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Serological Evidence of Arboviruses in Horses During West Nile Fever Monitoring Surveillance in Southeastern Brazil

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Many human arboviruses are also pathogenic for horses, and some of these have emerged recently. A descriptive cross-sectional observational study was conducted to assess the prevalence of West Nile virus (WNV) and other arboviruses among 77 horses on the rural properties of the Espirito Santo state, Brazil. Serum samples were screened for arbovirus-reactive antibodies using the hemagglutination inhibition technique and subsequently a plaque reduction neutralization test for the confirmation of exposure from sera was used to detect heterotypic immune reactions. Overall, the total antibodies against at least one arbovirus of *Alphavirus*, *Flavivirus*, and *Orthobunyavirus* genera were detected in 39 (50.6%) animals. The antibodies to *Phlebovirus* were not detected in any sample. When the 24 WNV hemagglutination inhibition (HI)-positive samples were tested by the plaque-reduction neutralization test 90%, 9 (32.1%) were positive for WNV antibodies and 14 (50%) for Saint Louis encephalitis virus. Our findings indicate that the region provides ideal conditions for the emergence of arboviruses, reinforcing the need for further surveillance of mosquito-transmitted diseases in domestic animals.

Keywords: equids, serology, flavivirus, arboviral disease, zoonoses

INTRODUCTION

Over the past four decades, the rate of infectious disease emergence has increased—in both humans and animals (1). Between a quarter and two-thirds of human infectious pathogens are zoonotic, and there is evidence to suggest that increased agricultural intensification is linked to the emergence of zoonotic diseases while it has expanded the geographic range of livestock diseases with major economic repercussions (2, 3).

In the Americas, several endemic and emerging arboviruses cause clinically indistinguishable systemic and neurologic diseases in equids and humans (4). As horses can become infected and seroconvert to the arboviruses of public health importance, they also serve as sentinels for these viruses (5).

The *Flaviviridae* family contains the largest number of viral species that can cause encephalitis in horses. The most significant are the *West Nile virus* (WNV) and *Japanese encephalitis virus* (JEV) (6). The serological evidence of flavivirus activity has been vastly reported in many countries, including South Korea (7), Canada (8), Spain (9), France (10), Argentina (11), and China (12).

The epidemic potential of this viral genus reflects many factors related to the unique characteristics of their insect vectors, the geographical expansion of vectors, changing environmental conditions, extensive global travel, and the consequences of poorly planned urbanization that creates ideal arthropod-breeding habitats (13).

Espírito Santo is a state located on the southeastern coast of Brazil, the second smallest region in the country but the most populous one, inserted in the Atlantic Forest biome (14). Due to a high degree of destruction and fragmentation, the Atlantic Forest is considered one of the most threatened biomes in the world and has been classified as one of the five largest hotspots (15).

On April 25, 2018, the first isolation of WNV in Brazil occurred from a horse with neurological disease from the region of Pedra Grande in the municipality of São Mateus, Espírito Santo (16). Due to the case, an extensive scientific expedition was carried out in the state, including different actors in the transmission chain, such as domestic and wild birds and equines. Here, we conducted a serosurvey to investigate the presence of arboviruses in horses from the State of Espírito Santo.

MATERIALS AND METHODS

Ethics Statement

The procedures were in accordance with fundamental ethical and scientific requirements contained in the Research Regulatory Guidelines and Norms of the Ethics Committee on the Use of Animals of Evandro Chagas Institute, Ananindeua, Pará, Brazil.

Regardless, no animal ethics approvals were required for animal samples, considering that they were obtained from a continuous public health surveillance of a mandatory reporting disease.

Study Site and Sample Collection

From July 4–13, 2018, serological surveys were conducted in Espírito Santo State, Brazil, and 6 municipalities were visited in areas belonging to 22 different rural properties distributed across different parts of the region (**Figure 1**).

