The Thai Journal of Veterinary Medicine

Volume 52
Issue 3 *52(3)*

Article 16

9-1-2022

Molecular determination of west nile virus, equine herpesvirus 1 and herpesvirus 4 in feral horses in Turkey

Irmak DIK

Oguzhan AVCI

Sibel YAVRU

H. Pelin ASLIM

H.Sercan PALANCI

Original Article

Molecular determination of west nile virus, equine herpesvirus 1 and herpesvirus 4 in feral horses in Turkey

Irmak DIK[1*] Oguzhan AVCI[1] Sibel YAVRU[1] H. Pelin ASLIM[1] H.Sercan PALANCI[1]

Abstract

West Nile Virus (WNV), Equine Herpesvirus 1 (EHV-1) and Equine Herpesvirus 4 (EHV-4) are among the important pathogenic viral factors for both domestic and feral horses. This study was carried out to determine the molecular presence of WNV, EHV-1 and EHV-4 infections in feral horses in Konya. Blood samples were taken from 36 horses selected by a randomized sampling method from a total of 250 feral horses bred together. The leukocyte samples obtained were examined virologically for the presence nucleic acid of WNV using the Nested reverse transcriptase-polymerase chain reaction assay (nested RT-PCR) method and using molecular methods for the presence of EHV-1 and EHV-4 by multiplex nested polymerase chain reaction assay (multiplex nested PCR) method. As a result, 6 (16.6%) feral horses were determined to be antigen positive for WNW, while all the horses were found to be antigen-negative for EHV-1 and EHV-4. Determining the presence of WNV infection in feral horses reveals the possibility that the sampled horses were exposed to infected birds and arthropods. Again, the failure to determine EHV-1 and EHV-4 in these horses was considered to be because these viruses could be in the latent period at the time of sampling. The presence of WNV in feral horses was revealed for the first time. It is thought that molecular characterization will also shed light on the transition of WNV between species by examining more samples.

Keywords: West Nile virus, Equine Herpesvirus 1 and Equine Herpesvirus 4, Feral horses

[1]*Department of Virology, Faculty of Veterinary Medicine, University of Selcuk, Konya, TURKEY*
***Correspondence**: irmakdik@selcuk.edu.tr* (I. Dik)
Received January 28, 2021
Accepted August 18, 2022
https://doi.org/10.14456/tjvm.2022.65

Introduction

West Nile virus (WNV) is a zoonotic factor that was first identified in the West Nile state of Uganda in 1937 by isolating it from the blood of a febrile woman and transmitted by arthropods (Donadieu *et al*., 2013). WNV is a single-stranded RNA virus and is located in the subgroup *Flavivirus* of the virus family *Flaviviridae*. The factor is also a member of the JE serocomplex, which includes the Japanese Encephalitis Virus (JEV), the St. Louis encephalitis virus (SLEV), the Murray Valley Encephalitis Virus (MVEV) and the Kunjin Virus (Castro-Jorge *et al*., 2019; Kramer *et al*., 2007). The viral genome contains three structural proteins, C, prM/M, E and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Macdonald *et al*., 2005; Khan *et al*., 2021). While in the past WNV caused sporadic meningoencephalitis in Africa, Asia and the Middle East, it spread globally 78 years after its discovery and is now considered one of the main viral causes of encephalitis (David and Abraham, 2016). Natural transmission of the factor in WNV infections is when Culex flies take the virus from infected birds (feral and domestic) and infect them again (David and Abraham, 2016; Shocket *et al*., 2020) while recent studies have stated that it may play a role in transmission by Aedes flies as well as Culex flies (Napp *et al*., 2018). WNV, which has a chain of transmission between birds and arthropods, infects various mammals, especially humans and horses, and domestic poultry such as chickens (Bosco-Lauth and Bowen, 2019). Humans and horses are also considered to be end/terminal hosts due to short-term viremia. However, the resulting infection can be fatal for them (Castro-Jorge *et al*., 2019; Chancey *et al*., 2015). The presence and prevalence of WNV infection are a concern among horse owners and veterinarians (Anderson *et al*., 1999; Bertram *et al*., 2020). Studies conducted have reported an increase in WNV infection in humans and horses, especially in the dry and hot summer and autumn (Baqar *et al*., 1993; Bertram *et al*., 2020).

