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SEROLOGIC EVIDENCE OF NATURAL TOGAVIRUS INFECTIONS IN PANAMANIAN SLOTHS AND OTHER VERTEBRATES*

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Abstract. Plasmas of sloths and other Central Panamanian wild vertebrates were tested for plaque-reduction neutralizing (PRN) antibodies against four flaviviruses and one alphavirus. Forty percent of 97 two-toed sloths, *Choloepus hoffmanni*, and 8% of 168 three-toed sloths, *Bradypus variegatus*, were specifically positive against St. Louis encephalitis (SLE) virus. The prevalence of antibody against SLE virus was considerably higher in sloths than in any other group of wild vertebrates tested, including birds, and was found mainly in adult sloths. Specific PRN antibody against yellow fever (YF) virus was found only in monkeys. A high prevalence of PRN antibody against Ilheus and Mayaro viruses was detected in agoutis, *Dasyprocta punctata*, and against Mayaro virus in howler monkeys, *Alouatta villosa*. No plasma was specifically positive against Bussuquara virus. The results are interpreted as evidence that sloths are probably not important hosts in jungle YF cycles, but may be significant amplifying hosts in tropical SLE virus cycles.

Tree sloths are New World arboreal mammals related to armadillos and anteaters. They occur from Honduras to Argentina. The two existing genera include *Bradypus*, the three-toed sloths, and *Choloepus*, the two-toed sloths. Although they share the same arboreal habitat and many convergent anatomical features, the behavioral and anatomical differences between the two genera are clear enough to place them in separate families.[1,2] Sloths are difficult or impossible to see when camouflaged in their forest canopy habitat, but they are nevertheless among the most abundant mammals of the Neotropical forest.[3–5] Both genera are characterized by low body temperature and metabolic rate,[6] and are highly evolved for a vegetarian existence.[7] *Choloepus* commonly survive in captivity for over 10 years,[7,8] and probably both genera are longlived in nature.

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Interest in sloths as hosts of flaviviruses initially centered on the search for alternate hosts in New World jungle yellow fever (YF) cycles, which are usually considered to involve monkeys and forest canopy mosquitoes.[9,10] In early experimental work, Colombian two-toed sloths *Choloepus didactylus* showed little potential as YF amplifying hosts.[9] In 1974, P. A. Webb and K. M. Johnson of the Gorgas Memorial Laboratory repeated this observation with the Panamanian species *Choloepus hoffmanni*; however, they also showed that some Panamanian *Bradypus variegatus* sustained remarkably long experimental YF viremias of unusually high titer, with no apparent ill effects.[11]

The hypothesis that sloths are natural amplifying hosts of YF virus was tested by field studies in Panamá in 1974–76, during and after a wave of jungle YF. These and other concurrent field studies also included observations on related flaviviruses, among them St. Louis encephalitis (SLE) virus, a human pathogen which is transmitted by mosquitoes among wild birds in most of North America.[12] In the tropics, SLE virus causes sporadic human illness, but its natural cycle is poorly understood.[13] As with YF virus, inoculation of sloths with SLE virus produces prolonged viremias of high titer.[14] Field studies of flavivirus infections of sloths were therefore a test of the role of these animals in tropical cycles of SLE virus as well as YF virus.

This article reports the results of the 1974–76

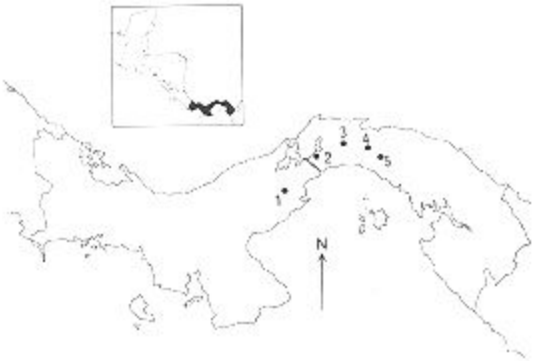

FIGURE 1. Location of study areas. 1. Aguacate. 2. Chilibre. 3. Cerro Azul. 4. El Llano-Carti road camp. 5. Majé.

