

Alphavirus serosurvey in domestic herbivores in Pará State, Brazilian Amazon

Soropesquisa de *Alphavirus* em herbívoros domésticos no Estado do Pará, Amazônia Brasileira

Estudio serológico de *Alphavirus* en herbívoros domésticos en el Estado de Pará, Amazonía Brasileña

Alexandre do Rosário Casseb
Instituto da Saúde e Produção Animal, Universidade Federal Rural da
Amazônia, Belém, Pará, Brasil

Jannifer Oliveira Chiang
Seção de Arbovirologia e Febres Hemorrágicas, Instituto Evandro
Chagas/SVS/MS, Ananindeua, Pará, Brasil

Lívia Carício Martins
Seção de Arbovirologia e Febres Hemorrágicas, Instituto Evandro
Chagas/SVS/MS, Ananindeua, Pará, Brasil

Sandro Patroca da Silva
Centro de Inovações Tecnológicas, Instituto Evandro Chagas/SVS/MS,
Ananindeua, Pará, Brasil

Daniele Freitas Henriques
Seção de Arbovirologia e Febres Hemorrágicas, Instituto Evandro
Chagas/SVS/MS, Ananindeua, Pará, Brasil

Livia Medeiros Neves Casseb
Seção de Arbovirologia e Febres Hemorrágicas, Instituto Evandro
Chagas/SVS/MS, Ananindeua, Pará, Brasil

Pedro Fernando da Costa Vasconcelos
Seção de Arbovirologia e Febres Hemorrágicas, Instituto Evandro
Chagas/SVS/MS, Ananindeua, Pará, Brasil
Departamento de Patologia, Universidade do Estado do Pará, Belém,
Pará, Brasil

ABSTRACT

Pará State comprises 26% of Brazilian Amazon Region where a large diversity of arboviruses has been described. This study aimed to assess the prevalence and distribution of hemagglutination-inhibition (HI) that detect antibodies against four units to *Eastern equine encephalitis virus* (EEEV), *Western equine encephalitis virus* (WEEV), *Mayaro virus* (MAYV) and *Mucambo virus* (MUCV), a subtype of the *Venezuelan equine encephalitis virus* (VEEV), in 2,191 serum samples of horses, cattle, sheep and water buffaloes in Pará State, Brazil. The goal was to identify the prevalence of antibodies in these domestic farm animals to determine which arboviruses are circulating and determine which farm animal is the most sensitive for detecting *Alphavirus*. Antibodies against all investigated arboviruses were detected in almost all animals species studied. Our results indicated that domestic herbivores are susceptible to the tested arboviruses and evidence of active *Alphavirus* in farm animals in the Brazilian Amazon. An analysis of HI antibody prevalence by animal species indicated significant difference between horses and water buffaloes, cattle and sheep. The horses showed higher prevalence of antibodies in heterotypic reactions showing to be the best species of domestic farm animal to serve as sentinel to detect the movement of arboviruses in the Brazilian Amazon.

Keywords: *Alphavirus*; Amazonian Ecosystem; Animals; Hemagglutination Inhibition Tests.

INTRODUCTION

With few exceptions, the arboviruses are zoonosis because they are maintained in nature in a cycle of non-human vertebrates and arthropods and the Pan-Amazonia is the largest arbovirus reservoir in the world. The Brazilian Amazon hosts the largest variety of known and isolated arboviruses¹. In Brazil, several species cohabitat between hematophagous diptera and

wild vertebrates, especially in Amazon. Those species may have high diversity among large species population sizes, sometimes are globally unique. Altogether provide favorable environmental for viruses, mainly some arboviruses^{1,2}.

Disequilibrium in this ecosystem is associated with factors such as deforestation, highways and dam constructions³, colonization and urbanization of new areas after railway construction⁴ and improper use of the land and subsoil^{5,6}. All of these factors contribute to the appearance of new arboviruses diseases.

The *Togaviridae* family comprises the *Alphavirus* and *Rubivirus* genus. The *Alphavirus* infect a variety of vertebrates, including man. Eleven types of virus have been associated with human disease, and at least eight have been responsible for outbreaks: *Eastern equine*

Correspondence / Correspondência / Correspondencia:

Livia Medeiros Neves Casseb
Instituto Evandro Chagas/SVS/MS
Seção de Arbovirologia e Febres Hemorrágicas
Rodovia BR 316, km 7, s/n. Bairro: Levilândia
CEP: 67030-000 Ananindeua-Pará-Brasil
E-mail: liviacasseb@iec.pa.gov.br

encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), Mayaro virus (MAYV), O'Nyong-nyong virus (ONNV), Ross River virus (RRV) Chikungunya virus (CHIKV), and Getah virus (GEV)⁷.

