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Characterization of two new rhabdoviruses isolated from midges (*Culicoides* SPP) in the Brazilian Amazon: proposed members of a new genus, Bracorhabdovirus

Brief Report

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Summary. Itacaiunas and Curionopolis viruses were isolated from *Culicoides* midges in Parauapebas municipality, Pará state, Brazil, in 1984 and 1985, respectively. Itacaiunas virus infected newborn mice and mosquito cells (C6/36), but did not replicate in some mammalian cell lineages; while Curionopolis virus infected only mice. Neither virus showed a serological relationship with any of the 195 known arboviruses circulating in Brazil, nor against 38 other rhabdoviruses isolated worldwide. Both virus particles are bullet-shaped and similar in morphology to that observed for other members of the family *Rhabdoviridae*. Partial nucleotide sequencing of the N protein showed that those two viruses constitute a separate clade in the family *Rhabdoviridae*, which we propose to be a new genus, designated Bracorhabdovirus.

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The Amazon region has a tremendous biological diversity, and is one of the richest sources of arboviruses in the world [4]. Many arboviruses have been identified in this ecosystem, some of which are important public health agents, such as Oropouche virus (OROV), Mayaro virus, and yellow fever virus [3, 21]. In March 1984, during ecologic studies performed in Serra Norte, municipality

of Parauapebas (50°15′W; 6°8′N), Pará state, Brazil, Itacaiunas virus (BE AR 427036) was isolated from a pool of *Culicoides* sp midges. In the next year, on March 12, Curionopolis virus (BE AR 440009) was also isolated from a pool of *Culicoides* sp midges [19].

Culicoides midges are members of the family *Ceratopogonidae*, which includes over 1400 species. About 50 species have been implicated in the transmission of human and animal pathogens [13]. According to Meiswinkel et al. [12], more than 50 arboviruses have been isolated from *Culicoides*; most of which are members of the families *Bunyaviridae* (20 viruses), *Reoviridae* (19 viruses), and *Rhabdoviridae* (11 viruses). In order to classify Curionopolis and Itacaiunas viruses in a taxon, we used serology, transmission electron microscopy, and nucleotide sequencing techniques. Our results indicate that Curionopolis and Itacaiunas viruses constitute a new genus in the family *Rhabdoviridae*, which we propose to be called Bracorhabdovirus for Brazilian Amazonian *Culicoides* rhabdoviruses.

The suckling Swiss mice used in our studies were obtained from the animal care facility at Instituto Evandro Chagas (IEC) and maintained in standard mouse cages. All procedures using animals were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, 2nd Edition (2002) and Brazilian laws, and performed in biosafety level 2 facilities, and were approved by the ethics committee of the IEC.

Newborn mice (2–3 days old) were intracerebrally inoculated with 0.02 ml of a viral suspension containing homogenized infected mouse brains diluted 1:10 (v/v) in phosphate-buffered saline (PBS) containing 0.75% bovine albumin and antibiotics, and cell cultures were infected using 1 ml of a 1:100 dilution [1]. Virus titrations were carried out by inoculation of newborn mice with 0.02 ml of serial 10-fold dilutions of the viral suspensions in PBS, and LD_{50} values were calculated by the method of Reed and Muench [15]. Newborn mice infected with Curionopolis virus became sick and died within 3–4 days post infection (dpi), while Itacaiunas virus killed newborn mice on 4–5 dpi. Titers for Itacaiunas and Curionopolis viruses were 4.7 $\log_{10}$ and 5.6 $\log_{10}$, respectively.

Vero (African green monkey kidney), RD (human rhabdomyosarcoma), and Hep-2 (human larynx epidermoid carcinoma) cells were cultivated according to the technique described by Lennette [11], and C6/36 cells were maintained as described by Beaty and colleagues [1]. Inoculations of Curionopolis and Itacaiunas viruses into Vero, RD, Hep-2, and C6/36 cell cultures did not produce a cytophatic effect (CPE).

Hyperimmune mouse sera (HMS) against Itacaiunas and Curionopolis were prepared in adult (4–6 weeks old) Swiss mice as described elsewhere [2]. These HMS were tested against their respective antigens by complement fixation (CF), indirect immunofluorescence assay (IFA), and mouse neutralization test (MNT), and showed a specific positive reaction.

