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Case report

Co-infection of *Peruvian horse sickness virus* and *West Nile virus* associated with neurological diseases in horses from Brazil

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ABSTRACT

In 2018, during the surveillance for West Nile virus (WNV) in horses with neurological clinical signs in the state of Espírito Santo (Brazil), 19 animals were investigated, and 52 biological samples were collected for WNV diagnostic. One brain sample was positive for WNV by RT-qPCR and the virus was isolated in C6/36 cell culture and sequenced. We obtained a nearly complete genome of WNV co-infected with Peruvian horse sickness virus (PHSV) in the cell culture. After confirmation of PHSV by next-generation sequencing, a new PHSV RT-qPCR protocol was developed, which was used to detect another horse positive only for PHSV. This assay provides a simple and direct method for easy identification of PHSV from biological samples from horses and may become a useful tool in the epidemiological surveillance of this virus. It is the first case of PHSV in Brazil, and only the third country overall to report, 23 years after the first confirmed notification in Peru. Moreover, it is the first reported co-infection of PHSV and WNV in a horse with neurological signs, confirmed by RT-qPCR.

1. Introduction

The genus *Orbivirus*, contains 22 distinct virus species within the family *Reoviridae*, based on the International Committee for the Taxonomy of Viruses (ICTV) classification. Orbiviruses are vector-borne viruses transmitted by arthropod vectors, such as Culicoides midges, mosquitoes, black flies, sandflies, or ticks, and can infect wildlife, domesticated animals, and/or humans [1, 2]. Viral particles are non-enveloped with icosahedral morphology and genomes composed of 10 segments of linear double-stranded RNA (dsRNA) that encode seven structural proteins (VP1-VP7) and at least three non-structural proteins (NS1-NS3) [3].

Two *Orbivirus* species *Bluetongue virus* (BTV) and *African horse sickness virus* (AHSV) are the most economically significant members of this genus, however, several others orbiviruses are potentially important, either regionally or globally, including *Equine encephalitis virus* (EEV), *Epizootic hemorrhagic disease virus* (EHDV), *Palyam virus*, and *Peruvian horse sickness virus* (PHSV) [1].

PHSV is an emerging orbivirus identified in 1997 associated with neurological disease in horses from Peru, causing outbreaks with a high fatality rate in this animal species. Two years later, a new strain of PHSV (*Elsey virus*, ELSV) was isolated from sick horses in Australia [4].

Besides these orbiviruses, other arthropod-borne viruses are notable for their characteristic of infecting equines, such as members of the family *Alphavirus* including *Venezuelan equine encephalitis virus* (VEEV), *Western equine encephalitis virus* (WEEV), and *Eastern equine encephalitis virus* (EEEV) [5]; and *Flavivirus* like *West Nile virus* (WNV)

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[6], *Saint Louis Encephalitis virus* (SLEV) [7] and *Japanese encephalitis virus* (JEV) [8].

WNV is an emerging neurotropic flavivirus (*Flavivirus* genus, *Flaviviridae* family), transmitted by *Culex* sp. to human, bird, and equine. WNV has disseminated broadly in the Western hemisphere and now poses a significant public health risk [9]. In Brazil, several studies indicated serologic evidence of WNV circulation in humans and animals [10, 11], however only in 2018, the first isolation was successful in Espírito Santo State and a genome sequence of WNV was obtained [6]. During this period, as part of the surveillance actions for WNV, several biological samples were collected, resulting in the first isolation and sequencing of the nearly complete genome of PHSV in Brazil from a horse with neurological signs, naturally co-infected with WNV.

2. Material and methods

2.1. Samples

During the WNV epidemiological survey conducted in February to September of 2018 in rural areas of Espírito Santo State, Brazil by Health Secretariat and Agricultural Defense Institute of Espírito Santo state, Arbovirus surveillance group of Health Surveillance Secretariat of Federal District and Department of Arbovirology and Hemorrhagic Fevers of Evandro Chagas Institute (IEC), Ministry of Health of Brazil, several biological samples of humans, horses, domestic and wild birds and mosquitoes were collected. For this analysis only horses were tested.

