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DETECTING EQUINE HERPESVIRUS-1 AND EQUINE HERPESVIRUS -4 INFECTIONS IN RACEHORSES USING POLYMERASE CHAIN REACTION

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Abstract: The aim of this study was to use polymerase chain reaction (PCR) to detect and differentiate Equine Herpesvirus Type-1 (EHV-1) and Type-4 (EHV-4) viruses within the racehorse population in Türkiye. The diagnostic sensitivity of PCR was also assessed. For this purpose, 98 nasal swab specimens from naturally infected racehorses aged 2 and above, displaying respiratory symptoms suggestive of EHV infections, and 26 aborted fetuses with various organ samples were collected. DNA extraction and PCR analysis were performed on these samples. The study started with PCR optimization using reference strains of EHV-1 (89c25p) and EHV-4 (TH20p) DNA. Finally optimized was completed and reference strains were used for this study. In conclusion, PCR successfully detected and differentiated 7 EHV-1 positive samples from the tissues of the 26 aborted fetuses, as well as one EHV-1 positive and two EHV-4 positive samples from the nasal swabs of the 98 cases. This study represents one of the pioneering works where PCR was firstly employed to detect and differentiate EHV-1 and EHV-4 strains in Türkiye. The study's findings reveal the presence of both EHV-1 and EHV-4 in Türkiye's racehorse population and is among the early reports to identify the existence of EHV-4 using PCR. These findings underscore the circulation of both viruses within the racehorse population. As a result of this study, it has been concluded that the PCR method is a sensitive, cost-effective, and time-saving diagnostic approach for detecting and distinguishing EHV-1 and EHV-4 infections.

Keywords: EHV-1, EHV-4, Abortus, Fetus, Horse, PCR

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1. Introduction

Herpesviruses belong to the Herpesviridae family and are classified into three subfamilies: Alphaherpesvirinae, Gammaherpesvirinae, and Betaherpesvirinae (Murphy et al., 1999; Newton et al., 2000; van Regenmortel et al., 2000). Extensive studies have identified herpesviruses across diverse animal species, including humans. EHV-1 primarily affects domestic horses, although there is serological evidence of sporadic and experimental infections in donkeys, domestic cattle and captive camelids and cervids. Conversely, EHV-4 exclusively infects domestic horses (Gupta et al., 2000; van Regenmortel et al., 2000).

Herpesviruses possess double-stranded, linear DNA with a G + C content of 32-75 mol% and molecular weight ranging from 80 to 150 × 10^6 (MW). The capsid exhibits an icosahedral structure, containing 150 hexameric and 12 pentameric capsomers (van Regenmortel et al., 2000). Envelope glycoproteins, prominent in alphaherpesviruses, play vital roles in infectivity and pathogenicity. Moreover, serve as primary antigenic structures for eliciting immune responses in the host animal (Packiarajah et al., 1998; Csellner et al., 2000). Notably, antibodies targeting EHV-1 and EHV-4 predominantly recognize envelope glycoproteins (Crabb et al., 1993; Telford et al., 1992; Tsujimura et al., 2001). For these reasons, envelope proteins are preferred in the development of recombinant subunit vaccines produced using DNA technology. (Packiarajah et al., 1998). Various viral proteins have specific functions. For instance, glycoprotein (gD), encoded by gene 72, is a membrane protein crucial for cell-to-cell fusion and virus entry in EHV-1 Glycoprotein (gE), arising from gene 74, is another membrane protein with a significant role in enhancing viral virulence and facilitating cell-to-cell spread. Notably, gE is dispensable for viral replication, attachment, and penetration in vitro environments (Damiani[a,b] et al., 2000; Matsumura et al., 1998). These studies highlight the multifaceted nature of herpesvirus proteins and their effects on virulence and infection progression.

