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PRELIMINARY INVESTIGATION OF CULICIDAE SPECIES IN SOUTH PANTANAL, BRAZIL AND THEIR POTENTIAL IMPORTANCE IN ARBOVIRUS TRANSMISSION*

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SUMMARY

In view of the high circulation of migratory birds and the environmental and climatic conditions which favor the proliferation of arthropods, the Brazilian Pantanal is susceptible to circulation of arboviruses. However, the amount of data concerning arbovirus vectors in this area is scarce; therefore the aim of this study was to conduct a preliminary investigation of Culicidae species in the Nhecolândia Sub-region of South Pantanal, Brazil and their potential importance in the arbovirus transmission. A total of 3684 specimens of mosquitoes were captured, 1689 of which caught in the rainy season of 2007, were divided into 78 pools and submitted to viral isolation, Semi-Nested RT-PCR and Nested RT-PCR, with a view to identifying the most important arboviruses in Brazil. Simultaneously, 70 specimens of ticks found blood-feeding on horses were also submitted to the same virological assays. No virus was isolated and viral nucleic-acid detection by RT-PCR was also negative. Nevertheless, a total of 22 Culicidae species were identified, ten of which had previously been reported as vectors of important arboviruses. The diversity of species found blood-feeding on human and horse hosts together with the arboviruses circulation previously reported suggest that the Nhecolândia Sub-region of South Pantanal is an important area for arbovirus surveillance in Brazil.

KEYWORDS: Arbovirus; Pantanal; Culicidae; Ticks; Nhecolândia.

INTRODUCTION

Arboviruses are maintained in nature due to their biological transmission from an infected vertebrate host to another through hematophagous arthropods, mainly by mosquitoes, ticks, sand flies and biting midges[56]. The Pantanal is a vast sedimentary plain of approximately 140,000 km² located in South America covering part of Brazilian, Bolivian and Paraguayan territories[26]. However, about 85% of the area is located in Brazil, where 65.5% is situated in Mato Grosso do Sul State (MS) and 35.5% in the Mato Grosso State (MT), respectively recognized as South and North Pantanal[4,36]. The region is a large floodplain whose dynamics are regulated primarily by the flood-pulse, whereby periods of flooding and drought alternate and constitute the primary factor governing the ecology of the plains[33]. The considerable annual and multi-annual variability affects the biota with different intensities and on different time scales[8,32,39,31]. The Pantanal wetland is classified into sub-regions that differ in their vegetation, flooding and physiognomy[1,2]. The Nhecolândia Sub-region in MS, which occupies 19.48% of the Pantanal area, is one of the largest of these floodplains[48]. Like other periodically flooded plains, the Brazilian Pantanal presents a set of factors that could be important for the circulation and maintenance of arboviruses. Among these factors, the richness of wildlife including the presence of migratory birds[38] and climatic conditions[49] that favor the proliferation of arthropods, sets the region a promising area for arbovirus surveillance in Brazil, in particular for the early detection of unrecognized or not previously reported species in Brazil, which may be circulating silently in the country through enzootic cycles in the Brazilian Pantanal[42].

The circulation of *Maguari virus* (MAGV), *Ilheus virus* (ILHV), *Tacaiuma virus* (TCMV), *Eastern equine encephalitis virus* (EEEV) and *Western equine encephalitis virus* (WEEV) was demonstrated, through arbovirus surveillance in horses from the Nhecolândia Sub-region in the 1990's[25]. Despite this, data concerning arbovirus vectors in the area are scarce, with the exception of a few reports such as an interesting study about the feeding habits of Culicidae species by means of the precipitin technique conducted in North Pantanal[3] and the description of Culicidae

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fauna that can be potential vectors of infectious diseases in an impacted area of MS, outside South Pantanal[23]. Although ticks have not commonly been detected as arbovirus vectors in Brazil[54], these arthropods have been reported as the most important vectors of disease-causing pathogens in domestic and wild animals[15] and their potential competence as arbovirus transmitters in Brazil has still to be considered. Some tick studies had been conducted in Pantanal, although most have focused on ecological aspects and wild fauna[9,32,11,12]. The aim of this study was to conduct a preliminary investigation into Culicidae species and their potential importance as arbovirus transmitters mainly to human and horse hosts during the rainy and dry seasons in the Nhecolândia Sub-region of South Pantanal, Brazil, and the detection of the presence of arboviruses in the Culicidae and Ixodidae specimens sampled in the area.