All animals sacrificed showed neurological diseases causing incapacity to eat and drink, that evolved to state of suffering. The procedure of euthanasia was followed by Veterinarians, using anesthetic overdose in all animals according to resolution n° 1000, of 11th May 2012 of Federal Council of Veterinary Medicine of Brazil, which provides procedures and methods for euthanasia in animals and gives other provisions. All procedures were supervised by an official officer for human and animal health surveillance of Brazil to investigate the cause of this neurological diseases. In this case, an approbation by an ethics committee from Brazil it is not necessary.

2.2. RT-qPCR for WNV and SLEV

RNA Extraction started with approximately 10 mg of tissue fragments using TRIzol® Reagent, and PureLink™ RNA Mini Kit (details describe in supplementary protocol). The RT-qPCR assay to detect WNV and SLEV genomes was performed following established protocols [12] on an ABI7500 Real Time PCR System (Applied Biosystems) using Superscript III Platinum One-Step qRT-PCR System kit (Invitrogen).

2.3. Sequencing and phylogenetic analysis

The sequencing process started with the extraction of RNA from the supernatant of virus isolation in cell culture (details describe in supplementary protocol) with QIAamp Viral RNA Mini Kit, synthesis of the cDNA and library preparation previously describe [6] on the MiniSeq (Illumina, Inc) platform, using 2 × 150 paired-end reads.

The contigs were generated from *De Novo Assembler* methodology using IDBA-UD [13] and SPAdes [14] to recover the viral genome. For annotation, all translated contigs were aligned and compared against the RefSeq database of virus proteins available in NCBI through the Diamond protein aligner [15], and the result visualized in the program Megan 6 [16]. The inspections of putative ORF genes were performed using the Geneious v.9.1.6 software (Biomatters, New Zealand).

Multiple sequence alignment (MSA) was performed using the Mafft v.7 [17] program using the complete amino acid sequence the segment 1 that encodes the polymerase protein of the sequences obtained in this study and other *Orbiviruses* from different vectors available in the NCBI database. Prior to the phylogenetic analysis, the ProtTest was applied to select the best-fit model of amino acid substitution [18]. The Maximum likelihood (ML) method [19] was applied for reconstruction of the phylogenetic tree, implemented in the RaxML v.8.2.4 software [20]. Furthermore, bootstrap analysis [21] was carried out on 1000 replicates for determining of the reliability of the tree topology.

2.4. Standardization of RT-qPCR for PHSV

2.4.1. Primers and probe

For the PHSV RT-qPCR assay two primers (forward and reverse) and a probe (FAM labeled dual quenched hydrolysis probe) were designed targeting a region of segment 1 of PHSV genome that encodes RNA-dependent RNA polymerase protein (reference accession number DQ248057). Two additional primers (PHSV-cloneF and PHSV-cloneR) were designed flanking the region used for the RT-qPCR primer/probe set in order to amplify a DNA fragment for cloning and in vitro transcription of the RNA standards (Table 1). All primer and probe design were performed using the Primer3 package that is part of the Geneious software v.9.1.6. (Biomatters, Auckland, New Zealand) and synthesized by Integrated DNA Technology (IDT, Iowa, USA).

Different combinations of the final concentrations between primers (0.1 μM, 0.2 μM, 0.4 μM, 0.6 μM, 0.8 μM and 1 μM) and probe (50 nM, 100 nM, 150 nM, 200 nM and 250 nM) were tested, using 0.04 ng/μL of PHSV RNA as a positive control and total RNA extracted from negative horse sample to find the best amplification result (lowest CT values and highest ΔRn values possible) with the lowest concentration of both primers and probe.

RNA was denatured at 94 °C for 2 min and immediately kept on ice for 1 min. The RT-qPCR was performed using 12.5 μL of 2x Reaction Mix, 0.5 μL of SuperScript™ III RT/Platinum™Taq Mix PCR, both components of the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen), 5.5 μL of nuclease-free water, 0.5 μL of each primer, 0.5 μL of probe and 5 μL of denatured RNA, totaling a final reaction volume of 25 μL. The passive reference dye (ROX) was used in all master mix reactions to normalize the fluorescence data. The amplification started with cDNA transcription at 50 °C for 30 min; initial denaturation at 95 °C for 2 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min using ABI7500 Fast Real-Time PCR System (Applied).

