A Saint Louis encephalitis and Rocio virus serosurvey in Brazilian horses

Jaqueline Raymondi Silva[1], Marilia Farignoli Romeiro[1], William Marciel de Souza[1], Thiago Demarchi Munhoz[2], Gustavo Puía Borges[3], Otavio Augusto Brischi Soares[4], Carlos Henrique Coelho de Campos[4], Rosângela Zacarias Machado[2], Maria Luana Cristiny Rodrigues Silva[5], Joice Lara Maia Faria[6], Juliana Helena Chávez[1] and Luiz Tadeu Moraes Figueiredo[1]

ABSTRACT

Introduction: Arboviruses are an important public health problem in Brazil, in especially flaviviruses, including the Saint Louis encephalitis virus (SLEV) and the Rocio virus (ROCV), are especially problematic. These viruses are transmitted to humans or other vertebrates through arthropod bites and may cause diseases with clinical manifestations that range from asymptomatic infection, viral hemorrhagic fever to encephalitis. Methods: A serological survey of horses from various regions of Brazil using an enzyme-linked immunosorbent assay (ELISA) with recombinant SLEV domain III peptides and ROCV E protein as antigens. Results: Overall, 415 (55.1%) of the 753 horses that were screened were seropositive for flavivirus and, among them, monotypic infection symptoms. Conclusions: Our results suggest that SLEV and ROCV previously circulated among horses in northeast, west-central and southeast Brazil.

Keywords: Arbovirus. Saint Louis encephalitis virus. Rocio virus. Serosurvey. Epidemiology. Brazil.

INTRODUCTION

Arboviruses are transmitted to humans or other vertebrates through hematophagous arthropod bites1. The emergence or reemergence of arboviruses is a natural phenomenon associated with species evolution and adaptation. Brazil is a large tropical country (8,514,215km²) with more than one-third of its territory still covered by rainforests and other natural ecosystems; it also has an extremely diverse flora and fauna2. Additionally, Brazil has a population of approximately 200 million people, most of whom live in large cities in the northeast and southeast regions of the country. These conditions are ideal for the existence of many arboviruses, which are maintained in a large variety of zoonotic cycles. In fact, more than 200 distinct arbovirus species have been isolated in Brazil, and approximately 40 of them cause human diseases3.

The genus Flavivirus, family Flaviviridae, comprises positive-sense, single-stranded ribonucleic acid (RNA) viruses and includes some of the most important arboviral pathogens. In South America, Saint Louis encephalitis virus (SLEV) and Rocio virus (ROCV), which are both flaviviruses that are closely related to the Japanese encephalitis serocomplex viruses, can produce human encephalitis4,5. ROCV was first isolated from a fatal human case during an epidemic of encephalitis in Ribeira Valley in the southeastern region of the State of São Paulo, Brazil, in 19754. This ROCV epidemic involved over 1,000 reported cases with a 10% fatality rate and sequelae in 20% of the cases5.

SLEV was first isolated in 1933 during a major epidemic in St Louis, Missouri, USA. The virus is widely distributed throughout the western hemisphere from Canada to Argentina and outbreaks or clusters of encephalitis cases associated with SLEV have been reported with fatality rates that range from 5% to 20%6,7. In Brazil, SLEV infections have been diagnosed during acute febrile illness or in meningoencephalitis patients, with 2 patients from State of Pará in the northern region of Brazil and 9 patients from State of São Paulo in the southeastern region exhibiting these symptoms8,9.
Additionally, SLEV was recently isolated from the brain of a horse that presented neurological signs in the countryside of State of Minas Gerais, Brazil\textsuperscript{13}. Moreover, serological evidence of ROCV circulation in horses was recently reported in the Pantanal, Brazil\textsuperscript{14}. Consequently, these large animals are suitable sources for serological studies of flaviviruses that can cause encephalitis. Here, we report a serological survey of horses from various regions of Brazil for SLEV and/or ROCV detection.

