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Investigation of *Alphavirus* of public health interest in equines hosts in Foz do Iguaçu, triple frontier region (Brazil-Paraguay-Argentina)

Investigação de Alphavirus de interesse em saúde pública em hospedeiros equinos em Foz do Iguaçu, região da tríplice fronteira (Brasil-Paraguai-Argentina)

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ABSTRACT

The *Alphavirus* genus encompasses arboviruses of great importance to public health. These viruses presents zoonotic potential and are associated with infections in humans and other vertebrates, including equines. Reverse transcription followed by polymerase chain reaction (RT-PCR) allows the early recognition of *Alphavirus* circulation, which is crucial for effective preventive measures adoption. This study aimed to investigate the presence of *Alphavirus* in equines hosts living in close contact with humans and other animals in the city of Foz do Iguaçu (Brazil), which is part of the Triple Frontier region of Brazil, Paraguay and Argentina. Methods: In 2015 and 2016, we collected equine serum samples (n=117), of which 53.9% (n=63) were female, and 56.4% (n=66) were employed as working animals for recyclable material collection. Subsequently, for RT-PCR analysis, 10 µL of equine RNA and the cM3W primer targeting gene NS1 (432bp) were utilized to screen for the presence of the viral genome. Results: All serum samples were negative for the presence of *Alphavirus in RT-PCR*. Conclusions: These data indicate that the equines examined were not infected and did not represent a risk of viral transmission to humans or other animals

during the period in which the samples were collected. This information is relevant because these animals may act as sentinels and urban disseminators of pathogens.

Keywords: Emerging infectious diseases; zoonoses; arboviruses; equines; RT-PCR.

INTRODUCTION

In recent decades, several species of arboviruses have become an important public health concern. These viruses have caused outbreaks and epidemics in humans, and comorbidities in wild and domestic animals (WEAVER, 2013).

Some arboviruses known to have zoonotic potential and which can detrimentally affect the health of humans and equines belong to the *Alphavirus* genus (*Togaviridae* family). This genus has 31 species, some of them, including Mayaro, Equine Encephalitis viruses (Eastern, Western and Venezuelan), Chikungunya, Rio Negro, Pixuna and Una, are very important for public health in Brazil and globally (ICTV, 2023).

Alphavirus are mainly transmitted by mosquitoes of *Aedes* and *Culex* genera, are distributed worldwide and predominate in tropical and subtropical regions. The global mobility of humans and climatic conditions conducive to the proliferation of vectors associated with the diversity of animals that can act as amplification and dispersion hosts are contributing to the propagation of these viruses (WEAVER and REISEN, 2010)

The detection of arboviruses in non-endemic areas or detection of new species requires the use of viable and effective laboratory tools that allow the rapid confirmation of cases of infection during surveillance activities (SÁNCHEZ-SECO et al., 2001; BRONZONI et al., 2004). Despite the importance of serological surveys in animals that can act as sentinels to public health, these tests have disadvantages that include cross-reactivity and low sensitivity (BRONZONI et al.2004)

In seeking an alternative, attention has turned to molecular biology methods such as reverse transcription followed by polymerase chain reaction (RT-PCR), which combines the high sensitivity of the test with rapid and simple implementation. These advantages are important in surveillance programs as well as in the clinical diagnosis of these infections (BRONZONI et al., 2004). Early and specific detection of Alphaviruses is paramount for public health authorities to adopt effective preventive measures and for the proper management of patients (SÁNCHEZ-SECO et al., 2001; BRONZONI et al., 2004).

The city of Foz do Iguaçu (State of Paraná, Brazil) is internationally known for the Iguazu Falls located in the Iguaçu National Park (one of the last native Atlantic Forest reserves in the State of Paraná) and also by the Itaipu Hydroelectric Dam (the largest hydroelectric plant in the world in terms of energy production). The region is the third most popular destination for foreign tourists in Brazil (BRAZIL, 2018). Due to high numbers of tourists transiting in this Triple Frontier region between Brazil, Paraguay, and Argentina, the city of Foz do Iguaçu is considered by Brazilian Ministry of Health a high-risk area for the introduction of arboviruses and/or new serotypes or strains, making the municipality strategically important for the investigation of the presence of these viruses.

