Serological evidence of arboviruses and coccidia infecting horses in the Amazonian region of Brazil

Fábio Alves Gomes, Ana Maria Jansen, Rosângela Zacarias Machado, Hilda Fátima Jesus Pena, Marcílio Jorge Fumagalli, Angélica Silva, Bruna Farias Alves, André Luiz Rodrigues Roque, Luiz Tadeu Moraes Figueiredo

1 Laboratory of Trypanosomatid Biology, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil, 2 Federal Institute of Education Science and Technology of Roraima, Cararacará, Roraima, Brazil, 3 School of Agricultural and Veterinary Studies of Jaboticabal, São Paulo State University, Jaboticabal, Brazil, 4 School of Veterinary and Animal Science, Department of Preventive Veterinary and Animal Health, University of São Paulo, São Paulo, Brazil, 5 Virology Research Center, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

* These authors contributed equally to this work.

Abstract

Background
Arboviruses and protozoans can cause neurologic disorders in horses. In Brazilian Amazon, several horses presenting signs compatible with disorders caused by these infectious agents have been observed.

Objective
To contribute to the knowledge of this epidemiological picture, we sought to construct a serological diagnostic panel for neurotrophic infectious agents in local horses.

Material and methods
A total of 213 blood samples from horses were collected from 29 farms in three municipalities. Samples were evaluated and considered positive when they met the following criteria: titers ≥ 1:80 with the indirect fluorescent antibody test (IFAT) for apicomplexan protozoans; positive recombinant enzyme-linked immunosorbent assay (ELISA) with subsequent titers ≥ 1:10 by the PRNT for viruses; and detection under direct microscopic examination for Trypanosoma evansi.

Results
No horses were found to be infected by T. evansi, and only two were infected Toxoplasma gondii and/or Neospora spp. The highest protozoan infection rate was observed for Sarcocystis neurona (40.3%; n = 86/213). Among the positive ELISA samples tested by the plaque reduction neutralization test (PRNT), 92% (n = 76/83) were positive for St Louis Encephalitis virus, 43% (n = 6/14) were positive for West Nile virus and 33% (n = 16/48)
were positive for Mayaro virus. Eighteen percent (n = 39/213) of horses were co-infected by S. neurona and at least one arbovirus, particularly SLEV and/or MAYV.

**Conclusion**

Samples positive for SLEV associated with S. neurona, including samples from horses that had recovered from neurological signs were frequent, and must be considered when investigating the possible causes of neurological diseases in South Roraima horses.

**Introduction**

*Sarcocystis neurona, Neospora caninum* and *Toxoplasma gondii* are related coccidians that are reported to cause encephalitis in horses [1]. North and South American opossums, *Didelphis virginiana* and *D. albiventris*, respectively, are the known definitive hosts of *S. neurona* [2]. Horses become infected by ingesting *S. neurona* oocysts or sporocysts [3,4] and some horses may develop equine protozoal myeloencephalitis (EPM), a clinical progressively debilitating neurologic disease that affects the central nervous system [2].

Arboviral infections in humans and animals have been increasing globally with dengue (DENV), West Nile (WNV), Zika (ZIKV), chikungunya (CHIKV), Schmallenberg and bluetongue viruses. This phenomenon has been associated with the increased transport of animals and people worldwide, environmental and climate changes, and human encroachment into natural habitats [5]. Most arbovirus infections are asymptomatic or may be present as a mild acute febrile illness. However, several arboviruses are important human and veterinary etiologic agents that can cause disease of the central nervous system, leading to coma and death [5,6].

In Brazil, there are favorable ecological characteristics (availability of vectors, hosts, and other factors) that support the introduction and maintenance of arboviruses, such as DENV, ZIKV, and CHIKV, with a high impact on public health [7]. There are densely populated cities infested by *Culex* and *Aedes* mosquitoes and ecological changes, such as deforestation due to human settlements can affect the transmission cycles of arboviruses [8].

More than 200 different arboviral species have been isolated in Brazil, including 40 viruses have been associated with human diseases [8]. Many of these viruses belong to two taxonomic families: the *Flaviviridae* (*Flavivirus* genus) and *Togaviridae* (*Alphavirus* genus). Flaviviruses such as St. Louis Encephalitis virus (SLEV) and WNV can infect horses and cause infection of the central nervous system, with encephalomyelitis with ataxia being the most common clinical presentation [9–12]. SLEV was previously isolated from a horse’s brain with neurological symptoms, and is widely distributed in the Americas, from Canada to Argentina. In Brazil, SLEV was first isolated in 1960, from a pool of *Sabethes belisarioi* mosquitoes captured at the Belém-Brasilia highway in the Amazonian region. In this same region, studies on the SLEV cycle showed that *Culex declarator* and *Culex coronator* were vectors and wild birds, monkeys, sloths, armadillos and marsupials were virus reservoirs [13–15].

In parts of North America, Europe and Asia, *Culex quinquefasciatus* has already been proven to be a capable vector for the transmission of WNV from birds to horses [9,16,17]. This mosquito species is widespread in the American continents (including the western Amazon region) and is able to colonize both urban and wild areas, similar to those areas encountered in the south of the Roraima state [18–20].
Disease in horses due to Rocio (ROCV) *Flavivirus* and Mayaro (MAYV, *Alphavirus*) has not been described. However, ROCV causes human encephalitis, and MAYV produces human febrile acute illness disease with arthropathy [8,21]. ROCV was isolated from a wild bird, *Zenothrichia capensis* and from the mosquito *Psorophora ferox* [14,22], and MAYV antibodies were found in birds from seven families [23]. Moreover, mammals from different orders (Xenarthra, Marsupiala, Rodentia, Carnivora and Artiodactyla) presented antibodies to MAYV in northern Brazil and French Guiana [24,25].