Blood was collected from 77 horses by jugular venipuncture in a microtube without an anticoagulant. After collection, the clot was retracted at room temperature and subsequently, each sample was subjected to centrifugation at 3,000 rpm for 5–10 min. The separated serum was transferred to another microtube, immediately stored in liquid nitrogen, then at -70°C freezer, and later transported on dry ice.

All of the animals enrolled in the serologic survey were apparently healthy and without a history of traveling outside of

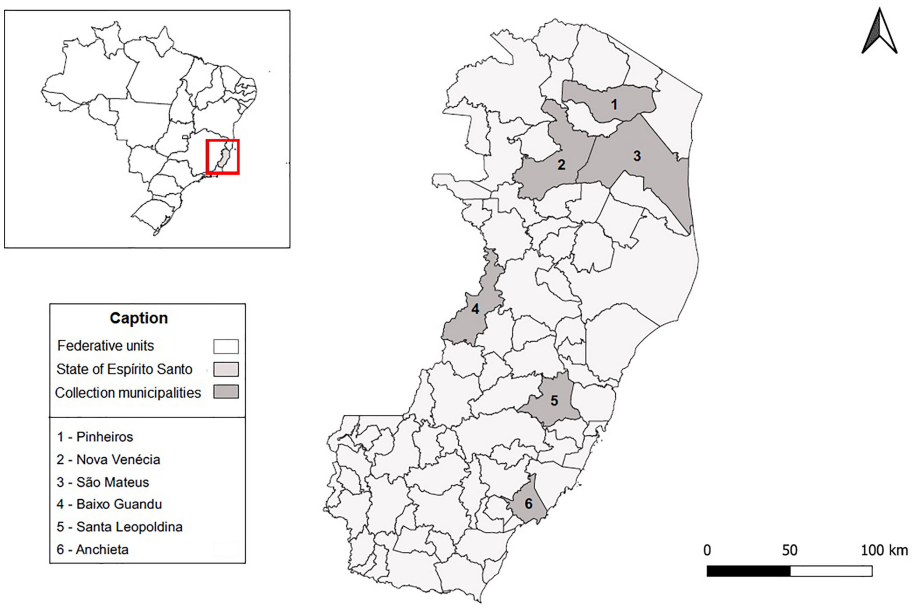

FIGURE 1 | Schematic representation of the Espírito Santo State. Espírito Santo located in southeastern Brazil is shown in light gray. Municipalities where sampling occurred are shown in dark gray.

the state. The data collected for each animal included the sex, age, vaccination history, and utility (work purposes).

Hemagglutination Inhibition Test

Arbovirus infections were evaluated by screening for total antibodies with the hemagglutination inhibition (HI) test (17) adapted to microplates (18), using sucrose-acetone (19).

The test was performed with a standardized panel of 19 arbovirus antigens, including four genera: *Flavivirus*: Yellow fever virus (YFV), West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), Cacipacore virus (CPCV), Bussuquara virus (BSGV), Rocio virus (ROCV), Ilheus virus (ILHV); *Alphavirus*: Eastern equine encephalitis (EEEV), Western equine encephalomyelitis virus (WEEV), Mayaro virus (MAYV), Mucambo virus (MUCV); *Orthobunyavirus*: Maguari virus (MAGV), Utinga virus (UTIV), Belem virus (BELV), Oropouche virus (OROV), Catu virus (CATUV), Caraparu virus (CARV), Tacaiuma virus (TCMV); and *Phlebovirus*: Icoaraci virus (ICOV).

Samples were initially treated with 100% acetone, aiming to remove possible non-specific inhibitors of hemagglutinant activity, and subsequently, the test was performed in two steps: screening and titration. Serum samples were placed to react in equal parts of serum and antigen, with the introduction of a revealing system [composed by white goose red cells (*Anser cinereus*) diluted in dextrose, gelatin, and barbital solution] to test for the formation of an antigen–antibody complex. The positive samples at the screening step were diluted with bovine serum albumin (0.4%) and titrated up to a dilution of 1:40 to 1:1,280.

