Kocatepe Veterinary Journal

Kocatepe Vet J. (2023):16(4):580-587
DOI: 10.30607/ kvj. 1185083

RESEARCH ARTICLE

Determining The Presence of Equine Herpesvirus-1 and Equine Herpesvirus-4 Infections by Real-Time PCR in Horses in Kars Region

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ABSTRACT

Horse herpesvirus infections are significant from a clinical, financial, and epidemiological standpoint. EHV-1 and EHV-4 are two of the ones that contribute to outbreaks. In this study, blood samples taken from 138 horses with or without symptoms in Kars region were examined for EHV-1 and EHV-4. EHV-1 was detected in 40 (29%) blood samples, EHV-4 was detected in 49 (35,5%) blood samples and both agents were detected in 24 (17,4%) blood samples by Real-Time PCR technique. The results suggest that, in addition to care-feeding and environmental factors, the strategic location of the Kars region should be considered in the fight against the viral agents infections.

Key words: Blood, EHV-1, EHV-4, Horse, Real-Time PCR

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Kars Bölgesindeki Atlarda Real-Tıme PCR ile Equine Herpesvırus-1 ve Equine Herpesvırus-4 Enfeksiyonlarının Araştırılması

ÖZ

Atlarda herpesvirus enfeksiyonları klinik, ekonomik ve epidemiyolojik açıdan önemli bir yere sahiptir. Bunların içerisinde EHV-1 ve EHV-4 salgınlara neden olan etkenler arasındadır. Bu çalışmada Kars bölgesinde semptom gösteren veya göstermeyen 138 adet attan alınan kan örnekleri EHV-1 ve EHV-4 yönünden incelenmiş olup EHV-1 40 (%29) adet kan örneğinde, EHV-4 ise 49 (%35,5) adet kan örneğinde, 24 (%17,4) adet kan örneğinde ise her iki etken Real-Time PCR tekniği ile tespit edilmiştir. Elde edilen bulgular neticesinde bakım-besleme, iklim koşulları yanında Kars bölgesinin konum açısından stratejik bir yerde bulunması EHV-1 ve EHV-4 enfeksiyonları ile mücadelede dikkat edilmesi gereken bir husus olarak ortaya çıkmaktadır.

Anahtar kelimeler: At, EHV-1, EHV-4, Kan, Real-Time PCR

To cite this article: Akman A. Akpınar R. Kızıltepe S. Determınıng The Presence Of Equıne Herpesvırus-1 And Equıne Herpesvırus-4 Infectıons By Real-Tıme Pcr In Horses In Kars Regıon. (2023):16(4): 580-587
Submission: 06.10.2023 **Accepted**: 05.12.2023 **Published Online**: 14.12.2023
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INTRODUCTION

Equine herpesvirus types 1 (EHV-1) and 4 (EHV-4) are the most prevalent types, and they can infect horses, donkeys and mules (Crabb et al., 1991). EHV-1 and EHV-4 are DNA viruses that are a part of the subfamily *Alphaherpesvirinae*. (Patel and Heldens, 2005).

Equestrian herpesvirus-related illnesses cause the worldwide equine sector to suffer considerable financial losses. Nine distinct varieties of equid herpesviruses (EHV-1 to EHV-9) have been found via investigations to far (Davison et al., 2009, Abdelgawad et al., 2016). In horses, the most significant pathogens in terms of clinical care, economics, and epidemiology are EHV-1 and EHV-4 (Patel and Heldens, 2005), both of which have the potential to spark severe outbreaks of respiratory diseases (Allen and Bryans 1986).

Despite being assumed to have less economical and veterinarian impact than EHV-1 and EHV-4 (Borchers et al., 1997), EHV-2 and EHV-3-related illnesses are more common (Palif et al., 1978, Browning and Studdert, 1987, Blanchard et al., 1992). According to reports, EHV-4 is only found in the lymph nodes that drain the lungs and respiratory epithelium. EHV-1, however, can result in systemic infections (Patel and Edington, 1983). EHV-1 and EHV-4 have latent infection potential, just like other herpesviruses. A key element in the transmission of illness to vulnerable animals is repeated viral shedding via asymptomatic carriers at irregular intervals (Patel and Heldens, 2004).

In EHV-1 and EHV-4, the glycoprotein B (gB) gene's nucleotide sequence was identified (Riggio et al., 1989, Whalley et al., 1989). The only gene in the *Herpesviridae* family that has not undergone mutation is one that is essential for viral replication (Wagner et al., 1992). Therefore, it is unlikely that the sequence will vary across isolates of the identical EHV type. While there is significant conservation in the first 500 nucleotides of the coding sequences of EHV-1gB and EHV-4gB, it was discovered that some sequence sections are specific to each EHV type (Wagner et al., 1992).