field studies in Panamá on natural flavivirus infections of sloths and other forest vertebrates. Other articles in this series describe experimental SLE infection of sloths and cormorants,[11] as well as the isolation from sloths of several viruses unrelated to flaviviruses.[15]

MATERIALS AND METHODS

Study areas

Sloths were captured at five Central Panamanian localities (Fig. 1). At Aguacate (elevation 300 meters) and Chilibre (100 meters), vegetation was mostly secondary, with patches of primary tropical humid forest (Holdridge classification).[16] At the other three areas the vegetation was predominantly primary, with the following Holdridge classifications: El Llano-Cartí road camp (elevation 300 meters), tropical wet forest; Cerro Azul (500–800 meters), tropical humid forest; Majé (less than 200 meters) tropical humid-to-dry transitional forest.

Animal capture and bleeding

Two-toed sloths *C. hoffmanni* (Fig. 2), and three-toed sloths *B. variegatus* (Fig. 3), were captured by hand; at Cerro Azul, they were fitted with radio transmitters[17] and periodically recaptured for serial blood samples. Sloth age was determined as infant when the animal was still attached to its mother, juvenile when it was independent but not fully grown, and adult when it had reached full size, (*Bradypus* forearm 180 mm; *Choloepus* forearm 170 mm). *B. variegatus* leave their mothers

FIGURE 2. *Choloepus hoffmanni*, the two-toed sloth. (Photograph courtesy of Dr. Howard Christiansen.)

at 6 months, and are fully grown at two years.[4] *Choloepus* sloths probably develop somewhat more slowly.[18]

Most other mammals and birds were either

FIGURE 3. *Bradypus variegatus*, the three-toed sloth.

trapped or netted routinely by the Gorgas Memorial Laboratory Bayano River Program, or rescued from impounded waters by the International Society for the Protection of Animals (ISPA). At El Llano-Cartí and Cerro Azul, howler monkeys and other mammals, apart from sloths, were shot. Mammals which were shot or which were captured by ISPA were bled by cardiac puncture. Otherwise, sloths were bled from the brachial vein; rodents, bats and marsupials from the retroorbital sinus, and birds from the external jugular vein. In the field, whole heparinized blood was frozen in liquid nitrogen within 2 hours of collection. Plasma was aseptically separated from heparinized blood either by centrifugation or by overnight settling, and then frozen in liquid nitrogen until transfer to electric freezers. Whole blood was stored at −60°C, and plasma at −20°C.

Virus isolation attempts

Heparinized whole blood (0.1 ml) was inoculated onto tube cultures of Vero cells, which were maintained at 35°C and observed for 14 days.

Neutralization tests

Test viruses included the four sylvatic flaviviruses known to occur in Panamá: YF virus (French neutrotropic strain); SLE virus, strain Buena Vista-7, isolated from Panamanian mosquitoes in 1956; Bussuquara (BSQ) virus, strain GA-7, from Panamanian mosquitoes captured in 1961; and Ilheus (ILH) virus, strain Guat-245, isolated in 1956 from Guatemalan mosquitoes. Mayaro (MAY) virus, an alphavirus, was included as an antigen to control for non-specific plaque reduction, since it is antigenically distinct from the four flaviviruses; it was also included because its natural transmission cycle is thought to be similar to that of Neotropical jungle YF virus. We used MAY strain Pan Ar 443 isolated in 1974 from Panamanian mosquitoes.

Plaque reduction neutralization (PRN) tests were basically performed as described elsewhere.[19] Briefly, plasmas were screened 1:8 against 40–250 (generally 50–100) plaque forming units (pfu) of challenge virus. Guinea pig serum at a final concentration of 1:60 was incorporated in the virus-plasma mixture as a source of fresh serum factor. The mixtures were incubated at 4°C overnight before inoculation onto 2 cm^2 Vero cell monolayers. A second overlay including neutral red followed 2 or 3 days after the initial overlay, which varied in composition for each virus. To enhance plaque clarity, Eagle's Minimal Essential Medium replaced the original basic nutrient medium in ILH virus tests; gum tragacanth was used in various combinations with either agarose or agar in flavivirus overlays.