Equine Herpesvirus 1 (EHV-1) and Equine Herpesvirus 4 (EHV-4) are in the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus-group *Varicellovirus* (Kapoor *et al*., 2014). EHV-1 and EHV-4 cause economically significant diseases in horses worldwide. They are double-stranded (ds) DNA viruses with a molecular weight of 150 kb. The capsid has icosahedral symmetry and is surrounded by a lipid membrane containing 12 different glycoproteins (Patel and Heldens, 2005; Li *et al*., 2021). Although EHV-1 and EHV-4 are very similar genetically, some important differences exist in their pathogenesis and epidemiology. While EHV-4 infection is most commonly observed in the upper respiratory tract, it can sometimes cause abortions. EHV-1 infection often causes systemic diseases such as upper respiratory tract infection and abortion, myeloencephalitis (Patel and Heldens, 2005; Vaz *et al*., 2016). EHV-1 and EHV-4, can be transmitted by direct contact, the aerosol route and semen. The agent isolation can be made from a nasal swab and whole blood. Serological diagnosis can be made from blood serum (Ma *et al*., 2013; Giannetto *et al*., 2022). Both EHV-1 and EHV-4 can remain latent throughout an animal's life and remain latent in the trigeminal ganglia or lymphocytes after natural infection. In animals with latent infection, the virus begins to actively re-spread during stress factors such as corticosteroid administration, birth, weaning, transport and vaccination. Detection of infected individuals is of primary importance in reducing the spread within the animal population (Foote *et al*., 2003; Taouji *et al*., 2002; Wang *et al*., 2007). It has been reported that Nested PCR is 100 times more sensitive than conventional PCR for EHV-1 and 1000 times more sensitive for EHV-4. It has been stated that this sensitivity may be due to the use of a smaller amplification product in the second reaction of Nested PCR (Borchers and Slater, 1993). Conventional PCR is a sensitive test that can distinguish between EHV-1 and EHV-4 (Hussey *et al*., 2013). However, while PCR tests can detect active nasal viral shedding or viremia, it has been reported that it is not sensitive enough to detect latent infection (Léon *et al*., 2008).

Since our country is advanced in terms of horse breeding and migratory birds are on the migration route, it is of great importance to prevent WNV infection, especially the WNV infection and the infections that may cause great economic and yield loss for the horse sector, such as EHV-1 and EHV-4. This study was carried out to determine the virological presence of WNV, EHV-1 and EHV-4 infections in wild horses Turkey.

Materials and Methods

Preparation of Whole Blood Samples: Blood samples were collected from feral horses of different genders and ages, which were brought from Karadağ (Karaman city of Turkey) to the Çumra (Konya city of Turkey). The 36 blood samples were randomly collected from a herd of approximately 250 feral horses in November 2017. Because of the the horses' difficulty in being caught the collection of blood samples was quite hard. After the collected blood samples were centrifuged, the buffy coat portion was separated and stored at -20 C until being analyzed.

RNA Extraction from Blood Samples and Nested RT-PCR for WNV: RNA was extracted from samples of the buffy coat using a QIAamp viral RNA (QIAGEN). All specimens were subjected to nested RT-PCR targeting, the E protein-coding region in WNV (Table 1.). For the first step of Nested RT-PCR, 50µL final reaction mix (10µl 5x, 1 µl dNTP, 2 µl forward primer (10pmol), 2 µl reverse primer (10pmol), 1 µl enzyme mix, 29 µl Nuclease free water 5 µl RNA was prepared for each sample with positive and negative controls. For the amplification, the following conditions were applied for the step-1 of nested RT-PCR: 50°C for 30 mins, 95°C for 10 mins followed by 33 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 60 s, and a final extension of 72°C for 5 mins. The 2nd step of nested RT-PCR was carried out using the obtained PCR products and 2nd step primers. For this purpose, 50 µl final reaction mix (5µl 10x, 1µl 10mM dNTP, 3 µl 25mM $MgCl_2$, 0,5µl Taq DNA polymerase, 2 µl forward primer (10pmol), 2 µl reverse primer (10 pmol), 1 µl enzyme mix, 33.5µl nuclease free water 3 µl DNA) was prepared. For the amplification,

the following conditions were applied for the step-2 of nested RT-PCR: 95°C for 10 mins followed by 33 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 60 s, and a final extension of 72°C for 5 mins followed by electrophoresis on a 1.5% agarose gel with ethidium bromide. All steps included a positive control and RNA-free water as a negative control. Bands formed at 248 bp indicated that WNV RNA was present in the samples (Figure 1). The positive control (WNV NY99 strain) was obtained from the Selcuk University Faculty of Veterinary Medicine Virology Department.