The most widely used method to detect equine herpesviruses by laboratory diagnosis is virus isolation in cell culture. The virus's stability is essential to the success of EHV isolation. Herpesviruses are unstable and generally undetectable in clinical specimens that have not been refrigerated or otherwise mishandled (Wagner et al., 1992).

MATERIALS and METHODS

This study was carried out in Samsun Veterinary Control Institute with the permission of Kafkas University Animal Experiments Local Ethics Committee with the letter dated 29.08.2022 and numbered 2022/137.

Blood was drawn into tubes containing EDTA from 138 unvaccinated symptomatic and asymptomatic equids up to the age of 2 years from the Kars region. The samples were randomly selected from privately owned unregistered horses in different regions of Kars in 2022. The buffy coat fraction from the centrifuged blood samples was removed using a capillary pipette after a ten-minute spin at 1500 g. Multiplex Real-Time PCR was utilized to recognize the DNA of EHV-1 and EHV-4 in buffy coat cells. Nucleic acid of all samples were initially extracted for this purpose using the Roche High Pure Viral Nucleic Acid Kit (Lot: 53059300). Afterwards, Real-Time PCR operations were applied using SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Cat:172-5017). EHV-1 and EHV-4 were

distinguished using PCR primers based on a difference in sequence between the two EHV species. It was decided to use the nucleotide sequence of the glycoprotein B (gB) genes from the viral agents (Riggio et al., 1989, Whalley et al., 1989). The *Herpesviridae* family's most conserved gene, gB, is necessary for viral replication (Wagner et al., 1992).

To find nucleic acids of the viral agents in the samples, real-time PCR was used. For this purpose, primers (Table 1) were used (Wagner et al., 1992).

The following combination was made according to the manufacturer's instructions, with a final reaction volume of 20 µl: 5 µl template DNA, 4 µl nuclease-free water, 0.5 µl of forward and reverse primer solution in 10 M, and 10 µl 2X SsoAdvanced universal SYBR® Green supermix (Bio-Rad Laboratories, USA) for Real-Time PCR testing. Using a CFX96 Touch Real-Time PCR Detection System (USA: Bio-Rad Laboratories), a 98 °C activation phase for three minutes was followed by 40 cycles of denaturation for fifteen seconds at 98 °C and annealing and elongation for thirty seconds at 60 °C. The melting curve was created using dwell times of 5 seconds at temperatures ranging from 65 °C to 95 °C. The mixtures prepared in different tubes for both agents were subjected to the same reaction.

Table 1. Primer sequences, target regions and sizes used in Real-Time PCR tests

Tablo 1. Real-Time PCR testlerinde kullanılan primer dizileri, hedef bölgeler ve büyüklükleri

Primer	Index (5'----- 3')	Target region	Size(bp)	Reference
EHV-1 F	TACTCCCACTTCCATGTCAACG	Glycoprotein B (gB)	135	Wagner et al., 1992
EHV-1 R	GCAGGTGTAAAAGTCTCCATCC			
EHV-4 F	CGTGTTCTCGCTTCTATACACC		326	
EHV-4 R	GTAAAAGTCTCCATCCTCTGCG			

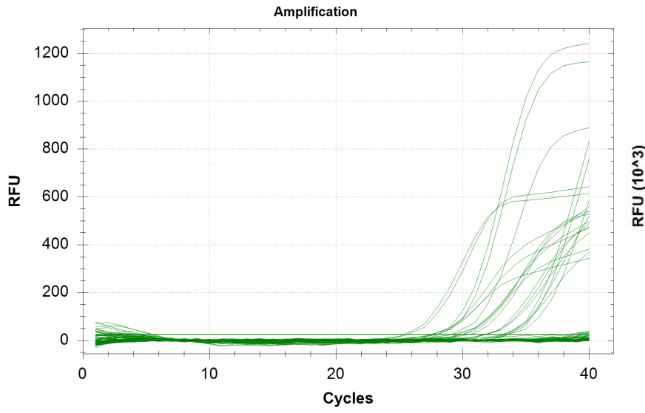
RESULTS

In this study, PCR tests were performed on DNA extracts obtained from blood samples collected from 138 horses in the Kars region (Figure 1, 2, and 3). As a result of PCR, EHV-1 was detected in 40 (29%) horse blood samples and EHV-4 was detected in 49 (35.5%) horse blood samples. There were 24 samples (17.4%) which EHV-1 and EHV-4 were both found (Table 2).

