Arquivos do Instituto Biológico
ISSN 1808-1657
https://doi.org/10.1590/1808-1657000462020

SCIENTIFIC ARTICLE
Animal Epidemiology

Seroprevalence and risk factors associated with seropositivity for equine encephalomyelitis virus in horses in Rio Grande do Norte, Brazil

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ABSTRACT

The present objective was to investigate the presence of anti-equine viral encephalomyelitis (EVE) antibodies and the possible risk factors for its dissemination in horses raised in the East and West Potiguar mesoregions of the state of Rio Grande do Norte, Brazil. Serological diagnosis for neutralizing antibodies against Eastern (EEEV), Western (WEEV) and Venezuelan (VEEV). Equine viral encephalomyelitis was performed using a seroneutralization technique on 811 blood samples from horses from ninety properties and sixteen municipalities between July 2018 and February 2019. Factors associated with EVE were evaluated using an investigative epidemiological questionnaire, and the data were statistically analyzed using the Epi Info 3.5.2 software with a confidence level of 95%. The seroprevalence of anti-EVE antibodies was 14.2% (115), with 10.36% (84) for EEEV, 6.9% (56) for WEEV, and null for EVE. When analyzing risk factors, it can be concluded that horses raised in properties that do not clean installations and/or rent out their pasture are more likely to have anti-EVE antibodies. These results show evidence that horses raised in the East and West Potiguar mesoregions were exposed to EEEV and WEEV, thus reinforcing the importance of vaccination and serological survey of nonvaccinated horses as a means of monitoring the disease.

Keywords: arbovirus; equine; viral encephalitis; seroneutralization; zoonosis.

INTRODUCTION

Equine viral encephalomyelitis (EVE) is widely distributed in the Americas, transmitted via mosquitoes of the *Culex*, *Aedes*, *Anopheles* and *Culiseta* genera, and is classified as a highly lethal zoonosis in accidental hosts such as equines and humans, while having rodents and passerines as its principal wildlife reservoirs (BARROS et al., 2007). There are currently seven known arboviruses that cause encephalitis that present a risk of human infection, such as the Eastern equine encephalomyelitis virus (EEEV), Western equine encephalomyelitis virus (WEEV), and Venezuelan equine encephalomyelitis virus (VEEV), which are three extremely relevant RNA viruses by epidemiological standards that belong

Received: Sept 16, 2020. Accepted: Jun 03, 2022
Associate Editor: Silvia Galleti
Peer Review History: Double-blind Peer Review.

to the genus *Alphavirus* and family *Togaviridae*, and that cause encephalomyelitis in horses and humans (FERNÁNDEZ et al., 2000).

Equine viral encephalomyelitis may have several clinical manifestations depending on the infecting variant and age of the animal and its immunological state. Generally, it has an acute course with anorexia, hyperthermia, difficulty walking, circling, tongue paralysis, lateral decubitus, and death (CAMPOS et al., 2013).

In human literature, the disease is called Eastern, Western, or Venezuelan encephalomyelitis and is considered the most invasive and virulent among the diseases caused by the family *Togaviridae*, resulting in high encephalitis, morbidity, and mortality rates. The viral invasion into the central nervous system occurs in 1/23 of individuals who develop viremia (ZACKS; PAESSLER, 2010). Mortality is around 75% among children and 50% among the elderly, where survivors may develop intellectual deficits, changes in personality, and spastic paralysis (LOPES et al., 2014).

Serological detection and isolation of the virus has been reported in several regions and demonstrates how widespread the virus is in Brazil, with studies from Pará (HEINEMANN et al., 2006; CAMPOS et al., 2013), Rondônia (AGUIAR et al., 2008), Ceará (SILVA et al., 2011), Paraíba (ARAÚJO et al., 2012), Pernambuco (PIMENTEL et al., 2009), Mato Grosso (MELO et al., 2012), Distrito Federal (SOUSA et al., 2015), Minas Gerais (LARA et al., 2014), São Paulo (KOTAIT et al., 1992; CUNHA et al., 2009) and Paraná (FERNÁNDEZ et al., 2000). The presence of these agents in domestic herds should be frequently monitored because of the possible economic and social losses incurred from infection (LARA et al., 2014).