The city was included in an extension project entitled 'CartHorses', which consists of educational activities for prevention and control of zoonotic diseases, and monitors the status of animal health, especially in horses, as these animals may act as sentinels and urban disseminators of infectious agents.

Considering the effects on public health, socioeconomic factors, and the limited information available about these viruses in horses in border regions, this study examined the presence of Alphavirus genus arboviruses in equines residing in close proximity to humans and other animals in the city of Foz do Iguaçu in the Triple Frontier region.

MATERIALS AND METHODS

Study Location

The serum samples were collected in the city of Foz do Iguaçu that borders on the west with the municipality of Ciudad del Este in Paraguay and to the south with the municipality of Puerto Iguazu (Argentina) (Figure 1). In the southwestern region of the municipality, the Iguazu and Paraná Rivers join together forming the Triple Border between Brazil, Paraguay, and Argentina.

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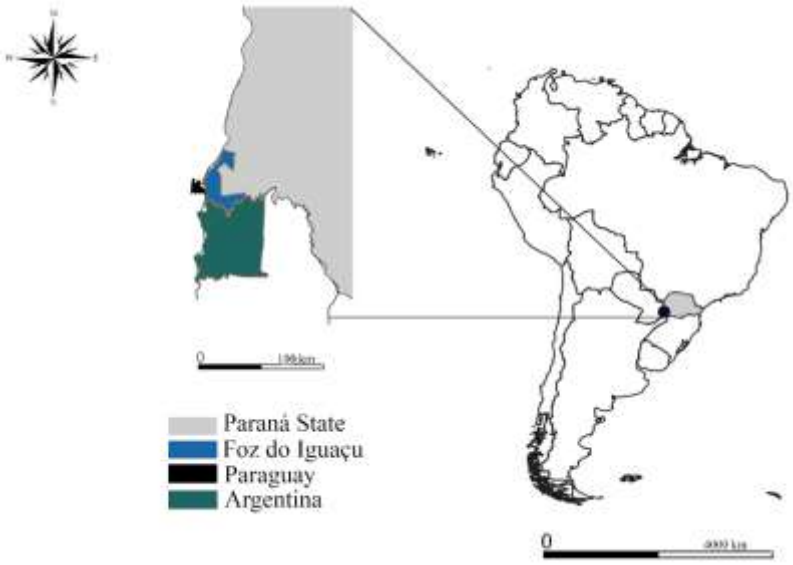

Figure 1. Geographical location of Foz do Iguaçu, Paraná, Brazil.

The city of Foz do Iguaçu (25° 32' 52"S and 54° 35' 17"W) is located in the extreme west of Paraná State, has an area of 617.701 km², and is situated at 174 m altitude. The estimated population in 2017 was 264,044. The city has a humid, subtropical, and mesothermal climate, with an average annual temperature of 20.4 ºC and average rainfall of 1,800 mm (Prefeitura Municipal de Foz do Iguaçu, 2018). The climate conditions are important risk factors for mosquito's proliferation, especially of *Aedes* spp., which can act as an *Alphavirus* vectors.

Sample Description

Blood samples were collected using 40 × 12 mm needles from 117 equines in 2015 and 2016 of the external jugular vein and were individually collected in sterile 10 mL tubes without anticoagulant. They were centrifuged at 1,500 × g for 5 minutes for clot retraction and the serum samples obtained were transported in liquid nitrogen to the laboratory, where they were kept at -70 ºC until processing.

Of the equines analyzed (n=117), 53.9% (n=63) were female, and 56.4% (n=66) were used as working animals for the collection of recyclable materials. Besides living with their owners, 76% (n=89) of the equines also lived with other animals, as 90% (n=80),

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57.3% (n=51), 42.7% (n=38), 21.3% (n=19), 16.8% (n=15) and 6.7% (n=6) with dogs, birds, cats, pigs, cattle, and goats or sheep, respectively. It is worth noting the occurrence of neurological symptoms in 5.1% of the equines (n=6) and bird deaths in 11 (19.3%) of the locales where the equines lived or near these locales.

