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Detection of equine herpesvirus-1 (EHV-1) in urine samples during outbreaks of equine herpesvirus myeloencephalopathy

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Abstract

Background: Real-time PCR is the diagnostic technique of choice for the diagnosis and control of equine herpesvirus-1 (EHV-1) in an outbreak setting. The presence of EHV-1 in nasal swabs (NS), whole blood, brain and spinal cord samples has been extensively described; however, there are no reports on the excretion of EHV-1 in urine, its DNA detection patterns, and the role of urine in viral spread during an outbreak.

Objectives: To determine the presence of EHV-1 DNA in urine during natural infection and to compare the DNA detection patterns of EHV-1 in urine, buffy coat (BC) and NS.

Study design: Descriptive study of natural infection.

Methods: Urine and whole blood/NS samples were collected at different time points during the hospitalisation of 21 horses involved in two EHV-1 myeloencephalopathy outbreaks in 2021 and 2023 in Spain. Quantitative real-time PCR was performed to compare the viral DNA load between BC-urine samples in 2021 and NS-urine samples in 2023. Sex, age, breed, presence of neurological signs, EHV-1 vaccination status and treatment data were recorded for all horses.

Results: A total of 18 hospitalised horses during the 2021 and 2023 outbreaks were positive for EHV-1, and viral DNA was detected in urine samples from a total of 11 horses in both outbreaks. Compared with BC samples, DNA presence was detected in urine samples for longer duration and with slightly higher concentration; however, compared with NS, detection of EHV-1 in urine was similar in duration with lower DNA concentrations.

Main limitations: Limited sample size, different sampling times and protocols (BC vs. NS) in two natural infection outbreak settings.

Ana Velloso Alvarez and E. Jose-Cunilleras should be considered joint first authors.

Conclusions: EHV-1 was detected in the urine from naturally infected horses. Urine should be considered as complimentary to blood and NS in diagnosis of EHV-1 infection.

KEYWORDS
diagnosis, EHV-1, horse, outbreak, PCR, urine

1 | INTRODUCTION

Equine herpesvirus type 1 (EHV-1) is an endemic viral disease in many countries around the world.[1,2] Infection with EHV-1 can result in respiratory disease, abortions and neonatal deaths, and outbreaks of neurological disease (equine herpesvirus myeloencephalopathy or EHM).[3,4] EHV-1 is highly contagious and can be easily transmitted between horses through respiratory contact and by contamination with fomites or organic material.[5] EHM typically affects less than 10% of infected horses in EHV-1 outbreaks.[3,6] However, EHM can cause devastating losses during outbreaks[7–11] and the international nature of equestrian sport can lead to rapid dissemination across national boundaries.[10,11]

In suspected EHV-1 outbreaks, rapid diagnosis is vital, and real-time PCR has become the technique of choice.[2,7,12] Accurate diagnosis in outbreaks also relies on obtaining the best possible sample at the best possible time.[13,14] It is recommended that nasopharyngeal swabs should be taken as soon as possible after the onset of symptoms, as EHV-1 nasal shedding is short, and generally greater in the first few days post-infection (1–5 days), when the horses are showing fever.[3] During the viraemic phase (4–10 days post-infection), peripheral blood can be collected in tubes with anticoagulant (lithium heparin or EDTA).[3] EHM may appear following the onset of viraemia, often following a secondary fever spike and in the absence of respiratory signs, typically 6- and 10-days post-infection, at the end of the viraemic phase.[15] Samples from the brain and spinal cord may also be used for viral isolation and PCR.[3]

It is also very important to be able to assess the viral shedding status of affected horses to ensure the absence of infection and allow the movement of negative horses.[2] Information on the presence of EHV-1 in samples other than those routinely used (nasopharyngeal swab, whole blood and brain and spinal cord samples) in the literature is limited; EHV-1 has been found in faecal samples from captive zebras[16] and from foals experimentally infected with EHV-1,[17] and viral DNA has been found in semen of stallions with natural infection,[18,19] but there is no information on the detection of the virus or its DNA in urine samples. Therefore, this study was conducted with the objectives to determine (1) if urine samples could be an option for the molecular detection of EHV-1 in an outbreak setting and (2) evaluate the EHV-1 DNA detection patterns (viral DNA load over time) in urine, blood and nasopharyngeal swabs (NS) from horses involved in EHV-1 outbreaks in Valencia in 2021 and 2023.

