Flaviviruses infections in neotropical primates suggest long-term circulation of Saint Louis Encephalitis and Dengue virus spillback in socioeconomic regions with high numbers of Dengue human cases in Costa Rica

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Summary:

The presence of neotropical primates (NPs) positive or with antibodies against different species of Flavivirus common in Latin America, and specifically in Costa Rica (i.e. Dengue virus) has been established. However, it is unclear if a maintenance of this and other Flavivirus in sylvatic cycles exists, as has been established for yellow fever, with the howler monkey as primary host. We determined the presence of NPs seropositive to Dengue virus (DENV), Saint Louis Encephalitis virus (SLEV), West Nile virus (WNV), and undetermined Flavivirus in the country. The circulation of DENV and SLEV was also determined over a long period of time in the neotropical primates

studied. This coincides with the regions and years with high seroprevalences, being reported with years and regions with a high number of cases of DENV in humans. Therefore, our work proves the circulation for at least fifteen years of these flaviviruses in NPs and bidirectional transmission between humans and non-human primates.

Abstract:

Arthropod-borne viruses belonging to the flavivirus genus possess an enormous relevance in public health. Neotropical non-human primates (NPs) have been proposed to be infected more frequently with flaviviruses due to their arboreal and diurnal habits, their genetic similarity to humans and their relative closeness to humans. However, the only known flavivirus in America that is maintained by sylvatic cycles involving NPs is Yellow Fever virus (YFV), and the NPs role as potential hosts of flaviviruses is still unknown. Here, we examined flavivirus exposure in 86 free range and captive NPs of Costa Rica to evaluate their involvement in flavivirus transmission cycles and their potential as flavivirus hosts. We used a highly-specific micro plaque reduction neutralization test (micro-PRNT) to determine the presence of antibodies against YFV, Dengue virus 1-4 (DENV), Zika virus, West Nile Virus (WNV) and Saint Louis Encephalitis virus (SLEV). We found evidence of seropositive NPs to DENV-1 8.2% (homotypic –3/86, heterotypic – 4/86), SLEV 15.1% (homotypic – 10/86, heterotypic – 2/86), WNV 2.3% (homotypic – 2/86) and 8.1% (7/86) undetermined Flavivirus species. No antibodies against YFV or ZIKV were found. This work provides compelling serological evidence of exposure in NPs of flaviviruses associated with urban cycles, i.e. DENV, and confirms decades of circulation of SLEV in the same environments. Also, the range of years of sampling and the socioeconomic region was statistically significant for the presence of Dengue and Flavivirus undetermined seropositive individuals, respectively. Both the years and socioeconomic regions with greater seroprevalence coincide with the years and socioeconomic regions with high numbers of Dengue human cases for the country. Our work suggests bidirectional? circulation of different flaviviruses between humans and wildlife with public health importance and underscores the necessity of further surveillance for flaviviruses in the humans/wildlife interface in Central America.

Keywords: Neotropical non-human primates; serology; sylvatic cycles; Flavivirus; Dengue Virus; Saint Louis Encephalitis Virus; West Nile Virus.

1. Introduction

Arthropod-Borne Viruses (acronym Arboviruses) are zoonotic viruses transmitted to vertebrates by hematophagous arthropods (Moreli et al. 2013). Arboviruses are maintained in natural cycles by transmission through arthropod vectors from reservoir vertebrate hosts to susceptible vertebrate hosts (Valentine et al. 2019). They belong to diverse viral families such as Togaviridae (genus Alphavirus), Flaviviridae (genus Flavivirus), Bunyaviridae (genus Orthobunyavirus and Phlebovirus) and Reoviridae (Weaver & Reisen 2010).

Specifically, the Flavivirus genus possesses an enormous relevance in public health exemplified by millions of cases, hospitalizations, and deaths every year (Holbrook, 2017). Viruses such as Yellow fever virus (YFV), Dengue virus 1, 2, 3, and 4 (DENV 1-4), and Zika virus (ZIKV) have triggered enormous outbreaks in Latin America causing millions of cases. Additionally, the non-systematic detection of West Nile virus (WNV), and Saint Louis encephalitis virus (SLEV) in Latin America create a more complex epidemiologic scenario complicating public health control measures (Kuno et al. 1998; Cleton et al. 2012).

Despite their worldwide distribution, countries in tropical and subtropical areas that possess vast forest reserves and high fauna diversity represent the most favorable environments for arbovirus diversity and ecology (Musso et al. 2018, Araújo et al. 2019). Neotropical non-human primates (NPs) have been studied for decades as hosts of flaviviruses since their genetic and physiological characteristics are similar to those of humans, which makes them susceptible to flaviviruses infections that can cross species boundaries through vectors (Wolfe et al. 1998). Although in Latin America the role of NPs in the maintenance in sylvatic cycles of zoonotic flaviviruses remains speculative, past research has shown that NPs are the only mammals classified as high risk to be part of maintenance in sylvatic cycles (Pandit et al. 2018). In Africa, YFV and ZIKV circulate between African non-human primates, and DENV was originally a non-human primate virus from Southeast Asia. All have been