Extraction of Viral RNA

Viral RNA was extracted from 140 µL of each serum sample using the QIAmp Viral Mini Kit (QIAGEN Inc., Hilden, Germany), following the manufacturer's specifications.

RT-PCR

For reverse transcription reaction, 10 µL of RNA and the cM3W primer (1 µM, gene NS1, 432pb) were placed in microtubes that were positioned in a thermocycler at 70 ºC for 5 minutes. The microtubes were then removed from the thermocycler and placed in an ice bath. After this stage, 4 µL of 5× buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), deoxyribonucleotide triphosphate (1 mM of each dNTP), dithiothreitol (10 µM), and 200 U M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, USA) were added. The final volume was completed to 20 µL with autoclaved Milli-Q water and reverse transcription was performed in a thermocycler at 42 ºC for 90 minutes followed by 70 ºC for 15 minutes.

The amplification reaction was prepared to a final volume of 50 µL with autoclaved Milli-Q water, using 3 µL of cDNA previously obtained, 5 µL of 10× buffer [200 mM Tris HCl (pH 8.4), 500 mM KCl], cM3W primer (1 µM), sM2W primer (1 µM), $MgCl_2$ (2 mM), dNTPs (0.2 mM of each dNTP), and 3 U of AmpliTaq Gold DNA polymerase enzyme (Thermo Fisher Scientific, Waltham, USA). The samples were placed in a thermocycler at 95 ºC for 5 minutes, and for 35 cycles of 95 ºC for 45 seconds, 53°C for 30 seconds, and 72 ºC for 1 minute, with a final polymerization stage at 72 ºC for 10 minutes.

The primers used in this study were previously described in the literature and were employed to amplify the genome region encoding the nonstructural protein 1 (NS1) of Alphaviruses (Gene sequence: ACA TRA ANK GNG TNG TRT CRA. ANC CDA YCC) (PFEFFER et al., 2017). The same RT-PCR procedure was used for the positive and negative controls. The amplified fragments were subjected to 1% agarose gel electrophoresis together with a molecular weight marker and the positive and negative controls.

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RESULTS

None of the 117 serum samples from equine species analyzed in the present study was positive for the presence of arbovirus viral genome of the *Alphavirus* genus.

DISCUSSION

Several studies have been carried out in South American countries to assess the presence of *Alphavirus*, including the Triple Border region countries of Brazil, Paraguay, and Argentina (CASSEB et al., 2012; MELO et al., 2012; DÍAZ et al., 2007; PISANO et al., 2013). However, some of these studies evaluated the distribution of seropositive animals, meaning the presence of antibodies would be detected only as a means of gauging the exposure of animals to pathogens. In Brazil, the presence of different *Alphaviruses*, including Mayaro virus (MAYV), Chikungunya virus (CHIKV), Oropouche virus (OROV), Rocio virus (ROCV), Una virus (UNAV), Pixuna virus (PIXV), and Rio Negro virus (RNV), has already been described in the five geographical regions of the country (ZUCHI et al., 2014; CUNHA et al., 2009; SILVA et al., 2011; FERNÁNDEZ et al., 2000, CASSEB et al., 2016; SALGADO et al., 2021).

The social and economic impacts attributed to the recent introductions of ZIKV and CHIKV, driven by the high number of human infections and associated pathologies in Brazil and the Americas, are well-documented. Additionally, the recent isolation of West Nile virus (WNV) from equine hosts represents an additional emerging virus circulating in Brazil. It is worth noting that in Brazil, the co-circulation of various flaviviruses (such as Dengue, Zika, Yellow Fever, Saint Louis encephalitis, Ilhéus, and others) complicates the serological diagnosis of these emerging infections due to extensive cross-reactivity of flaviviruses in serological assays (SALGADO et al., 2021).

The Mayaro virus has caused epidemics in the Northern region, but cases have been described in other states of the Midwest and Southeast regions of the country (ZUCHI et al., 2014; IVERSSON et al., 1981; COIMBRA, 2007). Serologic studies have detected the presence of antibodies against the Mayaro virus in equines in the State of Pará (CASSEB et al., 2012), in the Pantanal region in the State of Mato Grosso do Sul (PAUVOLID-CORREA et al., 2015), and in free-living non-human primates also in the State of Mato Grosso do Sul (BATISTA et al., 2013). Serological and molecular studies have suggested that horses are unlikely to be dead-end hosts and could potentially be amplifying hosts (YUEN el al 2021).