2 | MATERIALS AND METHODS

2.1 | Sampling procedures and data collection

2.1.1 | 2021 outbreak

In Madrid, following the arrival of horses from an equestrian event in Valencia, 10 horses with clinical signs were hospitalised at the Complutense Veterinary Clinical Hospital (HCVC). For the current study, samples were collected from six horses between 1 and 15 March 2021 at different time points from the first day of fever shown either at the equestrian premises or at the hospital. Two tubes of 10 mL heparinised blood were collected via jugular venipuncture from all sampled horses at four different time points (Day 6–9, 10–13, 14–17 and 18–22) whereas urine samples were collected from the six sampled horses via urethral catheterisation (horses 1–4) or during spontaneous urination (horses 5, 6) at one to four different time points (Day 0–4, 5–8 and 9–14), depending on the sampled horse.

In Barcelona, nine horses showing clinical signs at the same equestrian event in Valencia were hospitalised at the Veterinary Clinic Hospital UAB (Universitat Autònoma de Barcelona). Samples were collected from five horses between 1 and 8 March 2021 at different time points from the first day of fever shown either at the equestrian premises or at the hospital. Two tubes of 10 mL heparinised blood were collected via jugular venipuncture from all sampled horses at two to six different time points, depending on the sampled horse, whereas urine samples were collected from two horses either by urethral catheterisation (horse 10) or during spontaneous urination (horse 11) at three and two different time points, respectively.

2.1.2 | 2023 outbreak

Ten horses that developed clinical signs at an equestrian event in Valencia and were hospitalised at CEU Cardenal Herrera University Veterinary Hospital (Valencia); all of them were sampled for this study between 21 February and 6 March 2023 at different time points from the first day of fever shown either at the equestrian premises or at the hospital. NS were collected and embedded in 1 mL sterile saline and placed in sterile 10 mL tubes without medium at two to six different time points, depending on the horse, whereas urine samples were collected during spontaneous urination (horses 12 to 21) at one to seven different time points, depending on the sampled horse.

Urine samples were collected into sterile cups, refrigerated and shipped to the VISAVET Health Surveillance Centre (Madrid) where they were analysed within 24–48 h. However, urine samples from the horses sampled in 2021 in Barcelona were frozen and stored at −80°C for 19 months before they were shipped to the laboratory. Since it was not possible to collect daily urine samples in all the horses, for data analysis all the samples taken during the study were grouped in 4 days periods.

Sample collection dates and the date of the first day of fever were recorded. Sex, age, breed, neurological signs (ataxia/recumbency/urinary incontinence), data regarding vaccination status for EHV-1 and the treatment carried out in the horses in both outbreaks were also collected.

2.2 | DNA extraction

Total genomic DNA was extracted from 200 µL neat buffy coat (BC) samples (EDTA/Heparinised). Blood was centrifuged at 600 G for 5 min, and the BC was manually extracted from the two heparinised blood tubes using a Pasteur pipette. The tubes containing NS in 1 mL sterile saline were initially vortexed for 10 s before removing the swab. Thereafter, 200 µL of saline containing nasal secretions was processed and the QIAamp DNA mini kit (Qiagen) was used for DNA extraction according to the manufacturer's protocol. Total genomic DNA was extracted from 250 µL urine using the Urine DNA Isolation kit (Norgen Biotek) following the manufacturer's instructions. DNA was eluted in 200 µL for NS and BC samples, and 250 µL for urine samples. Extracted DNA was preserved at −80°C until quantitative real-time PCR was carried out.

