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Absence of active Equine Herpesvirus Type 1 detected by qPCR in asymptomatic high-risk horses from

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Short Report

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Abstract

Equine herpesvirus type 1 (EHV-1) is a highly contagious pathogen of major clinical and epidemiological importance in horses, capable of establishing lifelong latency with periodic reactivation under stress. This study investigated EHV-1 circulation in horses exposed to increased epidemiological risk, particularly those participating in large-scale equestrian events, through clinical evaluation, hematological analysis, and molecular detection. Between July and September 2025, 111 unvaccinated horses from southern Brazil were clinically assessed, and blood and nasal swab samples were collected. Hematological analyses were performed to support clinical interpretation. Viral DNA was extracted using a magnetic bead-based protocol, and EHV-1 was detected by quantitative polymerase chain reaction (qPCR) targeting the glycoprotein B (gB) gene. No clinical or hematological abnormalities consistent with active EHV-1 infection were identified. Positive controls yielded significantly lower cycle threshold (Ct) values (mean Ct = 20.09) than field samples (plasma: 38.56; buffy coat: 37.93; nasal swabs: 37.53; $P < 0.05$), indicating the absence of detectable viral replication in the sampled population. Although EHV-1 was not detected at levels consistent with active infection, the elevated Ct values observed in field samples may reflect low-level viral circulation or latent infection below the assay's detection threshold. These findings highlight the value of continuous molecular surveillance and sensitive diagnostic strategies to support biosecurity programs and reduce the risk of EHV-1 outbreaks in horse populations subjected to transportation, crowding, and other stress-associated conditions.

Introduction

Equine herpesvirus type 1 (EHV-1) is one of the most clinically and epidemiologically significant viral pathogens affecting horses and is associated with respiratory disease, abortion, neonatal mortality, and equine myeloencephalopathy (Pusterla et al. 2009; Soboll-Hussey et al. 2024). Its high transmissibility, ability to establish lifelong latent infections, and potential for reactivation under stress make EHV-1 a constant threat to equine health and to the productivity of the equine industry. Stressful situations such as transportation, intensive training, and parturition favor viral reactivation and shedding, often in the absence of evident clinical signs, making subclinically infected horses a critical factor in maintaining viral circulation within equine populations (Mori et al. 2003).

Recently, researchers have reported ten neurological outbreaks in the state of Pará in Brazil, where 88% of clinically affected horses tested positive for EHV-1 by PCR, confirming viral circulation in the Amazon region and emphasizing the importance of including this pathogen in the differential diagnosis of neurological disorders in horses (Barbosa et al. 2022). In the state of Rio Grande do Sul (RS), the equine population is estimated at approximately 820,000 animals, while at Brazil hosts about 5.7 million equids (IBGE). The regions selected for this study represent roughly 11% of the equine population in RS and are located in an area strongly associated with cultural, sporting, and work-related equine activities. Given the substantial equine population and the intense animal movement associated with recurrent medium- and large-scale events in these regions, there is a pressing need for continuous epidemiological

surveillance and the implementation of reliable diagnostic methods to support effective field monitoring and mitigate viral spread.

Real-time polymerase chain reaction (qPCR) is currently considered the diagnostic method of choice for EHV-1 due to its high sensitivity and specificity (Kambayashi et al. 2026; Pusterla et al. 2022a). Detection of viral DNA in nasal secretions and blood allows identification of different stages of infection and contributes substantially to epidemiological surveillance and outbreak control (Pusterla et al. 2023). In Brazil, vaccination against EHV-1 is not mandatory, and available national studies have primarily focused on diagnostic findings and outbreak reports rather than vaccination practices or preventive strategies. In this context, the present study aimed to investigate the circulation of EHV-1 in horses exposed to large-scale events and crowding in the Sinos and Paranhana Valley regions of Southern Brazil through clinical monitoring, hematological evaluation, and qPCR molecular diagnosis detection.

Material and methods

Study design and sample collection

The methodological design is a prospective cohort study, with systematic observation in a field environment and primary data collection through structured questionnaires and laboratory analyses. Animals included in the study were selected based on specific epidemiological criteria: horses not vaccinated against herpesvirus, housed in facilities (such as barns, hotels, and training centers), and with a history of recent participation in events with large animal population, especially sporting and reproductive events. The study was conducted between July and September 2025, covering the municipalities located in The Sinos and Paranhana Valley in the state of Rio Grande do Sul (RS), Southern Brazil (Fig. 1). The properties' location was identified by region to preserve anonymity and to facilitate epidemiological analysis (Supplementary Fig. 1). A total of 111 horses were used in the study, and all animals had recently participated in large-scale events, except for the pregnant mares. Sample collection was performed in each animal and comprehends, two nasal swab samples (one per nostril) and one whole blood sample, in compliance with the sanitary and operational management of the properties. Additional information, such as breed, sex, age, location, clinical signs, and health history, are demonstrated in Table 1.