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The Venezuelan Equine Encephalitis virus has been isolated from vectors in Brazil (VASCONCELOS, 1998), but evidence of infection in equines was also observed in the States of Mato Grosso, São Paulo and in the Pantanal region of the State of Mato Grosso do Sul (MELO et al., 2012; CUNHA et al., 2009; PAUVOLID-CORREA et al, 2015).

With regard to Eastern Equine Encephalitis virus, evidence of the circulation of the virus in humans in the State of São Paulo has been reported through antibody detection (IVERSSON et al., 1981), in birds and vectors in the State of Pará by antibody detection and virus isolation (VASCONCELOS et al., 1991), and in equines in the States of Pará by antibody detection (CASSEB et al., 2012 ; CASSEB et al., 2016; CAMPOS et al., 2013; HEINEMANN et al., 2006), São Paulo by antibodies against viral agents in equids (CUNHA et al., 2009), Pernambuco, Ceará and Paraíba by antibody detection (SILVA et al., 2011), Paraná by virus isolation (FERNÁNDEZ et al., 2000), Mato Grosso by antibody detection (MELO et al., 2012) and in the Pantanal region in the State of Mato Grosso do Sulby antibody detection (PAUVOLID-CORREA et al, 2015).

The Western Equine Encephalitis virus was reported infecting equines in the States of Rio de Janeiro (BRUNO-LOBO et al., 1961), Pará (CASSEB et al., 2012; CASSEB et al., 2016; HEINEMANN et al., 2006) in the Pantanal region in the State of Mato Grosso do Sul (PAUVOLID-CORREA et al, 2015), and in humans in the State of São Paulo (IVERSSON et al., 1981).

The first cases of Chikungunya virus infection in South America occurred in 2014, with 3,655 autochthonous cases registered in Brazil, according to data from the Ministry of Health (Brazil) (Brazil, 2018). Between 2017 and 2020, Brazil had a higher number of Chikungunya fever cases (644,761) compared to Zika (102,035). The year with the highest number of Chikungunya cases was 2017 (247,692), with the highest monthly numbers occurring in May (134,254) and April (111,916). Regarding Zika, 2017 (32,684) showed a peak in the number of cases, followed by 2019 (30,500). In Bahia, there were 75,782 cases of Chikungunya and 12,337 of Zika. Both diseases showed higher prevalence in 2020 with 46,422 and 4,692 cases respectively (FARIAS et al., 2022; JORNAL DA USP, 2024).

In Argentina and Paraguay, a numbers of cases of Chikungunya virus infection have been reported. Argentina reported 1,336 cases of chikungunya in 2023, after a six-year gap since its first epidemic in 2016. The most affected country is Paraguay, which recorded its

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worst epidemic in history with 138,730 cases in 2023 (PAHO, 2016; PAHO, 2017; PAHO 2023).

Other Alphaviruses, such as the Rio Negro, Pixuna and Una viruses are considered emerging pathogens in the Americas. These viruses are able to infect humans and animals, and are investigated with greater frequency in Argentina and Paraguay.

Evidence of infections caused by the Rio Negro virus was found in humans in Argentina in the provinces of Corrientes, Salta, and Cordoba (Pisano et al., 2012, 2013). The virus has also been isolated from mosquitoes in these provinces, as well as in Chaco and Tucumán, confirming the endemic circulation of the virus in the neotropical region of Argentina. Additionally, the Pixuna virus has been isolated from mosquitoes in Chaco and Tucumán provinces, and human infections have been reported in the provinces of Salta and Chaco (Pisano et al., 2010a, 2010b, 2013).

The Una virus was first detected in non-human primates (Alouatta caraya) in Paraguay, marking the first record of the virus in this country. Furthermore, in Argentina, evidence of infection in humans in the province of Cordoba was the initial indication of the virus circulating among humans in the country (Díaz et al., 2003, 2007). The fact that there were more adults infected than juveniles suggests that the virus is endemic in the region (DIAZ et al., 2003).