The primary mode of transmission for EHV-1 and EHV-4 is through the aerosol route, and foals typically become infected within their first year of life (Carvalho et al., 2000). Both viruses are also capable of establishing latent infections, with infected horses becoming carriers. These

carriers can readily transmit the viruses to susceptible individuals during subsequent infections (Carvalho et al., 2000). EHV-1 and EHV-4 infections in horses present with similar clinical signs. These include fever, coughing, rhinotracheitis, and tracheobronchitis (Murphy et al., 1999). Additionally, symptoms such as runny nose, excessive tearing, and swollen mandibular lymph nodes are common (Walker et al., 2000). In cases where EHV-1 targets the central nervous system, clinical signs like unsteady gait and impaired coordination can arise. EHV-1 and EHV-4 infections are often associated with abortion in mares (Başkaya et al., 1968; Donaldson et al., 1998; Gilkerson et al., 1998; van Maanen et al., 2000). Clinical signs include sudden abortion, with or without preceding symptoms, occurring most frequently in the last 4 months of pregnancy (Başkaya et al., 1968; Gilkerson et al., 1998). Notably, EHV-1 causes a significant percentage of herpesviral abortions, with around 98% attributed to this virus (Allen and Bryans, 1986). Abortion cases may sporadically occur or escalate into an outbreak, affecting several mares within a short time span. For example, an outbreak caused by EHV-1 resulted in 52 mare abortions at TJK Izmit pension stud (Yilmaz et al., 1995). There are different studies that report that EHV-1 and EHV-4 infections are observed in Türkiye (Başkaya et al., 1968; Mengi et al., 1996; Gür and Yapici, 2008; Ataseven et al., 2010, Turan et al., 2012; Yildirim et al., 2015).

Symptoms like upper respiratory tract disorders, coughing, sneezing, nasal discharges, weakening, neuropathogenic symptoms, imbalance, incoordination, and nodding have been observed in animals affected by herpesviruses (Newton et al., 2000; Yilmaz et al., 2018). Histopathological examinations of fetuses following EHV-1-related abortion cases have revealed necrotic lesions in the liver and lung, intranuclear inclusion bodies in cells, germinal center necrosis, eosinophilic inclusion bodies, petechial hemorrhage in the brain, bronchiolitis, and pneumonia, clear yellow fluid accumulation in the brain, and pulmonary congestion and edema (Murphy et al., 1999). Effective drug treatments for EHV-1 and EHV-4 infections in horses are lacking. Administering antibiotics to horses with Rhinopneumonitis can help prevent secondary infections and their spread, usually spanning a 4-6 day period. Furthermore, isolating infected horses in warm and quiet environments while providing laxative foods is recommended (Murphy et al., 1999). Hygiene practices are crucial for infection control. Isolating aborted mares in entirely separate locations and discontinuing their contact with other horses is of utmost importance. Pregnant mares should receive vaccination, proper care, and nutrition at different intervals during pregnancy, and they should be quarantined. In cases where this approach is not feasible, vaccinating all horses within the herd becomes crucial (Robinson et al., 1997). Adequate stable hygiene and well-ventilated shelters are also essential to prevent the rapid spread of infection within populations. The diagnosis of infection involves both direct and indirect methods to identify antibodies formed at the later stages of the disease. In recent years, the Polymerase Chain Reaction (PCR) technique, noted for its sensitivity and practicality by various researchers, has gained prominence in disease detection (Drummer et al., 1995; Ishiyama et al., 1996; Yasunaga et al., 2000).

The primary objective of this study was to use polymerase chain reaction (PCR) to detect and differentiate Equine Herpesvirus Type-1 (EHV-1) and Type-4 (EHV-4) viruses within the racehorse population in Türkiye. For this purpose, a total of 98 nasal swab specimens were collected from naturally infected racehorses aged 2 and above, originating from various breeding farms and racehorse hospitals. These specimens were obtained specifically from horses displaying respiratory symptoms suggestive of EHV infections, subjected to DNA extraction, and examined using PCR. In addition to nasal samples, a total of 26 aborted fetuses were examined, with multiple organ and tissue samples subjected to DNA extraction and PCR analysis.

2. Materials and Methods

2.1. Samples and Sample Preparations

Within the scope of this study, a total of 96 nasal swab samples and 26 aborted fetus samples were collected from horses exhibiting natural equine herpesvirus infection symptoms from various racehorse farms and hospitals between 1997-2002. Liver, lung, and spleen tissues were used to prepare homogenized tissue emulsions for the aborted fetus samples. Additional tissues such as kidney, heart, fetal membranes, and umbilical cords were included in the emulsion whenever available. Additionally, 98 nasal swab samples were obtained from different breeding farms and racehorse hospitals, specifically from horses aged 2 and older, exhibiting natural equine herpesvirus infection symptoms. These horses displayed clinical symptoms such as nasal discharge, coughing, mild fever, and epistaxis (nosebleeds), with variations in symptom presentation observed among different animals. Nasal discharge from suspected animals was collected using sterile swabs and diluted in 2 ml of PBS, followed by vortexing for 30 seconds at high speed. The tissues adhered to the cotton swabs were released into the liquid by crushing them with sterile injector tips. The liquid in the tube was drawn using a syringe and transferred to sterile sample collection containers, then stored at –20°C until further examination.