Positive reactions were classified as either monotypic reactions, referring to the presence of antibodies with titers ≥1:20 for one arbovirus; heterotypic or cross reactions were the samples with the presence of antibodies with titers ≥1:20 for more than one arbovirus of one same viral genus.

Plaque Reduction Neutralization Test (PRNT90)

For samples with cross-reactive results in the HI test to SLEV (ChimeriVax - SLEV) and WNV (BeAn854747 strain), we performed the plaque-reduction neutralization test 90% ($PRNT_{90}$)test, since it is more specific than HI, and due the purpose of the surveillance action in the study area. It was executed using Vero cells at a concentration of $1.6 \times 10^5/cm^2$ in 6-well plates, as previously described (20).

Horse sera were inactivated at 56°C for 30 min and diluted to 1:5 in Medium 199 with Earle's salts. Samples were tested at twofold serial dilutions (1:10 to 1:80), mixed with viruses and allowed to incubate for 1 h at 37°C, at which point the samples were added onto Vero cells, overlaid, and incubated for 5– 7days (21, 22).

Serum samples were considered positive when a serum dilution of 1:10 or greater reduced the viral formation of plaques by at least 90%. In heterotypic patterns, specific virus exposures were assigned if a fourfold or greater titer to one virus than to all other flaviviruses tested for simultaneously was demonstrated.

Statistical Analysis

Results were tabulated by simple percentage and descriptive statistics implemented by GraphPadPrism 6.0 for Windows (GraphPad software, San Diego, CA, USA). They were also analyzed using the BioEstat 5.0 software (23), assuming statistical significance for $p \leq 0.05$.

RESULTS

The seroepidemiological survey was carried out in 77 horses, 34 (44.1%) of which were males and 34 (44.1%) females, 9 did not have detailed sex data. Their ages ranged from 5 months to 19 years. Moreover, those animals were used in many different ways, such as for riding, leisure, sports, reproduction, and general work. Most horses were used for work (48.0%) (**Table 1**).

Of the serum samples analyzed by the HI test, 39/77 (50.6%) had total antibodies to at least one arbovirus tested in the study, considering both monotypic and heterotypic reactions, and 38 (49.3%) were seronegative. Overall, 8 (20.5%) samples had

TABLE 1 | Result of statistical analysis of horses attributes tested for arbovirus in Espírito Santo, Brazil.

Characteristics		Frequencies		Chi-square
		n	%	*p*-value
Sex	Female	34	44.15%	21.075
	Male	34	44.15%	*p* <0,0001
	NI	9	11.69%	
Age groups	<1 year	3	3.89%	41.438
	1–5 years	12	15.58%	*p* <0.0001
	5–10 years	32	41.56%	
	≥10 years	20	25.97%	
	NI	10	12.99%	
Function/ Activity	Riding	8	10.39%	81.498
	Leisure	8	10.39%	*p* <0.0001
	Sport	16	20.78%	
	Reproduction	2	2.6%	
	Work	37	48.05%	
	NI	6	7.79	

NI, not informed.

antibodies to the arboviruses of the *Alphavirus* genera, 28 (71.8%) to *Flavivirus*, and 21 (53.8%) to the *Orthobunyavirus* genus.

Monotypic HI reactions occurred in 19/39 (48.7%) of the samples, 5 (12.8%) for EEEV (with titers ranging from 1:40 to 1:160), 8 (20.5%) for MAGV (with titers ranging from 1:20 to 1:80), 3 (7.6%) for TCMV (with titers ranging from 1:20 to 1:640), 3 (7.6%) for SLEV (with titers ranging from 1:20 to 1:80) and 1 (2.5%) for WNV (with a titer of 1:20).

On data collection about equine vaccination, it was reported that 43 (55.8%) animals had been immunized against equine encephalitis. From those, we could observe that 04 (9.3%) and 02 (4.6%) were seropositive for EEEV and WEEV on HI, respectively.