Between June 2014 and May 2016, in the Amazon biome in the south of Roraima state, North Brazil, 25 horses died after presenting neurological symptoms (i.e., ataxia, poor motor skills, and torsion and bending in the neck). In addition to the economic impact caused by livestock deaths, the clinical and epidemiological characteristics of such cases suggest the involvement of infectious agents. Almost all horses have close contact with ruminants and considering the absence in the horses of respiratory, ocular or reproductive symptoms such as conjunctivitis, nasal discharge, abortion in the final third of pregnancy or death of neonate foals; or abortion and teratogeny between the ruminants that have contact with horses, we consider that Equine Herpes virus, Equine Arteritis virus and Bunyamwera virus have no relevance in the local epizootiological context. Regarding the encephalitic alphaviruses, we observed that there was a discrepancy in the information obtained in the studied farms about horse’s vaccination status. There was a strong divergence in the information provided by the owners and the farm staff about the vaccination for Eastern Equine Encephalitis virus and Western Equine Encephalitis virus, which could lead to misinterpretation of the results.

We, thus, performed a serological survey of horses from the southern region of the Roraima State to determine their levels of antibodies to some selected arboviruses (SLEV, WNV, ROCV and MAYV) and protozoa (*S. neurona*, *Neospora caninum* and *T. gondii*), and we performed microscopic blood examination for *Trypanosoma evansi* detection, aiming to construct a serological diagnostic panel for neurotrophic infectious agents in local horses.

**Material and methods**

**Horse blood samples**

A total of 213 native horses and without history of travelling outside the state of Roraima were enrolled in the serologic survey. The criteria employed to define the farms for the survey were as follows: (i) the horses on the farm had contact with dead or recovered horses from neurological symptoms between 2014/2016 (F1, F9, F11, F12, F14, F16, F17, F18, F19, F23, F24, F25, F26, F27, F28, F29), and (ii) the horses that frequented or dwelled on farms where there was a huge agglomeration of horses from other properties (F2, F3, F4, F5, F6, F7, F8, F10, F13, F15, F20, F21, F22). All farms were located in late-70’s settlements. Horses that participated in the study were apparently healthy and did not have a history of central nervous (CNS) infection, except for four animals that had recovered from a neurological disease.

Horse blood samples were collected by external jugular vein and were stored in 2 flasks, one flask without preservatives that was used for the arbovirus and coccidiosis survey and another containing EDTA that was used for hemoparasite direct testing. The tubes were stored in containers with ice and transported to the field laboratory.

**Parasitological and serological assays**

Blood smears were stained by Panòtico® (Laborclin, São Paulo, Brazil) and microscopically visualized for hemoparasites. After that, blood was centrifuged in microhematocrit tubes and the buffy coat was directly observed between the glass and cover slide by optical microscopy [26].
An indirect immunofluorescence antibody test (IFAT) was employed as previously described [27] to search for *Toxoplasma gondii*, *Neospora* spp. and *S. neurona* infections. The strains used as antigens were RH for *T. gondii*, and NC-1 for *Neospora* spp. and SN-138 for *S. neurona* [28,29], and a cut-off dilution of 1:80 was used for both parasites. The reactions were revealed by an anti-horse IgG conjugate, with the whole molecule produced in rabbit (Sigma Aldrich, Saint Louis, USA) at a 1:64 dilution following the manufacturer’s instructions. The *S. neurona* merozoites were grown and maintained in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1) in RPMI media supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. Supernatants with free merozoites were passed through a sterile 3-μm filter and then centrifuged at 1,500 g, for 10 minutes at 4°C. The pellet was resuspended in phosphate buffered saline (PBS), centrifuged and washed again with PBS. The concentration of merozoites in the filtrates was determined by counting on a hemocytometer and standardized to a concentration of 10⁷ merozoites/ml. Aliquots of 20 μl of SN-138 merozoites in PBS solution were dispensed to each well of a Teflon-coated antigen slide. Slides were air-dried at room temperature, fixed in 100% methanol, air-dried again, and stored at -22°C. For the assay, serum samples were diluted 1:80 in PBS for screening. Diluted samples were incubated for 30 minutes at 37°C. After three 5-minute washes in PBS, slides were air-dried, fluorescein isothiocyanate-conjugated anti-horse IgG was applied to each well, slides were incubated again, washed, air-dried and examined with a fluorescence microscope. Samples found positive with the 1:80 dilution screening were further diluted at two-fold increments to obtain the final titer. Positive and negative horse serum controls were included in each slide.

Arbovirus infections were evaluated in duplicates of horse serum samples by an indirect IgG enzyme-linked immunosorbent assay (ELISA) using anti-horse IgG peroxidase conjugate, whole molecule produced in rabbit (Sigma Aldrich, St. Louis, MO, USA). The reaction was quantified by adding 2,2-azinobis (3-ethylbenzthiazolinesulfonic acid) (ABTS) (KPL, Milford, MA, USA).

The ELISA used recombinant antigens of domain III peptides (rDIII) of the envelope proteins of West Nile virus (WNV), of Saint Louis encephalitis virus (SLEV) and of Rocio virus (ROCV), or a recombinant envelope protein 2 (rE2) of Mayaro virus (MAYV). All antigens were produced in an *Escherichia coli* (*E. coli*) system as previously described [30,31]. Mouse hyperimmune sera to WNV, SLEV, ROCV and MAYV were used as positive control, and extracts of *E. coli* proteins as negative control. The cut-off value in the assay was calculated as the mean optical density (O.D.) of the negative controls plus three standard deviations. Samples with an average O.D. above the cutoff value were considered positive.

