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Investigation of *Equine herpesvirus-1* and *4* infections in equine population of Iran by real-time PCR

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Abstract. Objective: To detect the presence or absence of *EHV-1* and *EHV-4* in North-East equine population of Iran. Material and methods: Blood samples of 200 adult horses located in 80 different rural areas of North-East of Iran, were examined for *Equine herpesvirus-1* and *4* presences. Absolute quantitation of *EHV-4* target molecules was performed using standard curves and the detection limit of the assay was shown to be six copies per reaction. Results: Our study showed a high prevalence of *EHV-4* (88%) in these regions. *EHV-1* DNA was not detected in any sample. Conclusion: In addition to previous serological study, our report is the first to detect the *EHVs* in blood samples of Iran's equine population by using a high sensitive real-time PCR diagnostic assay and it provides new information for the virus distribution map.

Key Words: *Equine herpesvirus*, real-time PCR, virus quantitation.

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Introduction

Equine herpesvirus, a linear double-stranded DNA virus, belongs to Herpesviridae family which is divided into 3 subfamilies known as *Alpha*, *Beta* and *Gamma- herpesvirinae* on genomic basis, host range and cytopathology. The *Equine herpesvirus* type *1* (*EHV-1*) and *Equine herpesvirus* type *4* (*EHV-4*) are included in *Alphaherpesvirinae* subfamily (Maclachlan *et al* 2011). The *EHV-1* type is frequently implicated in abortions, respiratory and neurologic diseases; on the other hand, the *EHV-4* type usually causes respiratory disease and, occasionally, abortions. Viral latency and reactivation are important features of *EHVs* epidemiology (Youngquist & Threlfall 2006).

Several techniques have been used for the diagnosis of *EHV* infection including: virus isolation as "gold standard", serological tests, nucleic acid detection techniques (PCR). Various PCR-based methods have been developed for detection and identification of *EHV-1* and *EHV-4* DNA in aborted fetuses or nasal swabs. PCR assays are frequently at risk of carryover contamination, mainly when a large amount of samples is included (Allen *et al* 2004; Sellon & Long 2007).

The quantitative real-time PCR (qPCR) assay is a quantitative and diagnostic tool for infectious diseases. It is faster, more flexible and especially well-suited for screening of a large number of samples with low risk of cross-contamination (Mackay *et al* 2002; Diallo *et al* 2006; Dorak 2006; Perkins *et al* 2008). The only evidence for the existence of *EHV*s in Iran equine populations was obtained indirectly through ELISA methods, in Chaharmahal & Bakhtiyari province and the prevalence rates of *EHV-1* and *EHV-4*, were reported to be 39.08% and 68.96% respectively (Momtaz & Hematzadeh 2003).

The goal of this study was to analyze the presence or absence of *EHV-1* and *EHV-4* in North-East equine population of Iran and to develop and validate a qPCR diagnostic assay in order to detect *EHVs* in equine blood samples.

Materials and methods

Samples

Two hundred samples were collected from Turkmen and cross breed horses at age of 1-19 years. These animals were with or without respiratory symptoms and located in 80 studs in North-East area of Iran. Sampling was done randomly during March 2011 to December 2012. Two ml blood from jugular vein was collected in EDTA tubes (Vacumed® K3 EDTA, FL medical, Italy). At the time of sample collection each horse was chosen for closer clinical examinations, according to the Goehring *et al* (2010) protocol. Some clinical signs such as cough, fever, nasal discharge, respiratory distress, anorexia and depression were included in the examination. To evaluate clinical disease a clinical score was determined for each case (Table 1).

DNA Extraction

DNA was extracted from 180 µl of each whole blood sample using a DNA extraction kit (DNA Extraction kit, MBST Inc., Iran). The quality of extracted DNAs was confirmed by the agarose gel electrophoresis and spectrophotometerical analysis.

Table 2. Nucleotide sequence of primers

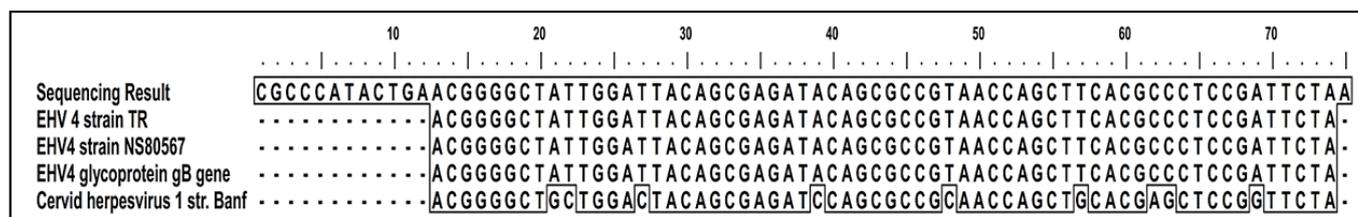| Virus	Target Gene	Genome Position	Primers (Forward, Reverse)	Amplicon size(bp)
EHV-4	*gB	2530 - 2549	TACCCCTGGAGGTTTACACG	100
(Accession#: M26171.1)		2629 - 2610	TAGAATCGGAGGGCGTGAAG	
EHV-1	*gB	990 – 1007	ATACTCGCTGAGGATGGA	113
(Accession#: M36298)		1102 - 1084	GTGAAGTTTCTCCCAAGGT	

*Glyco protein B

Figure 1. Sequence alignment of the 75 bp amplified fragment of real-time PCR products.