To determine primer and probe specificity, in-silico analysis was performed to check if the primer had a match with other organisms using Primer-Blast (online tool) from NCBI. In addition, the PHSV primers and probe were tested against RNA of other arboviruses of genus *Alphavirus* (*Chikungunya virus* [CHIKV], *Mayaro virus* [MAYV], *Madariaga virus* [MADV] and WEEV); *Flavivirus* (*Dengue virus 1* to *4* [DENV], *Ilheus virus* [ILHV], *Rocio virus* [ROCV], *Yellow Fever virus* [YFV], *Zika virus* [ZIKV], WNV and SLEV); *Orbivirus* (*Changuinola virus* [CGLV]) and *Orthobunyavirus* (*Oropouche virus* [OROV]) in separate real-time RT-PCR's.

Table 1. PHSV primers and probe designed in this study for the RT-qPCR assay.

Primer/ Probe	Sequences	*Position	Amplicon
PHSV-1713F	5′- ATT ATT ATT GGA GAT TTA GAG TCA ACG G -3′	1713	86
PHSV-1798R	5′- CAA ATG GTT GGA TCT GAT GTG TTC -3′	1798	
PHSV-1775S	5'-/**56-FAM**/TCT CGA GTT/**ZEN**/ATT GAT GCA GGA GAT ACA TTT CG/**3IABkFQ**/-3′	1743	
PHSV-cloneF	5′- GAA GCA GAA AGA CAT GGG TA -3′	1293	922
PHSV-cloneR	5′- AAG CTT CTC CGG AAA GAT GAG TTG AA -3′	2214	

* Genome positions based on a PHSV reference sequence (accession number DQ248057).

2.4.2. PHSV RT-qPCR reaction efficiency and limit of detection evaluation

To determine the efficiency and limit of detection an amplicon was synthetized from PHSV RNA from a template of a one-step RT-PCR using primers PHSV-cloneF and PHSV-cloneR (details describe in supplementary data). In order to evaluate the PHSV RT-qPCR efficiency, a standard curve was constructed using known concentrations (in copies/μL) of PHSV in-vitro transcribed (IVT) RNA. Eight serials 1:10 dilutions of IVT RNA (ranging from 10^1 to 10^8 copies/μL) were used for RT-qPCR amplification. Each dilution was tested in triplicates. For the LoD evaluation, eight 1:10 dilutions of IVT RNA (ranging from 10^1 to 10^8 copies/μL) were tested. Each dilution was tested eight times in a single run and the results were used to calculate the 95% LoD of the assay by probity regression analysis, using SPSS Statistics version 25 (IBM, USA). We then calculated the analytical sensitivity defined as the lowest amount of genome copies per reaction detected by the assay with 95% probability.

3. Results

3.1. Animals, virus isolation, sequencing and phylogenetic analysis

The epidemiological surveillance conducted in Espírito Santo in 2018 identified 21 counties (Figure 1[A, B]) with at least one case of horse with neurological signs such as easel position, ataxia, muscle fasciculation/tremors, excitability, lateral decubitus, difficulty in keeping sternal, pedaling movements, loss of consciousness and central blindness. However, it was only possible to collect samples from 11 counties, where 19 animals were sacrificed, resulting in a collection of 52 biological samples (Supplementary Table 1).