MATERIAL AND METHODS

Area of study and collections - A sampling of mosquitoes authorized by the Brazilian Institute of Environment and Natural Resources (Licence Number IBAMA 002/2007) was conducted, during the rainy season (February) and dry season (November) of 2007, on the Nhumirim Ranch (18° 58´ S and 56° 37´ W), a property of Embrapa Pantanal (Brazilian Agricultural Research Company). The ranch presents a mosaic of vegetation areas including forest, savanna, floodable grassland areas and shallow lakes that display varying degrees of salinity and coalescence with the system in flood located in the Nhecolândia Sub-region of South Pantanal, approximately 150 km east of Corumbá city, a municipality of MS, Brazil. Specimens of ticks detected blood-feeding on horses of the property were also collected, but only in February. Adult mosquitoes were collected from several sites randomly selected using CDC automatic light traps and Shannon light traps, both without bait, and suction tubes while landing to blood-feed on horses and on research team members, as usually described[35,52].

Culicidae and Ixodidae sampling on equines - Three Culicidae collections were undertaken for one hour at different times (5:00 pm, 7:00 pm and 8:00 pm) in February, and three manual collections of Ixodidae were made on different days in the same month from 6:00 am to 9:00 am. In the dry season, the only catch of Culicidae species was undertaken from 6:00 pm to 7:00 pm.

Culicidae sampling on team members - In February, six one-hour collections were made starting at different times (10:00 am, 3:00 pm, 7:00 pm and 8:00 pm). In November, three collections of approximately two hours each were made starting at 8:00 am, 9:00 am and 1:00 pm.

Culicidae capture using CDC light trap - In the rainy season, only one capture was performed, from 6:00 pm to 6:00 am. In the dry season, four catches were conducted in the same period.

Culicidae capture using Shannon light trap - In February, one capture was undertaken, from 9:00 pm to 10:00 pm. During the dry season, only one capture was performed, from 6:00 pm to 7:30 pm.

Identification of arthropods - Specimens were placed in coolers and transported to the field station where they were immobilized by chilling and identified through direct observation of the morphological characters. The arthropod identification was carried out at the Oswaldo Cruz Institute (IOC), by the Diptera Laboratory according to the dichotomous keys for South American mosquitoes[27,28,24,17,14,20] and by the Ixodides Laboratory according to the dichotomous key for Brazilian ixodological fauna[7]. After identification, 1689 specimens of mosquitoes and 70 specimens of ticks captured during the rainy season were separated by trap, date and point of capture and up to 100 specimens were pooled by species. The pools were placed in sterile cryovials and transported in liquid nitrogen to the Enterovirus Laboratory of IOC for further processing. The arthropods were stored at -70°C in a freezer until trituration, RNA extraction and RT-PCR. Mosquitoes sampled in the dry season were identified but were not investigated for arboviruses.

Processing and virus isolation - In the laboratory, pools from one up to 50 mosquito specimens were placed in polypropylene capped culture tubes with four copper-clad steel beads (BBs) and 2mL of modified BA-1diluent (5% heat-inactivated fetal bovine serum in Medium 199 with glutamine, NaHCO3, penicillin [100U/mL], streptomycin [100mg/mL], fungizone 1000x and TRIS 0.5M). Because of the anatomical characteristics of the ticks, their trituration was carried out with sterile mortar and pestle in the presence of 2mL of modified BA–1. The suspensions were vortex and clarified by centrifugation at 6000 RPM for 15 minutes at 25 °C. Aliquots of 200µL of each pool were inoculated into VERO cell monolayer tubes [5 x 10^5 cells/mL]. The tubes were kept at 36°C for one hour with gentle motion at each 15 minutes to optimize virus adsorption. At the end of this period 1800µL of Medium 199 containing 2% of bovine fetal serum, was added followed by incubation at the same temperature for seven days. Tubes were observed daily under an inverted microscope for evidence of cytopathic effects (CPE). The non-observation of CPE after three consecutive passages of seven days each was considered as demonstrating the absence of cytopathogenic viruses in a given pool.