**Plaque reduction neutralization tests (PRNTs)**

Serum samples that were positive for only one of the flaviviruses in the ELISA were submitted to a 90% plaque reduction neutralization test (PRNT₉₀) [32], in Vero cells with the same virus. For MAYV, all positive samples in the ELISA assay were tested, even though they were also positive for flaviviruses. Viruses used in the PRNT₉₀ were WNV NY99, SLEV SpAn 11916, ROCV SpH 34675 and MAYV Be Ar 20290. Serum samples were considered as positive by PRNT₉₀ when a serum dilution of 1:10 or greater reduced the viral formation of plaques by at least 90%.

**Statistics**

We calculated the correlation between seropositivity rates and: i) contact of horses with dead or recovered horses from neurological symptoms between 2014/2016 and ii) the habit to visit or dwell on farms where are a recurrent huge agglomeration of horses from other properties.
We use the chi-square contingency (BioEstat, version 5.0), adopting an $\alpha = 0.05$ level of significance. These analyses also encompassed the 4 horses that had recovered from neurological disease. Considering that WNV (6 positives), \textit{T.gondii} (1 positive) and \textit{Neospora} spp. (2 positives) had a low number of positive samples, these agents were not included in the statistical analyses.

**Geospatial analysis**

The satellite images from study area were provided by the Landsat 8 OLI sensor platform, corresponding to scenes 232/59, 231/59 and 231/60 https://earthexplorer.usgs.gov. The map with the location of the area in South America and the state of Roraima was made with the USGS National Map Viewer ttp://viewer.nationalmap.gov/viewer/.

**Ethical statements**

Sample collections and handling procedures were approved by the Animal Ethics Committee of the Instituto Oswaldo Cruz (No. L-009/2017), Brazil, and the University of São Paulo Animal Ethics Committee, Brazil (No 66/2018).

**Results**

The highest infection rates observed in horses from southern Roraima state, Brazilian Amazon was for \textit{S. neurona} followed by SLEV (Table 1 and Fig 1).

**Protozoans**

Out of 213 horses 40.4\% (N = 86) were positive for coccidiosis: one was positive for the three tested protozoans, one was positive for \textit{Neospora} spp. and \textit{S. neurona} and eighty-four (39.4\%) were positive for \textit{S. neurona} only, with titration ranging 1:80 to 1:5120 by IFAT, being 47 (1:80–1:320), 39 (1:640–1:5120) (S1 Table). For \textit{S. neurona}, no difference was observed in positivity regarding contact of horses with dead or recovered horses from neurological symptoms ($p = 0.9$) and the habit to visit or dwell on farms where are a recurrent huge agglomeration of horses from other properties ($p = 0.3$). In theuffy coat evaluation for hemoparasites, including \textit{Trypanosoma evansi} included, all samples were negative.

**Viruses**

One hundred and forty-nine horses (70\% seropositivity) had IgG antibodies to SLEV, 75 (35.2\%) to WNV, 48 (22.5\%) to MAYV and 19 (8.9\%) to ROCV. Regarding flaviviruses, 97 samples presented a monotypic reaction (to only one virus) and were selected for confirmation by a virus-specific neutralization assay (PRNT$_{90}$). Other sera presented polytypic reactivity (the same serum sample reacted to two or more viruses), and the most common association was SLEV-WNV in 37 of 87 sera samples (42.5\%). All 19 horses with seropositivity test for ROCV by ELISA also were positive for another Flavivirus (Fig 2A). A total of 48 sera were positive for the Alphavirus MAYV, and curiously 38 of them were positive for at least additional Flavivirus (Fig 2B).

\textbf{St. Louis encephalitis virus (SLEV).} A total of 92\% (n = 76/83) of the monotypic positivity for SLEV were also positive in PRNT$_{90}$, with titers ranging from 1:10 to 1:5120 (S1 Table). Regarding categories agglomeration and contact with dead or recovered horses the results were distributed in the following way: i) with contact 15 (1: 10–1:80), 29 (1:160–1:640), 0 (1:1280–1:5120), without contact 11 (1: 10–1:80), 18 (1:160–1:640), 3 (1:1280–1:5120); ii) with
Table 1. Results of the PRNT for viruses and IFAT for protozoans in horses (n = 213) from Roraima state, Brazilian Amazon.

| AGENT / POSITIVES / INFECTION RATE (%) | CONTACT | AGGLOMERATION |
|-------------------------------------|---------|---------------|
|                                     | Yes     | No            | P value | Yes     | No     | P value |
| SLEV / 76 / 35.7                    | 44      | 32            | 0.2     | 34      | 42     | 0.4     |
| MAYV / 16 / 7.5                     | 4       | 12            | 0.08    | 10      | 6      | 0.4     |
| WNV / 6 / 2.8                       | 6       |               |         | 1       | 5      |         |
| Sarcocystis neurona / 86 / 40.4     | 44      | 42            | 0.9     | 38      | 48     | 0.3     |
| Toxoplasma gondii / 1 / 0.5         |         |               |         |         |        |         |
| Neospora spp. / 2 / 0.9             |         |               |         |         |        |         |

Contact—contact of horses with dead or recovered horses from neurological symptoms; Agglomeration—the habit to visit or dwell on farms where are a recurrent huge agglomeration of horses from other properties. IFAT—immunofluorescence antibody test; PRNT plaque reduction neutralization test; P value—result of chi square test; St. Louis Encephalitis Virus (SLEV), West Nile Virus (WNV), Mayaro virus (MAYV).
agglomeration 10 (1: 10–1:80), 22 (1:160–1:640), 2 (1:1280–1:5120), without agglomeration 16 (1: 10–1:80), 25 (1:160–1:640), 1 (1:1280–1:5120).