Table 1 Primers used in nested RT-PCR analysis for WNV (Johnson *et al.*, 2001).

	Oligonucleotide	Gene Zone	Location	Amplicon Length (bp)
1. Step Primer Set	5'-ACCAACTACTGTGGAGTC-3' 5'-TTCCATCTTCACTCTACACT- 3'	E protein	1401-1845	445bp
2. Step Primer Set	5'-GCCTTCATACACACTAAAG-3' 5'-CCAATGCTATCACAGACT-3'	E protein	1485-1731	248bp

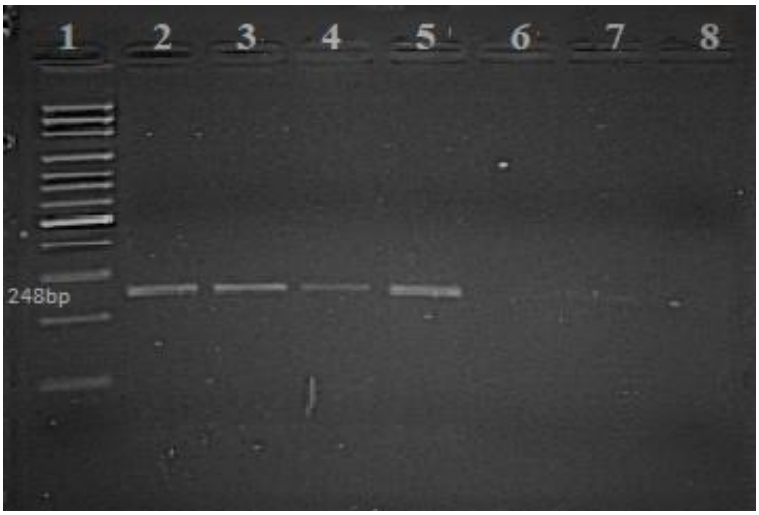

Figure 1 The results of nested RT-PCR in WNV.
1: 100bp DNA marker,
2: Positive control,
3,4,5: Positive samples (S1, S9, and S30),
6,7: Negative samples (S35 and S36),
8: Negative control.

DNA Extraction from Blood Samples and multiplex-nPCR for EHV-1 and EHV-4: The blood samples collected in this study were centrifuged at 3000 rpm for 5 mins. before DNA extraction, and the leukocyte layers were partitioned into eppendorf tubes. Commercial kits obtained viral DNA from portioned leukocyte samples (Qiagen Dneasy, Cat. No: 69506, Germany). Extractions were performed according to the manufacturer's instructions. In the study, the nested PCR investigated the presence of the DNA extracted samples EHV-1 and EHV-4, glycoprotein B (gB). The lists of primers used in this study are given in Table 2.

A commercial test kit (Solis BioDyne 5x HOT FIREPol Blend master mix, Cat no: 04-25-00115) was used in the PCR reactions. For nested PCR 1. Step reaction mixture: DNA 5µl was added to 5x master mix 10ul, forward and reverse primer 1.4 µl, Nuclease free water, 32.2 µl and the total mixture was prepared in a volume of 50 µl. The first reaction mixture was subjected to the PCR procedure. At the end of the procedure, 5µl of the 1st stage reaction mixture was taken and then added to the 2nd step reaction mixture containing 45µl. 2. Step reaction mixture: prepared per 5x master mix 10 µl, FC3 primer 1.6 µl R1 primer 1,4 µl, R4 primer 1,4µl, Nuclease free water 30.6 µl. 1. and 2. step PCR cycles of these mixtures;

PCR 1 and PCR 2 cycles: After the enzyme activation temperature was achieved at 95°C for 13 mins., the products were subjected to PCR for 30 reactions, Denaturation at 94 C for 20 seconds, Annealing at 59 C for 1 min., elongation at 72 C for 1 min., and the final extension was performed at 72 °C for 7 mins., and the PCR procedure was terminated.

As a result of the reaction, the 2nd step PCR products were transferred to 2% agarose gel and exposed to electrophoresis for 50 mins. The products were examined on the gel imaging device at the end of the procedure and the samples giving a band of 188 bp for EHV-1 and the samples giving a band of 677 bp for EHV-4 were accepted as positive (Figure 2). The positive controls for EHV-1 and EHV-4 were obtained from the Hatay Mustafa Kemal University Faculty of Veterinary Medicine Virology Department.

Ethical approval: All procedures and animal care complied with the guidelines of the Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2019/96 on 19/12/2019).

