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

Ali Sarani, Gholamreza Mohammadi, Ashraf Mayameei, Masoud Akbari

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 infections. 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-4 DNA was not detected in any sample. Conclusion: In addition to previous serological study, our report is the first to detect the EHV's 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 neurological diseases; on the other hand, the EHV-4 type usually causes respiratory disease and, occasionally, abortions. Viral latency and reactivation are important features of EHV's 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 EHV's in equine blood samples.

Materials and methods

Samples

Two hundred samples were collected from Turkmen and cross-breeds 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 spectrophotometrical analysis.
Table 1. Clinical scores were determined for each horse at the time of physical examination

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<tr>
<th>Clinical sign</th>
<th>Description</th>
<th>Score</th>
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<tr>
<td>Coughing</td>
<td>No cough</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cough</td>
<td>1</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>No discharge</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Serous discharge</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Mucopurulent discharge</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Profuse mucopurulent discharge</td>
<td>2</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>No dyspnea (≤36 breaths/min)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild dyspnea (&gt;36 breaths/min)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Severe dyspnea (&gt;36 breaths/min)</td>
<td>2</td>
</tr>
</tbody>
</table>

Priming
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 (89C25 strain) or EHV-4 (TH20p strain) using QIAGEN DNA Blood Mini Kit. 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 1. Sequence alignment of the 75 bp amplified fragment of real-time PCR products.

Figure 2. Post-amplification melting curve analysis of EHV-4 positive samples (A)

Figure 2. Agarose gel Electrophoresis of nine test sample EHV-4 real-time replicates (B)
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 $\text{eff} = 10^{(-1/\text{Slope})} - 1$ that amplification of the log dilution series showed linearity with slope $= -3.360$ and coefficient of determination ($R^2$) = 0.9977 for EHV-1 and slope $= -3.222$ and $R^2 = 0.997$ in EHV-4 (Fig. 3).

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**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 and subsequent latency than other areas. 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.

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References


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