Plasmas reducing plaque counts by at least 80% were retested in serial dilutions; 90% plaque reduction by plasma diluted 1:16 was considered positive. Plasmas positive against any one flavivirus were subsequently titrated against all four flaviviruses with approximately equal test virus doses, for comparison of PRN titers. Low plasma quantities reduced the number of screening antigens in tests of some animals.

RESULTS

Virus isolation attempts

No flaviviruses were isolated from 45 *Bradypus* and 14 *Choloepus*. Other viruses isolated from these animals are described elsewhere.[15]

Antibody titers and test specificity

Ninety percent plaque reduction serum titers of 1:8 were considered negative because they were not reproducible. Also, in other studies,[15] titers of 1:8 have been associated with low level inhibition of two or more unrelated viruses, suggesting broad-spectrum non-specific activity. Ninety percent reduction by plasmas diluted 1:16 was therefore considered positive, but most positive titers were at least as high as 1:64. The frequency distribution of sloth antibody PRN titers against SLE virus are shown in Table 1, and are representative of positive reactions seen in this study against other togaviruses and in other vertebrates.

A possible problem of cross-reactions between the four serologically related flaviviruses was resolved by experimental demonstration of the specificity of sloth antibodies against SLE virus,[14] and by the infrequency of multiple reactions and confusing titers in this study. In multiple positives, differences in titer as little as 4- or 8-fold occurred so rarely as to be insignificant, and in these cases, antibody was considered specific against the antigen of higher titer for simplicity.

Sloth plasmas were never positive against more than two of the four test flaviviruses. Even taking into account reactions at 1:8 (a level considered

TABLE 1

Distribution of neutralizing antibody titers against St. Louis encephalitis virus, and neutralizing reactions against three other flaviviruses in animals seropositive against St. Louis encephalitis virus, in sloths from Aguacate, Cerro Azul, and Majé

Species	Sloths positive against SLE virus		PRN reactions against other flaviviruses of sloths positive for SLE virus antibodies*					
			Titer† 1:8 against:			Titer ≥1:8 against		
	PRN titer	No.	ILH	YF	BSQ	ILH	YF	BSQ
Bradypus variegatus	1:32–1:64	3	0	0	0	0	0	0
	1:128–1:256	5	0	0	0	0	0	0
	1:512–1:1,024 (Median 1:256)	4	1	0	0	1‡	0	0
Choloepus hoffmanni	1:16–1:64	6	0	0	0	0	0	0
	1:128–1:256	21§	3	1	5	1	0	0
	1:512–1:1,024 (Median 1:256)	6§	1	0	1	0	0	0

* Abbreviations: PRN, plaque-reduction neutralization; SLE, St. Louis encephalitis; ILH, Ilheus; YF, yellow fever; BSQ, Bussuquara.
† Titers are the weakest plasma dilution reducing plaques 90%.
‡ SLE titer 1:1,024; ILH titer 1:256.
§ Two sloths not tested against BSQ virus.
‖ SLE titer 1:256; ILH titer 1:32.

negative in this study), 73% of the positive animals reacted against a single flavivirus. Of 14 multiple flavivirus reactions involving sloths positive against SLE virus, 12 consisted of only a 1:8 reaction against another flavivirus, with the SLE virus titer higher by at least 16-fold (Table 1). Only two sloth plasmas were considered to be positive against both SLE and another flavivirus. However the titers were 4- and 8-fold respectively higher against SLE virus than the other agent (ILH virus).

The specificity of PRN reactions was as clear in most cases involving other flaviviruses and forest vertebrates as it was for SLE virus and sloths. No multiple positives were seen in three sloth plasmas positive against ILH virus and two anteaters and a bird positive against SLE virus. Of 12 monkeys positive against YF virus (titers 1:32–1:512, median 1:128), eleven did not react with any other flavivirus even at a 1:8 plasma dilution. The twelfth monkey reacted at 1:128 against YF virus and 1:64 against SLE virus, and probably was infected by both agents.