Due to the absence of studies on this disease in the state of Rio Grande do Norte, the present objective was to perform a serological survey to determine the prevalence and risk factors associated with the presence of horses with anti-EVE antibodies in the East and West Potiguar mesoregions.

MATERIAL AND METHODS

This research was performed with horses in the state of Rio Grande do Norte (5°45'0 S, 36°30'0 O), in the Northeast region of Brazil. Municipalities were included if they had at least 500 equids registered in the Institute of Agricultural Defense and Inspection of Rio Grande do Norte and were located in the West Potiguar mesoregion (n = 409) and East Potiguar mesoregion (n = 402), totaling sixteen municipalities (Fig. 1). Samples were collected from 3 to 9% of the total number of equines in the municipalities. The investigated properties were selected according to the ease of access and availability for sample collection.

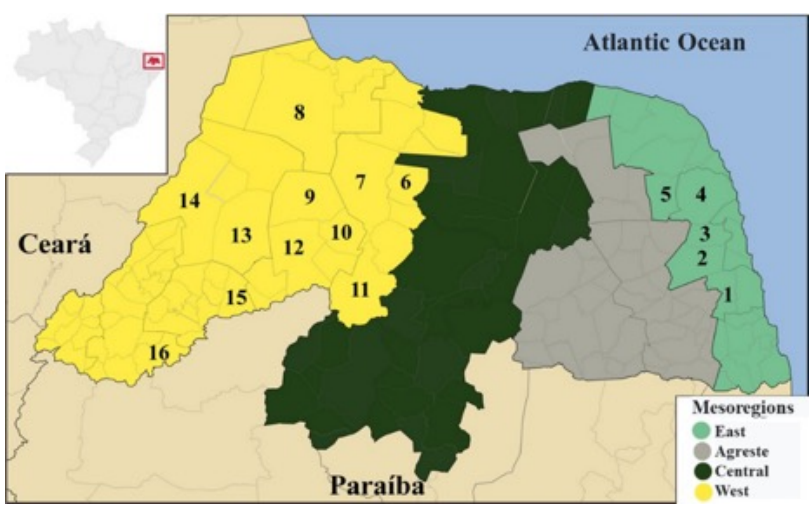

Figure 1. Map of the state of Rio Grande do Norte is divided into four mesoregions.
Note. Numbers indicate the municipalities where blood samples were collected from horses for serum diagnosis via seroneutralization for Eastern, Western, and Venezuelan equine encephalomyelitis. Mesoregion East: 1) São José de Mipibú, 2) Macaíba, 3) São Gonçalo do Amarante, 4) Ceará-Mirim, 5) Taipú. Mesoregion West: 6) Ipanguacu, 7) Assu, 8) Mossoró, 9) Upanema, 10) Paraú, 11) Jucurutu, 12) Campo Grande, 13) Caraúbas, 14) Apodi, 15) Patu e 16) Alexandria.

Blood samples were collected between August 2018 and February 2019, via aseptic venipuncture of the jugular vein using a dry vacuum tube, from randomly selected male and female horses. The horses were healthy, older than six months of age, unvaccinated for EVE and had different zootechnical standards. A total of 811 blood samples from 90 properties were centrifuged at 3000 rpm for 15 min to obtain serum samples and stored in 2-mL Eppendorf centrifuge microtubes at –20 °C until serum analysis. In each investigated property, an epidemiological research questionnaire was used containing questions about the owner, the property, the animal, and the sanitary, reproductive, and nutritional management.

Serum samples were sent to the Rabies and Viral Encephalitis Laboratory of the São Paulo Biological Institute for serological diagnosis by investigating neutralizing antibodies against EEEV, WEEV, and VEEV using the seroneutralization technique described by CUNHA et al. (2009). Sera were tested at dilutions of 1:5 for detection of antibodies against EEEV, WEEV, VEEV using a 100 DICT50/25 µL suspension of the Eastern (Tatuí sample), Western (TR 25717 sample), and Venezuelan complex (SP AN 15600 sample) viruses stored at the Biological Institute of São Paulo. After incubation for one hour at 37 °C, 100 µL of a VERO cell suspension containing 250,000 cells/mL was added for the EEEV, WEEV, and EEV tests. Plates for the seroneutralization technique were observed after a 72-h incubation period in a laboratory oven with 5% CO_2 at 37 °C, where the neutralization of the cytopathic effect was observed. Neutralizing antibody titers were expressed as the inverse of the dilution that neutralized 100 DICT50 of the virus, where sera were considered reactive with a titer ≥ 5 for the EEEV, WEEV, and VEEV viruses.