For neutralizing antibody detection, $PRNT_{90}$ tests were performed for all samples of animals that showed antibodies to WNV (24/39) or SLEV (27/39) in the HI, and 28 samples were selected (**Table 2**).

Regarding the reactivity by HI and the sex of the individuals, when informed, 19 (48.7%) were females and 16 (41%) were males. Considering the detection of total antibodies and the age group, it was possible to observe that 1 (2.5%) animal with <1 year was reactive, while in the age group of 1–5 years, there were 4 (10.2%), in the 5–10 years, there were 17 (43.5%), and in those aged ≥10 years, there were 10 (25.6%), which suggests greater reactivity to arbovirus in animals from 5 years of age.

When comparing the use of animals in samples reactive by HI, a higher percentage of reactivity was observed in those used for work practice (46.1%). However, this utility also represents the majority of those investigated.

DISCUSSION

Serological monitoring has been carried out in recent years in domestic animals to assess whether these species can act as sentinels for specific arboviruses (8). Horses are large domestic animals that live outside the protection of homes and are therefore common victims of mosquito bites. Consequently, they are a suitable source for studies on the viruses transmitted by these arthropods (24).

In horses, the age pattern for morbidity reflects a high risk of disease in the very young and middle-aged, that is, during the ages of use or more intense activity. Indeed, the prevalence of diseases is high in the population of animals aged ≥15 years, with the majority presenting multiple anomalies with increasing age (25). Herein, we could observe that the seropositivity in the HI was higher in animals from 5 years of age and in spite of those used for the work practice. Furthermore, an increase of flavivirus seroprevalence with age in horses has been justified by low-level enzootic transmission, where the risk of infection increases over time (26).

The HI test has often been used in serological research because it can detect antibodies for a long time after natural infection. It is considered a test of high sensitivity and low specificity when compared to other serological tests (27). The heterotypic reactions found can be explained by the greater sensitivity than specificity present in the HI test (28).

The *Flavivirus* genus was responsible for the majority of seropositivity in the investigated equine population. Similar results of seropositivity were previously observed in Brazil (24, 29). In addition to the presence of the hemagglutination inhibitors of the HI test, monotypic reactions were observed for WNV and SLEV, which suggests that these viruses circulate among this population, corroborating with what was observed in the northern (30), northeast (31), and central-west regions (32). These results provide an evidence of the circulation of these closely related flaviviruses in different regions of the country.

With the recent increase in suspected cases of West Nile fever and the first isolation of WNV from an infected horse, there were concerns with the possibility that this virus would establish itself (33). However, it was determined that WNV has been circulating silently in Brazil for many years, probably between 2001 and 2005 (34).

In April 2018, epizootics in horses with meningoencephalitis were notified to the Ministry of Health by the Espírito Santo State Health Department–which culminated in the first isolation of WNV in the country, from an equine with neurological disease (16). Between March 2018 and June 2019, there was a confirmation of the occurrence of infection in equines in the municipalities of São Mateus, Nova Venécia, and Baixo Guandu (35). This report, together with our description of $PRNT_{90}$ titers against WNV in the same region, suggests that measures should be taken to monitor WNV activity in Brazil, especially with a permanent surveillance program of domestic birds and horses.

Furthermore, the possibility of the circulation of other flaviviruses cannot be excluded since it was observed cross-reactions for this genus. In addition, the HI test is characterized by a high cross-reactivity that generally allows only a qualitative conclusion about the presence of antibodies against flaviviruses (36).

It was possible to observe that the percentage of animals investigated with antibodies to the genus *Alphavirus* showed differences when comparing previous studies with 66.6% (37) and 14.6% (38).The divergence between the percentages observed between these studies may have occurred due to the different inclusion criteria adopted in the sampling, such as the

TABLE 2 | Results of $PRNT_{90}$ for WNV and SLEV in horses of Espírito Santo, Brazil (n = 28).