No difference in positivity was observed regarding contact of horses with dead or recovered horses from neurological symptoms ($p = 0.2$) and the habit to visit or dwell on farms where are a recurrent huge agglomeration of horses from other properties ($p = 0.4$). Among the 76 PRNT$_{90}$ positive horses, 7.8% ($n = 6$) were also positive also for MAYV, and 41% ($n = 31$) were positive for $S$. neurona.

**West Nile virus (WNV).** A total of 43% ($n = 6/14$) of horses displaying monotypic positivity for WNV were also positive by PRNT$_{90}$, the highest titer was 1:80, being 1 (1:10), 2 (1:20), 1 (1:40) and 2 (1:80). Half of the WNV PRNT$_{90}$ positive horses lived in properties located in the center of Rorainópolis the municipality with the most extensively human-modified landscape out of all the study area.

**Mayaro virus (MAYV).** A total of 33% ($n = 16/48$) of horses were positive in PRNT$_{90}$ for MAYV with low titers 12 (1:10), 3 (1:20) and 1 (1:40); six horses 4 (1:10) and 2 (1:20) were also positive for SLEV ($S$ Table). No difference in positivity was observed regarding contact of horses with dead or recovered horses from neurological symptoms ($p = 0.08$) and the habit to visit or dwell on farms where are a recurrent huge agglomeration of horses from other properties ($p = 0.4$) (Table 1). Of the four recovered horses, two of them had coinfections by SLEV (PRNT$_{90}$) and $S$. neurona, one was positive for SLEV and the other was positive for $S$. neurona ($S$ Table).

**Discussion**

Protozoan infection was investigated because neurological symptoms in horses may be related to these parasites, such as *Neospora hughesi*, *Trypanosoma evansi* and $S$. neurona [1, 33, 34]. Our results demonstrated that *Neospora* spp. and *Toxoplasma gondii* had low circulation, and *T. evansi* was absent among the studied horses since all horses tested negative for trypanosomiasis, and only two of them (0.9%) were positive for these coccidiosi. The infections by *Neospora* spp. are probably related to herd management, since the two infected horses derived from beef and dairy farms, were in close contact with cattle and dogs, a known risk factor for infection by *Neospora* spp. [35–38]. A high infection rate by $S$. neurona in our study provides
evidence to suppose that in Roraima there are definitive hosts, probably opossums, maintaining the *S. neurona* biological cycle; since the variation in the seroprevalence has typically been attributed to climate and the density of competent hosts [2], our infection rate was lower than that observed in another region of the Amazon biome, in the Rondônia state, where among 192 horses, 84.4% were positive by recombinant ELISA assay [39].

South Roraima state presents some features, such as floodplain areas, primary forest and a small densely populated area that could enhance the dissemination and maintenance of arboviruses. We found at least two closely related flaviviruses that induced cross-reactive antibodies. The cross-reaction of antibodies between related viruses is a phenomenon commonly observed flaviviruses of the Japanese encephalitis serocomplex, as is the case for SLEV and WNV [40,41]. Horse sera were screened using a recombinant rDIII and rE2 antigen ELISA for flaviviruses and Mayaro, respectively. Monotypic Flavivirus-monotypic positive results were confirmed using a PRNT threshold of 90%, to optimize the virus-specific diagnosis.

In the present study, 23% of the horses (n = 49/213) presented positive results for more than one Flavivirus by ELISA, suggesting that one or more viruses of this genera infected horses in the area. Our data also suggest that cross-reactions occurred in the sera of most of the studied horses. Cocirculation of more than one Flavivirus was detected on one farm only (F15), where the cocirculating viruses were probably SLEV and WNV. Considering that 92% (n = 76/83) of monotypic samples for SLEV by ELISA were confirmed by the PRNT, which is considered the gold standard for detecting neutralizing antibodies [41], it is unlikely that the low WNV titers by the PRNT were due to cross-reactivity between these two viruses; however, it does not rule out that this result could be due to another phylogenetically closely related virus. It is important to point out that the rDIII ELISA has not been evaluated for its sensitivity/specificity for IgG detection, meaning that we might have not detected some positive samples in the initial screening, and consequently, these samples were not evaluated by a specific PRNT. For a satisfactory accuracy, it would be necessary to analyze Flavivirus polytypic samples with ELISA screening. The rE2 ELISA for MAYV has been reported as having 100% sensitivity and approximately 80% specificity for IgG detection [31].

Considering the ELISA for SLEV, the studied horses presented an infection rate higher than the mean infection rate in the other five regions of Brazil (38.9%, n = 83/213 vs. 12.3%, n = 93/753 in monotypic horses) [42]. Our infection rate for SLEV based on the PRNT90, 35.6% (n = 76/213), is similar to infection rates previously described in the Central Region of Brazil (40% to 45%) [43]. In another serological survey of 1401 horses from five municipalities (four in the Amazonian region), the mean infection rate for SLEV was 50.9% [44]. Thus, the detection of horses infected by St. Louis encephalitis virus in our study confirms that this virus is enzootic in Roraima state.