CF tests were performed according to the modified microtechnique described by Fulton and Dumbell [6]. Itacaiunas and Curionopolis antigens were initially tested by CF against HMS prepared for 195 different arboviruses and subsequently for 38 rhabdoviruses (Table 1).

Table 1. Rhabdovirus antigens and HMS used and with negative results in CF tests for the characterization of Curionopolis and Itacaiunas viruses

Family	Genus	Antigenic group	Virus
Rhabdoviridae	*Vesiculovirus*	VSV group	*Carajas* *Chandipura* *Cocal* *Isfahan* *Maraba* *Piry* *Vesicular stomatitis Alagoas* *Vesicular stomatitis Indiana* *Vesicular stomatitis New Jersey*
	Tentative species in the genus *Vesiculovirus*	–	Bote Calchaqui Gray Lodge Jurona Klamath Kwatta La Joya Malpais Spring Mt Elgon bat Perinet Porton Radi Yug Bogdanovac
	Lyssavirus	– –	*Mokola* *Rabies*
	Unassigned member in the family	Bahia Grande group	Bahia Grande
		Hart Park group	Hart Park Flanders Kamese Mosqueiro
		Kern Canyon group	Kern Canyon Nkolbisson
		Le Dantec group	Keuraliba Le Dantec
		Sawgrass group	Sawgrass New Minto
		ungrouped	Inhangapi

Using the CF test, cross-reactivity was not observed between Itacaiunas and Curionopolis viruses or between them and other arboviruses tested. Reactions were only observed against their specific HMS, with a CF titer for Curionopolis virus of 64/128 and for Itacaiunas virus of 512/64 (Reciprocal of the highest dilution of antibody/highest dilution of antigen).

IFAs were performed on all types of cell cultures used in this work, following the protocol described by Gubler et al. [7]. Curionopolis antigen was not detected by IFA in either mammalian or mosquito cells; however, antigen for Itacaiunas virus could be detected, but only in C6/36 cell cultures. Cross IFA using Curionopolis and Itacaiunas antigens and HMS showed that these viruses only reacted with their respective antiserum.

MNT was made by intracerebral inoculation of newborn mice, following the technique reported by Casals [2]. Itacaiunas and Curionopolis viruses were tested against 15 serum samples collected from wild animals (one carnivore, two birds, two rodents, four marsupials, and six non-human primates of the New World) that were captured in the same place and same year as the infected *Culicoides* were collected. LD_{50} values were also calculated as described by Reed and Muench [15]. Neutralizing antibodies for Curionopolis virus were found in serum samples of two animals, one monkey (*Cebus apella*; $LD_{50} \geq 3.3$ $\log_{10}$) and one coati (*Nasua nasua*; $LD_{50} \geq 3.6$ $\log_{10}$). However, none of the animal sera demonstrated a positive reaction by MNT to Itacaiunas virus. By cross MNT, Curionopolis and Itacaiunas viruses reacted only against their specific homologous antisera.

A test for sensitivity to deoxycholic acid (DCA) was used to determine the virus sensitivity or resistance of these viruses to DCA, a lipid solvent. DCA tests, performed as described elsewhere [18, 1], demonstrated that both viruses were sensitive to the DCA action. When Itacaiunas and Curionopolis virus were treated with DCA, both viruses showed a titer of 0.6 $\log_{10}$, while for controls, without DCA treatment, the titers were 3.4 $\log_{10}$ and 3.7 $\log_{10}$, respectively.

Mouse brains infected with Curionopolis and Itacaiunas viruses were processed to obtain ultrathin cuts for visualization with a Zeiss EM 900 transmission electron microscope [8]. Ultrastructural analysis of infected mice brains with both Itacaiunas and Curionopolis viruses showed "bullet-shaped" virions budding from the plasma membrane of central nervous system cells toward the extracellular space. The virions were approximately 180 nm in length and 80 nm in diameter (Fig. 1). In some sections, viral particles were also seen budding and at the same time being engulfed by the neighbor cells (Fig. 1b and d).