Infections in domestic herbivores by arboviruses belonging to the genus *Alphavirus* manifest themselves as systemic, encephalic and hemorrhagic syndromes. The encephalic form is more common in horses, which are mostly affected by EEEV and WEEV⁸.

There are approximately 210 arboviruses isolated in Brazil, with large majority of them in the Brazilian Amazon^{9,10,11}, however, a few studies have focused on identifying the prevalence of antibodies in domestic animals to determine which *Alphavirus* are circulating and which farm animal is the most sensitive for detecting these *Alphavirus*. Those are the goals of this study.

MATERIALS AND METHODS

ETHICAL FEATURES

All procedures which involved newborn (2-3 days old) of Swiss albino mice and domestic animals were done to avoid undue suffering. This study was approved by the Animal Research Ethics Committee (CEPAN) of the Instituto Evandro Chagas (IEC) (protocol 054/2009 CEPAN/IEC) in November 27, 2009.

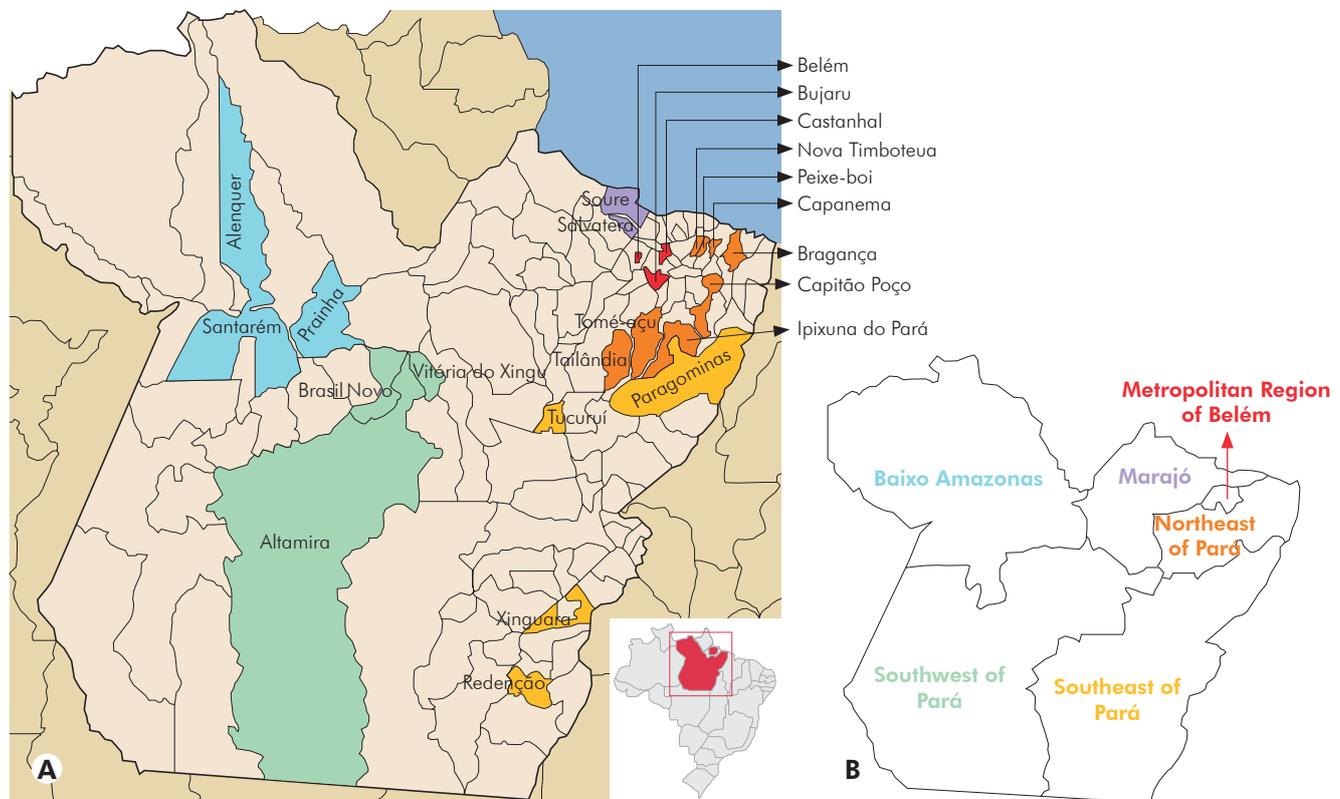
ANIMALS AND SAMPLES

Inclusion Criteria: Animals aged from 2 years old, without arbovirus vaccination, born and raised at the collection site.