transmitted by mosquitoes inhabiting treetops (Valentine et al. 2019). In the American Continent, the only flavivirus recognized and maintained in sylvatic cycles is YFV (Hanley et al. 2013). The virus circulates between *Alouatta* spp. and is transmitted by mosquito vectors of the genera *Haemagogus* spp. and *Sabethes* sp. (Hanley et al. 2013, Moreira-Soto et al., 2018). These mosquitoes accidentally infect humans as well, causing sporadic cases or even large outbreaks as observed in Brazil in 2017. (Valentine et al. 2019). ZIKV and DENV only circulate in urban cycles which has facilitated their rapid spread throughout the Americas (Hanley et al., 2013), and no sylvatic cycle is known to date. Nevertheless, possible maintenance through sylvatic cycles has been suggested in the Americas with NPs of different species, i.e. *Alouatta* sp., *Cebus* sp., *Ateles* sp., being the primary wild host involved (Valentine et al. 2019). However, it has not been conclusively demonstrated and it is believed that detection on NPs is due to spillback, i.e. humans being the primary source of the virus and spilling the virus into wildlife (Morales et al. 2017, Catenacci et al. 2018, Valentine et al. 2019, Moreira-Soto et al., 2017).

For flaviviruses that are only sporadically detected, such as WNV and SLEV, the transmission cycle involves mosquitoes of the genus *Culex* complex (family Culicidae) as the primary vectors, and birds as the natural reservoir or amplifying hosts (Colpitts et al. 2012, Kopp et al. 2013). For those viruses, positive NPs of the genus *Alouatta* sp, and *Cebus* sp; have been detected positive by molecular or serological techniques in the Neotropics (Morales et al. 2017; Dolz et al. 2019). However, their role as putative WNV or SLEV hosts remains unknown (Valentine et al. 2019).

It is essential to deepen the understanding between flavivirus transmission cycles involving humans and wildlife as humans could become involved in a sylvatic transmission cycle if they invade the natural habitat commonly linked to land-use changes (deforestation, agriculture and urbanization), or to the invasion of natural landscapes (tourism, hunting). NPs could also be involved in urban transmission cycles for the same reasons stated above. When these infected humans or NPs enter urban settings, infections may spread rapidly, transmitted by highly anthropophilic urban mosquitoes (Weaver & Barret 2004). It is well documented in the case of YFV in Brazil, that amplifications of infected NPs precede and lead to outbreaks of short

duration in human populations (Hanley et al. 2013; Chippaux & Chippaux 2018, Moreira-Soto et al., 2018). Therefore, an increased direct and indirect contact between NPs and humans might alter flaviviral transmission cycles that lead to outbreaks in human and NPs populations equally. In Costa Rica the outbreaks of DENV and other flaviviruses are reported in geographic areas with adequate climatic conditions, favoring the presence of vectors. However, in these geographic areas, marginalized socio-economic regions and with high poverty index favor and increase the number of cases (Troyo et al. 2011). Due to the mosquito life cycle and viral replication within the mosquito depend greatly on climatic conditions, in particular temperature and rainfall (Hamlet et al. 2018). Understanding how the climate propels the presence of these flaviviruses allows us to determine the regions where the disease is likely to persevere, emerge or reappear at a later time (Harris et al. 2019). However, presuming a spillback of flaviviruses towards the NPs, a similar pattern is expected between NPs and humans. With strong prevalence in NPs captured in geographic zones known with high human incidence, and in particular for marginalized socio-economic regions.

Due to their arboreal and diurnal habits, NPs can be infected more frequently by flavivirus than other animals (Jones et al. 2008; Pandit et al. 2018) and therefore could be used as sentinels to detect circulating flaviviruses that might enter human populations. They may become infected while feeding in the treetops, using the same feeding schedule as the vectors (Valentine 2019). Having genetic and physiological characteristics similar to those of humans, they are susceptible to flavivirus that can cross species boundaries through vectors (Wolfe et al. 1998). This work determined the presence of antibodies against YFV, DENV1-4, ZIKV, WNV and SLEV in the NPs of Costa Rica and evaluated their involvement in flaviviral transmission cycles.

2. Results

We analyzed a total of 86 NPs serum samples. 83.7% (72/86) of the total samples were wild caught individuals belonging to three species: 68 howler monkeys, three squirrel monkeys, and one white-faced monkey. The remaining 16.3% (14/86) were captive individuals, all spider monkeys (Table 1). Captive individuals belong to a

population of rescued NPs that live near human settlements and receive visitors on a regular basis.

2.1. Serological screening

Of the total 86 NPs evaluated, 39.5% (34/86) had evidence of prior flavivirus infection, irrespective of the flavivirus detected or if it was homotypic or heterotypic reaction, suggesting high exposure to flaviviruses. However, no antibodies against YFV were detected. Additionally, no antibodies against ZIKV were detected. However, this was expected since the NPs samples were collected before the introduction of ZIKV in 2015 to Latin America.

24.4% (21/86) of the total NPs analyzed showed homotypic reactivity. The homotypic reactivity detected was specifically against DENV-1 in 3.5% (3/86) of individuals (one howler monkey, one spider monkey, and one squirrel monkey), DENV-2 in 7% (6/86) of individuals (five howler monkeys, and one spider monkey), SLEV in 11.6% (10/86) of individuals (nine howler monkeys, and one spider monkey), and WNV in 2.3% (2/86) of individuals (all howler monkeys) (Table 2, Figure 1). 8.1% (7/86) of the NPs showed heterotypic reactivity, specifically against DENV-1 in 4.6% (4/86) of individuals (all howler monkeys), and SLEV in 3.5% (3/86) of individuals (one howler monkeys, and two spider monkey) (Table 3, Figure 1). 8.1% (7/86) of individuals did not show a four-fold difference in titers and were classified as undetermined (six howler monkeys, and one spider monkey) (Table 4, Figure 1).