2.3 | Quantitative real-time PCR assay

The extracted DNA from BC, NS and urine samples were screened by quantitative real-time PCR using primers and TaqMan probes validated to at the UK World Organisation for Animal Health (WOAH) Reference Laboratory according to the ISO 17025 quality system. The target region for amplification of EHV-1 is in a conserved type-specific area of the gene for glycoprotein B (gB) based on the method published by Hussey et al.[12] This qPCR, validated at our laboratory had a limit of detection of 1 EHV-1 copy/µL extracted DNA (95% IC 0.9–1.1). A DNA standard curve was used to quantify the levels of viral DNA, comprising at least three standards (dilutions from a plasmid containing EHV-1 target DNA) at known concentrations. The standards were stored at −40°C and not subjected to multiple rounds of freeze–thaw.[12] An internal control included in the PCR mastermix [Quantifast Pathogen +IC (Qiagen)] was used to check for samples causing inhibition of the reaction, and RNase-free water was used as a negative (non-template) control. The results for the qPCR carried out in the extracted DNA from BC, NS and urine samples were expressed as EHV-1 gB copies/µL extracted DNA as this quantification method has already been validated for EHV-1 in nasal swabs[20] and was deemed to be the best method to compare cellular and acellular specimens. For DNA sequencing and identification of genotype, samples from both outbreaks were submitted to an external laboratory.

2.4 | Data analysis

Data collected from the horses included in the study (sex, age, breed, presence of neurological signs, EHV-1 vaccination status and treatment) were evaluated using descriptive statistics. Within each outbreak (2021 and 2023), real-time PCR results (positive/negative) for BC samples versus urine samples and NS samples versus urine samples were analysed using Chi square test (χ^2), and kappa coefficient (k) was calculated to determine the level of agreement of the real-time PCR between sample types in each outbreak. Wilcoxon test was used to compare the median number of gB copies/µL DNA between sample types in each outbreak. All the analyses were performed using commercially available software (IBM SPSS Statistics software version 20) with a p-value for significance set at <0.05.

3 | RESULTS

In total, 21 horses were sampled by means of heparinized blood and urine or NS and urine. The characteristics of the horses included in this study are presented in Table S1. A total of 11 horses were sampled in 2021, and 10 horses were sampled in 2023. Three stallions, nine geldings, and nine mares were sampled, and the distribution by sex between both outbreaks was similar. The mean age was 9.6 years (median 10 years), with horses in 2021 outbreak being older than horses in 2023 outbreak (mean age 10.6 vs. 8.9, respectively). Breeds were French Saddle horse (SF) (8/21, 38.1%), Dutch Warmblood (KWPN) (3/21, 14.3%), Belgian Warmblood (BWP) (2/21, 9.5%) and Holsteiner (HOLS) (2/21, 9.5%). Regarding EHV-1 vaccination status, from the 11 horses in the 2021 outbreak, 4 horses were vaccinated, 1 had not received any EVH-1 vaccination and the vaccination status of 6 horses was unknown. From the 10 horses hospitalised in the 2023 outbreak, 9 horses were vaccinated against EHV-1 and 1 horse was not vaccinated. However, 3 out of 9 vaccinated horses had incomplete vaccination records and had not received an EVH-1 vaccination booster at least once a year. The neurological signs observed and scored according to Mayhew et al.[21] were: grade 1–4 ataxia (17/21, 81%), grade 5 ataxia with recumbency (2/21, 6.8%) and urinary incontinence (6/21, 28.6%).[21] In the 2023 outbreak in Valencia, three horses with ataxia also had seizures (3/21, 14.3%). All the hospitalised horses were treated with flunixin meglumine and valacyclovir; while dexamethasone and dimethyl sulfoxide (DMSO) were given depending on clinicians' preferences but were used in most cases (18/21, 86% and 15/21, 71% respectively).

Individual data for nasal shedding, viraemia and urinary shedding and the onset of fever are presented in Table S2.