The number of horses needed for the study was calculated considering expected prevalence of 50%, with a confidence level of 95%, and statistical error of 5% (THRUSFIELD, 2007), resulting in a minimum sample of 385 animals per mesoregion. For the study of risk factors associated with the presence of equids with anti-EVE antibodies, a univariate analysis of the variables of interest was performed for the variables of interest using Pearson's chi-square test. A logistical regression analysis was then performed, with serology (positive or negative) considered a dependent variable. Variables considered independent or explanatory in the model were those that had statistical significance < 0.05% (HOSMER; LEMESHOW, 1987). The software Epi Info 3.5.2 was used for statistical calculations.

The research project was approved by the ethics committee for the use of animals of the Federal Rural University of Pernambuco, under number 100/2018, on August 22, 2018.

RESULTS AND DISCUSSION

In this study, seroprevalence for EVE was 14.2% (115/811; IC: 0.1222–0.1614), which is lower than the 56% prevalence observed by ARAÚJO et al. (2012) in the state of Paraíba. This suggests that this elevated number may be related to a higher mean precipitation in the state in 2009 (1345 mm) compared to the mean for the previous 15 years, 864 mm, which would increase vector density. During that same year, after the rainy season, nine positive animals for EVE were detected via RTPCR in five municipalities in Paraíba (SILVA et al., 2011).

Results for seroprevalence in other regions of the country, such as those reported in the state of São Paulo, 2.26% (CUNHA et al., 2009), and Rondônia, 11.3% (AGUIAR et al., 2008), were lower than in this study. However, reported frequencies of seropositive equines in Minas Gerais, in the south of the state of Paraná, and in the Pantanal region of Mato Grosso were 32.1%, 27.37%, and 21.0%, respectively (HEINEMANN et al., 2006; AGUIAR et al., 2008; LARA et al., 2014). This suggests there is no uniform distribution for the disease in Brazil since results also differ between regions that possess a similar climate. These results are apparently associated not only with the presence of the virus but also with environmental and climatic characteristics, as well as with the presence of effective hosts and vectors at the location (ARAÚJO et al., 2012).

Despite a seroprevalence rate of 14.2%, approximately 66.7% of the investigated properties had at least one positive animal (Table 1), similar to what was observed in Pará, where 56.24% of properties had positive equines (HEINEMANN et al., 2006). This raises the hypothesis that some intrinsic factors to the animals and/or factors related to their care may influence the variability within the same property, as observed by LARA et al. (2014), where 70% of positive horses were over four years of age, and because of the longer exposure time, these horses were more often positive than other younger horses in the same property.

Anti-VEEV antibodies were not observed in this study, which differs from findings obtained in Paraíba, a state that borders Rio Grande do Norte, with a prevalence of 63.7% (ARAÚJO et al., 2012), from research in Paraná between 1996 and 1999, with a prevalence of 54.5%, (FERNANDEZ et al., 2000), and from a survey in the Pantanal region of Mato Grosso, with 6.7% and 47.7% neutralizing antibodies for EEEV (IVERSSON et al., 1993; PAUVOLID-CORRÊA et al., 2010). The fact that antibodies were not detected for VEEV does not lessen the importance of serological monitoring for this variant since it is a reemerging virus in Brazil and contrary to EEEV and WEEV, transmission of VEEV to humans and horses can occur via oral and nasal secretions, direct contact, aerosols, as well as via vectors, namely mosquitoes of the genera *Aedes* and *Culex*, which represent a greater risk of dissemination of the disease (KOTAIT et al., 2008).

Table 1. Detection of antibodies for equine encephalomyelitis virus using seroneutralization in equine sera, displayed according to property, animals, and mesoregion within the state of Rio Grande do Norte, Brazil. 2018–2019.