Table 1
General characteristics of the study population of horses in The Sinos and Paranhana Valley in Southern Brazil.

Characteristics		**Number (n)**	**Percentage (%)**
Age	< 5 years	13	11.71
	5–10 years	53	47.75
	> 10 years	45	40.54
	Total	111	100
	Male	34	30.63
Gender	Female	77	69.37
	Total	111	100
Reproductive Status	Not Pregnant	53	47.75
	Pregnant	24	21.62
	Gelding	32	28.83
	Stallion	2	1.80
	Total	111	100
Clinical Signs	Nasal Secretion	45	56.76
	No Nasal Secretion	63	40.54
	Others*	3	2.70
Vaccination Status	Total	111	100
	Vaccinated**	12	10.8
	Not Vaccinated	99	89.2
	Total	111	100

* Musculoskeletal system (Sole abscess; 3rd Metatarsus Fracture, and Joint Injury); **Animals were vaccinated against Leptospirosis.

Aliquots of whole blood were aseptically collected by jugular vein puncture using a vacuum system and tubes were stored in coolers containing recyclable ice to maintained at a temperature of approximately 8°C during transport to the Feevale University Veterinary Hospital. In the laboratory, samples for hematological analysis were stored refrigerated (4°C) and performed at the Clinical Pathology Laboratory at Feevale University. Samples for molecular detection of EHV-1 by qPCR were processed on the same day of collection. Whole blood was centrifuged at 3000 x *g* for 15 minutes to obtained biological fraction of plasma, buffy coat, and red blood cells. Nasal swab samples were aseptically

collected per animal, one from each nostril, using sterile swabs. Immediately after collection, the swab shaft was cut using disinfected scissors to avoid contact with the collected biological material. The segment containing the sample was then inserted into sterile 15 mL Falcon tubes containing 2 mL of sterile saline solution (0.9% NaCl) as a transport medium. Samples were stored in coolers containing recyclable ice at a temperature of approximately 8°C. In the laboratory, each sample was vortexed for 30 seconds to homogenize the collected material. After fractionation, each sample was transferred to sterile 2 mL microtubes and stored at -20°C until viral DNA extraction and qPCR analysis.

Molecular analysis

DNA was extracted and purified using the MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher, USA) following a magnetic bead-based protocol involving sample lysis, nucleic acid binding to magnetic beads, sequential washing, and elution. The extracted DNA was quantified and assessed for purity by NanoDrop 2000 spectrophotometry (A260/A280) and stored at -20°C until further molecular analysis. The target sequence for qPCR was a region of the EHV-1 Glicoproteína (gB) gene (GenBank #M36298) to achieve selective amplification of a 106 base pair sequence from only the EHV-1 gB gene (Hussey et al. 2006). Forward and reverse PCR primers and a fluorogenic probe were manufactured by Exxtend Biotechnologies Ltda (Paulina, SP, Brazil). The sequence of the forward primer annealing to EHV-1 gB was 5'-CATACGTCCCTGTCCGACAGAT-3'. The sequence of the reverse primer annealing to EHV-1 gB was 5'-GGTACTCGGCCTTTGACGAA-3'. The sequence of the 25-oligonucleotide probe annealing to EHV-1 gB was 5'-TGAGACCGAAGATCTCCTCCACCGA-3', and included the 5' reporter dye 6-carboxyfluorescein (FAM) and 3' Black Hole Quencher dye (BHQ1). Primers and probe were diluted to 100 mm in RNase/DNase free sterile water, aliquoted, and stored at -20^{O}C. qPCR assays included DNA from the EHV-1 Kentucky reference strain as a positive control and nuclease-free water as a negative control. Reactions were performed in a final volume of 20 µL containing 10 µL of 2× qPCR Probe Master Mix, 1 µL each of forward and reverse primers (20 pmol/µL), 1 µL of probe (10 pmol/µL), 2 µL of nuclease-free water, and 5 µL of template DNA. Thermal cycling consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. Amplification and fluorescence detection were performed using a CFX™ Real-Time PCR System (Bio-Rad, USA).