Duplex RT-PCR followed by Semi-Nested PCR - RNA was extracted by using a commercial kit (QIAamp© Viral RNA Mini Kit QIAGEN), in accordance with the manufacturer's instructions. Reverse transcription was conducted in extracted RNA with Superscript II (Invitrogen) system and random primers [500µg/mL] (Random Primers PROMEGA). Aliquots of 10µL of cDNA served as templates in subsequent Duplex RT-PCR followed by Semi-Nested PCR assay for *Venezuelan equine encephalitis virus* (VEEV), *Western equine encephalitis virus* (WEEV), *Mayaro virus* (MAYV), *Ilheus virus* (ILHV), *Rocio virus* (ROCV), *Yellow fever virus* (YFV) and *Saint Louis encephalitis virus* (SLEV) detection, as previously described[10].

Nested RT-PCR for flaviviruses - Five microliter aliquots of each cDNA underwent PCR amplification using a set of degenerate *Flavivirus* specific primers. The first round was conducted with external primers **FlagR2** (5`tgt cca cts ccc ctt tgr tct 3`) and **FlagF1** (5`aca tga tgg gra aam gwg aga 3`) for 40 cycles of 20 sec at 94°C, 45 sec at 50°C and one min at 68°C. A second round was conducted with 5µL of the first amplicon using flavivirus-specific inner primers **FlagR1** (5`tcc cai ccg gck gtg tca tc 3`) and **FlagF2** (5`gcc atw tgg twc atg tgg 3`). These primer sets were engineered to match sequences of the NS5 gene of SLEV, YFV, *Japanese encephalitis virus* (JEV), *Dengue virus* (DENV) and *West Nile virus* (WNV) genome fragments. The same cycling parameters were used as in the first reaction.

RESULTS

A total of 3684 Culicidae specimens captured were identified as belonging to 22 species and 70 Ixodidae specimens were identified as

Amblyomma cajennense and *Anocentor nitens*. Culicidae specimens of 18 species were identified blood-feeding on research team members and at least six species on horses. Of that total of mosquitoes captured, 17 species were identified among the 2139 specimens captured during the rainy season and nine among the 1545 specimens captured during the dry season (Table 1). *Ps. albigenu* was the most abundant Culicidae species in the rainy season (55.91%) of which 96.91% were caught blood-feeding on research team members indicating anthropophily. *Ma. titillans* was the most abundant species in the dry season (78%) of which the majority (60.5%) were caught in CDC automatic light traps. Interestingly, the light traps show higher abundance during the dry season than in the rainy season. Ninety-four mosquitoes were caught per hour of capture (mq/hr) in the rainy season and 299 mq/hr in the dry season with the Shannon light trap while the CDC automatic light trap caught 10 mq/hr in the rainy season and 20 mq/hr in the dry season. On the other hand, the suction tubes used to capture host-seeking female mosquitoes on horses and research team members were more efficient during the rainy season. The captures on horses were 126 mq/hr in February and 53 mq/hr in November, while on research team members the numbers were, respectively, 262 mq/h and 24 mq/h.

Table I

Distribution of Culicidae and Ixodidae specimens captured in Nhecolândia Sub-region of South Pantanal in 2007, by species, season and trap

