Molecular Detection and Phylogenetic Analysis of Equine Herpes Virus-1 in Horses with History or Clinical Signs in Four Provinces of Iran

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Abstract

BACKGROUND: Equine herpes virus-1 (EHV-1) is a major cause of economic loss in horse industry and is well recognized as a cause of abortion, respiratory disease, neurologic disorders and death of neonatal foals.

OBJECTIVES: The aim of this study was to evaluate the frequency of EHV-1 in horses with clinical signs and/or history associated with this virus from four provinces of Iran (Golestan, Tehran, Khuzestan, West Azerbaijan) that have considerable horse population, followed by phylogenetic study of positive cases and compare them with herpes viruses in other parts of the world.

METHODS: Blood samples and nasal swabs were taken from 150 horses from four aforementioned provinces. DNA of samples was extracted and used for detection in real-time PCR TaqMan assay. Finally, phylogenetic trees were designed based on neighbor joining method.

RESULTS: Out of 150 sampled horses, a total of 14 (9.33%) were found to be positive for EHV-1. The results indicated that positive cases of EHV-1 from this study were clustered to herpes virus cases in other parts of the world with a noticeable similarity.

CONCLUSIONS: This study confirmed the presence of EHV-1 in these provinces of Iran, thus consideration should be given to preventive and control programs to prevent dissemination and outbreak of this virus.

KEYWORDS: Equine herpes virus-1, Horse, Molecular detection, Phylogenetic analysis

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How to Cite This Article
Introduction


The biologic source of such virus originates from a primary lytic infection or the shedding of virus after reactivation of a latent state (Pusterla, et al. 2009; Ma, et al. 2013; Jhonstone, et al. 2016; Laabassi, et al. 2017). Viral latency and reactivation are important features of EHV-1 epidemiology; this virus can establish latent infection in the host, then intermittent recrudescent and viral shedding from asymptomatic carrier to susceptible horses (Ataseven, et al. 2009; Dami ani, et al. 2014; Sarkar, et al. 2015; Sarkar, et al. 2016; Oladunni, et al. 2018). Therefore, different techniques have been used to detect and differentiate EHV-1 and there is a clear need for a specific and sensitive technique that allows the rapid diagnosis of clinical disease, as well as surveillance of susceptible populations (Hussey, et al. 2006).

Real-time PCR is a flexible, rapid, sensitive, specific and quantitative method for diagnosis and surveillance of herpes viral diseases (Hussey, et al. 2006; Diallo, et al. 2007; Yilmaz, et al. 2012). Although there are some reports of EHV-1 infection in Iran, this study indicated the presence of EHV-1 in horses with signs associated with this virus. Based on the authors’ findings, this is the first study using real-time PCR TaqMan assay on nasal swab and blood samples of horses with clinical signs or clinical history associated with this virus and phylogenetic study in Iran.

This study focuses on estimating the frequency of EHV-1 in nasal swab and blood samples of horses from four provinces of Iran using real-time PCR TaqMan assay and phylogenetic study of positive samples.

Materials and Methods

Samples and sample preparation

Blood samples and nasal swabs were taken from 150 horses (from different sex, breed and age) with clinical signs or history associated with this virus, including respiratory signs (fever, coughing and nasal discharge), neurologic signs (depression, ataxia, recumbency and urinary incontinence) and abortion from different studs of four provinces of Iran that contain large horse population. Provinces include Golestan (north of Iran), Tehran (north of Iran), Khuzestan (south of Iran) and West Azerbaijan (northern west of Iran). The experimental procedures have been carried out in accordance with The Code of EU Directive 2010/63/EU for animal experiments.

Number of horses has been sampled from each province: Golestan: 47, Khuzestan: 45, Tehran: 37 and West Azerbaijan: 21. Sampling was done between December 2015 and December 2016. Five milliliters of blood were collected from jugular vein in EDTA tubes. Nasal swabs were taken from both nostrils then transferred to 1ml virus trans-
port media containing PBS, penicillin 800 IU ml-1, streptomycin 800 µg ml-1 and 0.1% w/v fetal calf serum (OIE, 2015). Samples were immediately transported on ice to Laboratory of Virology in Faculty of Veterinary Medicine at University of Tehran.