2.2. Data analysis

According to their origin, 42.8% (6/14) of positive individuals belong to captive environments and 40.3% (29/72) of positive individuals belong to free-living environments. Regarding the percentage of positives by sex and age, 40% (15/46) were females, and 14.6% (20/38) males, and 17.7% (11/62) of the adults, and 15.4% (2/13) of the juveniles were seropositive, two and 11 individuals were sex and age not determined respectively (Table 1). None of these variables were statistically significant.

Two variables were statistically significant. The sample year was statistically significant ($p \leq 0.03092$) for NP with antibodies against DENV (DENV-1, DENV-2, with

the highest seroprevalences found in the years 2014 to 2015 (Figure 2, Figure 3A). Also, the socio-economic region was statistically significant ($p \leq 0.03931$) for NPs with antibodies against flavivirus in total, with the highest seroprevalences found in the Chorotega and Central Pacific region (Figure 3B).

2.3. Data analysis

Figure 1. Map of Saint Louis encephalitis virus (SLEV), Dengue virus (DENV), West Nile virus (WNV) and undetermined flavivirus (FLAV) in seropositive Neotropical primates from Costa Rica, 2000–2015: (A.) SLEV, (B.) DENV, (C.) WNV, (D.) undetermined FLAV.

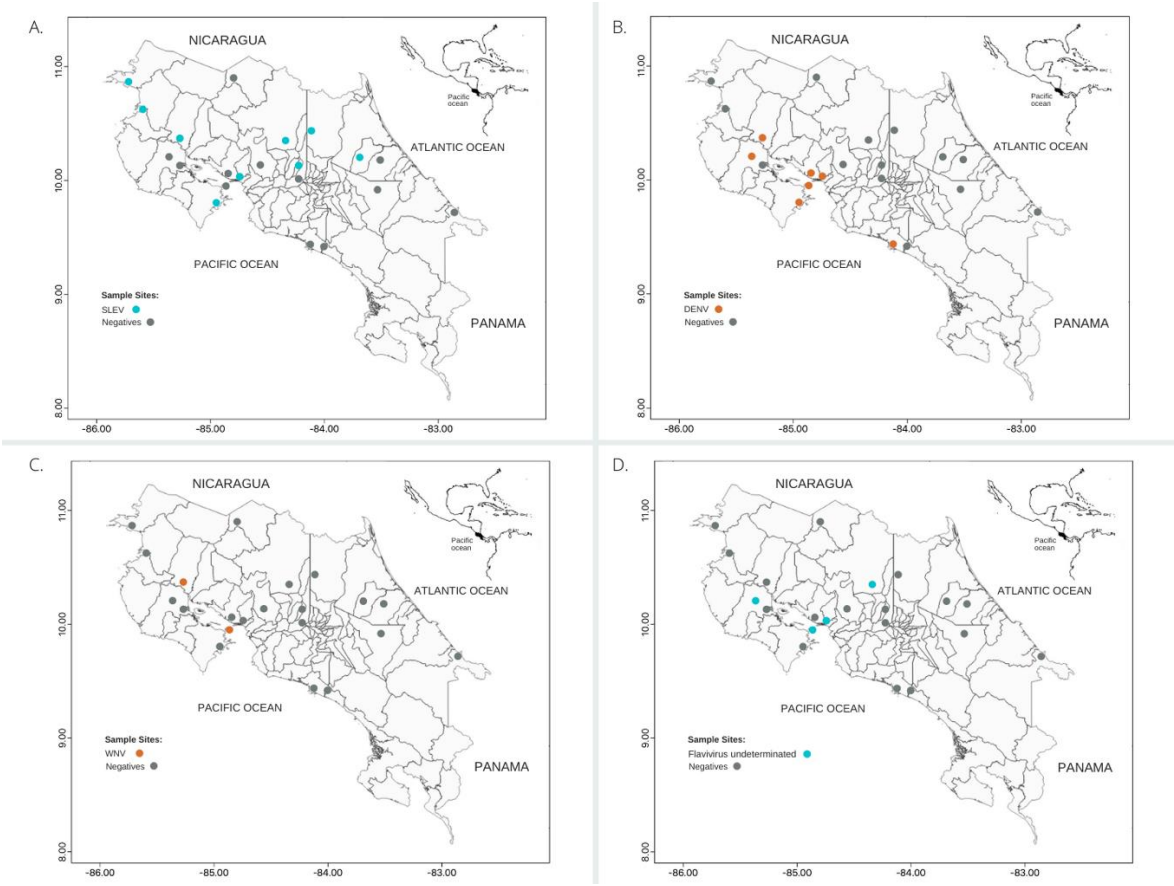

Figure 2. Seroprevalences of Saint Louis encephalitis virus (SLEV), Dengue virus (DENV), West Nile virus (WNV) and undetermined flavivirus (FLAV) of Neotropical primates from Costa Rica, 2000–2015: (A.) SLEV, (B.) DENV, (C.) WNV, (D.) undetermined FLAV.