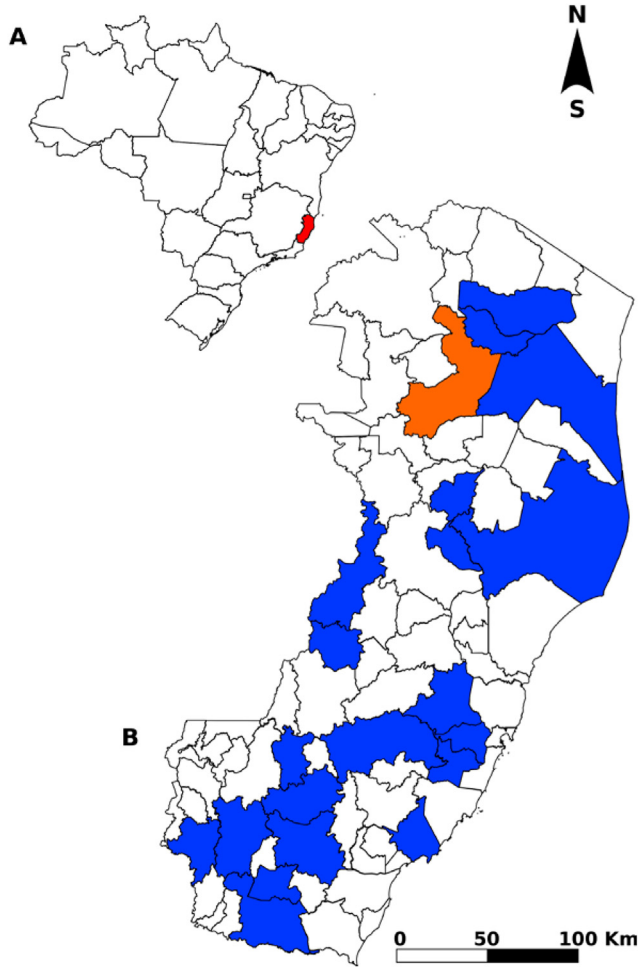

Figure 1. Cases of animals with neurological clinical signs in horses from Espírito Santo, Brazil. (A) Map of Brazil, state of Espírito Santo in red. (B) State of Espírito Santo, counties with cases of sick horses in blue, and the orange correspond to the county of Nova Venécia. Maps created using library rgdal and maps inside of R software.

From all of these samples inoculated in C6/36 cells, only the brain sample of one animal (BeAn854747) produced a CPE after the fourth day post-inoculation (dpi), which was characterized by cell death and formation of syncytia. *Flavivirus* infection was confirmed by immunofluorescence assay using polyclonal antibodies, and the supernatant was tested for WNV and SLEV by RT-qPCR, showing a positive signal for WNV. After this confirmation the total RNA was sequenced to recover the WNV genome (GenBank accession numbers: MH643887), as described previously [6].

A few months after the analysis of WNV was performed, analysis of the whole data set identified evidence for a second virus genome in this cell culture with 10 complete open reading frames (ORF) showing high nucleotide and amino acid identity to all segments of PHSV isolated in Peru in 1997 (Table 2). The Brazilian PHSV strain sequences were deposited in the GenBank database under accession numbers MN428629 to MN428638.

The PHSV strain isolated in Brazil was grouped in a monophyletic mosquito-borne clade (Figure 2), and was closely related to PHSV isolated in Peru, together with *Mobuck virus* (MOBV) and *Yunnan orbivirus* (YUOV), and showed lower relationship with other orbiviruses in a monophyletic clade with *Sathuvachari virus* (SVIV), *Koyama Hill virus* (KHV), *Umatilla virus* (UMAV), *Parry's Lagoon virus* (PLV) and *Corriparta virus* (CORV).

3.2. PHSV RT-qPCR assay

One aim of this study was to design and optimize a RT-qPCR assay to detect the PHSV genome. Bioinformatics analysis did not reveal any primer dimer, hairpin structures or match with other organisms like Archaea (taxid: 2157), Bacteria (taxid:2), Equine (taxid: 9796), *Homo sapiens* (taxid: 9606) and Viruses (taxid: 10239). This analysis returned only matches to PHSV.

The result of different primer concentration didn't show big differences in the Ct values (Supplementary Figure 1). However, the concentrations of 50 nM, 100 nM and 250 nM of probes increased the Ct values when compared to 150 nM and 200 nM (Supplementary Figure 2). In a total of 30 combination (primers plus probe) 19 had values down to 6 and 11 up to 6 (Supplementary Table 2) to ΔRn. The threshold of 0.1 was used in all RT-qPCR runs.

From these values, the best final concentrations of primers and probe to use were 0.8 μM of each primer (forward and reverse) and 200 nM of TaqMan probe. These combinations showed the lowest cycle threshold (Ct) and highest ΔRn in comparison to other concentrations of primers and probe.

The linear dynamic range (LDR) was performed to define the range of target concentrations that may be amplified with acceptable linearity. For this a serial dilution ranging from 10^1 to 10^8 copies/reaction of IVT was tested. The LDR assay was determined at 10^2 to 10^8 copies, with assay's limit of detection (LoD) at 4.87×10^2 copies/reaction (CI 95% $p < 0.001$, [lower 1.01×10^2; upper 5.4×10^4]) showing R^2 and slope values of 0.997 and -3.322, respectively. The RT-qPCR efficiency was around 100% (Supplementary Figure 3). The endpoint LoD of the assay was 10^2 copies/reaction (Ct: 37.5), with a limit of quantification (LOQ) of 10^3 copies/reaction.