For molecular analysis, viral RNA was extracted from fluid of a suspension containing homogenized infected mouse brains using the QIAmp® Viral RNA mini kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Amplification of the partial nucleocapsid (N) genes of Itacaiunas and Curionopolis viruses was made by a standard 2-step RT-PCR protocol [16] using the reverse degenerate primer Rab NR (GTCARTTGYCCCCAGAARTG) for the RT step and a combination of the Rab NR and the forward degenerate primer Rab NF (CCIGMAATGARGAYCCWGT) during the PCR step. Primers were designed

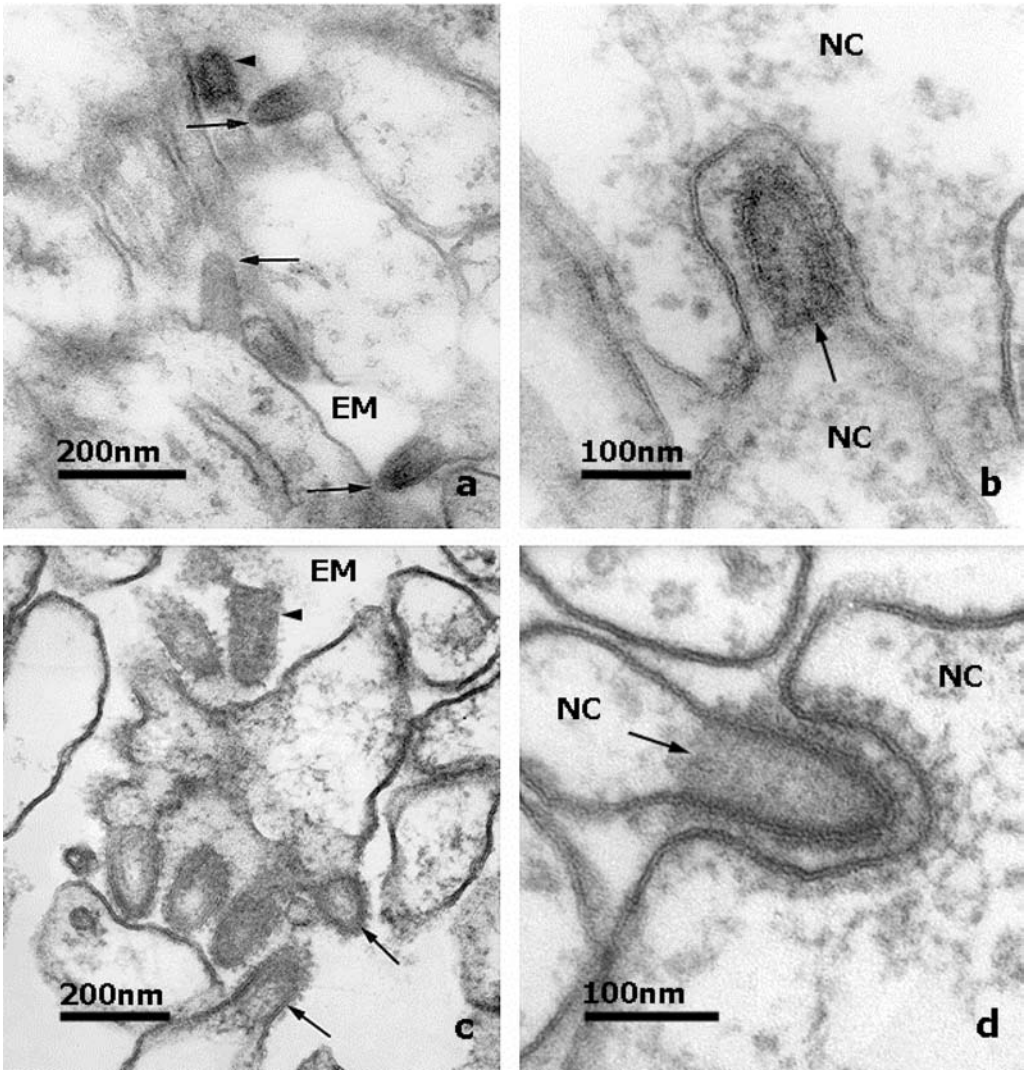

Fig. 1. Electron micrographs of Curionopolis virus (**a**, and **b**) and Itacaiunas virus (**c**, and **d**) showing their bullet-shaped morphology in the extracellular matrix (arrowheads) and budding from the plasma membrane (arrows) of suckling mouse central nervous system cells. At high magnification, Curionopolis virus (**b**) and Itacaiunas virus (**d**) budding from nervous cells and at the same time being engulfed by the neighbor cell (arrows). *NC* central nervous system cells, extracellular matrix (*MC*)