Figure 3. A. Map of Dengue Virus seropositive Neotropical primates captured during 2014 and 2015, and principal counties for DENV positive humans for the same years in Costa Rica. B. Map of the flavivirus family seropositive Neotropical primates, in seroprevalence socioeconomic regions of Costa Rica.

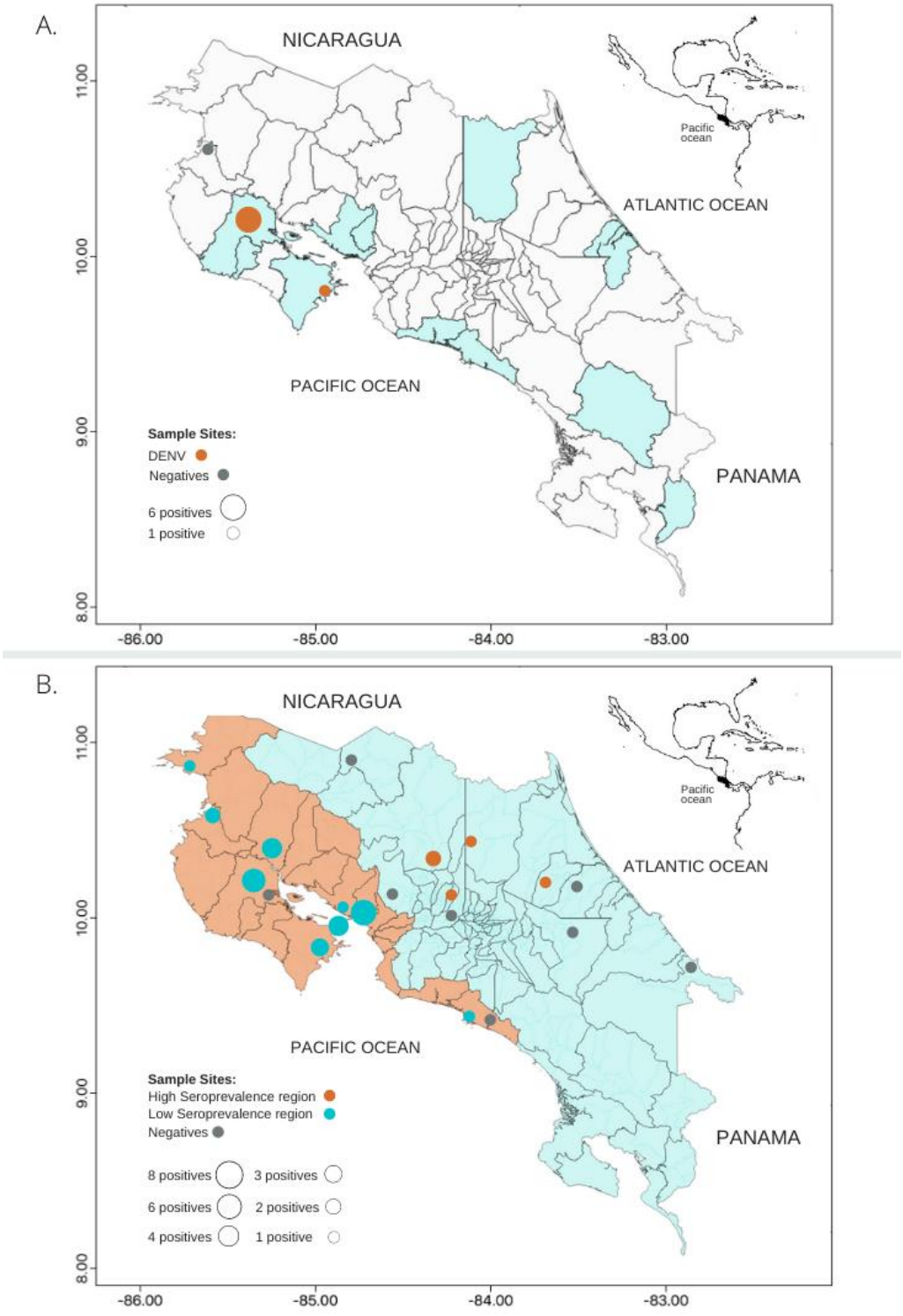

Table 1. Number of Neotropical Primate of each species, age, sex, origin, mean annual rainfall, mean annual temperature, capture year from which Saint Louis Encephalitis virus, Dengue virus, West Nile, and undetermined flavivirus micro PRNT positive in Costa Rica, 2000-2015.