The specificity test with other arbovirus RNAs showed no amplification for the PHSV primer/probe set with viral RNAs from genus *Alphavirus* (CHIKV, MAYV, MADV and WEEV); *Flavivirus* (DENV 1-4, ILHV, ROCV, YFV, ZIKV, WNV and SLEV); *Orbivirus* (CGLV) and *Orthobunyavirus* (OROV).

Using this assay, we were able to detect two horse samples (UN9219 and UN9227) positive for PHSV indicating the presence of PHSV in all

Table 2. Nucleotide and amino acid identity of PHSV from Brazil against several other orbivirus from the mosquito-borne clade (Figure 2).

Virus	VP1	VP3	VP2	VP4	NS1	VP5	NS2	VP7	VP6	NS3
	nt/aa	nt/aa	nt/aa	nt/aa	nt/aa	nt/aa	nt/aa	nt/aa	nt/aa	nt/aa
PHSV	98.3/99.3	98.8/99.3	97.5/98.4	99.0/100	98.4/98.7	98.5/99.3	98.9/99.8	99.0/99.4	98.3/97.9	98.6/98.8
MOBV	68.2/70.3	68.3/69.2	48.7/29.9	61.7/60.1	59.1/54.6	61.5/57.6	56.0/44.4	71.3/74.2	54.2/38.7	63.8/56.5
YUOV	67.5/69.5	64.1/65.2	47.3/26.9	62.7/61.6	57.7/51.8	62.6/58.8	55.4/45.9	68.0/71.6	55.7/38.4	59.0/45.5
SVIV	57.0/50.6	56.7/47.6	44.2/14.5	56.3/46.1	46.5/25.0	50.8/34.8	49.8/29.0	48.6/31.4	49.3/28.4	44.1/18.6
KHV	57.6/52.4	53.6/43.5	44.4/14.0	53.5/45.4	45.0/26.6	50.0/37.8	45.6/26.6	49.8/31.2	50.5/27.9	42.3/15.2
UMAV	58.3/52.8	53.9/43.3	43.7/15.8	54.5/43.1	43.8/24.5	52.0/36.6	45.4/26.6	49.7/30.6	49.3/26.8	41.3/17.0
PLV	56.9/52.0	55.7/44.8	43.8/14.0	52.5/44.4	44.6/25.7	51.7/41.2	46.0/26,7	47.9/34.2	47.1/24.7	45.1/17.4
CORV	56.3/51.6	54.6/45.1	44.9/13.5	52.0/43.4	46.6/27.0	51.1/40.3	44.9/26.4	47.6/33.6	48.2/24.7	43.4/18.6

Nucleotide (nt); Amino acid (aa); *Peruvian* horse sickness virus (PHSV; DQ248057 to DQ248066); Mobuck virus (MOBV; KF296322 to KF296331); Yunnan orbivirus (YUOV; AY701509 to AY701518); Sathuvachari virus (SVIV; KC432629 to KC432638); Koyama Hill virus (KHV; AB894484 to AB894493); Umatilla virus (UMAV; HQ842619 to HQ842628); Parry's Lagoon virus (PLV; KU724110 to KU724119) and Corriparta virus (CORV; KC853042 to KC853051).