3.1 | EHV-1 real-time PCR results—2021 outbreak

Horses were tested upon admission to the hospital (Barcelona/Madrid). All horses in Barcelona tested positive for EHV-1 DNA in BC samples and remained viraemic up to 12 days from the onset of fever (mean 9 days, range 6–12 days to last positive). The EHV-1 strain causing the outbreak was reported as genotype N752 (A2254). Neither of the two horses sampled in urine yielded positive real-time PCR results. In horses hospitalised in Madrid, four horses were EHV-1 positive in BC samples on admission and remained positive for 8 to 13 days (mean 11.7 days); these four horses tested positive in urine samples and remained positive for even longer (13 to 18 days, mean 16.5 days to last positive). Two horses in Madrid in 2021 were EHV-1 negative and remained negative in BC and urine samples throughout their hospital stay; however, these horses had ataxia and had been in contact with positive horses from the outbreak.

The number of EHV-1 gB copies/μL DNA of positive results in BC and urine samples for the 2021 outbreak throughout hospitalisation are shown in Figure 1A, while Table 1 shows the percentage of positive samples (BC and urine), the range and median number of gB copies/μL DNA during hospitalisation. The percentage of positive BC samples peaked during the 6th–9th day and decreased throughout the following period (10–13 days) until Day 14, when no BC samples were positive. This pattern differed substantially in the urine samples, as the percentage of positives increased at Day 10 and reached 100% from Day 14 to Day 22. Consequently, there were significant differences between the percentages of EHV-1 positives in BC and urine samples towards the final sampling period (Days 14 to 17 [$p = 0.008$], Days 18 to 22 [$p = 0.005$]). Interestingly, the number of gB copies/μL DNA showed significant differences between BC and urine samples only in the final periods of hospitalisation (Days 14 to 17 [$p = 0.02$], Days 18 to 22 [$p = 0.04$]). Regarding the two periods when no significant differences were encountered in the percentage of positives and number of gB copies/μL DNA (Days 6–9, Days 10–13), there was a lack of agreement between the results in both sample types (kappa = 0.000 for both periods).[22]

3.2 | EHV-1 real-time PCR results—2023 outbreak

In 2023, all but two horses (8/10, 90%) tested positive for EHV-1 DNA in NS samples upon admission and remained positive for 4–14 days from the first day of fever (mean 9.6 days). The EHV-1 strain causing the outbreak was reported as genotype N752 (A2254). From these horses, three (3/8, 38%) yielded a positive result in urine samples, with a range of 4 to 5 days and a mean of 4.7 days to last positive. There were two horses that tested negative for EHV-1 upon admission and remained negative; these horses (horses 18 and 21; Table S2) showed fever, and ataxia, and had been in contact with positive horses. Horse 18 also seizured during hospitalisation.

The number of EHV-1 gB copies/μL DNA of positive results in NS and urine samples from the 2023 outbreak throughout hospitalisation are shown in Figure 1B, while Table 2 shows the percentage of positive samples (NS and urine), the range and median number of gB copies/μL DNA during hospitalisation. The percentage of positive NS samples was high from the beginning (71.4%) and increased during the 5th–8th day, decreasing to 62.5% in the final period (9th–14th day). In urine samples, the percentage of positives was similar during the first two periods (25.0% in the first period, 28.6% in the second