2.2. Positive Controls

Positive controls and optimization of PCR stages were conducted using vacuum-dried DNA from the EHV-1 89c25p strain (52) and EHV-4 TH20p strain (Matsumura et al., 1991), with approximately 50 µg each DNA. The DNA was provided by Dr. Tomio Matsumura from the Epizootic Research Center, Equine Research Institute, The Japanese Racing Association.

2.3. PCR Core Kit

DNA amplification was carried out using a PCR Core Kit (MB-345) from Sigma company. This kit, along with the standard strain DNAs used in the study, was employed for optimization and PCR amplification of the extracted viral DNA from the study samples.

2.4. Mineral Oil

Mineral oil was used to the PCR master mix to prevent evaporation during the PCR process (Fluka BioChemika-69794).

2.5. Ethidium Bromide

Ethidium bromide (Fluka BioChemika-46065) was employed to visualize the PCR products generated during electrophoresis under UV light. An Ethidium bromide (EtBr) solution was prepared by placing 1g of EtBr into a 100 ml glass cylinder, adding 1ml of 95% ethanol, and gently stirring for 5 minutes on a magnetic stirrer. The solution was allowed to dissolve, followed by adjusting the final volume to 100 ml with distilled water.

2.6. Agarose Gel Preparation

1% agarose gel required for electrophoresis was prepared using agarose (A-9539) supplied by Sigma.

2.7. DNA Ladder (Marker)

A DNA ladder (50-2000 bp) from Bio-Rad (170-8200) was used to determine the molecular weights of PCR products (DNA bands) generated during gel electrophoresis.

2.8. DNA Extraction Kit

For DNA extraction from aborted fetus organs, the GenElute Mammalian Genomic DNA kit (Sigma MB-660/G1N70) was employed. Meanwhile, the InstaGene-Matrix DNA isolation kit (Bio-Rad 732-6030) was used for DNA extraction from nasal swab samples.

2.9. Loading Buffer

10X loading buffer, obtained from Takara (A152), was used to load PCR products into the gel pits and was formulated as follows: Tris Base: 48.4 g, EDTA: 7.4 g, Sodium acetate: 16.4 g, Glyceal acetic acid: 17 ml. The mixture was made up to 1000 ml with distilled water.

2.10. Taq Polymerase Enzyme

The Taq DNA polymerase enzyme was obtained from Sigma company (MB-300 / D1806) at a concentration of 5u/µl and used in the study. This enzyme was added to the PCR reaction as a component of the master mix to amplify specific DNA sequences during the PCR reaction,

2.11. Primers

Synthetic oligonucleotide primer pairs were designed to exhibit complete homology for both EHV-1 and EHV-4 viruses. These primer pairs were selected from the gB gene and had the following sequences:

- Forward Primer: 5'-GGA TGC CAT GGA GGC ACT ACA-3'
- Reverse Primer: 5'-GTT TGG CGG TGA CGT TGG AAG-3'

These primers were utilized in the polymerase chain reaction to determine the genomic DNA of EHV-1 and EHV-4 viruses. The anticipated product sizes were 840 bp for EHV-1, 649 bp for EHV-1 using the gC primer sequences, and 507 bp for EHV-4 using the gC gene primers.

2.12. Polymerase Chain Reaction (PCR)

The reconstitution of reference EHV-1 and EHV-4 DNA followed the protocol established by Matsumura. The vacuum-dried DNA extracts of EHV-1 and EHV-4 strains from Japan were reconstituted by adding 100 µl of autoclaved double-distilled sterile water to each tube, resulting in a concentration of 0.5 µg/µl in each tube. The initial solutions were further diluted with distilled water to create DNA dilutions. The diluted DNAs were divided into 10 aliquots of 100 µl each to minimize the risk of contamination and were subsequently stored at –20 °C alongside the original solutions.