We highlight a property (F1; n = 16) with a high infection rate by SLEV that also reported the deaths of many horses with neurological symptoms from 2014 to 2016. This farm had 1000 bovines, 150 sheep, 200 pigs, 200 ducks and 33 equines with free contact, and particularly, the horses there lived there in precarious conditions of hygiene. The same farm also reported many young ducks with neurological disease. Thus, SLEV, an avian zoonotic virus, could have been introduced into the area causing neurologic disease in ducks and horses.

WNV has been reported in equines in South America since 2006 [45]. In Brazil, there was a recent report of WNV isolation from a horse’s brain with neurological symptoms [46] in the Espírito Santo state, southeastern Brazil. An infection rate of 33.9% for SLEV, WNV and ROCV was detected with the rDIII antigen ELISA in 753 horses from three geographic regions of Brazil (southeast, northeast and central) [42,47], a similar result to the 35.2% observed in Roraima. Nevertheless, monotypic reactions to WNV were confirmed by the PRNT in only 11.4% (n = 9/79) of the animals whereas in Roraima, it was almost four-fold higher (43%);
n = 6/14). All 19 Roraima horses with antibodies to ROCV also reacted to other flaviviruses preventing a confirmation of a previous infection by this virus.

In 2011 and 2014, horses in the midwestern region of Brazil were tested using a blocking ELISA followed by a confirmatory PRNT. In 2011, the infection rate for WNV was 3% and 3.2% in 2014 (based on the CDC positivity criterion) [48,49], both results are similar to those observed in Roraima (2.8%). In the present study, all six horses infected by WNV were derived from areas with a strong anthropogenic disturbance, a spatial distribution pattern similar to that found in the Central Region of Brazil [50]. Half of the horses were derived from the Rorainópolis municipality, which is the largest focus of human occupation in the south of the Roraima state (S1 Table and Fig 1). None of the infected horses travelled outside of Rorainópolis municipality, confirming the autochthony of these infections. Therefore, our results agree with those of other authors, suggesting that WNV has been circulated over the last decade in large areas of Brazil and infecting horses.

MAYV is an endemic virus in the Amazon region where it infects nonhuman primates [51], in our work a high proportion (33%) of the studied horses had neutralizing antibodies to MAYV, however PRNT \textsubscript{90} values are quite low in these animals ($\leq 1:40$), suggesting possible cross-reaction with other alphaviruses. six horses presented antibodies to MAYV and SLEV suggesting that both viruses could cocirculate in the studied region.

Three out of the four recovered horses presented PRNT\textsubscript{90} SLEV titers higher than 1:160, and two presented titers higher than 1:640 without positive results in ELISA screenings for other viruses. Infection by \textit{S. neurona} was also detected in three of the four recovered horses, suggesting that SLEV (perhaps associated with a coinfection with \textit{S. neurona}) may be responsible for neurological diseases in south Roraima horses.

Conclusions

Horses were probably infected by WNV, suggesting that this virus reached this Amazonian region of Brazil. ROCV was not confirmed and is probably not circulating in the area. SLEV and \textit{S. neurona} were found to individually and coinfect studied horses, including two of them that recovered from neurological symptoms.

Surveillance of new cases as well as virologic and protozoologic diagnoses are necessary to clarify the possible correlation of SLEV/\textit{S. neurona} infections and the genesis of neurologic disease presented in Roraima horses.

Supporting information

S1 Table. Indirect serological diagnosis for arboviruses, in horses of the South of Roraima state.
(XLSX)

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Author Contributions

Conceptualization: Fábio Alves Gomes, Ana Maria Jansen, Hilda Fátima Jesus Pena, André Luiz Rodrigues Roque, Luiz Tadeu Moraes Figueiredo.

Data curation: Fábio Alves Gomes, Bruna Farias Alves, André Luiz Rodrigues Roque, Luiz Tadeu Moraes Figueiredo.

Formal analysis: André Luiz Rodrigues Roque, Luiz Tadeu Moraes Figueiredo.

Funding acquisition: Luiz Tadeu Moraes Figueiredo.

Investigation: Fábio Alves Gomes.

Methodology: Ana Maria Jansen, Marcílio Jorge Fumagalli, Angélica Silva, André Luiz Rodrigues Roque, Luiz Tadeu Moraes Figueiredo.

Project administration: Bruna Farias Alves, André Luiz Rodrigues Roque.

Supervision: Ana Maria Jansen, Rosângela Zacarias Machado, Hilda Fátima Jesus Pena, Marcílio Jorge Fumagalli, André Luiz Rodrigues Roque.

Validation: André Luiz Rodrigues Roque, Luiz Tadeu Moraes Figueiredo.

Writing – original draft: Fábio Alves Gomes, Ana Maria Jansen, André Luiz Rodrigues Roque, Luiz Tadeu Moraes Figueiredo.

Writing – review & editing: Fábio Alves Gomes, Ana Maria Jansen, André Luiz Rodrigues Roque, Luiz Tadeu Moraes Figueiredo.