The blood samples were centrifuged at 1500 g for 10 min, the buffy coat fraction was removed and stored at -70 °C. Nasal swabs with their transport media was stored at -70 °C (3). DNA was extracted from 100µl of each whole blood and nasal swab samples using DNA extraction kit (MBST, Iran) by the protocol described by the manufacturer. The quality of extracted DNA was confirmed by the agarose gel electrophoresis.

Reference strain used in this study was purified DNA of EHV-1 strain 89c25 (Kawakami, et al. 1962).

**Primers and probes**

EHV-1 specific real-time PCR (TaqMan assay) was performed on extracted DNA, isolated from buffy coat fraction and nasal swab solution. The glycoprotein B of Alpha Herpesvirinae was a conserved region and was selected because it contained highly specific sequence that could allow discrimination between the closely related equid herpes viruses EHV-1 and EHV-4 (Wanger et al., 1992).

Primers and probe targeting the glycoprotein B gene of EHV-1 were used from OIE terrestrial, 2015 (OIE, 2015). (Tables 1)

Primers and probe sequence specificity was confirmed by nucleotide – nucleotide Blast search in national reference for biotechnology information (NCBI) database and were synthesized by Sinacolon Ltd. Iran.

The monoplex EHV-1 real-time PCR was performed as a 20µl reaction containing 0.4mM of each dNTPs, 3mM MgCl2, 1 unit TaqDNA polymerase, 0.3µm of each primers and 5 µl of DNA template. The real-time was performed on Rotor-Gene (Qiagene, Germany) machine.

**Cycling parameter**

Real-time cycling parameters were used for initial denaturation at 95 °C for 5 min, the cycling consisted of 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Results were interpreted based on CT values as follows: CT values below or equal to 35 were considered positive, CT values above 35 were considered negative (Diallo et al., 2006). Four positive samples were sequenced for confirmation of results and phylogenetic study.

**Phylogenetic analysis**

In order to establish phylogenetic relationship between EHV-1 strains, sequences results of EHV-1 strains obtained from this study were used for phylogenetic analysis using MEGA version 7.0 software.

**Statistical analysis**

Statistical analysis was performed by SPSS software version 20. Statistical significance was determined using Chi square and Fisher’s Exact test. A p≤0.05 was considered statistically significant.

<table>
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<tr>
<th>Primers and probe</th>
<th>Sequences</th>
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<td>Forward primer</td>
<td>GGG-GTT-CTT-AAAT-GTC-ATT-CAG-ACC</td>
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<tr>
<td>Reverse primer</td>
<td>GTA-GGT-GCG-GTT-AGA-TCT-CAC-AAG</td>
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<td>Probe</td>
<td>{FAM}TCT-CCA-ACG-ACC-TGT-CCG-GGC-TGT-ACC{BHQ1}</td>
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**Table 1.** Real-time PCR primers and probe

Primers used for sequence analysis on positive samples described by kirasawa et al.,1993
Results

Out of 150 sampled horses, a total of 14 (9.33%) were found positive for EHV-1, 7 (4.6%) buffy coat samples, 5 (3.33%) nasal swab samples and 2 (1.33%) of both nasal swab and buffy coat samples were found positive for EHV-1. There was not any statistically significant difference between the nasal swab and blood samples with EHV-1 infection (p>0.05). Out of 14 positive samples of EHV-1, 10 (71.42%), 1 (7.14%), 3 (21.42%) were found with history or clinical signs of respiratory, abortion and neurologic disease respectively. Out of 47 samples from Golestan, 45 samples from Khuzestan, 37 samples from Tehran and 21 samples from West Azerbaijan, 7 (14.89%), 0 (0%), 4 (10.8%) and 3 (14.28%) were positive for EHV-1 respectively. There was statistically significant difference between EHV-1 infected samples from Golestan and Khuzestan. There was no significant association among the other provinces (Table 2).

Sampled horses were divided into 3 different age groups (≤ 5 years, 6–15 years and ≥16 years). Although frequency of infection with EHV-1 was higher in 6–15 years group, there was no significant association among different age groups. Out of 14 positive samples of EHV-1, 9 were taken from crossbred, 1 from KWPN, 1 from Holstein, 3 from Thoroughbred and 1 from Turkmen. There was significant difference between Turkmen and crossbred but there was not any significant difference among other breeds. Out of 14 positive samples for EHV-1, 8 belonged to female horses and 6 belonged to male horses. There was not any significant correlation between the male and female horses infected with EHV-1 (Table 2).