Exclusion Criteria: Animals younger than 2 years old, vaccinated against any arbovirus or originating from any other places than the collection site.

Blood was collected throughout 2009 from animals living in the six mesoregions of the Pará State (Figure 1). The samples were collected by jugular vein vacuum puncture, and the following domestic herbivores were independent of sex and race: horses (*Equus ferus*), cattle (*Bos spp.*), water buffalo (*Bubalus bubalis*) and sheep (*Ovis aries*).

The animals were restrained and the local asepsis was done, jugular vein was punctured, without anticoagulant, using a vacuum system. From 5 to 10 mL of blood was collected and waited approximately for 90 min to allow coagulation and serum separation and it was subjected to centrifugation at 2,000 rpm for 5 min. The separated serum was transported on ice and then stored at -70° C until being analyzed by serologic testing. A total of 2,191 serum samples were collected: 385 from sheep, 399 from cattle, 654 from water buffaloes and 753 from horses.



A: collecting municipalities; **B:** the division into mesoregions of Pará State.

Figure 1 – Schematic representation of the Pará State indicating were collected from the domestic herbivores serum samples

HEMAGGLUTINATION-INHIBITION (HI) TEST

HI test was performed following protocol described by Shope¹². This test was performed with antigens from EEEV, WEEV, MAYV and *Mucambo virus* (MUCV) isolated in Brazil. This virus belonged to the collection of the Arbovirology and Hemorrhagic Fevers Division of the IEC, Ananindeua, Pará State, Brazil. These antigens were prepared from infected brain, liver or serum of newborn mice, and sera were tested against four antigen units¹³. The criteria for positivity as monotypic (reaction against only one antigen) or heterotypic (reactions against two or more antigens of the same virus genus, that may indicate cross reaction) was used¹⁴.

STATISTICAL ANALYSIS

The proportion method recommended by the Brazilian Ministry of Health was used to evaluate data⁹. Susceptibility tests were conducted on Lowenstein-Jensen medium. Antibiotics were applied in the recommended critical concentrations of 0.2 µg/mL for H, 40.0 µg/mL for R, 2.0 µg/mL for E, 25.0 µg/mL for Z, 4.0 µg/mL for S and 20.0 µg/mL for Et.

The analysis was done using the Chi-square test and the sample scores were measured using a significance level of 0.05 (BioEstat v.5.0 software)¹⁵.

RESULTS

All equine, bovine and water buffaloes presented high serologic prevalence to the four alphaviruses used in this study; the sheep did not present HI antibodies to MAYV. The total reactions (TR), heterotypic reactions (HR) and monotypic reactions (MR) are shown in table 1.

The HR correspond to 65.7% while MR represented 34.7% of all positive reactions. The HR of animal species comparing to the TR were: equine 77.9%, water buffaloes 42.8%, bovine 11.4% and ovine 9.1%. The equine showed HR higher than other animal species ($p < 0.0001$). The water buffaloes have also presented HR significantly higher than other ruminant species ($p < 0.0001$), but no differences were found among bovine and ovine prevalences.

The MR of animal species comparing to the TR were: ovine 90.9%; bovine 88.67%; water buffaloes 57.8% and equine 22.1%. No statistic difference was found in the ovine and bovine ($p = 0.9203$) analysis, but significant differences were obtained in the other species analyzed ($p < 0.001$).

When was observed each species of arboviruses and studied animal, the HR showed the following results: equine - EEEV (67.4%), MUCV (85.7%), WEEV (86%) and MAYV (90%); water buffaloes - EEEV (28.3%), WEEV (41.2%), MUCV (53.1%) and MAYV (69.2%); bovine - EEEV (33.3%), MUCV (25%), WEEV (7.40%) and MAYV (0%); ovine - EEEV (33.33%); the other alphaviruses did not show positive HR. The MR were: equine - EEEV (32.6%), WEEV (13.95%), MAYV (10.0%) and MUCV (14.3%); water buffaloes - EEEV (71.7%), WEEV (60.8%), MAYV (30.8%) and MUCV (46.9%); bovine - EEEV (66.7%), WEEV (92.6%), MAYV (100%) and MUCV (75%); ovine - EEEV (66.7%), WEEV (100%) and MUCV (100%).