References

1. Dubey JP, Mitchell SM, Morrow JK, Rhyan JC, Stewart LM, Granstrom DE, et al. Prevalence of antibodies to Neospora caninum, Sarcocystis neurona, and Toxoplasma gondii in wild horses from central Wyoming. Journal of Parasitology. 2003; 89: 716–720. https://doi.org/10.1645/GE-66R PMID: 14533680

2. Dubey JP, Howe DK, Furr M, Saville WJ, Marsh AE, Reed SM, et al. An update on Sarcocystis neurona infections in animals and equine protozoal myeloencephalitis (EPM). Veterinary Parasitology. 2015; 209: 1–42. https://doi.org/10.1016/j.vetpar.2015.01.026 PMID: 25737052

3. Fenger CK, Granstrom DE, Gajadhar AA, Williams NM, McCrillis SA, Stamper S, et al. Experimental induction of equine protozoal myeloencephalitis in horses using Sarcocystis sp. sporocysts from the opossum (Didelphis virginiana). Veterinary Parasitology. 1997; 68: 199–213. https://doi.org/10.1016/s0304-4017(96)01112-0 PMID: 9066066

4. Dubey JP, Lindsay DS. Isolation in immunodeficient mice of Sarcocystis neurona from opossum (Didelphis virginiana) faeces, and its differentiation from Sarcocystis falcata. International Journal for Parasitology. 1998; 28: 1823–1828. https://doi.org/10.1016/s0020-7519(98)00166-0 PMID: 9925260

5. Chapman GE, Baylis M, Archer D, Daly JM. The challenges posed by equine arboviruses. Equine Veterinary Journal. 2018; 50: 436–445. https://doi.org/10.1111/evj.12829 PMID: 29517814

6. Weaver SC, Barrett ADT. Transmission cycles, host range, evolution and emergence of arboviral disease. Nature Reviews Microbiology. 2004; 2: 789–801. https://doi.org/10.1038/nrmicro1006 PMID: 15378043

7. Gould E, Pettersson J, Higgs S, Charrel R, de Lamballerie X. Emerging arboviruses: Why today? One Health. 2017; 4: 1–13. https://doi.org/10.1016/j.ohnet.2017.06.001 PMID: 28785601

8. Figueiredo LTM. Emergent arboviruses in Brazil. Revista da Sociedade Brasileira de Medicina Tropical. 2007; 40: 224–229. https://doi.org/10.1590/s0037-86822007000200016 PMID: 17568994

9. Castillo-Olivares J, Wood J. West Nile virus infection of horses. Veterinary Research. 2004; 35: 467–483. https://doi.org/10.1051/vetres:2004022 PMID: 15236677

10. Auguste AJ, Pybus OG, Carrington CVF. Evolution and dispersal of St. Louis encephalitis virus in the Americas. Infection, Genetics and Evolution. 2009; 9: 709–715. https://doi.org/10.1016/j.meegid.2008.07.006 PMID: 18708161
11. Terzian ACB, Mondini A, de Moraes Bronzoni RV, Drumond BP, Ferro BP, Cabrera EMS, et al. Detection of Saint Louis Encephalitis Virus in Dengue-Suspected Cases During a Dengue 3 Outbreak. Vector-Borne and Zoonotic Diseases. 2011; 11: 291–300. https://doi.org/10.1089/vbz.2009.0200 PMID: 20645866

12. Carrera J-P, Forrester N, Wang E, Vittor AY, Haddow AD, López-Vergés S, et al. Eastern Equine Encephalitis in Latin America. New England Journal of Medicine. 2013; 369: 732–744. https://doi.org/10.1056/NEJMoa1212628 PMID: 23964935

13. Travassos da Rosa J.F.S., Freitas E.M., Travassos da Rosa A.P.A., Pinheiro F.P. Epidemiologia do vírus da encefalite de São Luís na Amazônia, Revista da FSES. 1980; 25: 73–80.

14. Travassos da Rosa J.F.S., Travassos da Rosa A.P.A., Vasconcelos P.F.C., Rodrigues S.G., Travassos da Rosa E.S., Dias L.B., Cruzi A.C.R., Arboviruses isolated in the Evandro Chagas Institute, including some described for the first time in the Brazilian Amazon region, their known hosts, and their pathology for man, in: Travassos da Rosa A.P.A., Vasconcelos P.F.C., Travassos da Rosa J.F.S. (Eds.), An Overview of Arbovirology on Brazil and Neighboring Countries, Instituto Evandro Chagas, Belém. 1998; 19–31.

15. Rosa R, Costa EA, Marques RE, Oliveira TS, Furtini R, Bomfim MRQ, et al. Isolation of Saint Louis Encephalitis Virus from a Horse with Neurological Disease in Brazil. Weaver SC, editor. PLoS Neglected Tropical Diseases. 2013; 7: e2537. https://doi.org/10.1371/journal.pntd.0002537 PMID: 24278489

16. Reisen WK, Fang Y, Martinez VM. Avian Host and Mosquito (Diptera: Culicidae) Vector Competence Determine the Efficiency of West Nile and St. Louis Encephalitis Virus Transmission. Journal of medical entomology. 2005; 42: 9.

17. Napp S, Petrić D, Busquets N. West Nile virus and other mosquito-borne viruses present in Eastern Europe. Pathogens and Global Health. 2018; 112: 233–248. https://doi.org/10.1080/20477724.2018.1483567 PMID: 29979950

18. Barbosa M das GV, Fé NF, Marciano AHR, Silva APT da, Monteiro WM, Guerra MV de F, et al. Record of epidemiologically important Culicidae in the rural area of Manaus, Amazonas. Revista da Sociedade Brasileira de Medicina Tropical. 2008; 41: 658–663. https://doi.org/10.1590/s0037-86822008000019 PMID: 19124448

19. Klein TA, Lima JBP, Tang AT, Klein TA, Lima JBP, Tang AT. Seasonal distribution and diel biting patterns of culicine mosquitoes in Costa Marques, Rondônia, Brazil. Memórias do Instituto Oswaldo Cruz. 1992; 87: 141–148. https://doi.org/10.1590/s0074-027619920000010021 PMID: 1364053