**METHODS**

**Samples**

Serum samples were collected from 753 horses from 2004 through 2009 in 5 Brazilian states, which included 183 serum samples collected at Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista, Jaboticabal, State of São Paulo; 15 serum samples collected at Universidade de Uberaba, Uberaba, Minas Gerais; 267 serum samples collected through an epidemiological surveillance in Mato Grosso do Sul; 200 serum samples collected from horses at the Military Academy of the Black Needles in Resende, Rio de Janeiro; and 88 serum samples collected at Universidade Federal de Campina Grande, in Campina Grande, Paraíba. All participant horses were healthy at the time of blood collection and had no history of central nervous system infections or of vaccinations against flaviviruses.

**ELISA**

Serum samples were tested in duplicate using an indirect immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) with domain III (rDIII) from SLEV and ROCV in \textit{Escherichia coli}. Briefly, high-binding affinity 96-well microtiter plates (Corning, USA) were coated with 300ng of rDIII antigen and incubated for 48h at 4°C. Blocking was performed by incubating the plates for 2h at 37°C with 10% nonfat milk in PBS. SLEV and ROCV mouse immune ascitic fluid (MIAF) were used as positive controls. The MIAFs were incubated at 37°C for 1h. An anti-mouse IgG conjugated with peroxidase (Sigma, USA) was used as the secondary antibody. The reaction was quantified by adding 2,2-azinobis (3-ethylbenzthiazolinesulfonic acid) (ABTS) (KPL, USA) after a 30 minute incubation at 37°C. Spectrophotometry was performed at 405nm using a Multiskan Spectrum (Thermo, USA)\textsuperscript{15,16}. The test cut-off value was determined using optical density (OD) means added to three times the standard deviations of ODs obtained from at least four negative-control sera. After the test, all positive samples were diluted 100- to 1,600-fold and tested using ELISA to determine serum titers.

**Ethical considerations**

The sample collection and handling procedures were approved by the University of São Paulo Animal Ethics Committee, Brazil (protocol # 161-2008).

**RESULTS**

A total of 415 (55.1%) of the 753 equine sera had IgG antibodies to SLEV and ROCV. Additionally, 271 (35.9%) sera reacted to SLEV and 144 (19.1%) to ROCV. When considering only the monotypic reactions, 93 (12.3%) serum samples had antibodies to SLEV and 46 (6.1%) to ROCV. The monotypic positive serum titers ranged from 100 to 800 in the horses from the States of Rio de Janeiro, Mato Grosso do Sul and Paraíba, with the exception of one serum sample from Mato Grosso do Sul state, which had a titer of 1,600. An elevated seropositivity to SLEV was found in State of Mato Grosso do Sul, which had 140 (52.4%) positive samples, and an elevated seropositivity to ROCV was found in State of Paraíba, which had 31 (35.2%) positive samples, as shown in Table 1.

**DISCUSSION**

Our results indicate that SLEV, ROCV or a very closely related virus from the Japanese encephalitis serocomplex had infected horses in Brazil. SLEV has been isolated from \textit{Culex declarator} and \textit{Culex coronator}\textsuperscript{17}. Presumably,

| TABLE 1 - Positive SLEV and ROCV samples according to the rDIII ELISA. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Brazilian States | Samples | total | monotypic | total | monotypic | total | monotypic |
| Mato Grosso do Sul | 267 | 63 | 23.5 | 13 | 4.9 | 140 | 52.4 | 56 | 21.0 |
| Minas Gerais | 15 | 0 | 0.0 | 0 | 0.0 | 3 | 20.0 | 3 | 20.0 |
| Paraíba | 88 | 31 | 35.2 | 12 | 13.6 | 32 | 36.3 | 4 | 4.5 |
| Rio de Janeiro | 200 | 34 | 17.0 | 13 | 6.5 | 41 | 20.5 | 12 | 6.0 |
| São Paulo | 183 | 16 | 8.7 | 8 | 4.4 | 55 | 30.0 | 18 | 9.8 |
| Total | 753 | 144 | 19.1 | 46 | 6.1 | 271 | 35.9 | 93 | 12.3 |