DISCUSSION

The current study determined the prevalence of HI antibodies against EEEV, WEEV, MAYV and MUCV in four species of domestic herbivores. Of the *Alphavirus*, all of them have already been isolated from arthropods and/or wild vertebrates and also from humans in Brazil. Only the EEEV and WEEV have been proven to cause disease in domestic herbivores (horses).

In Brazilian Amazon, EEEV and WEEV have been isolated from birds, horses and mosquitoes in Belém Metropolitan Region and Southern Pará. Antibodies against these arboviruses have also been found, with a low prevalence in wild mammals¹⁶. The results of this study indicated a high prevalence of HI antibodies against EEEV and WEEV in horses, which showed a frequent exposure of this population to mosquitoes that carry these arboviruses, in order to confirm even the MR, it is necessary to conduct more specific tests such as the plaque reduction neutralization test (PRNT) or molecular studies as the next-generation sequencing (NGS).

Despite the lack of reports on clinical disease in horses, only one epizootic outbreak by EEEV has been reported in the Municipality of Bragança, Pará State, in 1962¹⁷; however there are no reported cases of encephalitis in human in the Amazon Region caused by those arboviruses¹⁸. But, EEEV caused neurological illness in human beings in the North America⁸, and two fatal cases had been reported in South America, one in Brazil, in Bahia State¹⁹ and the other one in Trinidad and Tobago²⁰. Aguilar et al²¹ report that the low incidence of cases of human encephalitis in the South America probably is due to the low infectivity and not the virulence of the isolated cases of EEEV that circulate in that region.

Table 1 – Prevalence of HI antibodies against four tested *Alphavirus* types in sera of domestic herbivores with heterotypic reactions (HR), monotypic reactions (MR) and total reactions (TR)

Virus	Horse			Cattle			Water buffalo			Sheep		
	%HR	%MR	%TR	%HR	%MR	%TR	%HR	%MR	%TR	%HR	%MR	%TR
EEEV	20.3	9.8	30.1	0.25	0.50	0.75	2.3	5.8	8.1	0.26	0.5	0.8
WEEV	19.6	3.2	22.8	0.50	6.7	6.8	3.2	4.7	7.8	–	1.8	1.8
MAYV	4.8	0.5	5.3	–	0.25	0.25	1.4	0.6	2.0	–	–	–
MUCV	7.2	1.2	8.4	0.25	0.75	1.0	4.0	3.5	7.5	–	0.25	0.25
Total	51.9	14.7	66.6	1.0	8.2	8.8	10.9	14.6	25.4	0.26	2.55	2.85

Conventional sign used: – Numeric data not equal to zero due to rounding.

Some epizootic strains of VEEV may be transmitted from horse to human by mosquitoes and the epidemic transmission cycle of VEEV involves horses which are the main source of the virus, namely, the source of infection for new hematophagous mosquitoes²². The MUCV subtype III of VEEV²³ is usually isolated in Amazonia and it has been associated with sporadic febrile syndromes in humans in Brazil, Trinidad and Tobago, Surinam and French Guiana.

The virus illness has evolved without important complications and without a record of epidemics²⁴. It has also been isolated in Southeastern Brazil, close to the Vale do Ribeira, in São Paulo State²⁵; however, there are no reports of that disease in domestic animals. Nevertheless, a 10 month-old equine experimentally infected via intramuscular route with high doses of MUCV has developed viremia which lasted for three days, with fever that lasts for 24 h and persistent leucopenia²⁶. Additionally, Iverson et al²⁷ found anti-MUCV antibodies in an equine that had symptoms compatible to encephalitis in the Brazilian Pantanal.

Ulloa et al²⁸ found 45% of positivity for antibodies by plaque reduction neutralization test (PRNT) for the cattle in VEEV in Mexico. These data contrasted with those observed in this current study, where MUCV prevalence in cattle was just 1% of positivity for antibodies HI. It is interesting to note a greater percentage of positive reactions to horse antibody HI (8.36%) and water buffalo (7.49%).

MAYV was first isolated in Trinidad and Tobago in 1954²⁹ and is frequently isolated in northern of South America from humans, wild vertebrates (mainly monkeys) and mosquitoes. Outbreaks in humans caused by the virus of that febrile exanthematous disease have been frequently reported in Amazonia^{11,30,31}. In this study, the prevalence of HI antibodies against MAYV in domestic herbivores was low. It should be noticed that antibodies against this virus were not detected in sheep during HI test, despite cohabitation with other animal species that did exhibit positive reactions against MAYV. There are no reports of any disease caused by this virus in domestic animals; however, the clinical syndromes that appear in

humans presents symptoms that may not be perceived in domestic animals such as horses and ruminants.