Statistical analysis

Statistical analyses compared qPCR Ct values among sample matrices using the Kruskal-Wallis test after non-normality was confirmed by the Shapiro-Wilk test. Descriptive statistics were used to summarize animal and farm characteristics, with continuous variables reported as mean ± SD and range, categorical variables as frequencies and percentages, and significance set at $P < 0.05$ using SPSS 20.

Results

Animals evaluated in this study had a mean body weight of 400.32 ± 26.39 kg and a mean age of 9.92 ± 5.73 years. Only 12 animals (10.8%) had been vaccinated against leptospirosis and none had been vaccinated against EHV-1. The hematology results were within the physiological ranges reported for horses, with no variations exceeding the established reference limits. Therefore, the findings do not suggest hematological deviations that could interfere with clinical interpretation. In addition, the findings indicated an absence of hematological alterations in the horses included in this study (Supplementary Table 2).

The EHV-1 positive control was reliably amplified using its specific primer pairs in qPCR, whereas all negative controls showed no amplification. In general, positive control consistently yielded low Ct values, with a mean of 20.09 ± 5.95, while quantitative field samples exhibited markedly higher Ct values, with an overall mean of 38.13 ± 2.29. Comparative statistical analysis of Ct values revealed significant differences in quantitative detection ($P < 0.05$) between the positive controls and all non-control sample types, including buffy coat, plasma, and swab samples (Table 2). The amplification plot further demonstrated a slight increase in Ct values among the field samples, with mean Ct values of 37.94 ± 3.45 for buffy coat fractions, 38.56 ± 0.93 for plasma, and 37.53 ± 2.44 for swabs, compared to the mean positive control (Fig. 2).

Table 2
qPCR Cycle Threshold (Ct) values are presented as mean ± standard deviation (SD) and range (Min-Max) across all relevant samples observations from horses in the Sinos and Paranhana Valleys of Southern Brazil.

Specimen types	**n**	**Mean ± SD**	**Min–Max**
Buffy Coat	10	37.94 ± 3.45[a]	28.21–39.79
Plasma	13	38.56 ± 0.93[a]	36.72–39.85
Swab	6	37.53 ± 2.44[a]	32.74–39.51
Positive Control	8	20.09 ± 5.95[b]	17.53–34.79
Different superscript letters denote statistically significant differences between sample types (Kruskal–Wallis indicated a significant group effect; $P < 0.05$).			

Discussion

The results from our study suggest that no active EHV-1 infection was present in the horses at the time of sampling, despite the presence of conditions typically associated with viral reactivation, including stress, crowding, immunosuppression, and recent involvement in events. The implementation of accurate and rapidly performing diagnostic methods is essential for the effective control of EHV-1, especially given its ability to establish latent infection and the wide range of clinical manifestations, which range from mild respiratory symptoms to abortions and equine herpetic myeloencephalopathy (de la Cuesta-Torrado et al. 2025; Pusterla et al. 2022b). In addition, Perkins et al. (2008) findings highlighted

that high Ct values often correspond to low viral DNA quantities, which may reflect latent or subclinical infections rather than active replication. Our observation of significantly higher Ct values in field samples compared to positive controls aligns with these reports.

In outbreaks, the lack of early diagnosis significantly limits the containment of viral spread, which can have substantial economic, and livestock impacts on the horse industry. Machado et al. (2023) emphasized that, despite the availability of vaccines, these do not prevent the neurological form of the disease, making strict biosecurity measures and contingency plans essential to contain outbreaks. In this scenario, the qPCR technique stands out as a highly sensitive and specific diagnostic tool, allowing the detection of viral loads even in the early stages of infection (Oladunni et al. 2019; Pusterla et al. 2023). The qPCR validation performed in this study, using a DNA of the Kentucky standard strain of EHV-1 extracted from RK13 cell culture, ensures the methodological robustness of the analysis and reinforces its applicability for EHV-1 screening in field samples.

In this study, descriptive analysis of EHV-1 qPCR results across the three matrices showed that swab, plasma, and buffy coat extracts produced similar high-cycle, low-template amplification profiles. Plasma samples were the most homogeneous, showing a tight cluster of Ct values near the upper detection limit. Buffy coat fraction and swab extracts displayed slightly broader variability, yet still within the same high-cycle range. These findings demonstrate that the assay performed consistently across all three sample types, yielding similar Ct profiles for low-concentration detections. The use of whole blood and nasal secretion samples is supported by the literature, which identifies these samples as the most suitable for detecting the virus at different stages of infection (Hussey et al. 2006; Perkins et al. 2008; Smith et al. 2018). Recently, Pusterla et al. (2023) reported that qPCR detects EHV-1 more reliably in nasal secretions from naturally infected horses, whereas experimental studies show similar sensitivities in nasal secretions and blood, with shedding and viremia starting 1–2 days post-infection and lasting up to a week or longer.