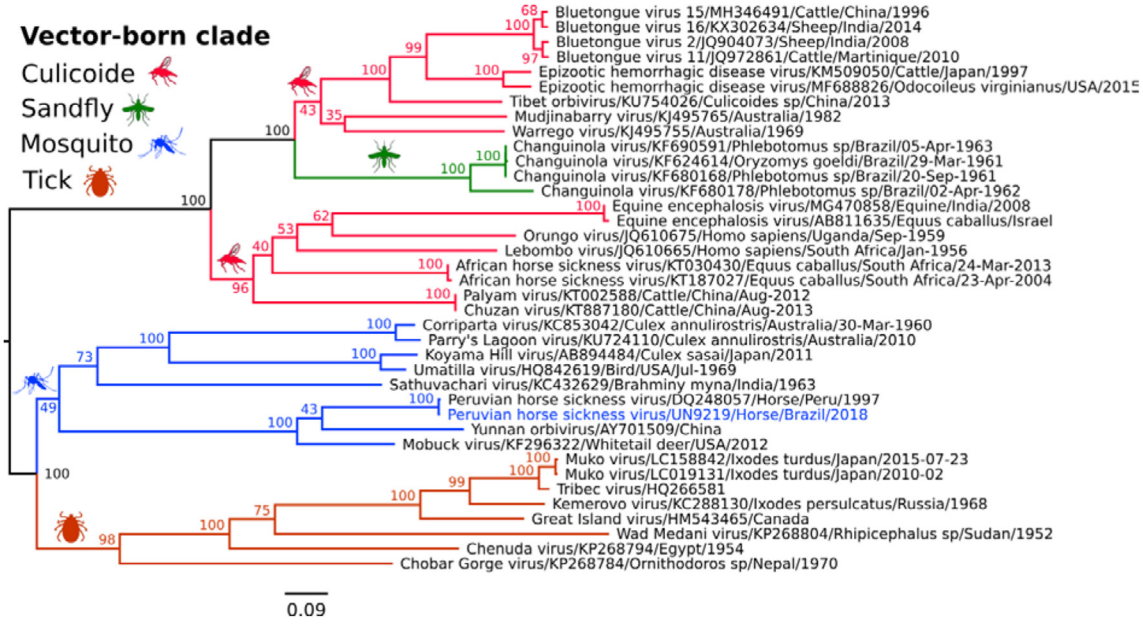

Figure 2. Maximum Likelihood (ML) Phylogenetic tree of different viruses belonging to the genus *Orbivirus* based on the complete amino acid sequences of the viral RNA polymerase (VP1). The LG matrix was the best-fit model to this dataset. Phylogenetic groups are labelled in different branch colors. Numbers at each main node of the tree correspond to bootstrap values in percent (1000 replicates). The scale bar corresponds to the genetic divergence among amino acid sequences.

tissues tested (Supplementary Table 1). Furthermore, a natural co-infection with WNV in clinical samples of horse UN9219 was confirmed in the RT-qPCR assay. These two animals came from Nova Venécia indicating that horses were exposed to infection with these viruses sometime before the date of collection. All biological samples tested were negative for SLEV.

4. Discussion

In Brazil, several arboviruses that can cause neurological signs in horses have been reported by serological tests. These include EEEV and WEEV with serological evidences of circulation in the Pantanal and Amazon regions [22, 23], causing neurological signs in horses from the northeast region of Brazil [24] and with presence of antibodies to VEEV in Minas Gerais, Brazil [25]. However, sometimes it is not possible to identify the cause of the disease by serological tests or PCR. In this case, it is important to try other methodologies like virus isolation and/or next-generation sequencing (NGS) analysis. This study describes the first isolation of PHSV from horses in Brazil, associated with co-infection of WNV in animals with neurological signs using NGS analysis.

In 1984 approximately 3000 horses in Peru showed neurological signs with 30 deaths, and in the following two years, 19 more animals died with the same clinical signs. In 1997 more severe epizootic cases were reported with similar clinical signs. When 800 animals were surveyed, a total of 132 horses showed neurological signs and 104 of them died. In some of these animals, a new *Orbivirus* was identified as PHSV. All these events were described by the Department of San Martin in the subtropical upper jungle of Peru. Two years later, two horses, from different locations in the Northern Territory of Australia showed neurological signs, caused by ELSV that showed serological and molecular similarity with PHSV [4].

The epidemiology of the infections caused by PHSV is poorly characterized, and it is uncertain how identical viruses came to be present in such distinct regions of the world [1]. However, the RT-qPCR assay, that was standardized in this study, provides a simple and direct method to identify PHSV from biological samples of horses and can be a good option for epidemiological surveys. These two cases of PHSV are the third worldwide description of this virus associated with neurological signs in horses from Espírito Santo state, Brazil almost 22 years after the first isolation of PHSV causing neurological disease in horses in Peru.