Three of four ILH virus antibody-positive plasmas from agoutis (*Dasyprocta punctata*) were multiple flavivirus positives. Although reactions against ILH virus were always strongest (1:128–1:1,024), titers against BSQ virus in three plasmas were only 2-, 4-, and 16-fold lower than against the corresponding ILH titers. Reactions against YF virus were 8- to 64-fold lower than those against ILH virus in two agouti plasmas.

The inclusion of MAY virus, an alphavirus, in the battery of test antigens proved useful in controlling for non-specific plaque inhibition. The number of double alpha- and flavivirus positive plasmas was not greater than that expected by chance double infections.

Yellow fever virus

Only primates reacted specifically against YF virus (Table 2). Of 14 howler monkeys, *A. villosa*, from Majé, only five juveniles did not have PRN antibody against YF virus; these five animals were born after the 1974 sylvatic YF wave had already passed through Majé. Other Majé monkeys specifically inhibiting YF virus antigen include two marmosets, *Saguinus geoffroyi*, and one night monkey *Aotus trivirgatus*. YF antibody was not detected in plasmas from 33 adult *Bradypus*, 16 adult *Choloepus* or any other non-primate species collected at Majé 4–26 months after active YF transmission there. At El Llano-Cartí, another forest locality which had suffered a sylvatic YF wave within 6 months previous to collection of plasmas, 9/15 adult howler monkey plasmas were positive against YF virus, in contrast with none of 10 adult *Bradypus* and none of three adult *Choloepus* sloths.

St. Louis encephalitis virus

At Majé (Table 2), most specific reactions against SLE virus were in plasmas collected from xenarthrans, particularly *Choloepus* sloths (46% posi-

TABLE 2

Frequency of specific neutralizing antibodies against four flaviviruses and Mayaro virus in animals collected near Majé, Panamá, 1974–1976

Species	Virus*				
	SLE	YF	ILH	BSQ	MAY
Birds (39 species)	1/75 (.01)†	0/61	0/61	0/1	0/61
Opossums:					
Didelphis marsupialis	0/13	0/13	0/13	nt‡	0/13
Metachirus nudicaudatus	0/2	0/2	0/2	nt	0/2
Marmosa sp.	0/2	0/2	0/2	nt	0/2
Bats:					
Artibeus jamaicensis	0/6	0/6	nt	nt	0/6
Artibeus lituratus	0/4	0/4	nt	nt	0/4
Carollia perspicillata	0/6	0/6	nt	nt	0/4
4 other species	0/4	0/4	nt	nt	0/4
Primates:					
Aotus trivirgatus	0/27	1/27 (.04)	0/27	0/6	0/6
Saguinus geoffroyi	0/40	2/40 (.05)	0/40	0/8	0/32
Cebus capucinus	0/2	0/2	0/2	0/1	0/1
Alouatta villosa	1/14 (.07)§	9/14 (.64)¶	0/14	0/13	3/5 (.60)
Carnivores:					
Nasua nasua	0/5	0/5	0/5	nt	0/5
Potos flavus	0/7	0/7	0/7	nt	0/7
Rodents:					
Agouti paca	0/2	0/2	0/2	nt	0/2
Dasyprocta punctata	0/8	0/8	4/8 (.50)§	1/8 (.13)§	3/5 (.60)
Sciurus granatensis	0/11	0/11	0/11	nt	0/11
Coendou rothschildii	0/1	0/1	0/1	nt	0/1
Proechimys semispinosus	0/22	0/22	0/22	nt	0/22
Sigmodon hispidus	0/13	0/13	0/13	nt	0/13
Rabbits:					
Sylvilagus brasiliensis	0/15	0/15	0/15	nt	0/15
Xenarthrans:					
Dasypus novemcinctus	0/4	0/4	0/4	nt	0/4
Cabassous centratus	0/1	0/1	0/1	nt	0/1
Tamandua tetradactyla	2/14 (.14)#	0/14	0/14	0/1	0/14
Choloepus hoffmanni	25/54 (.46)†	0/54	1/54 (.02)†	0/50	0/28
Bradypus infuscatus	9/58 (.16)	0/58	0/58	0/50	0/36

* Virus abbreviations: SLE, St. Louis encephalitis; YF, yellow fever; ILH, Ilheus; BSQ, Bussaquara; MAY, Mayaro.