	Host Abundances	SLEV	DENV	WNV	undetermined FLAV
		Positive (%)			
Specie					
Alouatta palliata	68	10 (14.7)	10 (14.7)	2 (2.9)	6 (8.8)
Ateles geoffroyi	14	3 (21.4)	2 (14.3)	0	1 (7.1)
Saimiri oerstedii	3	0	1 (33.3)	0	0
Cebus imitator	1	0	0	0	0
Age					
adult	62	11 (17.7)	13 (20.9)	2 (3.2)	6 (9.7)
juvenile	13	2 (15.3)	0	0	1 (7.7)
not determined	11	0	0	0	0
Sex					
female	46	7 (15.2)	4 (8.7)	0	4 (8.7)
male	38	6 (15.8)	9 (23.7)	2 (5.2)	3 (7.9)
not determined	2	0	0	0	0
Origin					
Captivity	14	3 (21.4)	2 (14.3)	0	1 (7.1)
Free-range	72	10 (13.9)	11 (15.3)	2 (2.8)	6 (8.3)
Mean Annual rainfall (mm)					
1000-2000	15	2 (13.3)	2 (13.3)	1 (6.7)	1 (6.7)
2000-3000	51	8 (15.7)	11 (21.6)	1 (2)	5 (10)
>3000-4000	20	3 (15)	0	0	1 (5)
Mean annual temperature (°C)					
20-22	1	0	0	0	0
22-24	6	1 (16.7)	0	0	0
24-26	11	3 (27.3)	0	0	1 (9.1)
26-28	68	9 (13.2)	13 (19.1)	2 (2.9)	6 (8.8)
Capture Year					
2000-2003	45	7 (15.5)	5 (11.1)	1 (2.2)	3 (6.7)
2005-2008	20	4 (20)	1 (5)	1 (5)	2 (10)
2014-2015	21	2 (9.5)	7 (33.3)	0	2 (9.5)
Socio-economic Region					
Chorotega	32	4 (12.5)	6 (18.7)	1 (3.1)	2 (6.2)
Central Pacific	31	5 (16.1)	8 (25.8)	1 (3.2)	2 (6.4)

Central Region	4	1 (25)	0	0	0
Huetar Atlantica	13	1 (7.7)	0	0	0
Huetar Northern	6	1 (16.7)	1 (16.7)	0	1 (16.7)

Table 2. Distribution of micro PRNT titers for flaviviruses in Neotropical Primates with monotypic immune pattern, Costa Rica, 2000-2015.

Animal identification	Species	Year	PRNT Titer						Result Interpretation
			DENV-1	DENV-2	DENV-3	DENV-4	SLEV	WNV	
AP-03	*A. palliata*	2000	<1:20	<1:20	<1:20	<1:20	**1:160**	<1:20	SLEV
AP-05	*A. palliata*	2000	<1:20	<1:20	<1:20	<1:20	**1:160**	<1:20	SLEV
AP-06	*A. palliata*	2001	<1:20	<1:20	<1:20	<1:20	**1:80**	<1:20	SLEV
AP-07	*A. palliata*	2001	<1:20	<1:20	<1:20	<1:20	**1:40**	<1:20	SLEV
AP-08	*A. palliata*	2001	<1:20	<1:20	<1:20	<1:20	**1:40**	<1:20	SLEV
AP-12	*A. palliata*	2001	<1:20	**1:20**	<1:20	<1:20	<1:20	<1:20	DENV-2
AP-19	*A. palliata*	2001	<1:20	**1:20**	<1:20	<1:20	<1:20	<1:20	DENV-2
AP-27	*A. palliata*	2001	<1:20	<1:20	<1:20	<1:20	<1:20	**1:20**	WNV
AP-30	*A. palliata*	2001	<1:20	<1:20	<1:20	<1:20	**1:40**	<1:20	SLEV
AP-58	*A. palliata*	2002	**1:20**	<1:20	<1:20	<1:20	<1:20	<1:20	DENV-1
AP-244	*A. palliata*	2004	<1:20	<1:20	<1:20	<1:20	<1:20	**1:20**	WNV
AG-45	*At. geoffroyi*	2006	**1:20**	<1:20	<1:20	<1:20	<1:20	<1:20	DENV-1
AG-46	*At. geoffroyi*	2006	<1:20	<1:20	<1:20	<1:20	**1:20**	<1:20	SLEV
AP-147	*A. palliata*	2006	<1:20	<1:20	<1:20	<1:20	**1:40**	<1:20	SLEV
SM-1	*S. oerstedii*	2006	**1:20**	<1:20	<1:20	<1:20	<1:20	<1:20	DENV-1
FPX-25	*A. palliata*	2014	<1:20	**1:20**	<1:20	<1:20	<1:20	<1:20	DENV-2
MP-45	*A. palliata*	2015	<1:20	<1:20	<1:20	<1:20	**1:80**	<1:20	SLEV
MP-48	*A. palliata*	2015	<1:20	<1:20	<1:20	<1:20	**1:40**	<1:20	SLEV
MC-32	*C. imitator*	2015	<1:20	**1:20**	<1:20	<1:20	<1:20	<1:20	DENV-2
FPX-21	*A. palliata*	2014	<1:20	**1:20**	<1:20	<1:20	<1:20	<1:20	DENV-2
FP-109	*A. palliata*	2014	<1:20	**1:20**	<1:20	<1:20	<1:20	<1:20	DENV-2

Table 3. Distribution of micro PRNT titers for flaviviruses in Neotropical Primates with heterotypic immune pattern, Costa Rica, 2000-2015.

Animal identification	Species	Year	PRNT Titer						Result Interpretation
			DENV-1	DENV-2	DENV-3	DENV-4	SLEV	WNV	

AP-22	*A. palliata*	2001	**1:80**	1:20	<1:20	<1:20	<1:20	<1:20	DENV-1
AP-249	*A. palliata*	2006	1:20	<1:20	<1:20	<1:20	**1:80**	<1:20	SLEV
AP-150	*A. palliata*	2007	<1:20	<1:20	<1:20	<1:20	**1:80**	1:20	SLEV
CI-273	*C. imitator*	2015	<1:20	<1:20	<1:20	<1:20	**1:160**	1:20	SLEV
FP-101	*A. palliata*	2014	**1:640**	1:20	<1:20	<1:20	<1:20	<1:20	DENV-1
FP-107	*A. palliata*	2014	**>1:1280**	1:20	<1:20	<1:20	<1:20	1:40	DENV-1
FP-108	*A. palliata*	2014	**1:80**	1:20	<1:20	<1:20	<1:20	1:20	DENV-1

Table 4. Distribution of micro PRNT titers for flaviviruses in Neotropical Primates without four-fold differences (undetermined flavivirus), Costa Rica, 2000-2015.