In contrast to PHSV, previous studies showed evidences of WNV circulating in Brazil for at least 8 years [26]. The presence of neutralizing antibodies for WNV was shown in eight infected horses from the state of Mato Grosso do Sul and one from the state of Paraíba in 2013 [11]. In 2014, a survey in horses and other animals from Pantanal region detected neutralizing antibodies in 172 equines from 15 ranches, where in some of these ranches neurological disorder outbreaks were reported [27]. In the same year, the first case of WNV in a human was detected in Brazil. IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA), hemagglutination-inhibition test (HI), and plaque-reduction neutralization test (PRNT90) showed high titers of antibodies against WNV in a Brazilian ranch worker with encephalitis and flaccid paralysis in Piauí State [10].

Furthermore, the first isolation of WNV in Brazil occurred from a CNS sample collected from a horse in a rural area of Espírito Santo state on 2018, with histopathologic alterations in the cerebral tissue of the horse compatible with encephalitis, similar to those previously described for WNV. Phylogenetic analysis of the full-length genome showed that this WNV isolate was included in lineage 1, which have greater genetic relationship with Argentine and North American strains [6].

A unique co-infection of WNV with another arbovirus has previously been reported with *Toscana virus* (TOSV) in two febrile patients identified via serology and/or viral RNA detection from Turkey [28]. Moreover, until this date no other cases of natural co-infection of WNV with other arboviruses in humans or animals have been reported. However, this study reports a second natural co-infection of WNV with PHSV. This low prevalence rate can represent a lower frequency of this co-infection and/or be a result of a limited capacity for diagnosis and identification of multi-infections in animals. In this regard, the new PHSV RT-qPCR assay developed here may become an important tool for the PHSV diagnostics and will help further investigation of PHSV co-infection with other arboviruses.

In other arboviruses, co-infection has been demonstrated for DENV/CHIKV (co-infection was 7.64%), DENV/ZIKV (6.37%), CHIKV/ZIKV (5.10%), and with DENV/CHIKV/ZIKV (1.91%) in patients with febrile syndrome at the Colombian-Venezuelan border [29]. Epidemiological synergy between outbreaks of viruses transmitted by *Aedes aegypti* mosquitoes, such as CHIKV, DENV, and ZIKV, has resulted in co-infection of humans with multiple viruses. Despite the potential impact on public health, little is known about the occurrence and consequences of such co-infections [30], and this scenario can be applied to other mosquitoes and animal species associated with other arboviruses.

There are still some open questions such as: How can these co-infections influence the virulence of these arboviruses and how can this interaction cause more damage and/or clinical signs in the horses? The clinical signs observed in the animals positive for WNV and PHSV didn't show any evidence of extension of the disease in comparison with cases of single infection of WNV or PHSV describe in the literature, however, additional studies are necessary to better understand the dynamics of these viral co-infections.

In addition, which vectors are related with the transmission of PHSV to horses and how could this virus spread in the Americas? There are no published studies about the transmission cycle of PHSV, however, it is probably transmissible by a mosquito like other arboviruses where horses are the vertebrate dead end hosts and birds might serve as the natural reservoir. To try to answer this question it is necessary to conduct mosquitoes surveillance to detect the genome of this virus.

5. Conclusions

Of the 52 clinical samples collected from 19 horses during a WNV surveillance in 2018 in the state of Espírito Santo (Brazil) which all showed neurological signs, one brain sample tested positive for WNV. Cell-culture isolation and whole genome sequencing yielded a nearly complete genome of WNV, but also showed a co-infection with PHSV in the same sample, making this the first reported case of PHSV in Brazil. A new PHSV RT-qPCR protocol was developed, which confirmed the co-infection, and which was used to detect another horse positive only for PHSV. In total, only two of the 19 horses tested positive for PHSV, therefore we hypothesis that the other 17 animals were past the viremic phase and so we were not able to detect the virus in serum samples. Our findings emphasize that it is important to conduct molecular and serological surveillance in horses from Espírito Santo and other states of Brazil to try to detect other cases of PHSV infection. This newly developed PHSV assay may become a useful tool in the epidemiological surveillance of this virus to analyze biological samples from horses with neurological symptoms as well as mosquito samples to identify the vector and transmission cycle.

Declarations

Author contribution statement

All authors listed have significantly contributed to the investigation, development and writing of this article.

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Data availability statement

Data associated with this study [The Brazilian PHSV strain sequences] has been deposited at GenBank database under the accession number MN428629 to MN428638.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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