Table 2 Primers used for multiplex nested PCR analysis for EHV-1 and EHV-4 (Ataseven *et al.*, 2009).

	Oligonucleotide		Gene Zone	Location	Amplicon Length (bp)
1. Step Primer Set	FC2	5′-CTTGTGAGATCTAACCGCAC-3′	EHV-1 gB EHV-4 gB	EHV-1: 2427–2446 EHV-4: 1737–1756	1118
	RC	5′-GGGTATAGAGCTTTCATGGG-3′	EHV-1 gB EHV-4 gB	EHV-1: 3607–3588 EHV-4: 2911-2892	1175
2. Step Primer Set	FC3	5′-ATACGATCACATCCAATCCC-3′	EHV-1 gB EHV-4 gB	EHV-1: 2699–2718 EHV-4: 2003–2022	
	R1	5′-GCGTTATAGCTATCACGTCC-3′	EHV-1 gB EHV-4 gB	EHV-1: 2886–2867	188
	R4	5′-CCTGCATAATGACAGCAGTG-3′	EHV-1 gB EHV-4 gB	EHV-4: 2679–2660	677

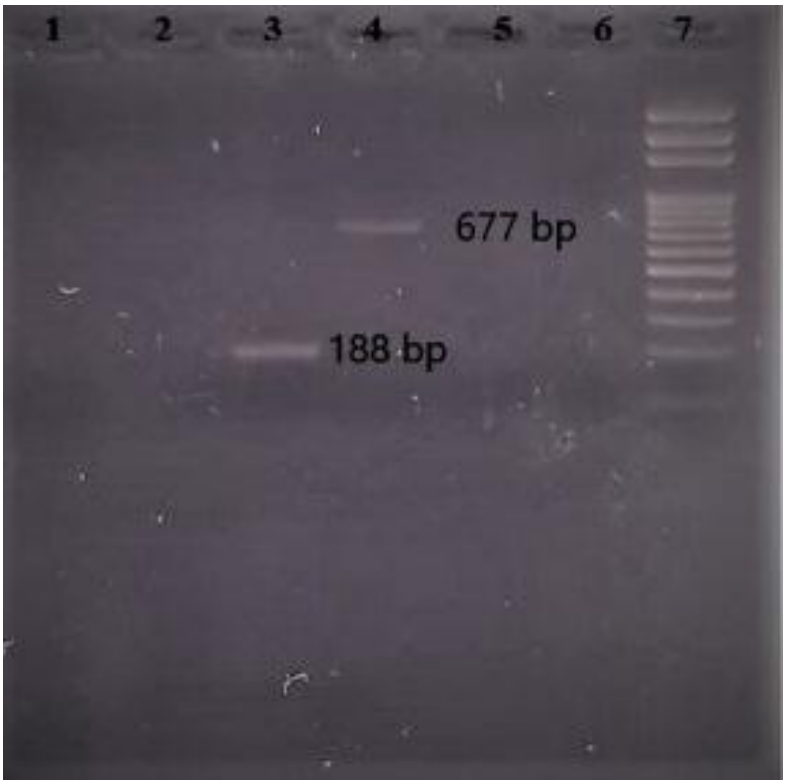

Figure 2 The results of multiplex nested PCR in EHV-1 and EHV-4.
1: Negative control,
3: EHV-1 Positive control,
4: EHV-4 Positive control
2,5,6: Negative samples
7: 100bp DNA marker,

Results

As a result of this study, 6 (16.6%) of 36 feral horse leukocyte samples were determined positive for the presence of WNV antigen by nested-RT PCR. In contrast all samples were determined to be negative for EHV-1 and EHV-4 by the multiplex nested PCR method (Table 3).

Table 3 WNV, EHV-1, and EHV-4 study results

	WNV	EHV-1	EHV-4
Number of samples examined	36	36	36
Number of positive samples	6 (16.6%)	0	0

Discussion

West Nile Virus was discovered in Africa about 80 years ago and is now important for human and veterinary medicine (David and Abraham, 2016). It causes infections in humans and various mammals, especially horses (Ergunay *et al.*, 2014; Loroño-Pino *et al.*, 2003). The incidence of the infection increases especially in hot periods when *Culex* flies are observed intensively and when migratory bird populations increase (David and Abraham, 2016; Duyum and Karaoğlu, 2019). Since feral horses can be reservoirs, it

is more important to reveal the presence of disease in these horses (Kapoor *et al*., 2014).