Table 2. Number of EHV-1, EHV-4 and mixed infections (EHV-1 and EHV-4) as a result of Real-Time PCR

Tablo 2. Real-Time PCR sonucunda EHV-1, EHV-4 ve miks enfeksiyonların (EHV-1 ve EHV-4) sayısı

Virus	Positive	Negative
EHV-1	40 (29%)	98 (71%)
EHV-4	49 (35,5 %)	89 (64,5%)
Mixed infection (EHV-1 and EHV-4)	24 (17,4%)	



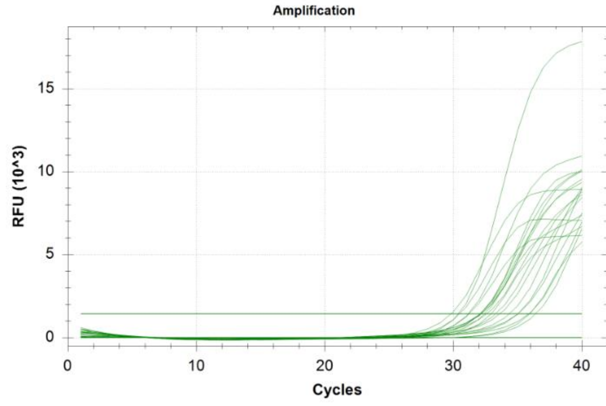


Figure 1. **Figure 2.**

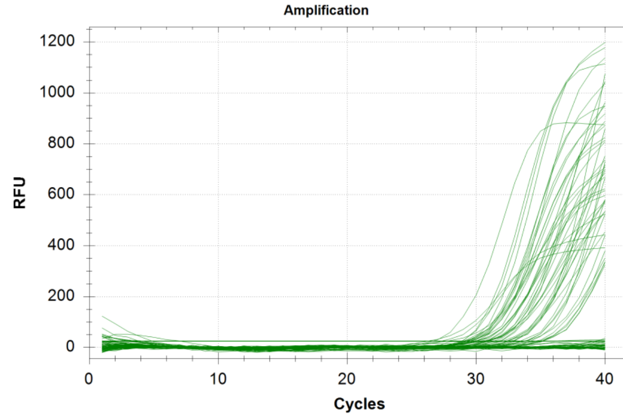


Figure 3.

Figure 1, 2, 3. Real-Time PCR image of EHV-1 and EHV-4

Şekil 1,2,3. EHV-1 ve EHV-4'ün Real-Time PCR görüntüsü

DISCUSSION

EHV-1 and EHV-4 have an endemic feature in the equine population in the world (Allen and Bryans, 1986, Allen et al., 1999). Many nations throughout the world have reported widespread the agents infections (Canada, New South Wales, Australia, India, Kyrgyzstan and Sweden). Seroprevalence rates for EHV-1 and EHV-4 are reported to be 8-85.2% and about over 90%, respectively, worldwide (Keane et al., 1988, Crabb and Studdert, 1993, Gilkerson et al., 1999, Nordengrahn et al., 1999, Singh et al., 1999, Avci et al., 2014). It has been emphasized that especially serotypes EHV-1 and EHV-4 are more important in herpesvirus infections in horses than other serotypes in terms of the frequency of occurrence, severity and effects of the infections they cause (Tekelioglu, 2016). EHV-4, on the other hand, causes sporadic cases in horses, upper respiratory tract infections in all seasons and rarely abortions (1%). Clinically, the disease caused by EHV-4 cannot be distinguished from infections caused by EHV-1. Latent infections, usually due to EHV-4, have been reported in horses. Contamination occurs by the ingestion of the agent, which infected horses emit as an aerosol through nasal discharge and respiratory tract, from the respiratory system by susceptible horses. In addition, contaminated feeders, drinkers, farm equipment, waste fetal membranes and personnel clothing are other important sources of contamination (Tekelioglu and Akay, 2003, Tekelioglu et al., 2006, Anonymous, 2022).