	February					November				
	Shannon light trap	CDC light trap	Blood feeding on horses	Blood feeding on team members	Total specimens of rainy season	Shannon light trap	CDC light trap	Blood feeding on horses	Blood feeding on team members	Total specimens of dry season
Species	N [% of specimens by specie according trap] (% of specimens by trap according species)									
Mansonia titillans Walker, 1848 *	29 [5.64%] (30.85%)	36 [7%] (40.45%)	212 [41.25%] (55.79%)	237 [46.11%] (15.04%)	514 [100%] (24.03%)	386 [32.03%] (85.97%)	729 [60.5%] (83.31%)	53 [4.4%] (100%)	37 [3.07%] (22.02%)	1205 [100%] (77.99%)
Psorophora albigenu Peryassu, 1908 *	2 [0.17%] (2.13%)		35 [2.93%] (9.21%)	1159 [96.91%] (73.54%)	1196 [100%] (55.91%)					0
Anopheles albitarsis Lynch Arribalzaga, 1878 *	19 [13.87%] (20.21%)	2 [1.46%] (2.25%)	56 [40.88%] (14.74%)	60 [43.80%] (3.81%)	137 [100%] (6.40%)	22 [73.33%] (4.90%)	8 [26.67%] (0.91%)			30 [100%] (1.94%)
Anopheles triannulatus Neiva & Pinto, 1922 *	4 [36.30%] (4.26%)		6 [54.55%] (1.58%)	1 [9.09%] (0.06%)	11 [100%] (0.51%)	35 [23.18%] (7.80%)	116 [76.82%] (13.26%)			151 [100%] (9.77%)
Culex spp. *	31 [24.80%] (32.98%)	42 [33.60%] (47.19%)	31 [24.80%] (8.16%)	21 [16.80%] (1.33%)	125 [100%] (5.84%)	1 [100%] (0.22%)				1 [100%] (0.06%)
Psorophora ferox Humboldt, 1819 *				1 [100%] (0.06%)	1 [100%] (0.05%)				82 [100%] (48.81%)	82 [100%] (5.31%)
Mansonia humeralis Dyar & Knab, 1916 *	5 [6.76%] (5.32%)	9 [12.10%] (10.10%)	20 [27.03%] (5.26%)	40 [54.05%] (2.54%)	74 [100%] (3.46%)					0
Psorophora discrucians Walker, 1856					0				43 [100%] (25.60%)	43 [100%] (2.78%)
Coquillettidia juxtamansonia Chagas, 1907 *	1 [2.94%] (1.06%)			33 [97.06%] (2.09%)	34 [100%] (1.59%)					0
Culex declarator Dyar & Knab, 1906					0	2 [9.09%] (0.45%)	20 [90.91%] (2.29%)			22 [100%] (1.42%)
Anopheles spp. *			20 [100%] (5.26%)		20 [100%] (0.94%)					0
Culex quinquefasciatus Say, 1823 *				7 [100%] (0.44%)	7 [100%] (0.33%)					0
Ochlerotatus scapularis (Rondani, 1848) *				6 [100%] (0.38%)	6 [100%] (0.28%)					0

Table I
Distribution of Culicidae and Ixodidae specimens captured in Nhecolândia Sub-region of South Pantanal in 2007, by species, season and trap (con.)

	February					November				
	Shannon light trap	CDC light trap	Blood feeding on horses	Blood feeding on team members	Total specimens of rainy season	Shannon light trap	CDC light trap	Blood feeding on horses	Blood feeding on team members	Total specimens of dry season
Species	N [% of specimens by specie according trap] (% of specimens by trap according species)									
Mansonia amazonensis Theobald, 1901					0	3 [60%] (0.67%)			2 [40%] (1.19%)	5 [100%] (0.32%)
Ochlerotatus argyrothorax Bonne-Wepster & Bonne, 1919 *				4 [100%] (0.25%)	4 [100%] (0.19%)					0
Ochlerotatus stigmaticus Edwards, 1922					0				4 [100%] (2.38%)	4 [100%] (0.26%)
Psorophora ciliata (Fabricius, 1794) *	1 [33.33%] (1.06%)			2 [66.67%] (0.13%)	3 [100%] (0.14%)					0
Culex chidesteri Dyar, 1921					0		2 [100%] (0.23%)			2 [100%] (0.13%)
Sabethes chloropterus Humboldt, 1819 *				2 [100%] (0.13%)	2 [100%] (0.09%)					0
Anopheles rondoni Neiva & Pinto, 1922 *	1 [100%] (1.06%)				1 [100%] (0.05%)					0
Aedeomyia squamipennis Lynch Arribalzaga, 1878 *	1 [100%] (1.06%)				1 [100%] (0.05%)					0
Sabethes albiprivus Theobald, 1903 *				1 [100%] (0.06%)	1 [100%] (0.05%)					0
Sabethes purpureus Theobald, 1907 *				1 [100%] (0.06%)	1 [100%] (0.05%)					0
Coquillettidia fasciolata Lynch Arribalzaga, 1891 *				1 [100%] (0.06%)	1 [100%] (0.05%)					0
Total Culicidae	94 [4.39%] (100%)	89 [4.16%] (100%)	380 [17.77%] (100%)	1576 [73.68%] (100%)	2139 [100%] (100%)	449 [29.06%] (100%)	875 [56.63%] (100%)	53 [3.43%] (100%)	168 [10.87%] (100%)	1545 [100%] (100%)
Anocentor nitens (Neuman, 1897) Schulze, 1937 *			40 [100%] (57.14%)		40 [100%] (57.14%)					
Amblyomma cajennense (Fabricius, 1787) Koch, 1844 *			30 [100%] (42.86%)		30 [100%] (42.86%)					
Total Ixodidae			70 [100%] (100%)		70 [100%] (100%)					

* Species that specimens caught during the rainy season (February 2007) were submitted to virological assays.