SLEV: Saint Louis encephalitis virus; ROCV: Rocio virus; rDIII: domain III; ELISA: enzyme-linked immunosorbent assay.
In Brazil, levels are high, indicating that flaviviruses may circulate widely throughout Brazil, most likely in cycles that involve migratory birds and mosquitoes (Culex spp.). These seropositivity and cross-reactions to ROCV. These seropositivity levels are high, indicating that flaviviruses may circulate widely in Brazil. ROCV was also isolated from a wild bird, Zonotrichia capensis, and wild birds are considered to be amplifier vertebrate hosts of this virus. Additionally, ROCV was also isolated from the mosquito species Psorophora ferox, and Ae. scapularis is likely another mosquito species involved in ROCV transmission. The factors responsible for the appearance and disappearance of this virus are unknown.

Many (55%) of the horses studied were seropositive for ROCV and/or SLEV. Moreover, 93 (12.3%) of the horses presented monotypic reactions to SLEV, and 46 (6.1%) presented monotypic reactions to ROCV. These seropositivity levels are high, indicating that flaviviruses may circulate widely in Brazil. We also show the first evidence of widespread ROCV infection among horses in Brazil. Horses are large domestic animals that live outside the protection of buildings and are therefore common mosquito bite victims. Consequently, these animals are a suitable source for sero-epidemiological studies on mosquito-borne viruses.

Most (82%) of the horse serum samples studied here were positive for both viruses, suggesting a tendency toward cross-reactions among flaviviruses that circulate in Brazil (Bussuquara, Cacipacore, Iguape, Ilheus, yellow fever, West Nile and dengue 1, 2, 3 and 4 viruses) and precluding a discrimination of the infecting virus. Another possibility is that the animals were infected by multiple flaviviruses during their lifetimes; however, 18% of the serum samples showed monotypic reactions in titers, suggesting that a subset of the horses were infected by either SLEV (12.3%) or ROCV (6.1%).

The highest monotypic positivity to SLEV was found in the horse serum samples from the States of Mato Grosso do Sul (20%) and Minas Gerais (20%), suggesting that higher circulating levels of the viruses might be found in the southeastern and central-western regions of Brazil. However, we have not analyzed horses from the southern and northern regions. The highest monotypic positivity to ROCV was found in the horse serum samples from State of Paraíba (13.6%), in the northeastern region. However, these higher rates of seropositivity were not significantly different from those observed in other Brazilian states.

Our results demonstrate that the indirect IgG-ELISA, which evaluated the SLEV and ROCV rDIII antigen, is suitable to screen a large amount of sera and could therefore be used during serological Flavivirus surveys. Unfortunately, due to the low volume of each serum sample, it was not possible to perform neutralization tests on the ELISA-positive sera to confirm the infections by SLEV and ROCV.

However, the serological evidence presented here, together with the lack of epizootic reports in horses, suggests the presence of asymptomatic, subclinical or pathognomonic infections in these animals. Interestingly, SLEV circulation may have coincided with human outbreaks of dengue-like diseases in São José do Rio Preto and with outbreaks in horses from Minas Gerais state. Furthermore, a case was reported recently in Ribeirão Preto City, São Paulo. However, other outbreaks of ROCV and SLEV may be occurring without being detected.

In conclusion, here, we report serological evidence of SLEV and ROCV infections in horses and the possible co-circulation of other flaviviruses. The data presented here are particularly important because many non-dengue acute febrile illnesses remain undiagnosed in Brazil. This report should serve as a warning to the Brazilian public health authorities that routine diagnoses of other (non-dengue) flaviviruses should be implemented and that surveillance should also be increased, particularly for SLEV and ROCV.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**FINANCIAL SUPPORT**

This work was supported by FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant Number: 08/50617-6 and Scholarships Numbers: 08/52605-9; 12/02836-6 and 12/24150-9), Brazil.

**REFERENCES**