Municipalities	Positives/Property (%)	Positives/Total (%)
Ceará-Mirim	5/6 (83.3)	16/80 (20.0)
Macaíba	9/9 (100)	25/136 (18.4)
Taipú	5/9 (55.5)	7/60 (11.7)
São Gonçalo do Amarante	4/5 (80.0)	9/60 (15.0)
São José de Mipibú	5/7 (71.4)	9/66 (13.6)
Total East Mesoregion	28/36 (77.8)	66/402 (16.4)
Alexandria	2/2 (100)	5/34 (14.7)
Apodi	3/3 (100)	5/29 (17.2)
Assu	4/8 (50.0)	8/66 (12.1)
Campo Grande	4/7 (57.1)	2/43 (4.7)
Caraúbas	4/6 (66.7)	5/46 (10.9)
Ipanguaçu	6/10 (40.0)	5/31 (16.1)
Jucurutu	2/2 (100)	5/28 (17.9)
Mossoró	2/4 (50.0)	5/67 (7.5)
Paraú	3/6 (50.0)	3/22 (13.6)
Patu	1/5 (80.0)	5/23 (21.7)
Upanema	1/1 (100)	1/20 (5.0)
Total West Mesoregion	32/54 (59.2)	49/409 (12)
Total	60/90 (66.7)	115/811 (14.2)

Seroprevalence for EEEV in this study was 10.36% (84), a lower rate than the one reported in a study in Mato Grosso, where 35.5% of equids had anti-EEEV antibodies; this result may be related to the fact that that state has an environment that facilitates the proliferation of the vectors (MELO et al. 2012). Higher rates than those in this study were also observed in Minas Gerais, where 30.2% of horses had serum reactive to the EEEV virus, and in the south of Pará (27.37%) (HEINEMANN et al., 2006). The main vectors for transmission of EEEV to horses and humans also belong to the genera *Aedes* and *Culex*. Therefore, like other arbovirus-caused diseases, they are also prone to seasonal variations which are influenced by temperature range, rainfall, and environmental factors that influence the vectors' presence. This may have played a role in the higher results found in areas with higher annual precipitation than the state of Rio Grande do Norte (SILVA et al., 2011). Exposure of the horses raised in the state to EEEV warns of the importance of equine vaccination because this is the most virulent of the three studied variants, with a mean lethality of 85% in animals and 65% in humans (KOTAIT et al., 2008).

The seroprevalence of WEEV found in this study was 6.9% (56), which differs from reports from other regions of the country that did not find serological evidence of contact with WEEV, such as those in Mato Grosso (MELO et al., 2012), in the southern state of São Paulo (CUNHA et al., 2009), and in Rondônia (AGUIAR et al., 2008). Antibodies for WEEV in horses have a low occurrence rate in Brazil, with the virus having been isolated for the first time in this species in Rio de Janeiro during an outbreak (BRUNO-LOBO et al., 1961) and antibodies detected in 1.09% of horses investigated in the state of Pará (HEINEMANN et al., 2006). However, in 2011, outbreaks of WEEV were reported in cities bordering Rio Grande do Norte in the states of Paraíba and Ceará (SILVA et al., 2011) and in the following year, 32% of animals studied in Paraíba had a positive serology test (ARAÚJO et al., 2012), which may explain the rate of positive animals for this variant in the studied region.

Among the 115 seropositive horses, 51.3% (59) had serological reactions only to EEEV and 27% (31) to WEEV, with parallel antibody detection for both variants in 21.7% (25) of the animals. The latter may result from the fact that these two viruses share vectors and hosts, as well as the similarity in the transmission cycle, which predisposes the animals to simultaneous infection (BARROS et al., 2007).

After univariate analysis based on the epidemiological questionnaires applied during sample collection (Table 2) and multivariate logistic regression analysis, it was possible to observe that properties that did not periodically clean their installations (odds ratio, OR = 3.7; confidence interval, 95%CI: 1.94–7.07, p = 0.000) and/or rented out their pasture (OR = 2.13; 95%CI: 1.24–3.64, p = 0.005) had a higher risk of horses with anti-EVE antibodies. The lack of cleaning of installations leads to accumulation of organic matter, which in turn leads to the proliferation of vectors, namely mosquitoes

(BARROS et al., 2007; LOPES et al., 2014). Renting out the pasture exposes animals from different properties, with varying sanitary management, to the same environment.