Animal identification	Species	Year	PRNT Titer						Result Interpretation
			DENV-1	DENV-2	DENV-3	DENV-4	SLEV	WNV	
AP-09	*A. palliata*	2001	**1:40**	**1:20**	<1:20	<1:20	<1:20	<1:20	DENV-1, DENV-2
AP-10	*A. palliata*	2001	**1:20**	**1:40**	<1:20	<1:20	<1:20	**1:20**	DENV-1, DENV-2, WNV
AP-11	*A. palliata*	2001	**1:40**	**1:40**	<1:20	<1:20	<1:20	<1:20	DENV-1, DENV-2
AP-247	*A. palliata*	2004	**1:40**	**1:20**	**1:40**	<1:20	<1:20	<1:20	DENV-1, DENV-2, DENV-3
AG-55	*At. geoffroyi*	2006	<1:20	**1:20**	<1:20	<1:20	**1:20**	<1:20	DENV-2, SLEV
FP-102	*A. palliata*	2014	**1:40**	**1:20**	<1:20	<1:20	<1:20	<1:20	DENV-1, DENV-2
FP-103	*A. palliata*	2014	**1:80**	**1:20**	<1:20	<1:20	<1:20	**1:40**	DENV-1, DENV-2

3. Discussion

In this study we evaluated the presence of neutralizing antibodies against YFV, SLEV, DENV1-4, WNV, and ZIKV in the NPs of Costa Rica across more than a decade and in diverse geographical regions. We detected a high presence of neutralizing antibodies against flaviviruses in general, as well as high detection of specific antibodies for SLEV, DENV-1, DENV-2 and WNV. Although the difference was not statistically significant, this suggests that wild-caught and captive individuals are being exposed to the same flaviviruses

Homotypic and heterotypic reactions detected were mainly to DENV irrespective of type and SLEV. In total, 91.9% of the flavivirus reactivity could be attributed to one of the flaviviruses analyzed, suggesting our flaviviral testing strategy was successful in detecting the majority of flaviviruses circulating in NPs. For the remaining 8.1% of

samples detected as undetermined, other flaviviruses that were not tested in our study might elicit a response. Alternatively, a decaying antibody response observed overtime in human patients might explain a basal antibody response without a clear determination of the flaviviral species (Moreira-Soto et al., 2020).

The striking high seropositivity to SLEV across years has important public health implications. In Costa Rica there is no SLEV monitoring program, even though since 1960 there is evidence of the presence of SLEV in Latin America and human cases have been reported sporadically in Brazil and Argentina (Díaz et al. 2006, Heinen et al. 2015). In the neotropics, a variety of mammals (NPs, ungulates, bats, folivores, rodents, marsupials) have been found infected with SLEV (Medlin et al. 2016; Morales et al. 2017) proposed as alternative transmission cycles involving mammals instead of birds, as well as vectors of atypical mosquitoes belonging to other genera (Reisen 2003; Kopp et al. 2013). Antibodies (80%) have only been determined in two sloth (two-toed sloths (*Choloepus hoffmanni*), and three-toed sloths (*Bradypus variegatus*) species, using Hemagglutination inhibition (HI) complement fixation (CF) tests and PRNT´s in Costa Rica (Medlin et al. 2016). Recent studies have characterized an SLEV clade identified in a primary tropical ecosystem in Central America, suggesting the discovery of ancestral SLEV (Kopp et al. 2013).

This increases the need to deepen research on the circulation of this virus in Central American forests. Specifically, in NPs, the presence of antibodies against SLEV was determined in the *Alouatta* genus, in Argentina (32% - 2001 and 1.85% - 2017 by PRNT), and Brazil (12% by HI test) (Contigiani et al. 2000, Svoboba et al. 2014, Morales et al. 2017). Although it is not clear if alternative cycles of SLEV including mammals may favor the maintenance of the virus. The wide diversity of mosquitoes suggested as vectors of these cycles (genera *Culex, Coquillettidia, Deinocerites, Mansonia, Psorophora, Sabethes*, and *Wyeomyia*) could favor a generalized distribution of SLEV in the country (Kopp et al. 2013).