Viral detection and isolation - No virus was isolated and viral nucleic-acid detection by RT-PCR was also negative in 78 pools containing a total of 1689 specimens of 17 mosquito species and in 18 pools containing a total of 70 specimens of two tick species.

DISCUSSION

Beyond the previously reported Culicidae species in the two most recent studies[3,23], we here report the presence of thirteen additional

unidentified species in North Pantanal and six species in MS. *Ma. titillans* was the most abundant species found in this present study. These data are in agreement with those of a previous study undertaken in North Pantanal[3]. However, despite the high prevalence of this species in the dry season (78%), *Ps. albigenu* accounted for more than half mosquitoes captured in the rainy season (55.91%). In spite of this and interestingly, *Ps. albigenu* was not found during the sampling performed in November. This absence suggests an ecological complexity of the sub-region and this should be taken into consideration by entomologists in arbovirus studies, since EEEV has already been detected in this species in the Peruvian Amazon[51].

The Ixodidae species identified in this study have commonly been reported for equine blood-feeding[21,19,47]. Despite a report on the experimental arbovirus transmission of *Amblyomma cajennense*[6], these species have not usually been reported in natural arbovirus cycles in Brazil[54]. Regarding the epidemiological and epizootiological relevance of the Culicidae species identified in the present study, at least ten out of the 22 had already been reported in the transmission of important arboviruses. In Brazil, *Ps. ferox* was found infected by MAGV, ILHV and MAYV and related to ROCV transmission during a serious outbreak which occurred in São Paulo State (SP) in 1975[30,46]. This Culicidae species was also reported as a vector in ILHV and VEEV cycles in the Peruvian Amazon and Central America, respectively[16,51].

Oc. argyrothorax was found naturally infected by ILHV and specimens of *Sa. chloropterus* were related to the transmission of ILHV and YFV in Guatemala and Panama in the 1950´s and were also found infected by YFV in Brazil[44,45,46]. In the same decade the transmission of VEEV in Ecuador and the Peruvian Amazon by *Ma. titillans* was reported[29]. Specimens of *Ps. albigenu* were reported as EEEV vector in the Peruvian Amazon[51] and *An. triannullatus* was found naturally infected by TCMV as well as *Oc. scapularis* by ILHV, MAGV and the *Mucambo virus* (MUCV)[46]. *Oc. scapularis* further demonstrated its ability to transmit ROCV when experimentally infected and is considered to be one of the main species involved in this arbovirus cycle in nature[34]. *Cx. quinquefasciatus* and *Cx. declarator* have been described in SLEV transmission[50,22,53,46] and *Cx. quinquefasciatus* and *An. albitarsis* have been reported in the transmission of WEEV in the US and Argentina respectively[43]. *Cx. quinquefasciatus* has further been found naturally infected by EEEV and the *Oropouche virus* (OROV)[46].

As for the absence of cytopathogenic viruses in the arthropod samples, a set of factors involving the capture of the samples and the way in which they have been analyzed should be examined. Factors such as the non epizootic periods of collection and possible loss of virus infectivity as a result of environmental conditions in the field, such as the high temperature-humidity indices in the rainy season, may have influenced the results. Besides, despite the existence of reports of equine encephalitis cases in the region in the 1990´s[25], collections and captures performed in February 2007 were conducted in the absence of recent official reports of epidemics, epizootics or even isolated cases of symptomatic infection by arboviruses. Epidemiological surveillance involving arthropods has shown that even during human or animal arbovirus outbreaks, the viral isolation in cell culture from mosquito samples is not easy.