Phylogenetic tree designed based on glycoprotein B gene of EHV-1 by using the real-time PCR sequence, neighbor joining method with bootstrap value equal to 1000 were used. Phylogenetic tree showed that the Iranian EHV-1 strains were homogenous and had a close relationship with the previously reported strains in gene bank (Fig. 1).

Figure 1. Phylogenetic tree of EHV-1 in Iran. It was generated by neighbor-joining method and Mega 7 software with 1000 bootstrap value. Black circles showed viruses detected in Iran and black diamond showed the out-group strain obtain from gene bank.
Table 2. Frequency of infection with EHV-1 based on type of disease, province, age and breeds*.

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C.S= Clinical sign, C.H= Clinical history, A= Affected, N= Non affected
The heterogenous letters showed significant statistic differences*

Discussion

Real-time PCR was employed for detection of EHV-1 based on its speed, high sensitivity and specificity (Hussy, et al., 2006; Diallo, et al., 2007; Pusterla, et al., 2009). Samples were taken from different studs in four provinces of Iran. These horses were used for race and jumping competition, breeding and riding. Based on the authors’ knowledge, this is the first study to present molecular detection of EHV-1 in nasal swab and blood samples of stud horses using real-time PCR TaqMan assay and phylogenetic study in Iran.

EHV-1 was detected from a total of 7.3% and 4.6% blood sample and nasal swab, respectively. Infection rate of EHV-1 in horses has been investigated worldwide and different results were found based on different regions and different detection methods were used (Ataseven, et al., 2009; Pusterla, et al., 2010; Yilmaz, et al., 2012; Turan, et al., 2012). In this study, some horses were positive only with nasal swab, the others with blood sample and some with both, therefore different kinds of discussion based on their clinical status (clinical signs or history) and type of sample (nasal swab or blood) could be considered.

In a serological survey in Chaharmahal-Bakhtiari, Iran, Momtaz and Hemmatzadeh, (2003) detected 39.08% EHV-1 positive horses. In molecular surveys the prevalence of EHV-1 in Isfahan, Chaharmahal-Bakhtiari and northeast of Iran were 13.2%, 8.1% and 0%, respectively (Sarani, et al., 2012; Tak-
Variation in detection rate among these studies could be due to climatic condition (moisture, temperature, etc.), frequency of EHV-1 infection in these provinces, immune status of horses, management factors and techniques used to detect this virus (Matsumura, et al., 1992; Gohering, et al., 2006).

The results of this study, like Friday et al., (2000), confirm the absence of significant association between different age groups, however Heninger et al., (2007) and Gohering et al., (2006) reported that, there is significant difference between age groups infected with EHV-1, nevertheless the results show that infection with EHV-1 in the age group of 5–16 years is higher than the other groups. We believe that with increase in age, possibility of EHV-1 infection increases. Basically, the possibility of encountering EHV-1 increases for adult horses used for competition, breeding and training (Friday, et al., 2000; Gohering, et al., 2006; Heninger, et al., 2007; Taktaz, et al., 2015).

The results of this study were similar to Gohering et al., (2006), Lunn et al., (2009) and Taktaz et al.’s, (2015) studies that showed different frequencies of EHV-1 in different breeds of horses, nevertheless there is only a significant difference between two breeds (Turkmen and crossbred); further, there is not any significant difference among other breeds sampled in this study. Higher use of crossbred horses in competitions with stressful condition (poor housing, transportation, gathering a large number of horses) that decrease function of immune system may be associated with higher frequency of EHV-1 infection in these horses.