20. Korte RL, Fontes G, Camargo J de SAA, Rocha EMM da, Araújo EAC de, Oliveira MZ de, et al. Survey of Bancroftian filariasis infection in humans and Culex mosquitoes in the western Brazilian Amazon region: implications for transmission and control, Revista da Sociedade Brasileira de Medicina Tropical. 2013; 46: 214–220. https://doi.org/10.1590/0037-8682-1708-2013 PMID: 23740057

21. Pinheiro FP, Freitas RB, Travassos da Rosa JF, Gabbay YB, Mello WA, LeDuc JW, Travassos da Rosa A. An outbreak of Mayaro virus disease in Belterra, Brazil. I. Clinical and virological findings. American Journal of Tropical Medicine and Hygiene. 1981; 30:674–81 https://doi.org/10.4269/ajtmh.1981.30.674 PMID: 6266263

22. Lopes OS, de Abreu Sacchetta L, Francy DB, Jakob WL, Calisher CH. Emergence of a new arbovirus disease in Brazil. II. Isolation of Rocio virus from Psorophora Ferox (Humboldt, 1819). American Journal of Epidemiology, 1981; 113:122–125. https://doi.org/10.1093/oxfordjournals.aje.a113075 PMID: 6110335

23. Hoch AL, Peterson NE, Le Duc JN, Pinheiro FP. An outbreak of Mayaro virus disease in Belterra, Brazil. II. Entomological and ecological studies. American Journal of Tropical Medicine and Hygiene. 1981; 30:689–98. https://doi.org/10.4269/ajtmh.1981.30.689 PMID: 6266265

24. Azevedo RSS, Silva EVP, Carvalho VL, Rodrigues SG, Neto JPN, Monteiro HAO, et al. Mayaro Fever Brazilian Amazon. Emerging Infectious Diseases. 2009; 15: 1830–1832. https://doi.org/10.3201/eid1511.090461 PMID: 19891877

25. de Thoisy B, Gardon J, Salas RA, Morvan J, Kazanjii M. Mayaro Virus in Wild Mammals, French Guiana. Emerging Infectious Diseases. 2003; 9: 1326–1329. https://doi.org/10.3201/eid0910.030161 PMID: 14609474

26. Woo PTK. The haematocrit centrifuge for the detection of trypanosomes in blood. Canadian Journal of Zoology. 1969; 47: 921–923. https://doi.org/10.1139/z69-150 PMID: 5343381

27. Camargo M. E. Introdução as técnicas de imunofluorescência. Revista Brasileira Patologia Clínica, 1974; 10: 87–107.

28. Dubey JP; Carpenter JL; Speer CA; Topper MJ; Uggl a A. Newly recognized fatal protozoan disease of dogs. Journal American Veterinary Medicine Association, 1988;192, 9: 1269–1285.
29. Duarte PC, Daft BM, Conrad PA, Packham AE, Gardner IA. Comparison of a serum indirect fluorescent antibody test with two western blot tests for the diagnosis of equine protozoal myeloencephalitis. Journal of Veterinary Diagnostic Investigation. 2003; 15: 8–13. https://doi.org/10.1177/1040638030150103 PMID: 1258288

30. Chávez JH, Silva JR, Amarilla AA, Moraes Figueiredo LD, Figueiredo LT. Domain III peptides from Flavivirus envelope protein are useful antigens for serologic diagnosis and targets for immunization. Biologicals. 2010; 38: 613–618. https://doi.org/10.1016/j.biologicals.2010.07.004 PMID: 20817489

31. Fumagalli MJ, de Souza WM, Rromeiro MF, de Souza Costa MC, Slessareno RD, Figueiredo LTM. Development of an Enzyme-Linked Immunosorbent Assay To Detect Antibodies Targeting Recombinant Envelope Protein 2 of Mayaro Virus. Tang Y-W, editor. J Clin Microbiol. 2019;57. https://doi.org/10.1128/JCM.01892-18 PMID: 30787146

32. Earley E, Peralta PH, Johnson KM. A Plaque Neutralization Method for Arboviruses. Experimental Biology and Medicine. 1967; 125: 741–747. https://doi.org/10.3181/00379727-125-32194 PMID: 19538255

33. Dubey JP, Barr BC, Barta JR, Bjerkås I, Björkman C, Blagburn BL, et al. Redescription of Neospora caninum and its differentiation from related coccidia. International Journal for Parasitology. 2002; 32: 929–946. https://doi.org/10.1016/s0020-7519(02)00094-2 PMID: 12076263

34. Silva RAMS, Arosemena NAE, Herrera HM, Sahib CA, Ferreira MSJ. Outbreak of trypanosomosis due to Trypanosoma evansi in horses of Pantanal Mato-grosso, Brazil. Veterinary Parasitology. 1995; 60: 167–171. https://doi.org/10.1016/0304-4017(95)00757-4 PMID: 8644453

35. Basso W, Venturini L, Venturini MC, Moore P, Rambeau M, Unzaga JM, et al. Prevalence of Neospora caninum Infection in Dogs From Beef-Cattle Farms, Dairy Farms, and From Urban Areas of Argentina. Journal of Parasitology, 2001; 87(4):906–907. https://doi.org/10.1645/0022-3395(2001)087[0906:POCIIJ2.CO;2] PMID: 11534656

36. Paré J, Fectue G, Fortin M, Marsolais G. Seroenpidiologic study of Neospora caninum in dairy herds. Journal of the American Veterinary Medical Association. 1998; 213: 1595–1598. PMID: 9838960