Dengue is the most important vector-borne disease in Costa Rica. Its incidence has increased significantly in the last 25 years, primarily due to lack of control in the proliferation of its main vector, *Ae. Aegypti* (Troyo et al. 2006). White-faced monkeys (1.4% at DENV-2, 1.4% at DENV-3 and 2.8% at DENV-4) have been determined

positive in Costa Rican NPs by polymerase chain reaction (PCR) (Dolz et al. 2019), and in other Latin American countries. The presence of individuals with positive serology against DENV1-4 is common in the neotropics (Catenacci et al. 2018). This justifies seeking greater scientific evidence and monitoring of wild environments and the human-wildlife interface, using NPs as sentinel species to assess the possible maintenance of the virus in sylvatic cycles. The proximity of forests to urban environments in neotropical countries has led to the entry of arboviruses from nature into cities (eg: the Mayaro virus (Alphavirus) and the Oropouche virus (Orthobunyavirus) of NPs in Brazil), which increases the risk of starting urban maintenance cycles (Mourão et al. 2009; 2012). However, in America a growing concern is the establishment of Flaviviruses sylvatic cycles like the DENV by spillback transmission from humans to wild animals (e.g., in the Atlantic Forest Reserve of Bahia in Brazil (2006-2014), low seropositivity to DENVs was found in lion tamarins (*Leontopithecus chrysomelas*) that were close to agricultural workers), increasing the possibility of spillback infection (Pandit et al., 2018; Valentine et al., 2019). The NPs analyzed inhabit patches of forests close to human activities, which we can classify with favorable conditions for the appearance of outbreaks or for the enzootic maintenance of vector diseases (Hanley et al 2013) and may represent a scenario for bidirectional transmission of flaviviruses. The average range of births in howler monkeys is 0.42 per female per year, even though they are markedly seasonal, it is common to observe births every year (Azkarate et al. 2017). Therefore, we could report that in the samples used there is evidence of presence of SLEV and Dengue in subsequent generations of primates.

When comparing the positive sites with the presence of DENV, the individuals with antibodies are concentrated in two classified regions with high rates of human cases during these two years (Ministerio de Salud, 2015). In Costa Rica, the most significant outbreak of Dengue occurred in 2013, since the first documented outbreak in 1993 (Soto-Garita et al. 2016). Our study did not have samples from that year, however the range of years with the highest number of cases is reported in the two years following 2014-2015. In addition, in all the identified flaviviruses we observed peaks in transmission events approximately every two years. In the country the infections by

Dengue are ever present, nevertheless the transmission peaks took place every 2 to 5 years (Soto-Garita et al. 2016).

Also, antibodies against WNV were determined in two howler monkeys from the Pacific coast of Costa Rica. In this same area, Dolz et al. (2019) detected by PCR the presence of howler monkeys positive to WNV (4.2%). In addition, antibodies against WNV (15%) were reported in two-toed sloths in the country (Medlin et al. 2016). Likewise, in South America (Argentina), neutralizing antibodies against WNV were determined in 22.2% of the howler monkeys analyzed (Morales et al. 2017). Although WNV is maintained in enzootic cycles among birds and mosquitoes, a wide variety of other vertebrate species appear to be susceptible to the infection, although very few alternative hosts appear to develop viremia with sufficient load to favor the transmission of the virus (Chancey et al. 2015). Little is known with respect to WNV's first appearance on the American continent in 1999 (Nash et al. 1999). It spread rapidly throughout the continent, infecting a wide range of species of birds and mammals (Pfeffer & Dobler 2010) which makes it necessary to learn more about the possible maintenance in sylvatic cycles.

Historically, the two regions with the highest reported incidence of dengue fever in humans are Chorotega and the Central Pacific (Marin & Diaz, 2012). The most relevant variables associated with the presence of dengue in the regions of greater incidence are situated near the coast, have elevated temperatures (maximum peaks of transmission between 26 and 29 C), and an elevated index of human poverty (Troyo et al. 2011). Unlike DENV and WNV, the detection of positive individuals was located in the Central and Northern Pacific coastal regions. SLEV was detected in all the evaluated socioeconomic regions, with varied altitude, environmental conditions and demographic aspects. While there was no association with mean annual temperature and mean annual rainfall.

None of the NPs was found positive to either YFV or ZIKV. This suggests either low circulation of both agents in the samples analyzed or that the high susceptibility of several species of NPs to YFV leads to a high mortality and therefore individuals get taken out of the population (Hanley et al. 2013). Central America has been free of YFV since the sixties after being controlled by *Aedes* sp. for a period of time, with the use

of DDT and massive vaccination. To date, there is no evidence of new cases in the area (Chippaux & Chippaux 2018). With respect to ZIKV, the first human cases reported in Costa Rica were in 2016 (Sanchez et al. 2019). Agreeing our results with the absence of individuals with antibodies against ZIKV and taking into account that the most recent year of the samples used in this study was 2015.

Additionally, the cross-reactivity observed suggests unidentified exposure to several flaviviruses that co-circulate in Costa Rica. Sequential infections with other flaviviruses cause strong cross-reaction anamnestic reactions, which can confer immunity, especially within members of the same serogroup, making specific serological differentiation difficult (Morales et al. 2017). Limitations of our study include heterogeneity in the number of individuals sampled per year and the number of NP species sampled. Our study suggests undetected circulation of SLEV for decades in Costa Rica, human samples from the same sites should be further tested to address if the same high detection is observed, and if NPs could be used as sentinels of undetected circulation of flaviviruses in Latin America. We find it necessary to determine in Central American forests the presence of competent vectors whose niche is in the wildlife/human interface. This knowledge will serve to identify local risk factors and determine whether human behavior or modified NPs behavior may be affecting the transmission cycle of flaviviruses (Pandit et al. 2018). This may indicate that the Costa Rican rainforests, the NPs and the vectors that circulate in these natural environments may be suitable for the sylvatic maintenance of these infectious agents.