2.12.1. PCR standardization

For PCR optimization, the protocol described by O'Keefe et al. (1991) was adapted and modified. A primer pair from the gB gene region, exhibiting homology for both EHV-1 and EHV-4 viruses, was utilized at a concentration of 50 pmol. The amounts of reference DNA template were systematically varied (1µl, 2µl, 3µl, 4µl, and 5µl) to ascertain the optimal quantity of DNA required for the PCR reactions (Table 1).

Table 1. 100 µl PCR reaction, the following components were mixed together

10 µl 10X PCR buffer
2 µl dNTP mix
2 µl Primer gB/F
2 µl Primer gB/R
0.5 µl Taq DNA polymerase enzyme
2 µl template DNA (average 10 ng)
81.5 µl distilled water
50 µl mineral oil

2.12.2. PCR amplification and gel electrophoresis

Following the outlined procedures, the mixtures were subjected to the following PCR conditions: 94°C for 5 minutes, 94°C for 75 seconds (30 cycles), 60°C for 90 seconds (30 cycles), and 72°C for 90 seconds (30 cycles) using a PCR thermocycler (Biometra) device. After amplification, the mixture was stored at 4°C until electrophoresis. The resulting PCR products were then subjected to gel electrophoresis at 100 Volts for 25 minutes. The final products containing ethidium bromide were visualized under UV light using a trans illuminator (Biometra) device and photographed. During this amplification process, the methods outlined by Lawrence et al. (1993) and Matsumura[a,b] et al. (1994) were followed.

2.12.3. PCR from type-specific oligonucleotide primer pairs

Type-specific oligonucleotide primer pairs, designed from the gC gene region of EHV-1 and EHV-4, were also employed to analyze the same samples. The contents of the mixture loaded into the thermocycler device are provided in Table 2. The amplification process was conducted with the following conditions in the PCR thermocycler (Biometra) device. Amplification was

performed with the following cycle conditions: 1 cycle at 94 ° C for 5 min, 30 cycles of 94 ° C for 75 secs, 60 ° C for 90 secs, and 72 ° C for 90 sec. The PCR products were then subjected to 25-minute horizontal gel electrophoresis at 100 Volt electric current. The final products containing ethidium bromide were examined using a trans illuminator (Biometra) device under UV light and photographed.

Table 2. The amplification mixture of using type-specific oligonucleotide primer pairs from the gC gene region of EHV-1 and EHV-4

For 50 µl PCR reaction for EHV-1	For 1 µl PCR reaction for EHV-1
5 µl 10X PCR buffer	5 µl 10X PCR buffer
1 µl dNTP mix	1 µl dNTP mix
1 µl Primer gC 1 F	1 µl Primer gC 4 F
1 µl Primer gC ¼ R	1 µl Primer gC ¼ R
0.25 µl Taq DNA polymerase enzyme	0.25 µl Taq DNA polymerase enzyme
1 µl template DNA	1 µl template DNA
40.75 µl dH20	40.75 µl dH20
50 µl mineral oil	50 µl mineral oil

2.13. DNA Extraction from Aborted Fetal Tissues

DNA extraction from aborted fetal tissues was conducted following the protocol recommended by the kit manufacturer. Various organs from each of the 26 aborted fetuses were sampled and documented. The collected tissues were stored in containers kept on ice. The tissues were subsequently diced using sterile scalpels and homogenized. A mixture of 25 mg of the organ was placed into sterile micro centrifuge tubes, and tissue homogenization was achieved using sterile single used toothpicks. Then, 180 µl of lysis buffer and 20 µl of proteinase-K were added to the tubes, followed by vortexing, and incubation at 55°C for complete digestion (3 hours). After adding 200 µl of melting solution and incubating at 70°C for 10 minutes, 200 µl of ethanol was introduced, vortexed, and the mixture was transferred to a holding column. Post centrifugation at 6500 rpm for 1 minute, the column was washed with 500 µl of wash solution. Subsequently, the column was dried by centrifuging at 12000 rpm for 3 minutes, and elution solution (200 µl) was applied to elute the purified DNA.