The results show there is not any significant association between sex and EHV-1 infection. In this category, our results are in agreement with reports of Momtaz and Hemmatzadeh, (2003), Friday et al., (2009) and Taktaz, et al., (2015), on the other hand Gohering et al., 2006 reported sex is a factor in the epidemiology of EHV-1 infection in the Netherlands. Different results between sexes in these studies may be due to differences in rate of exposure to the infection, health status, age, previous vaccination or immune status of the horses sampled in these studies (Momtaz and Hemmatzadeh, 2003; Gohering, et al., 2006; Friday, et al., 2009; Taktaz, et al., 2015). In our study there is no significant association between blood and nasal swab samples of EHV-1 infected, however frequency of EHV-1 in blood was higher than nasal swabs. Results obtained from other parts of the world in this category are different (Brown, et al., 2007; Ataseven, et al., 2009; Turan, et al., 2012; Yilmaz, et al., 2012).

In surveys performed in Turkey, Ataseven et al., (2009), identified frequency of EHV-1 in nasal swabs 30% and in blood 14.3%, Yilmaz et al., (2012), reported frequency of EHV-1 in nasal swabs 13.6% and in blood 20%. Turan et al., (2012) and Brown et al., (2007) reported that analysis of swabs is more sensitive than blood to detect EHV-1 infection.

Higher frequency of EHV-1 in blood samples could be due to the higher tendency of this virus to produce viremia and subsequently formation of latency in PBMC. However, it seems that based on sampling time, type of infection (lytic or latent), sensitivity of laboratory methods and sample size, frequency of this virus in nasal swab and blood samples is variable and could not definitely conclude that frequency of EHV-1 is always higher in blood or nasal swab samples (Brown, et al., 2007; Ataseven, et al., 2009; Turan, et al.,
2012; Yilmaz, et al., 2012). In a survey in Turkey Turan et al., (2012) identified EHV-1 that were homogenous and correlated with European EHV-1. Our results are also agree-able with this survey and EHV-1 obtained from Iran correlated with the EHV-1 ob-tained from other parts of the world.

Phylogenetic tree showed that the Iranian EHV-1 strains were homogenous and had a close relationship with the previously report-ed strains in gene bank. Generally, herpes vi-ruses are DNA viruses that use intra nuclear self-proofing system, so the mutation rates in this group of viruses are low, therefore ge-netic differences among them are low.

Acknowledgments

The authors appreciate Dr. Gharddan Mashhadi, Dr. Ghaliani-chi, Dr. Sarani and Dr. Ashrafi for their support and guidance.

Conflict of Interest

The authors declare that there is no con-flict of interest.

References


Sarani, A., Mohammad, G., Mayameei, A., Ak-
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چکیده
تزئین مطالعه: هرپس ویروس تیپ ۱ در سه نمونه یکی از اصل مهم خسارت‌های اقتصادی در صنعت گوسفند است و به عنوان علت اصلی و تعویضی سایر بیماری‌های داخلی و خارجی از جمله آنالیز فیلوژنی و تشخیص مولکولی با توجه به ضرورت کنار رفتن و جلوگیری از این بیماری مورد بررسی قرار داده شده است.

هدف: هدف از این مطالعه ارزیابی فراوانی هرپس ویروس تیپ ۱ در سه نمونه یکی از اصل مهم خسارت‌های اقتصادی در صنعت گوسفند است و به عنوان علت اصلی و تعویضی سایر بیماری‌های داخلی و خارجی از جمله آنالیز فیلوژنی و تشخیص مولکولی با توجه به ضرورت کنار رفتن و جلوگیری از این بیماری مورد بررسی قرار داده شده است.

روش کار: نمونه‌های سنجش شامل انجام PCR real-time با ماده آزمایش DNA توسط گروه بیماری‌های داخلی دانشکده دامپزشکی دانشگاه تهران از ۱۵۰ نمونه خون و سوپاب از ۳ استان در استان اردبیل در ایران در ماه مهر و اردیبهشت سال ۱۳۹۸ ماه آورده شدند.

نتایج: از مجموع ۱۵۰ نمونه ارسال شده در این مطالعه، هرپس ویروس تیپ ۱ در سه نمونه سنجش کردند. در نهایت بررسی خودشوندی با ترکیب درخت فیلوژنی بر اساس کورنیک اتصال همسایه، تطبیق جمعیت در واقع تأیید و ثبت ناکامی ویروس در استان‌های اردبیل و تهران در ایران را نشان داد.

واژه‌های کلیدی: اشکال نقلی، فیلوژنی، تشخیص مولکولی تیپ ۱ هرپس و ویروس گوسفند، استان اردبیل و تهران