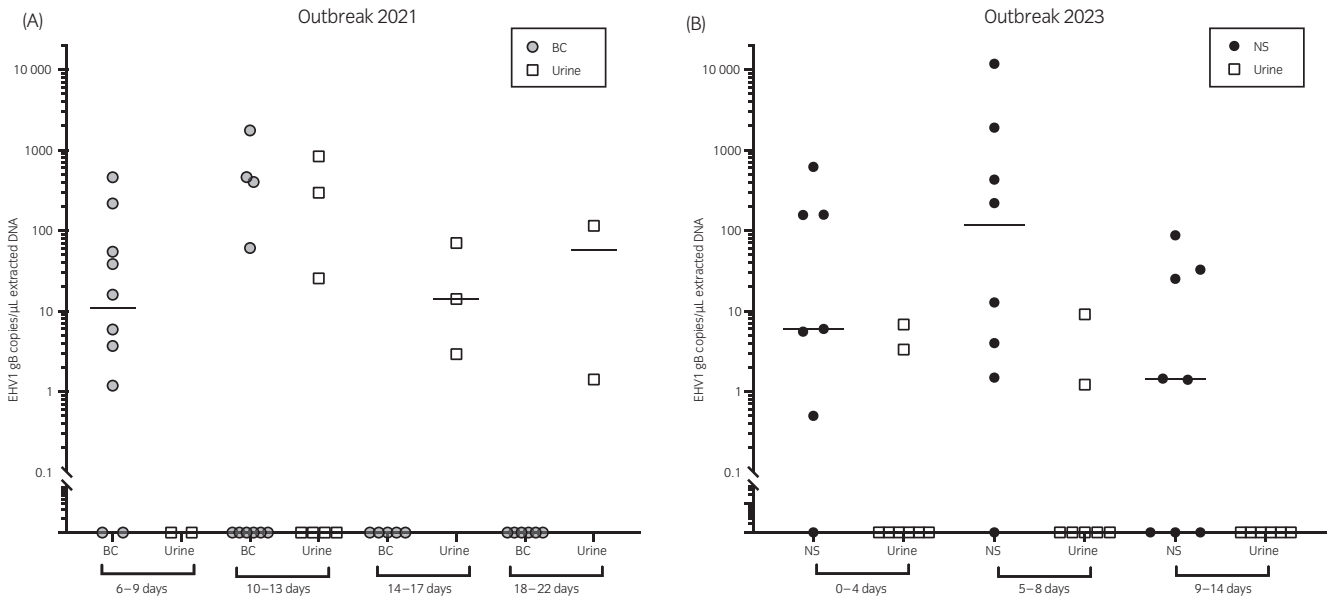

FIGURE 1 Detection of EHV-1 (gB copies/μL DNA) over time in buffy coat (BC), nasal swab (NS) and urine samples; (A) data from BC and urine samples collected in 2021, sampling periods 6–9, 10–13, 14–17 and 18–22 days after the onset of fever; and (B) data from NS and urine samples collected in 2023, sampling periods 0–4, 5–8, 9–14 days after the onset of fever.

TABLE 1 Quantitative PCR results in BC and urine samples from the horses sampled in 2021.

	qPCR results Buffy coat samples		qPCR results urine samples	
Day from first fever	Pos/Total (%)	EHV-1 gB copies/µL DNA range (median)	Pos/Total (%)	EHV-1 gB copies/µL DNA range (median)
6–9	8/10 (80.0%)	1.2–460.3 (27.3)	0/2 (0%)	0
10–13	7/10 (70.0%)	60.8–1756.9 (432.8)	3/7 (42.8%)	25.6–837.6 (296.3)
14–17	0/4 (0.0%)	0	3/3 (100%)	2.9–70.2 (14.2)
18–22	0/6 (0.0%)	0	2/2 (100%)	1.4–114.7 (58.1)

TABLE 2 Quantitative PCR results in NS and urine samples from the horses sampled in 2023.

	qPCR results nasal swab samples		qPCR results urine samples	
Day from first fever	Pos/Total (%)	EHV-1 gB copies/µL DNA range (median)	Pos/Total (%)	EHV-1 gB copies/µL DNA range (median)
0–4	5/7 (71.4%)	5.5–620.0 (156.0)	2/8 (25.0%)	3.3–6.8 (5.1)
5–8	7/8 (87.5%)	2.13–11800.0 (219.7)	2/7 (28.6%)	1.2–9.1 (5.2)
9–14	5/8 (62.5%)	1.4–87.5 (25.2)	0/6 (0.0%)	0

period) and decreased to 0% in the final period (9th–14th day). The number of gB copies/µL DNA in NS peaked during the 5th–8th day, while urine samples showed lower gB copy numbers than NS in all periods ($p = 0.25$ for Day 0–4, $p = 0.05$ for Day 5–8 and $p = 0.01$ for Day 9–14).