2.13.1. PCR from extracted fetal organs DNA

In order to identify the presence of EHV-1 and EHV-4 DNA in potentially affected organs, the primer pairs targeting the gB gene region were employed. The approach, adapted from O'Keefe et al. (1991), was employed. The ensuing protocol was implemented for a 100 µl PCR reaction as prescribed in Table 1. Subsequently, the mixtures underwent the following temperature, time, and cycle amplification in a PCR thermocycler (Biometra) device: 32 cycles of 94 °C for 5 minutes, 94 °C for 1 minute, 55 °C for 2 minutes, and 72 °C for 3 minutes. The amplified products were then stored at 4°C until the electrophoresis step. Gel electrophoresis was performed on the PCR products for 1 hour at 90 Volts electric current. The resulting products containing ethidium bromide were visualized under UV light using a trans illuminator (Biometra) device and subsequently photographed.

2.14. DNA Extraction from Nasal Swab Samples

For the purpose of DNA extraction from nasal swab samples, the extraction method recommended by the manufacturer Bio-Rad was followed. During testing, 200 µl of the samples were taken into sterile tubes and centrifuged at 12000 rpm for 1 minute. The upper liquid was discarded, and the sediment was suspended in 1 ml of PBS, with subsequent removal of the upper liquid. The sediment was then suspended in 20 µl of distilled water. To this mixture, 200 µl of InstaGene matrix was added and incubated at 56°C for 30 minutes. The mixture was vortexed for 10 seconds at high speed, followed by an 8-minute incubation in a water bath at 100°C. After re-vortexing for 10 seconds and centrifuging at 12000 rpm for 3 minutes, 20 µl of the supernatant was used in PCR experiments. This final step was repeated with each new PCR trial.

2.14.1. PCR from nasal swab samples

The nasal swab samples were subjected to examination using the gC oligonucleotide primer pairs specific to EHV-1 and EHV-4. The amplification process adhered to the methods established by Lawrence et al. (1993) and Matsumura[a,b] et al. (1994). The contents of the mixture loaded into the thermocycler device are provided in Table 2. The amplification of these samples was carried out in PCR thermocycler device (Biometra), following the temperature, time, and cycle conditions were 1 cycle at 94°C for 5 minutes, 94°C for 75 seconds, 60°C for 90 seconds, and 72°C for 90 seconds at 30 cycles. Stored at 4°C until electrophoresis was performed. The PCR products obtained were then subjected to 25-minute horizontal gel electrophoresis treatment at 100 Volt electric current. The final product containing ethidium bromide was examined using a trans illuminator (Biometra) device under UV light and photographed.

3. Results

3.1. PCR Results of Aborted Fetuses

Based on the PCR test results, genomic DNAs of EHV-1 were identified in 7 out of 26 samples derived from tissue and organ homogenates of aborted fetuses, yielding a product size of 649 bp. This identification was achieved using the gC1 F and gC¼ R primers (Fetus

samples No: 1, 2, 5, 6, 15, 16) (Table 3). However, the genomic DNA of EHV-4 could not be detected among the 26 samples examined (Figure 1).

Table 3. List of samples with detected EHV-1 and EHV-4 genomic DNA

Samples	Results
26 aborted fetal organs and tissues	*EHV-1:* 7 viral DNA were detected *EHV-4: No* viral DNA detected
98 nasal swab samples	*EHV-1*: 1 viral DNA were detected *EHV-4*: 2 viral DNA were detected

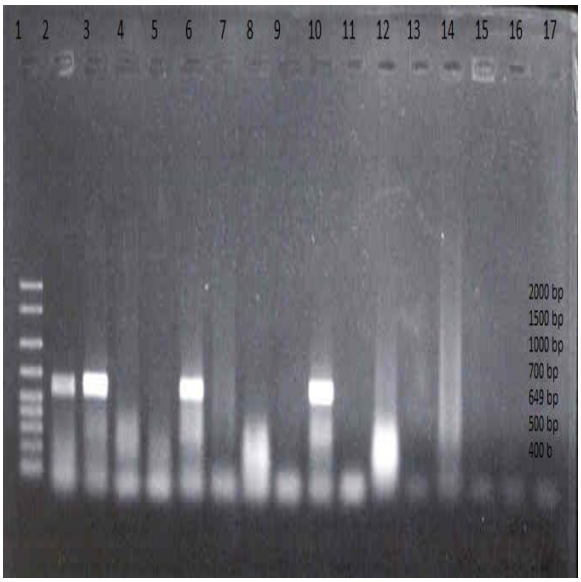

Figure 1. PCR Results: EHV-1 genomic DNA, detected in aborted fetus samples, yielded a 649 bp product using gC1 F and gC¼ R primers, as verified through horizontal gel electrophoresis. The lanes on the gel are as follows: Lane 1 - DNA ladder, Lane 2 - EHV-1 Positive control (89c25p/TH20p reference DNA at 10 ng), Lanes 3, 6, and 10 - EHV-1 positive fetal DNA samples, Lanes 4, 5, 7, 8, 9, 11, 12, and 13 - EHV-1 negative samples, and Lane 14 - Negative control.