In the present study, 6 (16.6%) (Table 3.) WNV RNAs were found in 6 (16.6%) horses as a result of nested RT-PCR performed from 36 blood samples randomly sampled from a farm with 250 feral horses adopted within the project's scope. Various studies have been conducted with WNV in Turkey. Ozkul *et al.* (2006) found WNV neutralizing antibodies in 1 of 40 mule samples (2.5%), 4 (4%) of 100 cattle samples, 43 (37.7%) of 114 dog samples, 35 (13.5%) of 259 horse samples, 18 of 88 human samples (20.4%) and 1 (1%) of 100 sheep samples as a result of the Plaque Reduction Neutralization Test (PRNT) they applied to serum samples collected from various mammals in different provinces in Turkey. With the data obtained, it was interpreted that a wide range of mammals in Turkey had been exposed to the virus. Ergunay *et al*., (2014) PRNT and WNV-specific antibodies made from blood samples collected from poultry and mammals in different provinces of Turkey were identified in 10.5% of 1180 samples detected in all examined and evaluated species. According to the results of nested RT-PCR performed, viral nucleic acids were reported to be positive in 5.9% of 522 samples, all of which were only in horses. Kale *et al*., (2017) scanned serum samples from 650 domestic horses and 234 domestic donkeys in different regions of Turkey for WNV IgG by ELISA. As a result, they reported that they had obtained 4.15% positivity in domestic horses, 1.28% in domestic donkeys and that the positivity detected in horses was only in Native type horses. Yildirim *et al*., (2018) collected blood samples by random sampling from horses, donkeys and Turkish native geese in the Northeastern Anatolia region of Turkey. When the researchers tested antibodies against WNV, they found that 0.8% (1/118) of horses, 20% (14/70) of donkeys and 1.1% (4/378) of geese were WNV seropositive. To determine the presence of WNV nucleic acid, when they tested positive blood sera with RT-PCR technique, they reported that while WNV nucleic acid was not found in horse and goose samples, 4 donkeys were positive. Duyum and Karaoğlu (2019), which they conducted to evaluated the serological and virological status of WNV in breeding horses resident in Şanlıurfa and subjected serum samples from 277 breeding horses to WNV antibody (Ab) and IgM ELISA, PRNT and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (rtRT-PCR). These samples were first examined for the presence of antibodies by subjecting them to the WNV/Ab ELISA test and 42 of the samples (15.16%), were considered as seropositive, 16 (5.77%) as suspicious, and 219 (79.06%) as seronegative. Existing seropositive and suspicious samples were subjected to PRNT and 11 of these 42 seropositive samples (26.19%) were found to have specific WNV antibodies. Specific WNV antibodies were found in 11 (3.97%) of 277 breeding horses sampled. No viral RNA was detected by rtRT-PCR in any of the 42 samples and 16 suspicious samples were detected as seropositive by the WNV Ab ELISA test. They explained this condition by the absence of any acute illness in the sampled animals and the short duration of the viremia period in horses.

In the current study, the fact that 16.6% positivity obtained by nested RT-PCR for WNV was determined to be higher than the results reported in Turkey was interpreted as because the sampled horses were exposed to more infected birds and arthropods due to their wildlife living in the wild and their presence in the open area, especially at night, where mosquito populations are intense.