Table 2. Analysis of the factors associated with seroprevalence for equine encephalomyelitis virus in horses from the East and West Potiguar mesoregions, Rio Grande do Norte, Brazil, 2018—2019.

Variable	N	Positive (%)	p-value
Mesoregion			
East	402	66 (16.4)	0.043
West	409	49 (12)	
Species			
Asinine	6	-	0.217
Equine	785	115 (14.7)	
Mule	17	-	
Pony	3	-	
Sex			
Female	291	37 (12.7)	0.209
Male	518	78 (15.1)	
Rent the pasture			
Yes	87	21 (24.1)	0.005
No	724	94 (12.9)	
Cleaning of facilities			
Periodically	764	99 (13.0)	0.000
No	45	16 (36.0)	
Disinfect of facilities			
Yes	135	14 (10.4)	0.099
No	674	101 (15.0)	

The presence of unvaccinated horses with antibodies for EVE (Eastern and Western) in the state of Rio Grande do Norte require attention because it is relevant socially and economically impactful zoonosis. Brazil registered the first human case in the state of Bahia in 1956, however, there are currently no reports of recent cases (ROVID-SPICKLER, 2017). The United States of America, which monitors the disease via its Centers for Disease Control and Prevention, registers around six human cases annually (LOPES et al., 2014).

A serological survey of unvaccinated horses is an option for monitoring the occurrence and exposure to the virus, and serves to alert health surveillance programs, thus functioning as a sentinel species that is important for preventing human cases. The high lethality rate and the absence of specific treatments in horses and humans suggest that prevention is the best form to avoid damage caused by EVE because although there are experimental human vaccines for VEEV and EEEV, these are only available for individuals at a higher risk of infection and have limited availability (ROVID-SPICKLER, 2017).

For horses, there is a vaccine produced with formalin-inactivated EEEV and WEEV, where the resulting neutralizing antibody titers remain within appropriate levels up to six months after immunization, with a booster needed every six months (ZACKS; PAESSLER., 2010).

CONCLUSION

There is evidence that horses in the East and West Potiguar mesoregions are exposed to EEEV and WEEV, reinforcing the importance of equine vaccination and of a serological survey of nonvaccinated horses as a monitoring tool. It was also observed that properties that did not periodically clean installations and rent out their pastures had a higher number of positive animals. Thus, these practices were factors associated with seroprevalence in the studied region.

AUTHORS' CONTRIBUTIONS
Conceptualization: Rizzo, H.; Diniz, D.D.M.; Rocha, L.L.L.; **Data curation:** Pinheiro Júnior, J.W.; Diniz, D.D.M.; Rocha, L.L.L.; Jesus, T.K.S.; **Formal analysis:** Pinheiro Júnior, J.W.; **Funding acquisition:** Diniz, D.D.M.; Rocha, L.L.L.; Lara, M.C.C.S.H.; Villalobos, E.M.C.; **Investigation:** Diniz, D.D.M.; Rocha, L.L.L.; Lara, M.C.C.S.H.; Villalobos, E.M.C.; **Methodology:** Rizzo, H; Lara, M.C.C.S.H.; Villalobos, E.M.C.; Pinheiro Júnior, J.W.; **Project administration:** Rizzo, H.; Diniz, D.D.M.; Rocha, L.L.L.; **Resources:** Rizzo, H.; Lara, M.C.C.S.H.; Villalobos, E.M.C.; Diniz, D.D.M.; Rocha, L.L.L.; **Supervision:** Rizzo, H.; **Writing – original draft:** Jesus, T.K.S.; Lima, G.S.; **Writing – review & editing:** Rizzo, H.; Jesus, T.K.S.; Lima, G.S.

AVAILABILITY OF DATA AND MATERIAL
All datasets were generated or analyzed in the current study.

FUNDING
This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

ETHICAL APPROVAL
The research project was approved by the ethics committee for the use of animals of the Federal Rural University of Pernambuco (Universidade Federal Rural de Pernambuco), (100/2018, on August 22, 2018).

ACKNOWLEDGEMENTS
Not applicable.

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