† Number specifically positive/number tested (*frequency specifically positive*). Positive: 90% plaque reduction by plasma 1:16 or weaker. Neutralizing titers at least four-fold higher against one flavivirus than against the other three are defined as specifically positive against the flavivirus inhibited to highest titer. Positive flavivirus reactions are not tabulated unless they are within one two-fold dilution of the highest titer.

‡ nt: not tested.

§ One *A. villosa* positive at 1:64 against SLE was also positive at 1:128 against YF; one *D. punctata* positive at 1:128 against BSQ was also positive at 1:256 vs. ILH.

¶ One positive serum not tested against BSQ.

tive). Other positive xenarthrans include *Bradypus* sloths and *Tamandua* anteaters (16% and 14% positive, respectively). Although birds are generally considered to be the natural amplifying hosts of SLE virus in North America, only one tropical kingbird, *Tyrannus melancholicus*, was seropositive out of 75 birds of 39 species from Majé tested.

Specific antibody against SLE virus is widespread in Panamanian sloths. A survey of sloths from five areas of central Panamá showed that SLE virus infections are common in both sloths species in Aguacate, Chilibre and Cerro Azul, as well as Majé (Table 3, Fig. 1). Antibody against SLE virus was more prevalent in *Choloepus* than in *Bradypus* sloths in all positive localities.

Antibody against SLE virus is rare in infant and juvenile animals and common only in adults (Table 4). Only two seropositive young sloths of either

TABLE 3

*Frequency of specific neutralizing antibody against St. Louis encephalitis virus in sloths of all ages from five central Panamanian localities**

Species	Locality					
	Aguacate	Chilibre	Cerro Azul	El Llano Carti Road	Majé	Total
Bradypus variegatus	2/68 (.03)†	1/5 (.20)	1/27 (.04)	0/10	9/58 (.16)	13/168 (.08)
Choloepus hoffmanni	5/27 (.19)‡	5/8 (.63)	3/5 (.60)	0/3	25/54 (.46)§	38/97 (.40)

* Plaque reduction neutralization test; 90% reduction of 50–150 pfu by plasma 1:16 considered positive.
† Number positive/number tested (*frequency positive*). Unless otherwise indicated positive titers were always ≥4-fold higher against St. Louis encephalitis virus than against yellow fever, Ilheus, and Bussuquara viruses.
‡ Three plasmas positive against St. Louis encephalitis were not tested against Bussuquara virus.
§ One plasma positive against St. Louis encephalitis virus was not tested against Bussuquara virus.

species were found. One was a baby *Choloepus* from Aguacate which was attached to its seronegative mother. The other was a radio-marked infant *Choloepus* at Cerro Azul; it developed specific PRN antibody against SLE virus between 17 September 1974, when its plasma titer was <1:8, and 6 November, when the titer was 1:32. Its mother also showed a significant rise in SLE antibody titer during this period, from 1:8 to 1:32. Plasmas were tested in parallel and were uniformly negative against the other three flaviviruses. The baby was still attached to the mother, which was not lactating at either capture. No other seroconversions against SLE virus or any other flavivirus were observed in other radiomarked sloths or sentinel rhesus monkeys at Cerro Azul.

Ilheus virus

One of 54 *Choloepus* from Majé, 1/27 *Bradypus* from Cerro Azul, and 1/65 *Bradypus* from Aguacate were positive for PRN antibody against this virus and negative against other flavivirus test antigens, although the positive *Choloepus* plasma was not tested against BSQ virus (Table 2). Three of eight agoutis, *Dasyprocta punctata*, from Majé were specifically positive against ILH virus; and a fourth reacted to high titer against both ILH and BSQ viruses (Table 2).

