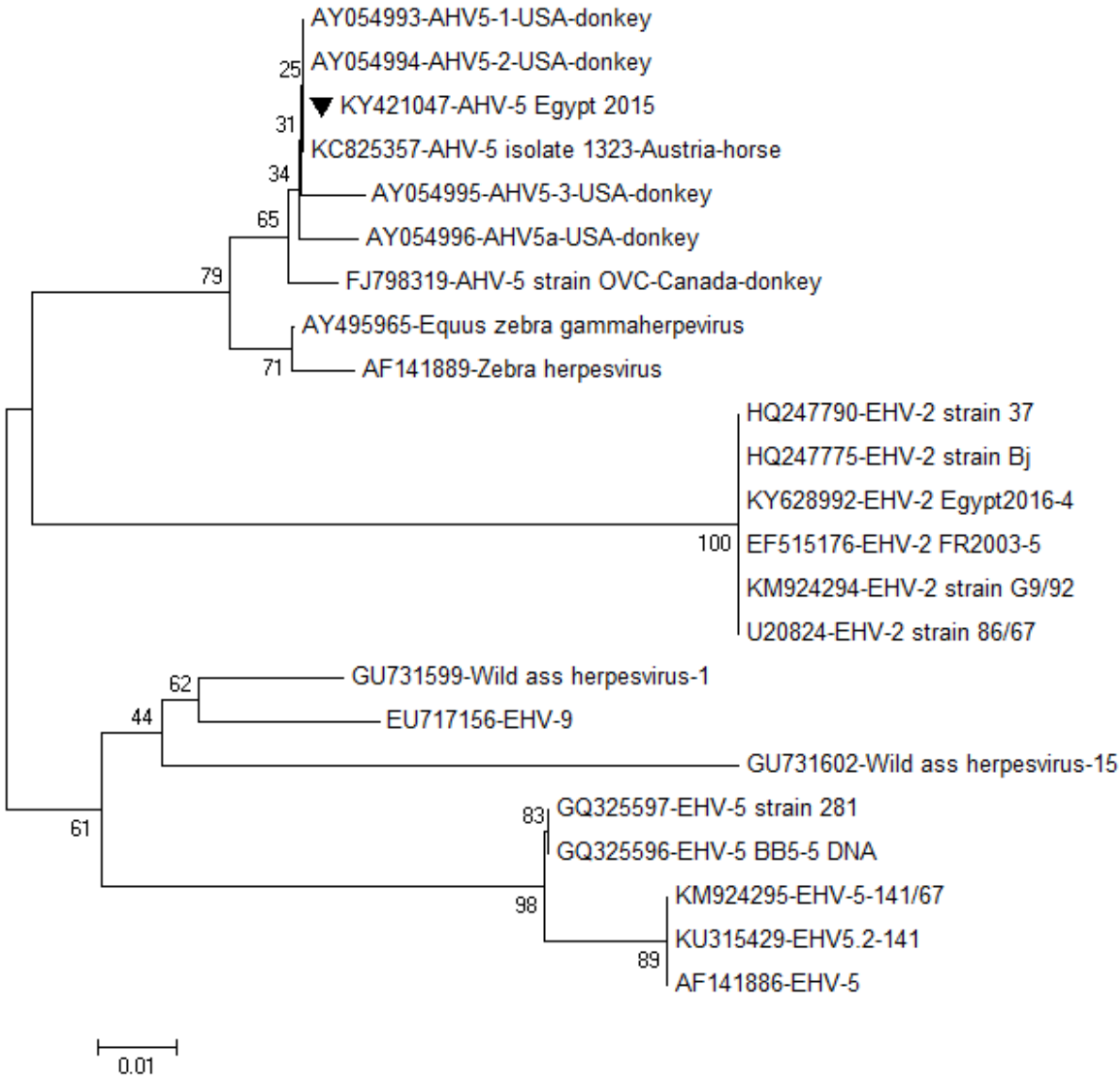

Figure 4 Phylogenetic tree for DNA polymerase gene partial nucleotide sequence of AHV-5 Egypt 2015 strain (Acc. no KY421047) (black triangular) compared with other AHV-5 reference strains obtained from GenBank database. The scale bar represents the number of substitutions per nucleotide.

We have successfully used consensus herpesvirus primers for detection of two different viruses from clinical samples by amplification of a region of DNA polymerase gene in herpes viral genomes (VanDevanter *et al*., 1996). Whilst the DNA polymerase gene is conserved among different herpesviruses, the phylogenetic analysis of DNA polymerase has not provided much information except for confirmation that the isolates were EHV-1 and AHV-5, respectively. Other authors have proposed that single-nucleotide polymorphisms observed in the region spanning approximately 600 bp of ORF68 may provide a molecular marker for tracking the geographical origin of EHV-1 (Nugent *et al*., 2006), although the utility of ORF68 for this purpose has been subsequently challenged by some authors (Malik *et al*., 2012 and Stasiak *et al*., 2017).

The limitations of this study include lack of histopathology, which gives valuable information about the pathogenesis of the virus in the host, due to the unavailability of samples. Moreover, there was no capability to follow-up the aborted mares and investigate other causes of abortions apart from EHVs.

Nevertheless, more intensification to imply the first report of AHV-5 in Egypt through an epidemiological survey and further studies is required. AHV-5 should be considered in investigations of equine abortion cases to better understand the association between this virus and fetal loss in horses and other equids.

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