EHV-1 and EHV-4 are responsible for serious economic loss in the horse industry as they cause many abortions and perinatal/neonatal deaths. For this reason, it is important to know the epidemiology of EHV-1 and EHV-4 infections, develop effective diagnostic methods and to take control with preventive measures (Vaz *et al*., 2016). Various studies conducted in our country reveal the prevalence of EHV-1 and EHV-4 infections. Gür and Yapici (2008) collected blood serum from a total of 188 animals from İzmir, Konya, Afyonkarahisar and Eskişehir provinces. They found that the EHV-1 antibody was 3.7% positive, EHV-4 was 56.9% positive. In Ataseven *et al*., (2009) which was conducted by collecting blood samples from 21 symptomatic horses and nasal swab samples from 40 symptomatic horses, when they evaluated the presence of EHV-1 and EHV-4 by multiplex nested PCR, they reported that a total of 14.3% (3/21) buffy coat sample and 32.5% (13/40) nasal swab samples contained EHV-1 DNA, and 19% (4/21) buffy coat sample and 22.5% (9/40) nasal swab samples were positive for EHV-4 DNA. In Ataseven *et al*., (2010) in which they examined the blood serum of 405 horses from the Eastern Anatolia Region of Turkey in terms of EHV-1 and EHV-4 antibodies by ELISA, they found EHV-1 antibody positive at the rate of 29.5% in Van, 6.1% in Bitlis, 5.1% in Muş, 24.0% in Iğdır and 20.6% in Erzurum and EHV-4 antibody positive in the rate of 87.2% in Van, 61.2% in Bitlis, 56.4% in Muş, 84.0% in Iğdır and 52.9% in Erzurum. Avci *et al*., (2014) collected blood serum samples from 150 horses from Adıyaman, Diyarbakır, Gaziantep, Kilis, and Şanlıurfa provinces of the Southeastern Anatolia Region of Turkey and tested them with ELISA for EHV-1 and EHV-4. They found positive 34.66% for EHV-1 and 67.33% for EHV-4. Yildirim *et al*. (2015), in Turkey, performed antibody screening for EHV-1 and EHV-4 by ELISA in blood serum samples obtained from 423 horses and 243 donkeys. They found 52.48% EHV-1 and 83.69% EHV-4 positive in horses serologically. 51.85% of the analyzed donkey samples tested positive for EHV-1 and 64.20% tested positive for EHV-4 antibodies. In another study, nasal swabs were collected from 36 aborted fetuses with suspected illness in different horse hospitals and breeding farms in Turkey, and 98 horses with symptoms of upper respiratory tract diseases 2 and over. When all the samples collected were evaluated by PCR, it was reported that EHV-1 positivity in 7 fetuses from 26 aborted fetuses and EHV-4 positivity in 2 horse samples from 98 nasal swab samples were found (Tekelioğlu, 2016).

Matsumura *et al*., (1992) revealed that EHV-1 could be detected especially in winter months such as January and February, while EHV-4 can be isolated throughout the year. They stated that this might be due to the activation of latent infection due to the cold

weather suppressing the horses' immune systems. They also stated that EHV-1 is mostly observed in animals older than 3 years old, EHV-4 is observed in animals younger than 2 years old.

In the present study, when EHV-1 and EHV-4 were evaluated by multiplex nested PCR in the animals sampled, all samples were determined as negative. The presence of the virus was determined per virological and serological studies performed for EHV-1 and EHV-4 in our country. However, EHV-1 and EHV-4 could not be detected virologically as a result of this study. This condition has revealed that due to the ability of EHVs to create a latent infection, it is important to sample while observing clinical findings, considering the age of the animals, and considering the season and the state of the region. For this purpose, it is recommended to perform sampling for these reasons in the diagnosis of both biotypes in future studies and to examine blood serum in terms of antibodies as well as virological studies from samples such as nasal swab and fetus in line with clinical symptoms.

Herewith, it was determined that the presence of WNV infection in Turkey is at a rate that cannot be ignored. It is thought that determining the presence of the disease in many mammalian species and performing phylogenetic analyses is important in determining its severity. Also, samples from mosquitos and birds need to be screened for WNV and thus the infection status determined in detail. Isolation studies that will be conducted in the future will contribute to vaccine development in horse and human health. In addition, the fact that EHV-1 and EHV-4 infections were determined as negative for all samples according to the current study results does not mean that these diseases are not common and it is necessary to perform more sampling and, if possible, to sample from horses with clinical symptoms.

Acknowledgements

We would like to express our sincere thanks to Prof. Dr. Veysel Soydal ATASEVEN for contributing to the provision of positive controls for EHV-1 and EHV-4, and to Prof. Dr. Bilal DİK and Assoc. Prof. Dr. Onur CEYLAN for their assistance in collecting samples.

Financial Support: This manuscript has not been supported by any organization.

Conflict of Interest: The authors declare that there are no conflict of interest.

References

Anderson, JF, Andreadis, TG, Vossbrinck, CR, Tirrell, S, Wakem, EM, French, RA, Garmendia, AE, and Van Kruiningen, HJ 1999: Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. *Science* 286, 2331-2333.

Ataseven, VS, Dağalp, SB, Başaran, Z, and Keskin, S 2010: Seroepidemiological studies of equine herpesviruses 1 (EHV-1) and 4 (EHV-4) infections in working horses from the eastern Turkey. *Ankara Univ Vet Fak Derg* 57, 39-42.

Ataseven, VS, Dağalp, SB, Güzel, M, Başaran, Z, Tan, MT, and Geraghty, B 2009: Prevalence of equine herpesvirus-1 and equine herpesvirus-4 infections in equidae species in Turkey as determined by ELISA and multiplex nested PCR. *Res Vet Sci* 86, 339-344.