In the International Catalogue of Arboviruses are registered approximately one hundred forty arboviruses infecting humans and farm animals, and some of these arboviruses infections only cause sub-clinical disease detected by the presence of antibodies³². In this study, domestic herbivores showed different prevalences of HI antibodies to the *Alphavirus* analyzed with the higher prevalence for horses, which may be related to the thickness of the skin which is thinner than those of the ruminants, thereby easing up the infection. On the other hand, the water buffalo has the keratin layer of the skin thicker than the cattle, because buffaloes have the habit of living in flooded regions where there is the greatest amount of arthropods, so increasing the probability of infection since there was higher prevalence of antibodies to the *Alphavirus* HI compared to other ruminants analyzed.

CONCLUSION

In the Brazilian Amazon, farm animals may have exposed for thousands of mosquito bites in areas where could be used as transmission for this virus. It only takes one infected mosquito to cause seroconversion. But there is no evidence that domestic farm animals are frequently exposed to arboviruses, it may represent a public health risk to humans that can be susceptible to develop disease from that virus. Thus, it is possible to infer that horses showed higher prevalence of antibodies in heterotypic reactions showing to be the best species of domestic farm animal to serve as sentinel to detect the movement of arboviruses in the Brazilian Amazon.

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RESUMO

O Estado do Pará compreende 26% da Amazônia brasileira, onde uma grande diversidade de arbovírus é descrita. Este estudo teve como objetivo avaliar a prevalência e distribuição de inibição da hemaglutinação (HI) que detecta anticorpos contra quatro tipos de vírus: vírus da encefalite equina do leste (EEL), vírus da encefalite equina do oeste (EEO), vírus Mayaro (MAYV) e vírus Mucambo (MUCV), um subtipo do vírus da encefalite equina venezuelana (VEEV), em 2.191 amostras de soro de equinos, bovinos, ovinos e bubalinos no Estado do Pará, Brasil. O objetivo foi identificar a prevalência de anticorpos nestes animais de fazenda para determinar quais arbovírus estão circulando e determinar que animal é mais sensível para detectar *Alphavirus*. Os anticorpos contra os arbovírus investigados foram detectados em quase todos os animais das espécies estudadas. Os resultados indicaram que herbívoros domésticos são suscetíveis aos arbovírus testados e evidencia *Alphavirus* ativos em animais de fazenda na Amazônia brasileira. Uma análise da prevalência de anticorpos HI por espécies de animais indicaram diferença significativa entre equinos e bubalinos, bovinos e ovinos. Os equinos apresentaram maior prevalência de anticorpos em reações heterotípicas, os quais demonstram ser as melhores espécies de animais de fazenda como sentinelas para detectar a circulação de arbovírus na Amazônia brasileira.

Palavras-chave: *Alphavirus*; Ecossistema Amazônico; Animais; Testes de Inibição da Hemaglutinação.

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RESUMEN

El Estado de Pará comprende 26% de la Amazonía brasileña, en la cual se describe una gran diversidad de arbovirus. Este estudio tuvo como objetivo evaluar la prevalencia y distribución de inhibición de la hemoaglutinación (HI) que detecta anticuerpos contra cuatro tipos de virus: virus de la encefalitis equina del este (EEE), virus de la encefalitis equina del oeste (EEO), virus Mayaro (MAYV) y virus Mucambo (MUCV), un subtipo del virus de la encefalitis equina venezolana (EEV), en 2.191 muestras de suero de equinos, bovinos, ovinos y bufalinos en el Estado de Pará, Brasil. El objetivo fue el de identificar la prevalencia de anticuerpos en estos animales de hacienda para determinar cuales arbovirus están circulando y determinar que animal es más sensible para detectar *Alphavirus*. Los anticuerpos contra los arbovirus investigados fueron detectados en casi todos los animales de las especies estudiadas. Los resultados indicaron que los herbívoros domésticos son susceptibles a los arbovirus testados y evidencia *Alphavirus* activos en animales de hacienda en la Amazonía brasileña. Un análisis de la prevalencia de anticuerpos HI por especies de animales indicó una diferencia significativa entre equinos y bufalinos, bovinos y ovinos. Los equinos presentaron mayor prevalencia de anticuerpos en reacciones heterotípicas y demuestran ser las mejores especies de animales de hacienda como centinelas para detectar la circulación de arbovirus en la Amazonía brasileña.

Palabras clave: *Alphavirus*; Ecosistema Amazónico; Animales; Pruebas de Inhibición de Hemaglutinación.



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