based on the most conserved region of the vesiculoviruses N gene. The amplified products were visualized on a 1.2% agarose gel, purified using the Quiack gel extraction kit (Qiagen, Valencia, CA), cloned into a plasmidial-bacterial system (PGMT-Easy Vector® system; Promega, Madison, USA), and sequenced in a automated sequencer ABI 377 (Applied Biosystems) using the ABI PRISM Dye Terminator 3.0 kit (Applied Byosystems, Foster City, CA). Universal T7 and SP6 primers were used to sequence the recombinant DNA in both directions. At least three plasmid clones were sequenced for each viral amplicon.

Table 2. Genetic relationship among N gene nucleotide and deduced amino acid sequences of Curionopolis and Itacaiunas viruses and selected vesiculoviruses

Viruses	Itacaiunas	Curionopolis	VSV indiana	Isfahan	Chandipura
Itacaiunas	***	**50.2**	**43.2**	**29.6**	**28.9**
Curionopolis	*52.2*	***	**34.1**	**27.9**	**24.6**
VSV idiana	*43.8*	*34.5*	***	**52.0**	**53.7**
Isfahan	*29.9*	*28.1*	*53.3*	***	**55.9**
Chandipura	*29.1*	*24.9*	*54.8*	*56.3*	***

Bold numbers: nucleotide sequence identity; Italicized numbers: amino-acid identity

Partial sequences of the N genes of Curionopolis (537 nt) and Itacaiunas (544 nt) viruses were obtained. The sequences showed low similarity (between 24.6 and 43.2%) to partial nucleotide sequences of the N genes of selected representative members of the genus *Vesiculovirus* (VSV Indiana virus, Isfahan virus, and Chandipura virus) and corresponded to the homologous nucleotide position from the nucleotide 895–1439. The N gene partial nucleotide sequence comparisons between Curionopolis and Itacaiunas viruses and selected vesiculoviruses are summarized in Table 2.

To establish phylogenetic relationships among Itacaiunas and Curionopolis viruses and other selected rhabdoviruses, phylogenetic trees were constructed using the neighbor-joining (NJ) method (17) implemented in the Mega 2.1 package [10]. The N gene partial nucleotide sequences determined for the Curionopolis and Itacaiunas viruses were aligned with homologous sequences of representative members of the genera *Vesiculovirus*, *Ephemerovirus*, *Lyssavirus*, *Novirhabdovirus*, *Nucleorhabdovirus*, and *Cytorhabdovirus*. For NJ analysis, a distance matrix was calculated from the aligned sequences in accordance with the Kimura two-parameter formula [9]. Bootstrap analyses using 2000 replicates were used to place confidence values on groupings [5].

Although these two Brazilian rhabdoviruses appear to be more related to members of the genus *Vesiculovirus* and to the Tupaia rhabdovirus, an unassigned virus in the family *Rhabdoviridae*, all trees generated from the N gene partial sequences showed that Itacaiunas and Curionopolis viruses grouped together (bootstrap value of 99%), and depicted them as a monophyletic group sharing a common ancestor (Fig. 2).

The isolation of the Itacaiunas and Curionopolis viruses from *Culicoides* midges demonstrates the importance of programs aimed at the isolation and characterization of new viruses. The fact that these viruses were isolated from midges is interesting, since *Culicoides* are the principal vectors of OROV, which is the etiologic agent of Oropouche fever, the second most important arboviral disease after dengue occurring in the Amazon [14]. Interestingly, serologic evidence of Curionopolis virus infection was found in a carnivore (*Nasua nasua*) and a non-human primate (*Cebus apella*), suggesting that this virus is a possible pathogen of mammals. Although Itacaiunas and Curionopolis viruses have not yet been