Avci, O, Yavru, S, Tokgoz, S, and Kale, M 2014: Detection of Antibodies against Equine Herpes Virus-1 and Equine Herpes Virus-4 in Horses in Southeast Anatolia by Indirect Elisa. *Acta Sci Vet* 42, 1-5.

Baqar, S, Hayes, CG, Murphy, JR, and Watts, DM 1993: Vertical transmission of West Nile virus by Culex and Aedes species mosquitoes. *Am J Trop Med* 48, 757-762.

Bertram, FM, Thompson, PN, Venter, M 2020: Epidemiology and clinical presentation of west nile virus infection in horses in south africa, 2016–2017. *Pathogens* 10, 1-16.

Borchers, K, and Slater, J 1993: A nested PCR for the detection and differentiation of EHV-1 and EHV-4. *J Virol Methods* 45, 331-336.

Bosco-Lauth, AM, and Bowen, RA 2019: West Nile Virus: Veterinary Health and Vaccine Development. *J Med Entomol* 56, 1463-1466.

Castro-Jorge, LAd, Siconelli, MJL, Ribeiro, BdS, Moraes, FMd, Moraes, JBd, Agostinho, MR, Klein, TM, Floriano, VG, and Fonseca, BALd 2019: West Nile virus infections are here! Are we prepared to face another flavivirus epidemic? *Rev Soc Bras Med Trop* 52, 1-9.

Chancey, C, Grinev, A, Volkova, E, and Rios, M 2015: The global ecology and epidemiology of West Nile virus. *Biomed Res Int*, 1-21.

David, S, and Abraham, AM 2016: Epidemiological and clinical aspects on West Nile virus, a globally emerging pathogen. *Infect Dis* 48, 571-586.

Donadieu, E, Bahuon, C, Lowenski, S, Zientara, S, Coulpier, M, and Lecollinet, S 2013: Differential virulence and pathogenesis of West Nile viruses. *Viruses* 5, 2856-2880.

Duyum, RA, and Karaoğlu, T 2019: Şanlıurfa'da Yerleşik Damızlık Atlarda Batı Nil Virüsü (BNV) Enfeksiyonu'nun Serolojik ve Virolojik Olarak Araştırılması. *Etlik Vet Mikrobiyol Derg* 30, 36-43.

Ergunay, K, Gunay, F, Kasap, OE, Oter, K, Gargari, S, Karaoglu, T, Tezcan, S, Cabalar, M, Yildirim, Y, and Emekdas, G 2014: Serological, molecular and entomological surveillance demonstrates widespread circulation of West Nile virus in Turkey. *PLOS Negl Trop Dis* 8, 1-10.

Foote, C, Gilkerson, J, Whalley, JM, and Love, D 2003: Seroprevalence of equine herpesvirus 1 in mares and foals on a large Hunter Valley stud farm in years pre-and postvaccination. *Aust Vet J* 81, 283-288.

Giannetto, C, Giudice, E, Piccione, G, Castronovo, C, Arfuso, F 2022: Immune and inflammatory response in horse vaccinated against equine herpesviruses 1 (EHV-1) and 4 (EHV-4) assessed by serum protein electrophoretic pattern and leukocyte population. *J Equine Vet Sci* 116, 1-8.

Gür, S, and Yapici, O 2008: Equine herpesvirus type 1 and 4 in individually reared horses in central and western Turkey. *Acta Vet Brno* 77, 609-613.

Hussey, GS, Goehring, LS, Lunn, DP, Hussey, SB, Huang, T, Osterrieder, N, Powell, C, Hand, J, Holz, C, and Slater, J 2013: Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions. *Vet Res* 44, 118.

Johnson, DJ, Ostlund, EN, Pedersen, DD, and Schmitt, BJ 2001: Detection of North American West Nile virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. *Emerg Infect Dis* 7, 739.

Kale, M, Sibel, G, Orhan, Y, Nuri, M, Sibel, Y, Sibel, H, Oya, B, and Metin, G 2017: Serological investigation of West Nile virus infection in domestic horses and donkeys in Turkey. *Pak Vet J* 37, 51-54.

Kapoor, S, Sharma, H, Singh, M, Kumar, P, Ranjan, K, Kumari, A, and Khirbat, R 2014: Equine herpesviruses: a brief review. *Nine* 2014, 06-25.