Various diagnostic methods can be used in the diagnosis of EHV-1 infection. The important point to be considered here is the cross-reactions that

will occur with EHV-4. Conventional serological testing is unable to distinguish between the viral agents because of substantial antigenic cross-reaction with EHV-4, prior immunization, cross-reactivity of antibodies and maternal antibodies against the viral agents (Yasunaga et al., 1998, Carvalho et al., 2000, Allen et al., 2004, Harless and Pusterla 2006, Diallo et al., 2007, Sellon and Long 2007). Type-specific diagnostic techniques should be utilized for this, and the PCR is the most sensitive of them all. (Tearle et al., 2003). Standard virological methods are considered the "gold standard", but examining large numbers of specimens in particular is both cumbersome and time consuming. Utilizing molecular diagnostic methods currently necessitates the laboratory detection of various animal viruses (Carvalho et al., 2000, Milic et al., 2010, Nisavic et al., 2010, Ohta et al., 2011, Nisavic et al., 2016). Additionally, taking into account the test's sensitivity, PCR is an appropriate screening method for detecting latent EHV-1 and EHV-4 infections in horses (Welch et al., 1992, Kirisawa et al., 1993, Borchers et al., 1997, Allen, 2006, Pusterla et al., 2012). The signal intensities and quantification findings are unaffected by changes within the sequence covered by the primers, making Real-Time PCR technology a precise, straightforward, sensitive, rapid, and quantitative tool for identifying infectious illnesses (Hussey et al., 2006, Watzinger et al., 2006, Diallo et al., 2007, Slater, 2007, Pusterla et al., 2009, Milic et al., 2010). The OIE Reference Laboratories use q-Real-Time PCR tests that seek for heterologous sequences of important glycoprotein genes to discriminate between EHV-1 and EHV-4 among herpesvirus strains (OIE, 2015). . Using primers that focused on the glycoprotein B (gB) gene region, known as the conserved area in *Herpesviridae*, multiplex Real-Time PCR was performed to precisely identify the EHV-1 and EHV-4 agents in our investigation.

In this investigation, blood samples from 138 horses in the Kars region were drawn, and EHV-1 and EHV-4 were found in 40 (29%) and 49 (35.5%) respectively. Both virus agents were detected in 24 blood samples (17.4%). Kars is located in the east of Turkey and has a strategic position in terms of bordering Armenia and very close to Iran. It is also very close to Igdır and Ardahan, where horse breeding is intense. Considering the animal movements with these regions, the prevalence rates in this study are similar to other studies. Yildirim et al. (2015) used an ELISA test to serologically investigate blood samples obtained from 423 horses in the Kars and Ardahan areas for the viral agents. Antibodies to EHV-1 and EHV-4 were detected in 222 (52.48%) and 354 (83.69%) equine blood samples, respectively. In addition, antibodies to both viruses were detected in 210 (49.65%) blood samples. The reason for the lower prevalence in our study is that detection of EHV-1 and EHV-2 agents from blood samples by PCR is only possible in animals in the acute infection period. Considering that the detection of viral agents in blood samples is only in the viremia phase, the rates we obtained can be considered high. Because it is the first molecular epidemiological study carried out in the Kars area, this work is significant.

As a result of their serological study on EHV-1 and EHV-4 in working horses in eastern Turkey, Ataseven et al. (2010) detected that the region's high seroprevalence rates were relatively high for the area's number of horses per household (≥10 heads), stressful working conditions, new herds in the area, changing climatic conditions, and illegal border trade. The researchers pointed out that illicit border trafficking of horses from Turkey to Iran was another reason for the high seroprevalence found. Equine herpesvirus disease development is correlated with elements including climatic conditions, population density, management practices, transportation, and other diseases (OIE, 2015). In a study conducted with

Real-Time PCR from 200 horse blood in Iran, EHV-4 was found at a rate of 88% and EHV-1 could not be detected (Sarani et al., 2013). Molecular studies on EHV-1 in Iran have reported positivity between 0% and 13.2% (Afshin et al., 2020, Sarani et al., 2013, Taktaz et al, 2015). No studies were found on EHV-1 and EHV-4 in Armenia. In our investigation, the prevalence rate of EHV-4 was found to be higher than that of EHV-1, which indicates that the factor of EHV-4 is more common in horses.

CONCLUSION

As a result, serological and molecular studies have demonstrated that EHV-1 and EHV-4 cases are widespread in horses, and new strategies need to be developed in the fight against these infections. Uncontrolled animal movements, unsuitable breeding conditions, vaccination procedures and climatic conditions are important for Equine Herpesviruses and strategic decisions should be made on these issues. For the eradication of these diseases, breeders should be informed about the course and control of these diseases. The epidemiological data obtained will shed light on the fight against *Herpesviridae*.

Conflict of interest: The authors declare that there are no real, potential or perceived conflicts of interest for this article.

Authors' Contribution: The authors declare that they have contributed equally to the manuscript.

Ethical approval: This study was approved by KAÜ-HADYEK with number 2022/137 and date 29.08.2022.

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