3.2. PCR Results from Nasal Swab Samples

The results indicated that two samples (Sample No: 51 and 57) tested positive for EHV-4, while the remaining 96 samples tested negative. Similarly, one out of the 98 samples (Sample No: 55) tested positive for EHV-1, with the other 97 samples being negative. The PCR products from EHV-4 positive samples presented a genomic DNA size of 507 bp, whereas those from EHV-1 positive samples displayed a size of 649 bp (Table 3 and Figure 2).

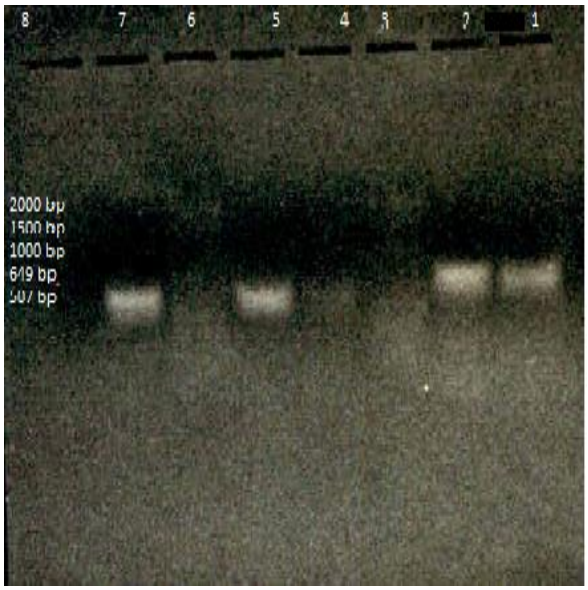

Figure 2. PCR Results: Genomic DNA of both EHV-1 and EHV-4 was detected in nasal swab samples at 649 bp and 507 bp, respectively, using gC1 F and gC¼ R primers. This detection was verified through horizontal gel electrophoresis. The lanes on the gel are as follows: Lane 1 - EHV-1 positive DNA, Lane 2 - EHV-1 Positive control gC1f/r89c25p reference DNA, Lanes 3 and 6 - DNA ladder, Lanes 4 and 7 - EHV-4 positive DNA, Lane 5 - Positive control TH20p reference DNA gC 4 f/r, and Lane 8 - Negative control.

4. Discussion

Herpesviruses play a significant role in causing abortions in horses (Başkaya et al., 1968; Gilkerson et al., 1998; O'Callaghan et al., 1999). Studies conducted worldwide (Matsumura et al., 1991; Murphy et al., 1999; van Maanen et al., 2000) have demonstrated that most abortion cases are linked to EHV-1 infections, while EHV-4 is rarely implicated. Past research in our country has also highlighted the occurrence of EHV infections (Başkaya et al., 1968; Yilmaz et al., 1995; Mengi et al., 1996). The EHV-1 and EHV4 infections were presented in the indigenous horse population in the Eastern, North Eastern, Central and Western Anatolia of Türkiye (Gür and Yapici, 2008; Ataseven et al., 2010, Turan et al., 2012; Yildirim et al., 2015). In diagnosing EHV-1 and EHV-4 infections in horses, samples are typically taken from fetal organs when nasal discharge, buffy coat, or abortion is observed. However, serological tests may not always reliable results. In such cases, alternative diagnostic methods are essential. PCR has gained prominence as a rapid and dependable diagnostic approach, as confirmed by various researchers (Carvalho et al., 2000; Kennedy et al., 1996).