Khan, MT, Islam, R, Jerin, TJ, Mahmud, A, Khatun, S, Kobir, A., Islam, MN, Akter, A, Mondal, SI 2021: Immunoinformatics and molecular dynamics approaches: Next generation vaccine design against West Nile virus. *Plos one* 16, e0253393.

Kramer, LD, Li, J, and Shi, P-Y 2007: West nile virus. *Lancet Neurol* 6, 171-181.

Léon, A, Fortier, G, Fortier, C, Freymuth, F, Tapprest, J, Leclercq, R, and Pronost, S 2008: Detection of equine herpesviruses in aborted foetuses by consensus PCR. *Vet Microbiol* 126, 20-29.

Li, Y, Liu, D, Wang, Y, Su, W, Liu, G, Dong, W 2021: The importance of glycans of viral and host proteins in enveloped virus infection. *Front Immunol* 12, 1-12.

Loroño-Pino, MA, Blitvich, BJ, Farfán-Ale, JA, Puerto, FI, Blanco, JM, Marlenee, NL, Rosado-Paredes, EP, García-Rejón, JE, Gubler, DJ, and Calisher, CH 2003: Serologic evidence of West Nile virus infection in horses, Yucatan State, Mexico. *Revista Biomédica* 14, 159-163.

Ma, G, Azab, W, and Osterrieder, N 2013: Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)—masters of co-evolution and a constant threat to equids and beyond. *Vet Microbiol* 167, 123-134.

Macdonald, J, Tonry, J, Hall, RA, Williams, B, Palacios, G, Ashok, MS, Jabado, O, Clark, D, Tesh, RB, and Briese, T 2005: NS1 protein secretion during the acute phase of West Nile virus infection. *J Virol* 79, 13924-13933.

Matsumura, T, Sugiura, T, Imagawa, H, Fukunaga, Y, Kamada, M 1992: Epizootiological aspects of type 1 and type 4 equine herpesvirus infections among horse populations. *J Vet Med* 54, 207-211.

Napp, S, Petrić, D, and Busquets, N 2018: West Nile virus and other mosquito-borne viruses present in Eastern Europe. *Pathog Glob Health* 112, 233-248.

Ozkul, A, Yildirim, Y, Pinar, D, Akcali, A, Yilmaz, V, and Colak, D 2006: Serological evidence of West Nile Virus (WNV) in mammalian species in Turkey. *Epidemiol Infect* 134, 826-829.

Patel, J, and Heldens, J 2005: Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)–epidemiology, disease and immunoprophylaxis: a brief review. *Vet J* 170, 14-23.

Shocket, MS, Verwillow, AB, Numazu, MG, Slamani, H, Cohen, JM, El Moustaid, F., Rohr, J, Johnson, LR, Mordecai, EA 2020: Transmission of West Nile and five other temperate mosquito-borne viruses peaks at temperatures between 23 C and 26 C. *Elife* 9, 1-67.

Taouji, S, Collobert, C, Gicquel, B, Sailleau, C, Brisseau, N, Moussu, C, Breuil, MF, Pronost, S, Borchers, K, and Zientara, S 2002: Detection and isolation of equine herpesviruses 1 and 4 from horses in Normandy: an autopsy study of tissue distribution in relation to vaccination status. *J Vet Med B* 49, 394-399.

Tekelioğlu, BK 2016: At Viral Hastalıkları ve Türkiye'de Neonatal Ölen Tayların Organlarında EHV-1 DNA'sı ile burun sürüntülerinde EHV-4 DNA'sının saptanması. *JTSER*, 8-15.

Vaz, P, Horsington, J, Hartley, C, Browning, G, Ficorilli, N, Studdert, M, Gilkerson, J, and Devlin, J 2016: Evidence of widespread natural recombination among field isolates of equine herpesvirus 4 but not among field isolates of equine herpesvirus 1. *J Gen Virol* 97, 747.

Wang, L, Raidal, S, Pizzirani, A, and Wilcox, G 2007: Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR. *Vet Microbiol* 121, 18-28.

Yildirim, Y, Yilmaz, V, and Kirmizigul, A 2015: Equine herpes virus type 1 (EHV-1) and 4 (EHV-4) infections in horses and donkeys in northeastern Turkey. *Iran J Vet Res* 16, 341.

Yildirim, Y, Yilmaz, V, Yazici, K, Ozic, C, and Ozkul, A 2018: Molecular and serological investigation of West Nile virus (WNV) infection in donkeys, horses and native geese in Turkey. *Revue Méd Vét* 169, 87-92.