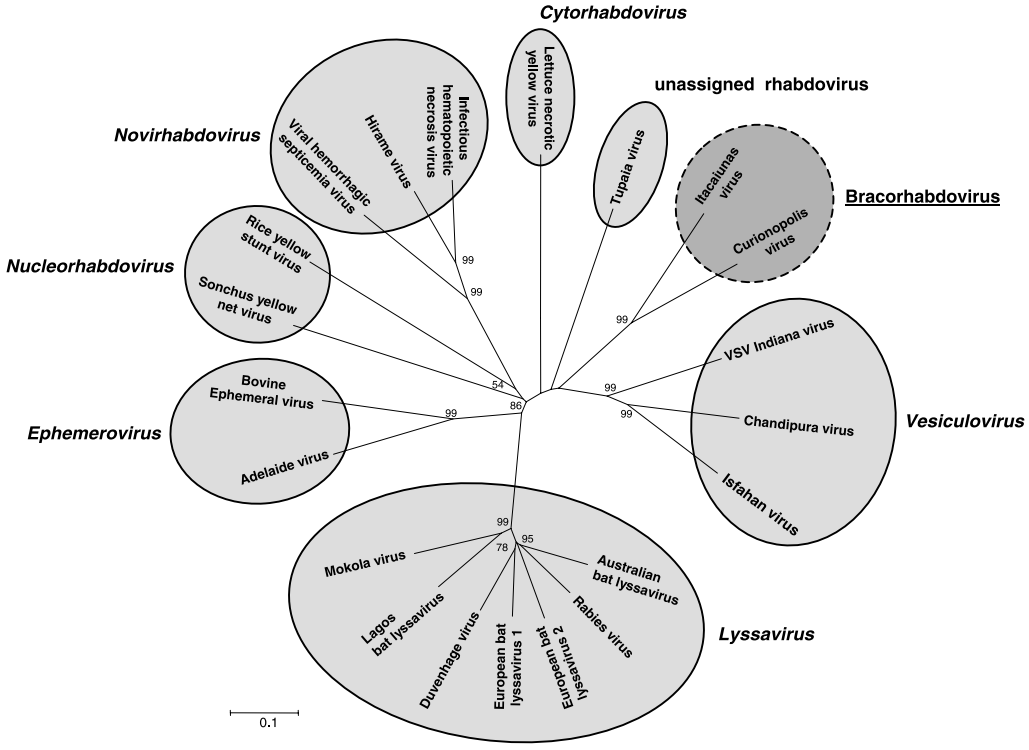

Fig. 2. Phylogenetic comparison of Itacauinas and Curionopolis virus in the proposed genus Bracorhabdovirus, not yet approved by ICTV and other selected rhabdoviruses, based on their N gene partial nucleotide sequences using the NJ method. Numbers adjacent to each branch represents the percentage bootstrap support calculated for 2000 replicates. Scale bar represent 10% nucleotide sequence divergence

associated with human illness, surveillance of febrile disease among people living in the Carajas area, as well as additional ecological studies should be done in order to acquire information on ecological and epidemiological aspects, including host range of these new viruses. Although serological tests have not revealed a viral taxon, ultrastructural analysis of infected mouse brain demonstrated the presence of bullet-shaped virions similar to those described for other members of the family *Rhabdoviridae* [20, 22], giving an important indication of the taxonomic status of these viruses.

Collectively, the isolation from arthropods, the experimental animal infection, and serologic and ultrastructural studies indicate that Curionopolis virus is a new rhabdovirus and a probable arbovirus transmitted by *Culicoides* sp to mammals, while Itacaiunas virus is a new rhabdovirus and a possible arbovirus isolated from *Culicoides* sp.

By nucleotide sequencing and phylogenetic analysis, a proximal relationship could not be observed among these viruses and others from different genera of the family *Rhabdoviridae* (Fig. 2). Since these two viruses were included in a same genetic clade, separated from representative genera of this family, we propose

that Curionopolis and Itacaiunas viruses should be provisionally considered as forming a new genus in the family *Rhabdoviridae*, which we suggest to be called Bracorhabdovirus, for Brazilian Amazonian *Culicoides* rhabdoviruses.

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