4 | DISCUSSION

Rapid identification of EHV-1 positive horses and implementation of appropriate biosecurity measurements are key to controlling the devastating effects of an EHM outbreak.[23,24] The gold standard for EHV-1 diagnosis is viral detection from nasopharyngeal swabs or blood.[3] The identification of EHV-1 DNA in faecal swabs and semen in wild and domestic horses has also been reported[16,18,19,25] and there is a study reporting the presence of EHV-1 DNA in the muzzle/nares area of horses.[25] Free-catch urine can be regarded as a non-invasive specimen, and its collection is less traumatic than blood drawing, especially in horses suffering from vasculitis as occurs in EHV-1 infected horses.[3] In both 2021 and 2023 outbreaks in Spain the EHV-1 strain causing the outbreak was reported as genotype N752 (A2254),[26] unpublished data from the National Reference Laboratory (Algete, Spain). This genotype has previously been related to non-neuropathogenic strains, although this classification might need to be revised since the appearance of a new genotype (C2254) in France and the United States.[27–30] Additionally, a recent publication describes the N752 strain as the leading genotype in horses presenting EHM.[31]

This is the first study reporting the presence of EHV-1 in urine in naturally infected horses. In horses, EHV-1 was detected in a horse with eosinophilic and haemorrhagic cystitis by real-time PCR in urinary bladder tissue, but the analysis in urine was not performed,[32] whereas in humans, Herpes simplex virus-1 has been found in urinary bladder tissue and urine.[33,34] In the current study, prolonged detection of EHV-1 DNA by real-time PCR in urine samples was observed in 2021 outbreak and, in the horses hospitalised in Madrid, for longer periods than it was found in blood. In addition, in some of these horses, viral DNA loads in urine were higher than those in the BC. Information on the white blood cells count in these urine samples is lacking and would have been useful to rule out clinical and subclinical cystitis as the presence of leukocytes in urine could be responsible for the positive result in urine. However, the possibility that blood contamination in these samples explains the results is not likely since it is well described that 10–20 mL of blood are needed to obtain enough BC to detect EHV-1 by qPCR.[12,35] The higher DNA load found in urine vs. the BC may indicate that the urinary bladder is a potential site of replication in the horse in accordance some reports,[32,33] but it could also be due to different viral persistence in these biological compartments.[3]

Laboratory confirmation of EHV-1 infection and EHM currently relies on compatible clinical signs, cerebrospinal fluid cytology, and molecular confirmation by PCR on nasal (or nasopharyngeal) swabs and blood (BC) samples. Difficulties in the collection of NS in fractious horses, inexperienced veterinary personnel, and/or viraemia of short duration are possible reasons for false negative results.[23,36] In experimental studies of EHV-1 infection, NS were positive up to 10–12 days post-infection, whereas BC samples were only positive from Day 5 to Day 10 post-infection.[12,37] In the horses hospitalised in Madrid in 2021, positive real-time PCR results with high DNA loads were observed in urine from Day 10 of the onset of fever, and for an additional 10–12 days after. High viral loads using qPCR has been related to the presence of live virus in other studies[38]; therefore, it is possible that urinary excretion of EHV-1 could perhaps involve contagious virus and represent a threat for further spread of the virus, an issue which needs to be further studied.

There were differences in viral loads between the horses hospitalised in Madrid (2021), Barcelona (2021) and Valencia (2023). The differences between horses in different locations in the 2021 outbreak could be due to the fact that samples obtained in Barcelona were sent to Madrid after prolonged storage at −80°C while the highest DNA loads in urine were obtained from hospitalised horses in Madrid, where immediate sample analysis was carried out. In our experience, immediate sample processing or short refrigeration at 4°C (<24 h) leads to higher detection of EHV-1; this has also been reported for Human papillomavirus.[39] The presence of urea, together with DNA nucleases and subproducts or bacterial growth potentially decreases DNA yield in urine samples subject to long storage times.[40] In humans, papillomavirus DNA detection in urine was not affected by the total amount of urine analysed but different sample preservation and DNA extraction methods showed interference in the final DNA load.[41] For detection of EHV-1 in urine, further studies on optimal collection, transport and storage methods are required.

