Evidence of Apeu Virus Infection in Wild Monkeys, Brazilian Amazon

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Abstract. Orthobunyaviruses are arboviruses in which at least 30 members are human pathogens. The members of group C orthobunyaviruses were first isolated in the Brazilian Amazon in 1950, since that time little information is accumulated about ecology and the medical impact of these virus groups in Brazil. Herein, we describe the evidence of Apeu virus (APEUV; an *Orthobunyavirus* member) infection in wild monkeys from the Brazilian Amazon forest. APEUV was detected by using a neutralizing antibody in serum and its RNA, suggesting past and acute infection of Amazonian monkeys by this virus. These results altogether represent an important contribution of orthobunyavirus ecology in the Amazon and an update about recent circulation and risk for humans with expansion of the cities to Amazon forest.

The *Orthobunyavirus* genus (*Bunyaviridae* family) comprises more than 170 species that are grouped into 18 serogroups.^{1,2} At least 30 orthobunyaviruses are related to human diseases that cause a range of clinical manifestations, including hemorrhagic fevers and infection of the central nervous system.^{2,3} Orthobunyaviruses are arboviruses, which are transmitted to vertebrates by blood-feeding arthropods.² In Brazil, orthobunyaviruses group C were first isolated in the 1950s from sentinel monkeys (*Cebus apella*) in Pará State, in the Brazilian Amazon.^{4,5} The characterization of these agents revealed new viruses belonging to orthobunyaviruses group C, including Caraparu complex viruses.^{4–6} Other Caraparu complex viruses have been isolated in the following decades in the Americas using rodents and monkeys as sentinels.^{4–6} Human infections have been described, including Caraparu virus (CARV) and Apeu virus (APEUV) infections, emphasizing the potential of these viruses as emerging agents.^{7,8} Since the discovery and reporting of these viruses decades ago, very little information concerning the circulation of APEUV in natural environments has been reported.² Herein, we describe serological and molecular evidence of the circulation of APEUV in wild monkey species found in the Brazilian Amazon. Our data raise questions regarding viral management and this virus's potential as a (re-) emergent agent.

In this study, we selected 173 serum samples collected from wild monkeys in the Amazon region of Brazil (Tocantins State) from 2001 to 2002, 49 samples from black-howler monkeys (*Alouatta caraya*), and 124 samples from capuchin monkeys (*C. apella*). Samples were collected in an overflow area of a fauna rescue program during the construction of a hydroelectric dam in Tocantins State (Supplemental Figure 1A). The samples were collected according to the guidelines of National Environment Council—CONAMA (6177-13-GL-830-RT-08027). After blood collection, the animals were released into areas selected by environmental conservation programs. Because of the low volume of serum, these samples were grouped into pools of 3–5 serum samples from animals of the same species, for a total of 37 pools (5–10 µL of each serum

sample). All monkey sera and virus samples were manipulated separately to avoid cross-contamination, and both serological and molecular tests were performed in duplicate.

To determine the presence of orthobunyaviruses neutralizing antibodies in the sera pools, we used a 70% plaque-reduction neutralization test (PRNT) for APEUV (ATCC-BeAn848). PRNT assays were performed in Vero cells seeded on 6-well plates and maintained in 2 mL minimal essential medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 2% fetal bovine serum and 1.5% carboxymethyl cellulose. After 72 hours, the cells were stained and fixed with crystal violet and formaldehyde, respectively, and viral plaques were counted. Positive samples were then tested for CARV and Itaquí virus (ITQV) for the detection of homotypic serologic responses with no indication of cross-reaction.^{7,9,10} Of the 37 pools analyzed using PRNT, 16 (43.2%) had neutralizing antibodies against APEUV; of these, nine (56.25%) had titers of 100 neutralizing units (NU)/mL, three (18.7%) had titers of 200 NU/mL, one (6.2%) had a titer of 400 NU/mL, and three (18.7%) had titers of 800 NU/mL (Table 1). When seropositivity was analyzed among the tested monkey species, we observed that three of the 10 pools from black howler monkeys (30.0%) and 13 of the 27 pools from capuchin monkeys (48.15%) were positive.

To analyze the neutralization capacity of sera against other orthobunyaviruses (cross-reactivity), 10 positive pools (PRNT assay with APEUV) were used in PRNT assay using CARV (ATCC BeAn 3994) and ITQV (BeAn 12797). Of 10 pools tested, four presented neutralizing antibodies against CARV (titer 100 NU/mL). Six APEUV-positive pools could not be tested for CARV and ITQV because of restriction of sera volume. Two of CARV-positive pools showed a titer of 400 NU/mL in PRNT against APEUV, which indicate either a level of cross reactivity, likely due to these viruses belonging to a same serogroup, or the exposure of the tested animals to both viruses. None of the sera positive for APEUV in PRNT assay was positive for ITQV (distinct serogroup, Oriboca virus complex) (Table 2), and no monotypic reactions were detected.

