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Short Communication

Cacipacore virus as an emergent mosquito-borne *Flavivirus*

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Abstract

Introduction: *Cacipacore virus* (CPCV), a possible bird-associated flavivirus, has yet to be detected in mosquitoes. Our purpose is examining CPCV in mosquitoes from the Amazon region of Brazil. **Methods:** Approximately 3,253 *Culicidae* (grouped into 264 pools) were collected from the Amazon region during 2002-2006 and analyzed using a *Flavivirus* genus-specific reverse transcription- polymerase chain reaction followed by nested polymerase chain reaction assay and by nucleotide sequencing of amplicons. **Results:** Nucleotide sequences from five mosquito samples showed high similarity to the those of CPCV originally isolated in the Amazon region. **Conclusions:** This is the first report of CPCV-infected mosquitoes which has implications on the arbovirus maintenance in nature and transmission to man.

Keywords: *Cacipacore virus*. Mosquitoes. Emerging *Flavivirus*.

Cacipacore virus (CPCV) is a member of the *Japanese encephalitis virus* (JEV) complex belonging to the genus *Flavivirus* of the family *Flaviviridae*[1]. It was originally isolated in 1977, from the blood of a bird (*Percnostola rufifrons*) in the Amazon region of Brazil[2]. Recently, antibodies against CPCV have been reported in equines, non-human primates, and water buffalo in the central region of Brazil, suggesting virus circulation[3-5]. In addition, one patient from the Amazon region exhibiting an acute febrile illness was found to be infected by CPCV in 2011[6]. Here, we present the first report of CPCV-infected mosquitoes collected from the environment.

As part of the arbovirus surveillance programs (2002 to 2006), approximately 3,253 mosquitoes (*Culicidae*) were collected from urban and rural areas in Manaus (Amazonas State) and Monte Negro County (Rondonia State), using attraction traps including the CO_2, the cluster of differentiation (CD4) night model, and the Shannon. Captured specimens were identified based on morphological characteristics and grouped into 264 pools (including 10-13 specimens per pool) and stored at −80 °C[7].

Reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing for arbovirus diagnosis and identification were performed using ribonucleic acid (RNA) extracts from the mosquito pools while taking precautions to avoid contamination. The mosquito samples were triturated at −4°C temperatures using plastic pestles and divided into two aliquots: one for RNA extraction and the other for future virus isolation. Aliquots were stored at −80°C until ready for use. RNA was extracted from the mosquito pools, using the PureLink Viral ribonucleic acid/deoxyribonucleic acid (RNA/DNA) Kit (Invitrogen, USA) according to the manufacturer's instructions. Subsequently, RT and Hemi-Nested-PCR *Flavivirus* assays that differentiate dengue, yellow fever, and other viruses based on amplicon size, were performed as previously reported[8].

The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany), following the manufacturer's protocol and subsequently sequenced with the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences obtained in this study were submitted to GenBank under access numbers GU811223 to GU811230, as follows: CPCVBR/RO/*Culex sp* 54C/2002

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- GU811223; CPCVBR/RO/*Anopheles sp* 43A5/2002 - GU811224; CPCVBR/AM/*Aedes aegypti* 17/2005 - GU811225; CPCVBR/AM/*Aedes aegypti* 46/2005 - GU811226; and CPCVBR/AM/*Aedes aegypti* 47/2005 - GU811227. CPCV and others flavivirus sequences retrieved from GenBank were aligned using the BioEdit v 7.09 program[9]. Phylogenetic trees based on Neighbor-joining (NJ) methods were constructed using the Mega 5 software[10].

Captured specimens were identified based on morphological characteristics as *Aedes aegypti*, *Aedes albopictus*, *Anopheles* sp., *Culex* sp., *Coquillettidia* sp., *Haemagogus janthinomys*, *Haemagogus leucocelaenus*, *Haemagogus spegazzinii*, *Psorophor albigenus*, *Psorophora albipes*, and *Psorophora ferox*[7], as shown in **Table 1**.