37. Dubey JP, Mitchell SM, Morrow JK, Rhyman JC, Granstrom DE, et al. Prevalence of antibodies to Neospora caninum, Sarcocystis neurona, and Toxoplasma gondii in wild horses from central wyoming. Journal of Parasitology, 2003; 89: 716–720. https://doi.org/10.1645/GE-66R PMID: 14533680

38. Ribeiro MJM, Rosa M, Bruhn FRP, Garcia A de M, Rocha CBM da, Guimarães AM. Seroepidemiology of Sarcocystis neurona, Toxoplasma gondii and Neospora spp. among horses in the south of the state of Minas Gerais, Brazil. Revista Brasileira de Parasitologia Veterinária. 2016; 25: 142–150. https://doi.org/10.1590/S1984-29612016029 PMID: 27334814

39. Hoane JS, Gennari SM, Dubey JP, Ribeiro MG, Borges AS, Yai LEO, et al. Prevalence of Sarcocystis neurona and Neospora spp. infection in horses from Brazil based on presence of serum antibodies to parasite surface antigen. Veterinary Parasitology, 2006; 136: 155–159. https://doi.org/10.1016/j.vetpar.2005.10.023 PMID: 16310955

40. Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, et al. Antigenic Relationships between Flaviviruses as Determined by Cross-neutralization Tests with Polyclonal Antibodies to Trypanosoma evansi in horses of Pantanal Mato-grossense, Brazil. Veterinary Parasitology. 1995; 929–946. https://doi.org/10.1016/s0304-4017(02)00094-2 PMID: 12076263

41. Kuno G. Serodiagnosis of Flaviviral Infections and Vaccinations in Humans, Advances in Virus Research. Elsevier. 2003; 3–65. https://doi.org/10.1016/s0065-3527(03)61001-8 PMID: 14714429

42. Silva JR, Romeiro MF, de Souza WM, Munhoz TD, Borges GP, Soares OAB, et al. A Saint Louis encephalitis and Rocovirus serosurvey in Brazilian horses. Revista da Sociedade Brasileira de Medicina Tropical. 2014; 47: 414–417. https://doi.org/10.1590/0037-8682-0117-2014 PMID: 25229279

43. Pauvolid-Corrêa A, Tavares FN, Costa EV da, Burlandy FM, Murta M, Pellegrin AQ, et al. Serologic evidence of the recent circulation of Saint Louis encephalitis virus and high prevalence of equine encephalitis viruses in horses in the Nhecolândia sub-region in South Pantanal, Central-West Brazil. Memórias do Instituto Oswaldo Cruz. 2010; 105: 829–833. https://doi.org/10.1590/S0074-27622010000600017 PMID: 20945001

44. Rodrigues SG, Oliva OP, Araujo FAA, Martins LC, Chiang JO, Henriques DF, et al. Epidemiology of Saint Louis encephalitis virus in the Brazilian Amazon region and in the State of Mato Grosso do Sul, Brazil: elevated prevalence of antibodies in horses. Revista Pan-Amazônica de Saúde. 2010;1. https://doi.org/10.5123/S2176-62232010000100012

45. Morales M, Barrandequy M, Fabbri C, Garcia J, Vissani A, Trono K, et al. West Nile Virus Isolation from Equines in Argentina, 2006. Emerging Infectious Diseases. 2006; 12: 1559–1561. https://doi.org/10.3201/eid1210.060852 PMID: 17176571

46. Martins LC, Silva EVP da, Casseb LMN, Silva SP da, Cruz ACR, Pantoja JA de S, et al. First isolation of West Nile virus in Brazil. Memórias do Instituto Oswaldo Cruz. 2019;114. https://doi.org/10.1590/0074-02760180332 PMID: 30672980
47. Silva JR, Medeiros LC de, Reis VP dos, Chávez JH, Munhoz TD, Borges GP, et al. Serologic survey of West Nile virus in horses from Central-West, Northeast and Southeast Brazil. Memórias do Instituto Oswaldo Cruz. 2013; 108: 921–923. https://doi.org/10.1590/0074-0276130052 PMID: 24037110

48. Pauvolid-Corrêa A, Morales MA, Levis S, Figueiredo LTM, Couto-Lima D, Campos Z, et al. Neutralising antibodies for West Nile virus in horses from Brazilian Pantanal. Memórias do Instituto Oswaldo Cruz. 2011; 106: 467–474. https://doi.org/10.1590/s0074-02762011000400014 PMID: 21739036

49. Pauvolid-Corrêa A, Campos Z, Juliano R, Velez J, Nogueira RMR, Komar N. Serological Evidence of Widespread Circulation of West Nile Virus and Other Flaviviruses in Equines of the Pantanal, Brazil. Michael SF, editor. PLoS Neglected Tropical Diseases. 2014; 8: e2706. https://doi.org/10.1371/journal. pntd.0002706 PMID: 24551266

50. Ometto T, Durigon EL, de Araujo J, Aprelon R, de Aguiar DM, Cavalcante GT, et al. West Nile virus surveillance, Brazil, 2008–2010. Transactions of The Royal Society of Tropical Medicine and Hygiene. 2013; 107: 723–730. https://doi.org/10.1093/trstmh/trt081 PMID: 24008895

51. Mackay IM, Arden KE. Mayaro virus: a forest virus primed for a trip to the city? Microbes and Infection. 2016; 18: 724–734. https://doi.org/10.1016/j.micinf.2016.10.007 PMID: 27989728