Table 1. Clinical scores were determined for each horse at the time of physical examination

Clinical sign	Description	Score
Coughing	No cough	0
	Cough	1
Nasal discharge	No discharge	0
	Serous discharge	0.5
	Mucopurulent discharge	1
	Profuse mucopurulent discharge	2
Dyspnea	No dyspnea (≤36 breaths/min)	0
	Mild dyspnea (>36 breaths/min)	1
	Severe dyspnea (>36 breaths/min)	2

Primers
The primers used in this study are listed in table 2.

Reference strains and housekeeping gene
Purified DNA of *EHV-1* strain 89C25 and *EHV-4* strain TH20p (Kawakami 1962) were used as reference strains in qPCR. The DNAs had been purified from fetal horse kidney cells infected with *EHV-1* (89C25p strain) or *EHV-4* (TH20p strain) using QIAamp DNA Blood Mini Kit (QIAGEN). For *EHV-1*, 113 bp and for *EHV-4*, 100 bp region of the gB gene was amplified for standard curve construction by using the primer pairs (Table 2). All the samples were tested for the presence of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described (Cappelli *et al* 2008; Hoffmann *et al* 2009).

qPCR assay
All samples were tested using Bio-Rad CFX 96 qPCR detection system. For *EHV-1* reaction cocktail we used Eva Green® qPCR Mix plus (ROX) (Solis Bio Dyne Inc., Estonia) and for *EHV-4* SYBR Green Maxima®SYBR Green/ROX (Thermo Fisher Scientific Inc.USA). Repeatability of the assay was tested in triplicate. Sample threshold and baseline values calculated automatically by the CFX manager software (Bio-Rad).

The following thermal cycler conditions were used: *EHV-1*: 95°C for 15min, followed by 40 cycles of 10 sec at 95°C denaturation, 30 sec at 64.1°C annealing, 30 sec at 72°C extension and for *EHV-4*: 95°C for 10min, followed by 40 cycles of 10 sec at 95°C denaturation, 30 sec at 59.3°C annealing, 30 sec at 72°C extension.
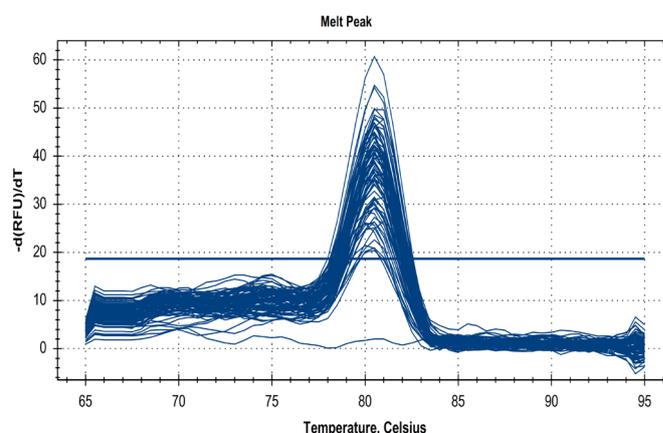
Figure 2. Post-amplification melting curve analysis of *EHV-4* positive samples (A)
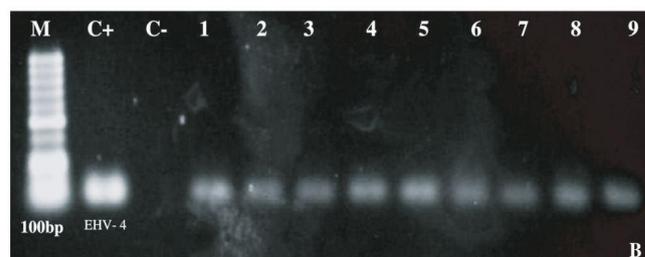
M   100bp DNA ladder.
C+   EHV4 positive control strain TH20p.
C-   Negative control.
Lanes 1-9   Real time PCR products.

Figure 2. Agarose gel Electrophoresis of nine test sample E*HV-4* real-time replicates (B).

The purified PCR products were then sequenced by Sanger's method on the ABI 3730XL DNA analyzer. The sequenced segment showed 100% identity to the gB sequence of *EHV-4* strains available in the Genbank database (Fig. 1).

Post amplification melt curve analysis of *EHV-4* positive sample consisting of raising the temperature in 0.5^0C increments from 65^0C to 95^0C every 0.05 seconds exhibited a peak melting temperature of 80.50^0C (Fig. 2A) confirmed by electrophoresis results (Fig. 2B).

Sensitivity and specificity of the real-time PCR assay

The sensitivity of the qPCR reaction was determined using a log dilution of the *EHV-1* and *EHV-4* positive DNA sample and the dynamic range of the assay was $8 \log_{10}$ dilutions as described by Hussey *et al* (2006).