For virus detection, we used a Hemi-Nested-PCR that did not include a specific primer for *Cacipacore virus*. Interestingly, CPCV amplicons were obtained using the primer for *dengue virus* (DENV) type 2 and the CACV specific origin of the amplicons was only recognized following nucleotide sequencing[8]. Amplicons ~200-300bp in size, corresponding to a section of the NS5 gene region of *Flavivirus* were obtained from five mosquito pools (1.89% positivity). Following further amplification and purification, it was possible to sequence the amplicons. The pools infected by *Flavivirus* included *Aedes aegypti*, *Anopheles* sp., and *Culex* sp., as shown in **Table 2**.

The sequences, ranging from 216 to 275 nucleotides, were aligned with other sequences retrieved from GenBank. The sequences exhibited 98-100% similarity with those of the CPCV originally isolated in 1977 from a bird[1]. The *Flavivirus* phylogenetic tree, shown in **Figure 1**, includes sequences of the viruses infecting our pools of mosquitoes, all of which cluster within the same CPCV branch, confirming this virus is the causative agent of the infections. The tree also shows that CPCV is related to *Japanese encephalitis virus*, corroborating a previous report by Kuno et al.[1] also based on the NS5 gene sequence. Unfortunately, despite numerous attempts that exhausted our samples, we were unable to obtain larger nucleotide sequences from our mosquito pools.

Japanese encephalitis virus (JEV), *Ilhéus virus* (ILHV), *Rocio virus* (ROCV), *Saint Louis encephalitis virus* (SLEV), and *West Nile virus* (WNV) are all zoonotic avian viruses transmitted by *Culicidae* mosquitoes. Thus, it is possible that CPCV, a closely related virus, shares the same zoonotic characteristics because it was originally isolated from a bird.

TABLE 1

Number of *Culicidea* (Diptera) collected for the study from 2002 to 2006.

Genus/species	Number of adults	Male specimens	Female specimens	Number of pools
Anopheles sp.	81	-	81	7
Culex sp.	867	140	727	75
Coquillettidia sp.	252	50	202	19
Aedes aegypti	950	45	905	78
Aedes albopictus	244	10	234	18
Psorophora albipes	97	-	97	8
Psorophora albigenus	55	-	55	5
Psorophora ferox	60	-	60	6
Haemagogus jantinomys	126	10	116	10
Haemagogus leucocelaenus	271	1	270	20
Haemagogus spegazzinii	250	241	9	18
Total	**3,253**	**497**	**2,756**	**264**

Aedes aegypti, an urban anthropophilic mosquito that lives around human houses, is the main vector for DENV in Brazil and has been involved in huge outbreaks over the last 30 years, with more than 10 million reported cases. Over the last three years, *Aedes aegypti* has been involved in the transmission of *Chikungunya virus* (CHIKV) and *Zika virus* (ZIKV), which have also resulted in large outbreaks and thousands of infected cases. In the present study, CPCV was detected in pools of *Aedes aegypti* collected in Manaus, a city of two million inhabitants located at the center of the Amazon rain forest. Therefore, it is possible that sylvatic CPCV could have been introduced into this city and infected *Aedes aegypti*; CPCV could have been transmitted to humans through this mosquito vector, but remained unnoticed amidst the widespread outbreaks of dengue and other tropical febrile diseases. It is important to note that

TABLE 2

Flavivirus amplicons obtained by reverse transcription- polymerase chain reaction followed by nested polymerase chain reaction assays for mosquitoes collected in the Brazilian Amazon region, 2002-2006.