Virus tested	Number of equines HI titer ≥ 1:20 (%)	Number of equines $PRNT_{90}$ titer ≥ 1:10 (%)	Number of equines seropositive by $PRNT_{90}$ using four-fold greater titer criterion (%)
WNV	24 (88.8)	18 (66.6)	9 (32.1)
SLEV	27 (96.4)	18 (66.6)	14 (50)
Inconclusive	–	–	5 (17.8)*

**Approximately 17.8% (5/28) of samples previously positive for SLEV or WNV in the HI test produced positive reaction in $PRNT_{90}$ for both viruses and were thus identified as positive for flavivirus.*

non-vaccination of those investigated against arboviruses or, finally, access to the forest.

As for the *Orthobunyavirus* genus, monotypic reactions were observed for MAGV and TCMV. Previously neutralizing antibodies to MAGV (28.2%) and TCMV (15.7%) were detected in horses under 2 years of age in the Pantanal, indicating recent circulation (39). The serological evidence for both viruses was also found in the state of Pará in buffaloes, with a higher prevalence for MAGV with 7.33%, while the antibodies to TCMV in these animals corresponded to 1.37% of the total reactions in the HI (40). Most of the monotypic reactions detected in the study were for MAGV and TCMV.

There was no positivity for the *Phlebovirus* genus, represented by ICOV, nor for MAYV, CPCV, BSGV, BELV, or CARV. Therefore, we assume that these viruses did not circulate in the population tested. However, among the viruses that were associated with seronegativity, some have already had serological evidence in Brazilian equids, such as MAYV (30, 41–43) and ICOV (44), and CPCV (26, 29).

Study limitations included the small number of animals per species; thus, a more extensive evaluation of the animals that developed antibody titers is needed. Moreover, we included the viruses that are part of a routine serological test and $PRNT_{90}$ was performed only for WNV and SLEV, considering the epidemiological importance of the viruses in the period evaluated in the study region.

For a PRNT titer to be considered specific for a given flavivirus, a fourfold or greater titer to that virus than to all other flaviviruses tested for simultaneously must be demonstrated (45). However, the interpretation of heterotypic patterns is complex and this conservative criterion could be limited to provide the distinction of the most recent infection (46).

One of the fundamental aspects in controlling the transmission of arboviruses is the early detection of the virus or the identification of the increase in its activity. For WNV, monitoring is carried out mainly in bird and equine epizootics (47). Moreover, many horses live in the region; according to federal data, the effective herd of horses in the state is of 47,503 (48). Additional surveillance methods and collaboration among veterinary and human health services are essential in providing early warning and adequate protection of human and animal health against arboviruses.

Mammals can be better sentinels than birds when the objective is the early detection of diseases that threaten humans (49). As an example, the Venezuelan equine encephalitis virus has an epizootic cycle during which the amplification of the virus in the horse is sufficient to result in mosquito infection and is believed to significantly increase the risk of human infection (50). Contrarily, for WNV, horses and humans are considered to be dead-end hosts, so the virus is not directly contagious from horse to horse or horse to human (51). Therefore, the results from laboratory-based experiments should not be viewed in isolation (52).

Because horses are more prone to WNV infection than humans, the signs of illness are often observed and reported quickly, demonstrating the importance of the horse as a sentinel of epizootic WNV activity (53).

Accordingly, long-term studies are needed to increase the understanding of the role of horses and other vertebrate hosts in arbovirus circulation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

No animal ethics approvals were required for stored animal samples, considering that they were obtained from a continuous public health surveillance of a mandatory reporting disease. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LC contributed in sample collection and carried out statistical analysis. MF and AS performed serological tests. AR, MC, GA, MS, and GR conducted the investigation process. MG wrote the article and did data curation. LM and JC took part in critically reviewing the study. All authors contributed to the article and approved the submitted version.

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