Data processing was based on standard curve method and efficiencies of each reaction calculated as efficiency $= 10^{(-1/slope)} - 1$ that amplification of the log dilution series showed linearity whit slope = -3.360 and coefficient of determination (R^2) = 0.9977 for *EHV-1* and slope = -3.222 and R2 =0.997 in *EHV-4* (Fig. 3).
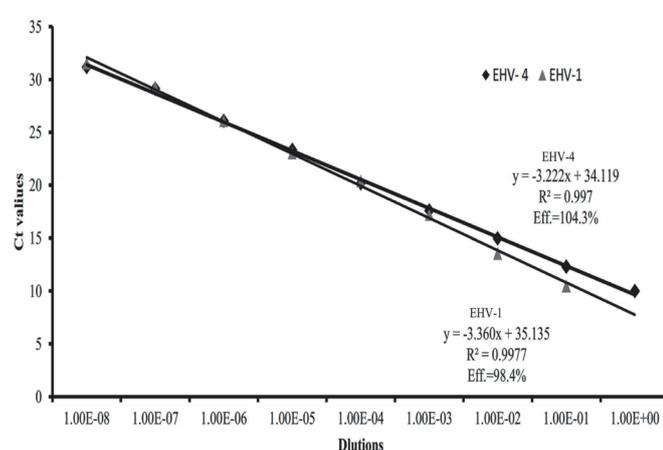

Figure 3. Standard curve of the *EHV-1* and *EHV-4* real-time PCR assay obtained from triplicates of 10-fold dilutions of standard DNA.

EHV-1 and *EHV-4* DNAs were used as positive and negative control reciprocally to determine the specificity of the assays. Any cross-reaction was not observed between them.

Statistical analysis

Statistical analyses were performed with the SPSS software (Chicago, IL, USA). The statistical significance was determined using the Student's t test. P<0.05 was considered significant.

Results

EHV-1 DNA was not detected in any of the samples. *EHV-4* DNA was identified in 88% of the blood samples. Based on standard curve, the detection limit of the test was as few as 6 copies of the gB gene per each PCR reaction.

The internal control (GAPDH) was detected in all samples; therefore, DNA losses did not occurred during nucleic acid extraction and DNA polymerase inhibition was not observed during real-time PCR amplification.

Based on PCR testing of samples, the threshold cycle (Ct) values range from 25.35 to 37.37(mean = 32.00, standard deviation (SD) = 3.83)

Results from physical examinations are summarized in table 3; the cough was the most common symptom (17%). 78.5% of examined horse did not have any clinical symptom. Statistical analysis did not show any significant differences between horses with symptoms and positive for *EHV- 4*.

Table 3 The summary of symptom results in horses

Symptom	Percent %
Cough	17
Serous discharge	5
Mucopurulent discharge	6
Profuse mucopurulent discharge	5
Mild dyspnea	7
Severe dyspnea	0.5
No symptom	78.5

Discussion

Our results showed that all samples were negative for *EHV-1* and positive for *EHV-4*. This indicates that *EHV-4* is more prevalent than *EHV-1* in North-East equine population of Iran, and confirms the earlier serological report (Momtaz & Hematzadeh 2003). Prevalence of this virus has been reported in nearby countries of Iran too (Teklioglu *et al* 2005; Ataseven *et al* 2009). Higher prevalence rate than results obtained in the previous serological study may have 2 reasons: higher sensitivity of molecular method for detection of viruses or because traditional housing or the transporting of racing horses for local competition, ceremonies and breeding in North-East of Iran can easily spread *EHV-4* between populations. This determined a higher incidence and subsequent latency than other areas.

Unlike the previous serological study in Iran, we could not detected *EHV-1* in our study. This absence of *EHV-1* detection may be due to strain variability or due to latency of infection and very low load of virus in blood samples. In clinical investigations, symptoms such as neurological signs and history of abortions in equine population of these districts did not observe either.

Confounding for factors such as: previous vaccination, maternal antibodies and cross reactivity of antibodies against *EHV-1* and *EHV-4* in many serologic tests, interpretation of serologic investigations for virus distribution map is not satisfying (Carvalho *et al* 2000; Allen *et al* 2004; Harless & Pusterla 2006; Diallo *et al* 2007; Sellon & Long 2007). The assay described in this report produces complete results within 2 hours and can be used as a rapid diagnostic tool. Although our test showed presence of *EHV-4* in horses, many of them did not have any clinical symptom, which might be due to establishment of latency and lack of virus in peripheral blood mononuclear cells (PBMCs) (Perkins *et al* 2008). The observed clinical symptoms in some horses are general and similar to common respiratory infections.

Conclusions

EHV-4 is a highly prevalent virus in horse population of Iran. *EHV-1* was not detected in our research but it is necessary to do more surveys in different regions of country. These viruses are potential causes for respiratory diseases and loss in equine industry and based on our study more attention must be paid to existence of these viruses in horse industry in Iran and its neighbors.

Acknowledgements

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