These results may reflect a period of viral latency with viral loads below the detection limit or an absence of active viral circulation in the sampled population, which may be associated with effective sanitary management, reduced regional antigenic pressure, or the restricted window for viremia detection (Giessler et al. 2020). However, the possibility of latent or subclinical infection reinforces the importance of continuous surveillance, combining molecular, serological and longitudinal assessments for a deeper understanding of the epidemiology of EHV-1 and supporting effective control and prevention strategies (Diallo et al. 2007; Lunn et al. 2024; Pusterla et al. 2022a). Our observation of significantly higher Ct values in field samples compared to positive controls is consistent with previous qPCR studies in horses, which demonstrated that elevated Ct values reflect low viral DNA loads characteristic of latent or subclinical EHV-1 infection (Perkins et al. 2008; Tallmadge et al. 2025).

It is important to highlight that, in the studied region, vaccination against EHV-1 is segmented and directed primarily to pregnant mares in the 5th, 7th, and 9th months of gestation, with low uptake among stallions, lactating mares, and competition horses, even in high-density events. Because vaccination is

not mandatory, immunological heterogeneity within the equine population is common and may facilitate subclinical or intermittent viral circulation (Silva et al. 2021). The absence of a strong positivity result may reflect low viral circulation during the study period, effective biosecurity practices implemented on the properties, or constraints related to infection stage or sampling timing. Notably, viral latency does not necessarily imply detectable viremia or active nasal shedding, which may hinder detection in single-point sampling strategies (Mori et al. 2009).

Another relevant aspect of this study was the inclusion of parallel hematological analyses. The findings revealed no alterations in the hematological profiles, confirming that the horses evaluated were clinically healthy. In veterinary medicine, hematological assessment, in combination with other diagnostic indicators, plays a central role in determining the health status of adult and young horses. Although molecular testing is considered the gold standard for detecting EHV-1, alterations in hematological parameters can assist in identifying potential cases of systemic infection or viral reactivation. Patterns such as leukopenia, lymphocytosis, or changes in red blood cell count, when interpreted in conjunction with clinical signs and epidemiological history, can support the selection of animals for targeted molecular testing in field conditions, thereby optimizing diagnostic resources in large horse management contexts. Given the complexity and dynamism of the Brazilian equine industry, there is a growing need for health surveillance strategies based in scientific evidence and supported by efficient diagnostic tools. The application of qPCR as a screening method for at risk animals, such as those involved in sporting, reproductive, or frequent transportation activities, enables a more rapid health response, contributing to outbreak prevention and the preservation of animals' health.

Conclusion

Our findings corroborate the established evidence that EHV-1 latency in horses is detectable by qPCR but typically yields high Ct values due to low viral DNA copy numbers. This study demonstrates that field samples with detectable viral or genetic material amplified at substantially higher Ct values in agreement with positive controls that consistently amplified at low Ct values. The magnitude of this difference supports the validity and sensitivity of the assay controls and further indicates that most Ct samples represent weak or low-level detections. Therefore, this study highlights the importance of active surveillance, validated molecular diagnostic methods, and health education as fundamental pillars for the control of emerging and re-emerging viral diseases and for strengthening biosecurity strategies within the equine production sector.

Declarations

Competing interests:

The authors declare no competing interests.

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Author Contribution

José L. A. Terra: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. Larissa Mallmann: Investigation, Methodology, Writing review & editing, Laboratory analysis, Data curation. Nilson J. S. Nunes: Methodology, Writing – review & editing, Data curation. Eduarda L. Pereira: Laboratory analysis, Data curation. Vitória Bandeira: Methodology, Laboratory analysis, Data curation. Ana C. Hugentobler: Methodology, Investigation, Validation. Camilly Braun: Methodology, Investigation, Validation. Luís A. G. Corrêa: Methodology, Investigation, Validation. Mariana S. Silva: Conceptualization, Methodology, Investigation, Writing review & editing. Gabriel R. Pereira: Writing – review & editing, Supervision, Formal analysis, Data curation, Conceptualization.

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Data Availability

The datasets generated during this study are available from the corresponding author upon reasonable request.