Gender/species	Number of detected pools	Amplicon size (bp)	Collection places/State	Date of collection
Aedes aegypti	3 (33 females)	~ 232	Manaus City/Amazonas State	2005-2006
Anopheles sp.	1 (9 females)	~ 216	Montenegro County/Rondonia State	2002
Culex sp.	1 (8 females)	~ 275	Montenegro County/Rondonia State	2002

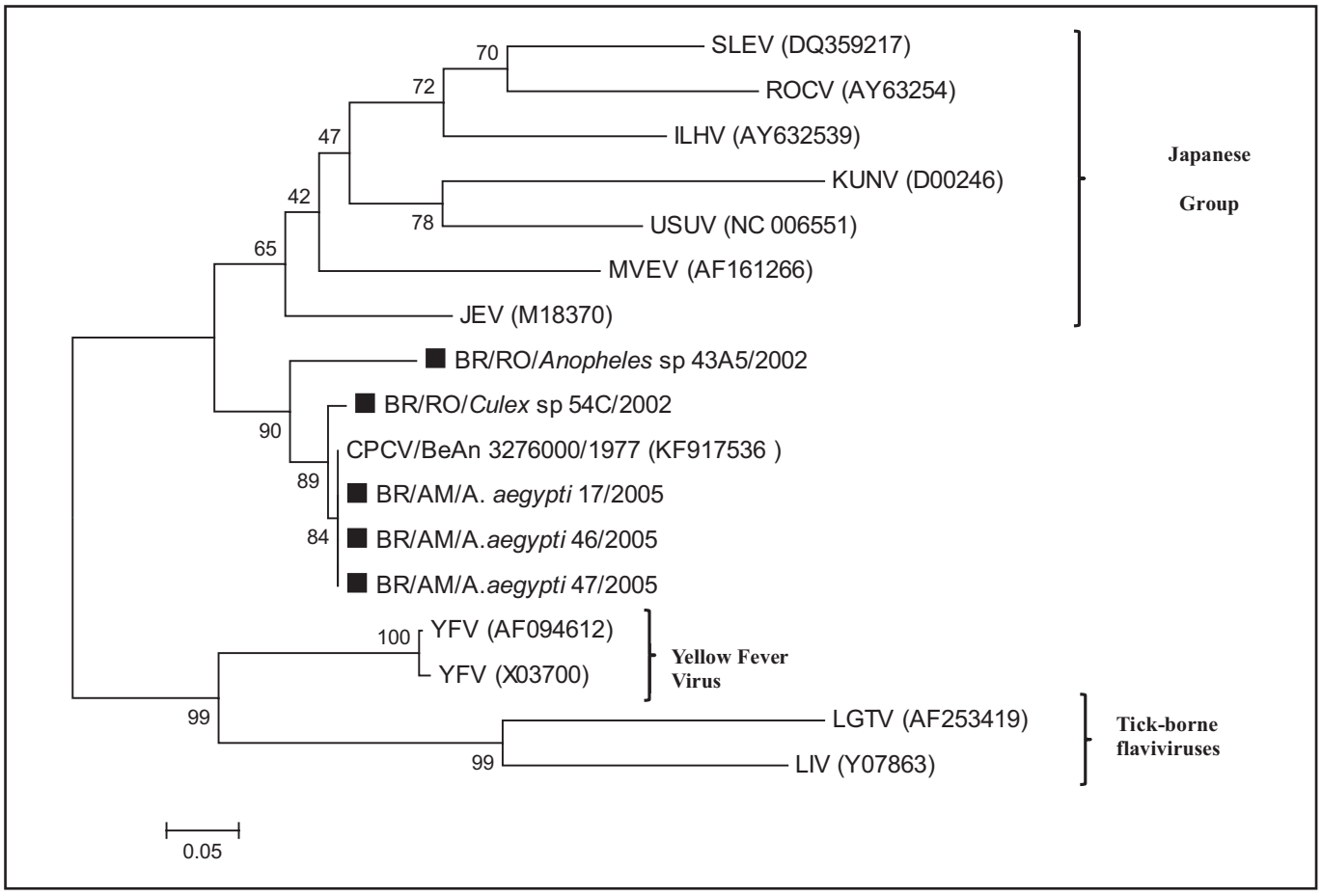

FIGURE 1 - Phylogenetic tree based on NS5 partial gene sequences. The tree was constructed using the Neighbor-joining method with 1,000 bootstrap replications. Branch lengths are proportional to the percentage of divergence. The Tamura-Nei nucleotide substitution model was used with a gamma distribution (shape parameter = 1). GenBank accession numbers, species, country of origin, and year of isolation are detailed in the tree. CPCVs isolated from mosquito samples are indicated with a square (■). **SLEV:** Japanese encephalitis; **ROCV:** Rocio virus; **ILHV:** lhéus virus; **KUNV:** Kunjin virus; **USUV:** Usutu virus; **MVEV:** Murray Valley encephalitis virus; **JEV:** Japanese encephalitis; **BR/RO:** Brazil/Rondonia; **CPCV:** Cacipacoré virus; **BR/AM/A:** Brazil/Amazonas/*aedes*; **YFV:** yellow fever virus;. **LGTV:** Langat virus; **LIV:** Louping ill virus .

serologic diagnostic tests for dengue could be positive for flavivirus cross-reactivity in patients with CPCV.

Culex pipiens are found worldwide, breeding in water contaminated with organic matter. The conditions in poorly sanitized urban areas of Brazil support the propagation of this mosquito. *Culex pipiens* feed at night and are zoophilic; they are particularly more ornithophilic. Alphaviruses such as *Western Equine Encephalitis* (WEEV), *Venezuelan Equine Encephalitis* (VEEV), and *Eastern Equine Encephalitis* (EEEV); Orthobunyaviruses such as *Caraparu virus* and *Oropouche virus*; and Flaviviruses, such as SLEV, have been isolated from *Culex* species in different regions of Brazil and Argentina. SLEV has also been isolated from birds, including migratory species, as well as rodents[2,11-13]. Thus, because CPCV has been found miles apart, it is conceivable that the virus has a natural cycle involving *Culex* species as vectors and birds, including those with migratory habits, as reservoirs.