Considering the PRNT results and aiming to detect APEUV RNA in the monkey serum pools, as an indicative of a possible acute infection, RNA was extracted (QIAamp Extraction Kit; Qiagen, Venlo, Limburgo, The Netherlands), followed by reverse transcription using random primers (M-MLV Reverse Transcriptase; Promega, Madison, WI). The obtained complementary DNAs were used in a real-time polymerase chain reaction (PCR) assay targeting specifically the L region

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TABLE 1
APEUV diagnosis in wild monkeys, Brazil

Species	No. of serum samples tested	Total no. of pools	No. of positive pools, by level of NU/mL against APEUV (Caraparu serogroup)*					No. (%) of positive pools by real-time PCR
			100	200	400	800	Total (%)	
Black howler monkeys (<i>Alouatta caraya</i>)	49	10	2	0	0	1	3 (30)	1†
Capuchin monkeys (<i>Cebus apella</i>)	124	27	7	3	1	2	13 (48)	1†
Total	173	37	9	3	1	3	16 (43)	2 (5)

APEUV = Apeu virus; PCR = polymerase chain reaction.

*NU/mL (neutralizing units/mL), in a plaque-reduction neutralization test (PRNT₇₀) assay, is calculated as the inverse of the dilution of serum that reduces 70% of virus plaques normalized to the volume of 1 mL.

†Animal was also positive by plaque-reduction neutralization test.

of APEUV (5'-CTTCTATCGAGACCCACGGC-3'/5'-TGCC TCCTGTAAACAGACACTG-3'), amplifying a target fragment of 204 bp from L region. When in silico test was performed using GenBank database, no other orthobunyaviruses were detected in this reaction mix. The in vitro amplification of CARV and ITQV has failed using this reaction. PCRs were performed using the SYBr Green Mix (Applied Biosystems, Carlsbad, CA), and the real-time PCR quality and sensitivity parameters were adjusted, including the efficiency (93.2%) and R^2 (0.991). To verify the results of the PCR, products were directly sequenced in both orientations (ABI-3730-Analyzer; Applied Biosystems),¹¹ and the obtained sequences (Supplemental Figure 1B) were compared (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with other sequences deposited in the GenBank. Among the monkey sera pools analyzed using real-time PCR, two (5.40%) revealed the amplification of specific fragments (named uncharacterized bunyaviruses Amazon 1 and 2). The sequencing of the PCR products of these samples demonstrated that there was 92.7% identity between each other and high identity (96.4%) with the other available APEUV sequences deposited in GenBank (FJ859039). Alignment of the L region of some bunyaviruses revealed unique substitutions in Amazon 1 and 2 compared with the available APEUV-BeAn848 sequence (FJ859039) (Supplemental Figure 1B). Despite high identity among amplified fragment and APEUV available sequences, it is important to highlight that the possibility of reassortment should not be overlooked. Therefore, although our analysis suggested the identification of a new APEUV sequence, future studies may be needed in this field.

During the 1950s, several orthobunyaviruses were isolated in the Brazilian Amazon forest, but we have accumulated little

knowledge about the distribution and endogenous life cycle of these viruses in the largest tropical forest of the world.^{4-6,8,12,13}

In this work, we suggest the circulation of APEUV in wild monkeys captured in the Brazilian Amazon forest (Table 1). Our data not only indicate possible APEUV hosts but also raise questions regarding the potential reemergence of APEUV among human populations that access the forest. Anthropogenic disturbance of the Amazon ecosystem and increases in agricultural and livestock areas result in more contact between wildlife and rural human populations. Every year, several cases of viral fevers occur in the analyzed region but are not reported, similar to practices in other remote areas in the Brazilian Amazon.¹³ Active surveillance is a sine qua non for predicting orthobunyaviruses as emerging agents, but epidemiologic studies of orthobunyaviruses are limited because the members of this group present immunologic cross-reactivity and few nucleotide sequences are deposited.

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TABLE 2

Titer of positive pools in PRNT assay for APEUV, CARV, and ITQV

Species	Pools	Titers by level of NU/mL*		
		APEUV	CARV	ITQV
Black howler monkeys (<i>Alouatta caraya</i>)	AC-E	800	—	—
	AC-G	100	—	—
	AC-V	100	—	—
Capuchin monkeys (<i>Cebus apella</i>)	C7-D	400	100	—
	C7-E	200	100	—
	C7-P	100	—	—
	C7-Q	100	—	—
	C7-S	200	—	—
	C7-AD	100	100	—
	C7-AE	400	100	—

APEUV = Apeu virus; CARV = Caraparu virus; PRNT = plaque-reduction neutralization test; ITQV = Itaquí virus.

*NU/mL (neutralizing units/mL), in a PRNT₇₀ assay, is calculated as the inverse of the dilution of serum that reduces 70% of virus plaques normalized to the volume of 1 mL.

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