Since 1999, the epidemiological surveillance for WNV in USA has reported reduced rates of viral isolation from Culicidae samples even in epidemic areas[37,5]. In a study accomplished to isolate viruses in the Peruvian Amazon, the presence of arboviruses was detected only in 1.09% of the more than half a million mosquitoes analyzed[51]. In the tropical forests the life expectancy of mosquitoes during the rainy season can be very low, which can greatly reduce the possibility of the isolation of arboviruses[13]. In the 1990´s, after an equine epizooty of encephalomyelitis in the Paraná State (PR) in Brazil, the attempt to isolate the agent in mice, from about 1800 mosquitoes, was unsuccessful[18].

The non-detection of viral RNA in the arthropod pools can also be attributed to the same factors considered for the results of viral isolation, such as the non-epizootic periods of collection and environmental conditions in the field. The results of several previous studies have demonstrated low levels of positive results even during epidemics. In 2000, during the mosquito surveillance for WNV in the USA, the presence of virus RNA was detected by RT-PCR in 3.6% of 9952 mosquito pools evaluated[55].

Another important issues are the environmental conditions prevailing during identification of mosquito species. Although carried out as described and recommended by several authors, the optimization of this step could minimize the reduction in the viral titer that may occur by virtue of abrupt changes in temperature and humidity. The use of triethylamine should be considered as an alternative to chilling for the immobilization of mosquitoes. In an arbovirus study in the Peruvian Amazon, the use of triethylamine showed some advantages such as immobilization with lesser humidity, considered an important factor in the reduction of the virus titer[41,40].

A total of 22 Culicidae species were identified, ten of which had been previously reported naturally infected with or as vectors of important human arboviruses. The diversity of species found blood-feeding on horses and mainly on human beings infers the potential susceptibility of these hosts to many arbovirus infections, and this must be taken into account in the epidemiological and epizootiological surveillance of arboviruses in Brazil. In this scenario, the advancement of environmental degradation in the region can lead not only to ecological but also health impact. For over two centuries, the Brazilian Pantanal has remained preserved, mainly because of the secular economic activity of the local population based on extensive beef cattle breeding that occupied large areas of native pastures with relatively low impact in the wild fauna and flora. However, the recent advancement of plantations of sugarcane around the floodplain and the arrival of cattle ranchers from other regions of Brazil, the management of Pantanal has been suffering drastic changes, as the deforestation for planting of exotic pastures, which may be compromising the fragile balance of flooding and drought in the floodplain. These changes can directly impact the fluctuations of vector populations, which could lead to the favoring of certain species of higher capacity of transmission of arboviruses resulting in outbreaks of arboviruses, as has been reported in the Amazon region[53]. Finally, the detection of arbovirus vectors associated with previous reports of the equine circulation of EEEV, WEEV, TCMV, MAGV and ILHV[25] suggests that the Nhecolândia Sub-region of Southern Pantanal is a key area for arbovirus surveillance.

RESUMO

Investigação preliminar das espécies de culicídeos do Pantanal Sul brasileiro e sua potencial importância na transmissão de arbovírus

Regiões como o Pantanal brasileiro, que apresentam fatores como riqueza de fauna silvestre incluindo circulação de aves migratórias e condições ambientais e climáticas favoráveis à proliferação de artrópodes estão potencialmente sujeitas à circulação de arbovírus. Entretanto, poucos trabalhos foram realizados acerca da presença de arbovírus em potenciais vetores no Pantanal. Neste sentido o principal objetivo deste trabalho foi conduzir uma investigação preliminar para presença de arbovírus em amostragens de culicídeos capturados na Sub-região da Nhecolândia no Pantanal Sul. Um total de 3684 mosquitos foi capturado, dos quais 78 grupos compondo uma amostragem de 1789 espécimes foram submetidos às técnicas de isolamento viral e RT-PCR para os mais importantes arbovírus no Brasil. Simultaneamente, 70 espécimes de carrapatos capturados durante hematofagia em cavalos também foram submetidos à pesquisa viral. Não houve isolamento viral em nenhuma amostra analisada e os resultados de detecção de ácido nucléico viral foram também negativos. Entretanto, foram identificadas 22 espécies de culicídeos, dez das quais previamente reportadas como vetores de importantes arbovírus. A competência vetorial de espécies capturadas durante hematofagia em humanos e cavalos aliada ao relato prévio de circulação de arbovírus sugerem a Sub-região da Nhecolândia como uma importante área de vigilância para arbovírus no Centro-Oeste do Brasil.

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