This study involved analyzing extracts obtained from homogenized tissues of various organs (lung, liver, spleen, fetal membranes, and kidneys) from 26 aborted fetuses originating from different studs. Polymerase chain reaction (PCR) was employed, and the genomic

DNA of EHV-1 was detected in seven samples, confirming EHV-1 infection. However, the focus of this study was solely on determining the genomic DNA of EHV-1 and EHV-4 viruses.

When considering the findings of this study alongside compatible fetal pathologies and the positive serological test results reported by Yilmaz (1971), where the isolated substance was neutralized by positive control serum and positive titers were detected through complement fixation, it indicates the presence of EHV-1 in Turkey. This presence has been established through both direct and indirect methods. In this study, genomic DNAs of EHV-4 and EHV-1 were detected in two and one of the nasal swab samples, respectively. The primary focus of the study was not to determine the infection's severity, thus there's no comparison made in this regard. However, considering that the DNAs of EHV-1 and EHV-4 used as positive controls were detectable with appropriate results, it can be concluded that the PCR detection of EHV-1 and EHV-4 is a suitable method, corroborated by findings from Sharma et al. (1992).

Throughout the study, it was observed that a 649 bp band was consistently formed in the PCR examination of homogenate prepared from organs like lung, liver, kidney, and spleen of the 26 fetuses. When these results were compared to reports from other researchers regarding PCR's efficacy, it became evident that the test exhibited high sensitivity. It was noted that variations in bp results could arise due to differences in primers and reaction conditions employed. Notably, the test was swift and dependable, yielding results within a short span of 24 hours. Ballagi et al. (1990) highlighted the concurrence between PCR and virus isolation results, stressing the economical, timesaving, and reliable nature of the test.

Carvalho et al. (2000) reported the detection of EHV-1 genomic DNA in various equine populations, including healthy-looking mares, fetuses, foals, stallions, and aborted fetuses. Consequently, a highly sensitive technique such as PCR is imperative for unveiling latent infections. Edington et al. (1994) reported identifying 87.5% of genomic viral DNA in bronchial and trigeminal ganglia of slaughtered horses through polymerase chain reaction. Their findings suggested that EHV-1 and EHV-4 latent infections were prevalent in the equine population, predominantly in respiratory lymph nodules.

Various studies have demonstrated that polymerase chain reaction can detect the genomic DNA of numerous disease-causing agents or microorganisms. In the context of this study, different primers specific to EHV-1 and EHV-4 were employed to differentiate between aborted fetuses and nasal swab samples. This polymerase chain reaction technique successfully revealed the genomic DNAs of EHV-1 and EHV-4 viruses within these samples.

This study marks the pioneering attempt in Türkiye to detect EHV-1 and EHV-4 using PCR. The findings affirm the presence of both viruses within Türkiye's equine population. Significantly, this study holds significant results as one of the pioneering works confirming the presence of the EHV-4 virus in Türkiye. Moreover, the research demonstrates the feasibility of using PCR as an effective means to differentiate between these two infectious agents, EHV-1 and EHV-4. Furthermore, the study concludes that the PCR technique not only provides economic benefits but also offers reliability and efficiency in diagnosing EHV-1 and EHV-4 infections. This research contributes to enhancing our understanding of the prevalence of these viruses in Türkiyes' horses and establishes PCR as a valuable tool for accurate diagnosis. Other studies conducted in our country confirm the existence of both viruses, in consistence with our findings, and indicated that they are still circulating among Türkiyes' horse population (Başkaya et al., 1968; Yılmaz et al., 1995; Gür and Yapici, 2008; Ataseven et al., 2010; Turan et al., 2012, Yildirim et al., 2015). Further studies are needed to investigate the prevalence and distribution of EHV-4 in the examined population.

Author Contributions

The percentage of the author(s) contributions is present below. All authors reviewed and approved final version of the manuscript.

	B.K.T.	Ö.A
C	90	10
D	100	
S	90	10
DCP	100	
DAI	100	
L	90	10
W	100	20
CR	90	10
SR	90	10
PM	50	50
FA	50	50

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Approval/Informed Consent

No animal experiments or tests were conducted in this study. Routine sampling and diagnostic procedures were carried out with the consent of the clients on naturally infected animals under veterinary supervision. According to the directive dated 28.06.2017 with reference number 138806 from the General Directorate of Nature Conservation and National Parks of the Ministry of Environment and Forestry of the Republic of Turkey, obtaining an ethics committee approval is not necessary for this study.

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