Anopheles mosquitoes, especially *Anopheles darlingi*, are important vectors of parasites of the genus *Plasmodium* in Brazil, which cause malaria. These mosquitoes have also been shown to be infected with alphaviruses, (VEEV), orthobunyaviruses (*Guaroa virus* and *Tacaiuma virus*), and the flavivirus SLEV[2,11-13]. Thus, it is possible that *Anopheles* mosquitoes could also transmit CPCV. As observed in the phylogenetic analysis, CPCV is closely related to JEV, which has also been reported in *Anopheles* and *Aedes* spp. However, further studies are necessary to check vector competence of these mosquitoes and the risk of CPCV emergence producing outbreaks[14].

The report of one patient from the Amazon region with an acute febrile illness caused by CPCV could resemble reports warning of ZIKV infections (only 13 reported human cases in six decades)[15]. However, over the past few years, ZIKV has emerged as an important pathogen causing worldwide epidemics associated with *Aedes* mosquitoes, especially in Brazil. Thus, CPCV may represent an important public health threat similar to ZIKV.

Cacipacore virus is a zoonotic flavivirus that infects different species of mosquitoes, including the urban anthropophilic *Aedes aegypti*, which possesses great potential to emerge in urban Brazilian areas and cause human disease. Thus, further studies are required to confirm the importance of CPCV and its

vectors. This study, using molecular biology technique, shows Aedes aegypti, Anopheles sp and Culex sp mosquitoes from the Amazon region can be infected by Cacipacoré virus, a Flavivirus until recently obscure but recently reported as causative of human acute febrile illness. These findings highlight a potential vectorial condition for emergence of this Flavivirus as a public health problem in Brazil.

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Conflict of interest

The authors declare that no competing interests exist.

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