Although there are fewer cases of infections by *Alphavirus* in Argentina and Paraguay, there is a risk of occurrence of outbreaks mainly in the northern region of Argentina, an area of more intense circulation of these viruses (PISANO et al., 2013; DIAZ et al., 2003).

The sylvatic cycle is responsible for the maintenance of most of the arboviruses in nature, which is a risk to the human population that comes into contact with the hosts and vectors that participate in these cycles. Anthropogenic actions on natural areas increase the risk of human exposure to arbovirus transmission, which may also result in the adaptation of arboviruses to new hosts and vectors, causing these viruses to develop new maintenance cycles (GUBLER, 2002).

In addition, the ability of some arboviruses to travel long distances by means of infected wild birds (migratory birds) and the ease of displacement of people and live cargo around the world can contribute to the emergence and/or reemergence of arboviruses (WOOLHOUSE and GOWTAGE-SEQUERIA, 2005).

In terms of public health, the most important arboviruses are those that have the ability to circulate mainly in urban cycles and that present epidemic potential. However, evolutionary evidence indicates that arboviruses most often associated with urban epidemics (e.g., Dengue, Zika, and Chikungunya viruses) have emerged from enzootic foci followed by transmission to humans in emergency areas, with subsequent dissemination to urban centers and different geographical locations (WEAVER, 2013).

Given the importance of animals, especially mammals, as hosts of human pathogenic agents, it is clear that the monitoring of these animals is essential for the understanding and management of threats of emerging and reemerging diseases (WOOLHOUSE and GOWTAGE-SEQUERIA, 2005). These animals, including equines, may act as hosts of several arboviruses, in particular, *Alphavirus*. The identification of the presence of these viruses in animals that may be acting as hosts is very important in the context of arbovirosis surveillance due to the zoonotic characteristic of these diseases and because arboviruses are an important human and animal public health concern.

CONCLUSION

The present study demonstrates that the equine species analyzed were not infected by *Alphavirus* at the time of sample collection. Although the equines were in close contact with humans and other animals, and so could potentially facilitate the transmission of arboviruses by vectors, the present findings reveal no risk of transmission of *Alphavirus* during the period in which the samples were collected. From the sanitary point of view, this data is relevant, given that these animals belong to a city that is considered a high-risk area for the emergence and reemergence of arboviruses.

Active surveillance activities in animals could lead to early recognition of circulation of arboviruses with zoonotic potential, functioning as an early warning system of *Alphavirus* circulation in humans. Thus, information on the natural occurrence of these viruses have fundamental importance to the public health services, since it would accelerate the response to possible outbreaks, consequently reducing the morbidity and mortality in animal and human populations. In addition, these activities may also contribute to the reduction of negative impacts on the main economic activities (trade and tourism) of this Triple Frontier region.

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Ethics approval

The use of animals samples this study was approved by the Animal Ethics Committee - Sector of Agricultural Sciences of the Federal University of Paraná (protocol number 046/2016).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Tatiana Carneiro Rocha: literature review, development of experimental steps, interpretation and analysis of results, writing, editing, approval of the final version; Beatriz Böger: literature review, writing, editing, critical review, approval of the final version and reviewer responsible for the manuscript; Mario Antonio Navarro-Silva: interpretation and analysis of results, writing, critical review and approval of the final version; Thállitha Samih Wischral Jayme Vieira: sample obtaining, member of the "CartHorses" project, writing, editing, critical review and approval of the final version; Alexander Welker Biondo: sample obtaining, member of the "CartHorses" project, logistical support, writing, editing, critical review and approval of the final version; Ivan Roque de Barros Filho: sample obtaining, member of the "CartHorses" project, logistical support, writing, editing, critical review and approval of the final version; Robson Michael Delai: logistical support, writing, editing, critical review and approval of the final version; André de Souza Leandro: logistical support, writing, editing, critical review and approval of the final version; Eliane Carneiro: interpretation and analysis of results, writing, critical review and approval of the final version; Walfrido Kühl Svoboda: logistical support, interpretation and analysis of results, writing, editing, critical review and approval of the final version.

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