4. Materials and Methods

4.1. Sampling protocol

This research employed serum samples collected for long-term NPs genetic variability project from the School of Biology of the University of Costa Rica. Samples belong to the four extant NPs species found in Costa Rica: howler monkey (*Alouatta palliata*), white-face monkey (*Cebus imitator*), spider monkey (*Ateles geoffroyi*) and squirrel monkey (*Saimiri oerstedii*). Samples were collected longitudinally from 2000 to 2015 in diverse geographical locations throughout Costa Rica (Figure 1).

This study was conducted under protocols established by the Institutional Committee for the Care and Use of Animals (Comité Institucional para el Cuidado y Uso de los Animales) of the University of Costa Rica, adhered to the legal requirements of Costa Rica and the American Society of Primatologists (ASP) Principles for the Ethical Treatment of NP (Collection permit number: MINAET-SINAC-Costa Rica: 042–2012-SINAC).

The NPs were captured using chemical immobilization with darts (Type P, 1ml, Pneu Dart Inc.), and compressed gas rifle (X-Caliber Gauged CO2, Pneu Dart Inc.) for individuals at long distances, or by blowgun for individuals at a close range. Anaesthetics used were Zoletil 50® (3.3–11 mg/kg, Virbac), or ketamine (5–20 mg/kg, Bremer Pharma GMbH), in combination with xylazine (0.5–2 mg/kg, Alfasan; Glander et al. 1991; Varela, 2006; West et al. 2007). As soon as the animal was anesthetized, a 2–4 mL blood sample was taken from the femoral, saphenous or cephalic vein and maintained at 4°C until arrival at the laboratory. The NPs were monitored until recovered from anesthesia and safely released at the capture site. Once in the laboratory, the samples were centrifuged at 2500 RPM for 5 min to separate the serum, and then transferred to sterile 1.5mL tubes at -20°C until processed.

4.2. Serological screening

Serum samples were tested for specific flavivirus-reactive antibodies by micro plaque reduction neutralization test (micro-PRNT) as described previously (Simões et al. 2012). All samples were heat-inactivated and tested at initial screening in a final dilution of 1:20 for each virus WNV (YFV 17D/WNV Flamingo 383-99), DENV 1-4 (YFV 17D/DENV-1 PUO 359, YFV 17D/DENV-2 218, YFV 17D/DENV-3 PaH881/88, YFV 17D/DENV-4 1228), ZIKV (ATCC VR-748) and YF (YFV 17D).

Briefly, samples were diluted 1:10 in 30 µl in Eagle's minimal essential medium (MEM) with 2% of FBS and mixed with an equal volume of each virus to produce an estimated 20 UFP/well (previously determined by viral titration). Virus-serum mix was incubated 1 hour at 37°C in a 5% CO_2 atmosphere. Then, 50 µl from the mix was inoculated into VERO cell monolayer (ATCC® CCL-81™) with a concentration of 2.5 x 10^4/ well and incubated for an hour as in previous conditions. The overlay was removed and 100 µl

of MEM with 2% of FBS and 1,5% of carboxymethyl cellulose (CMC) were added. Each plate contained virus and positive serum control (determined by other methods). After 3 days of incubation, the monolayers were fixed with formalin 10% for an hour and stained with crystal violet 1%. Then, plates were counted, and samples were classified. Serums were considered positive to a virus when reduced to at least 90% of the formation of plaques of the virus compared to the control virus.

Due to the known cross-reactivity between flaviviruses, all sera were tested and titrated individually for each virus. A plaque reduction of ≥90% was considered positive, with the titer measurement as the highest serum dilution showing ≥90% of plaque reduction relative to the viral control. For titrations, serial two-fold dilutions that ranged from 1:20-1:1280 were done. If a serum sample showed reaction to only one flavivirus, the reaction was considered homotypic. However, if the reaction was to several flaviviruses, the reaction was considered heterotypic. A 4-fold difference in titer between the different flaviviruses was required for identification. In case that 4-fold dilution difference was not reached the serum was considered as flavivirus positive (Morales et al. (2017).

4.3. Data analysis

Fisher's exact contingency table analysis were used to compare flavivirus seropositivity to climatic, social and epidemiological variables. The tested variables included mean annual rainfall (1000-2000, 2000-3000, 3000-4000 mm), mean annual temperature (20-22, 22-24, 24-26, 26-28 °C), sex, age, origin of NPs (free-range, captivity), sample year (2000-2003, 2005-2008, 2014-2015), and Costa Rican socio-economic regions (Chorotega, Central Pacific, Central Atlantic, Huetar Atlantica, Huetar Northern). A P-value < 0.05 was considered statistically significant. These statistical analyses were conducted in R (R-Development Core Team 2016). Primary data for mean annual rainfall and mean annual temperature were obtained from WorldClim (Hijmans et al. 2005).

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Abbreviations

The following abbreviations are used in this manuscript:

NPs: Neotropical primates
DENV: Dengue virus
SLEV: Saint Luis Encephalitis virus
WNV: West Nile virus
ZIKV: Zika virus
YFV: Yellow Fever virus
Micro-PRNT: micro plaque reduction neutralization test
HI: Hemagglutination inhibition
CF: Complement fixation
mg/kg: milligram per kilogram
mL: milliliter
µl: microliter
°C: centigrade
RPM: revolutions per minute
min: minutes
MEM: minimal essential medium
CMC: carboxymethyl cellulose

Reference

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Sample Availability: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.