Differences in viral load between horses hospitalised in Madrid (2021) and Valencia (2023) were also evident. Again, these differences might be related to the fact that samples in Valencia were refrigerated and sent to Madrid while samples in Madrid were immediately analysed. However, the samples from Valencia were not stored for longer than 48 h before the analysis. The administration and timing of certain medications could affect the viral DNA load in NS, BC and urine samples. Administration of valacyclovir may lower viral load by direct inhibition of viral replication when it is administered early in the course of the disease before neurological signs appear.[29,42] When used in an EHV-1 outbreak in the United States in 2021, valacyclovir treated showed a sharp decrease in viraemia with a reduction in the number of horses shedding EHV-1 and in the amount of virus shed within the first week of treatment.[29] This might explain the low viral loads in NS and urine samples in the horses hospitalised in Valencia, where EHV-1 was detected earlier and the administration of valacyclovir was instituted more promptly than in the 2021 outbreak. However, the viral loads from horses hospitalised in Madrid were high in both BC and urine samples even 2 weeks after the onset of fever, agreeing with an experimental study evaluating the effect of valacyclovir in EHV-1 infection in ponies which failed to show a lower nasal viral load.[43] The effect of valacyclovir on the urine viral load was not evaluated in either of the previous studies[29,42] and differences may also be due to the strain susceptibility to valacyclovir.[42] In contrast, DMSO, excreted in urine, has been shown to improve reaction yield during real-time PCR[40,44]; the majority of horses in this study received DMSO, however it is unclear whether DMSO could have enhanced EHV-1 DNA detection.

A recent experimental study on the environmental persistence of EHV-1 showed that viable virus was recovered up to 48 h post-infection in leather, polyester-cotton fabric, wood shavings, straw and plastic.[45] Wood shavings showed had lower DNA concentrations compared with the straw and the rest of materials.[45] Our results showed that EHV-1 DNA was present in urine samples; and although viral survival in urine has not been reported yet, it might be advisable to use wood shavings rather than straw for bedding in an EHV-1 outbreak setting.

This study has limitations including inconsistent sampling times and sampling and handling protocols. Ideally NS, blood samples and urine should have been collected at the same time in all horses to obtain a clearer DNA detection pattern. The method for the collection of urine samples was not the same in all horses, although there are no reports in any species showing a difference in viral DNA concentration between urine collected by catheterisation or free-catch urine samples.

In conclusion, EHV-1 DNA was detected in urine in horses with natural infection resulting in EHM neurological signs. The possible role of this finding in outbreak transmission remains to be elucidated, but the diagnostic usefulness of urine as a non-invasive specimen for PCR testing is confirmed. Optimal urine storage time and preservation protocols to achieve better yields require further study and it has yet to be determined whether valacyclovir affects urine viral excretion.

AUTHOR CONTRIBUTIONS

Fatima Cruz-Lopez, E. Jose-Cunilleras and Ana Velloso Alvarez conceived and designed the work. Ana Velloso Alvarez, E. Jose-Cunilleras, Lucas Troya-Portillo, Maria de la Cuesta-Torrado, Isabel Santiago-Llorente and Valentina Vitale acquired clinical data, organised and collected clinical samples. Fatima Cruz-Lopez, Abel Dorrego-Rodriguez and Belen Rivera processed and analysed all samples. Fatima Cruz-Lopez, E. Jose-Cunilleras and Lucia de Juan analysed results and carried out statistical analysis. All authors collaborated in the preparation and editing of the manuscript for submission. E. Jose-Cunilleras had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FUNDING INFORMATION

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14007.

DATA AVAILABILITY STATEMENT

The data that support findings of this study are available from the corresponding author upon reasonable request: Open sharing exemption granted by the editor due to lack of provision in the owner informed consent process.

ETHICAL ANIMAL RESEARCH

Research ethics committee oversight not currently required by this journal: the study was performed on archived material collected previously during clinical procedures.

INFORMED CONSENT

Explicit owner informed consent for inclusion of samples from animals in this study was not sought but owners were made aware that data and excess material from clinical samples would be retained for research in general.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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