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Figures



Figure 1

Geographical location of the study area in Southern Brazil. (A) Position of Rio Grande do Sul State within Brazil (slight brown); (B) Detailed municipal boundaries of Rio Grande do Sul and the highlighted (green) Sinos and Paranhana Valley regions representing the study area. Souce: MapasBR

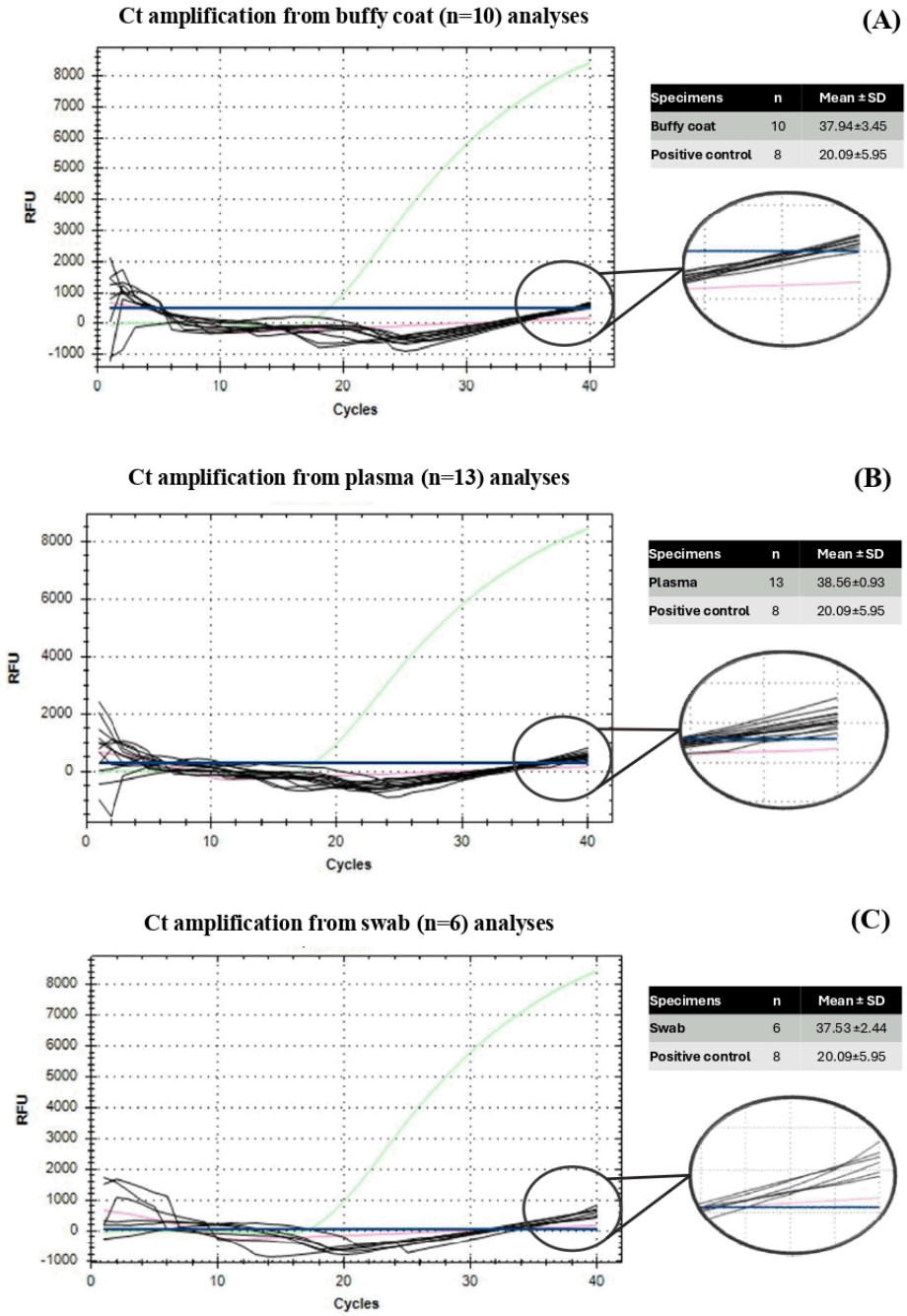


Figure 2

Amplification plots of the Ct standard curve for the probe-based qPCR assay targeting EHV-1 in samples collected from horses. The green line represents the positive control (genomic DNA from the Kentucky strain of EHV-1), the light red line represents the negative control (DNase/RNase-free water), and the black lines represent sample curves with detectable Ct values. (A) Ct amplification plot from buffy coat fractions (n= 10); enlarged view showing increased Ct values (mean Ct = 37.93). (B) Ct amplification plot from plasma (n= 13); enlarged view showing increased Ct values (mean Ct = 38.56). (C) Ct amplification plot from swabs (n= 6); enlarged view showing increased Ct values (mean Ct = 37.53).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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