TABLE 4

Frequency of specific neutralizing antibody against St. Louis encephalitis virus in sloths from Chilibre, Cerro Azul, Aguacate, and Majé, by age class

Species	Age class*		
	Adult	Juvenile	Infant
Bradypus variegatus	12/130 (.09)†	0/16	0/8
Choloepus hoffmanni	30/62 (.48)	0/10	2/9 (.22)

* Infant: still attached to mother; juvenile: independent but not fully grown.
† Number positive/number tested by plaque reduction neutralization test (*frequency positive*). Positive: 90% plaque reduction by plasma diluted at least 1:16. Titers against St. Louis encephalitis virus were always at least four-fold higher than against yellow fever, Ilheus, and Bussuquara viruses.

Bussuquara virus

The only plasmas positive against BSQ virus were also positive to higher titer against another flavivirus.

Mayaro virus

The only species collected at Majé with detectable neutralizing antibody against this virus were agoutis, *D. punctata*, and howler monkeys *A. villosa* (Table 2). The median positive titer was 1:128 (range 1:32–1:512). At Aguacate, none of 14 *Bradypus* or 15 *Choloepus* was positive against Mayaro virus.

DISCUSSION

Interpretation of these results is facilitated by the paucity of flavivirus cross-reactions. Positive tests were usually monospecific in all species except agoutis, and in the few cases of multiple positives, titers were almost always clearly higher against one particular flavivirus than against the other three. The rarity of heterologous flavivirus reactions in sloths experimentally infected with SLE virus[14] fits well with these field data.

Our results support the working hypothesis that New World sylvatic YF virus is a monkey virus. No evidence was found to suggest alternate hosts.

Prolonged non-fatal experimental YF viremias of high titer in *B. variegatus* have been uniformly followed by the development of neutralizing antibody,[11] but no neutralizing antibodies were detected in adult *Bradypus* from two areas recently affected by sylvatic YF. Therefore, it is logical to infer that if any *Bradypus* sloths were naturally infected, their viremias were not long nor of high titer, and that this species did not amplify the transmission of YF virus.

On the other hand, our results indicate a high prevalence of natural SLE virus infection in sloths of both species. The vertebrate hosts in tropical SLE cycles are not well understood. Previous isolations from tropical vertebrates include strains from birds, an opposum, armadillos, two rodent species and a three-toed sloth,[20] and from mites combed from a rice rat.[21] At Majé, specific antibody against SLE virus was found more frequently in sloths than in any other species or group of vertebrates tested. By itself, this observation suggests that sloth antibody is useful as the best index of the past occurrence of SLE virus in a given area. However, in combination with the demonstration of high prolonged SLE viremias in both sloth species,[11] the serological evidence of natural infection suggests that sloths are important natural hosts of SLE virus. Their remarkable abundance in tropical forests is little recognized but is epidemiologically important. Their density on Barro Colorado Island in Panamá has been estimated at 8.5 *Bradypus* and 1.1 *Choloepus* per hectare,[3] and our unpublished data using identical techniques at Cerro Azul indicate similar densities and proportions.

The lack of antibody against SLE virus in young animals may be interpreted as evidence that sloths are infected very infrequently, and gradually accumulate a high percentage of positives in a population only because of their long life span and slow population turnover. However, lack of antibody in young animals may also be interpreted as evidence that SLE virus had not been active in the areas studied for 2 years or more. Little is known of the epidemiology of Neotropical SLE virus, and it may follow the pattern of intermittent waves or wandering foci as classically seen for Neotropical YF virus.[22] In this case, dense sloths populations would be important sources of SLE virus. The recent isolation of SLE virus by Dr. F. P. Pinheiro from a Brazilian *Bradypus tridactylus* is interesting in this respect.[20]

The observations of antibody against ILH and MAY viruses in agoutis *D. punctata* are intriguing but difficult to interpret. Antibody against MAY virus in howler monkeys agrees with previous observations on the ecology of this virus.[23] The high titers and lack of double positives at a frequency higher than predicted by chance indicate that the results are specific. ILH virus has previously been isolated from birds in Panamá.[24]

A final intriguing point found in these field results is the apparently nearly simultaneous SLE virus infections of the radio-marked *Choloepus* mother-baby pair. Either they were both infected by mosquito(es), or one infected the other with SLE virus by contact or aerosol, as observed for a baby *Bradypus* and its experimentally infected mother.[14]

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