ARAGUARI VIRUS, A NEW MEMBER OF THE FAMILY ORTHOMYXOVIRIDAE: SEROLOGIC, ULTRASTRUCTURAL, AND MOLECULAR CHARACTERIZATION

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Abstract. This paper reports the results of serologic, structural, biochemical, and genetic studies indicating that Araguari virus, a previously unassigned viral agent, is a member of the family Orthomyxoviridae and genus Thogotovirus. Araguari virus has six RNA fragments; biologically, it shares several properties with other viruses in the family Orthomyxoviridae. Nucleotide sequencing of the RNA segments 4 (glycoprotein) and 5 (nucleoprotein) of Araguari virus aligned with the orthomyxoviruses, showing the closest relationship with Thogoto virus (sequence similarity = 61.9% and 69.1%, respectively, for glycoprotein and nucleoprotein), but also sharing a more distant similarity with Dhori and Influenza C viruses, especially for the glycoprotein gene. Based on these results, we propose that Araguari virus should be assigned as a new member of the family Orthomyxoviridae and genus Thogotovirus.

INTRODUCTION
Araguari virus strain BE AN 174214 was originally isolated from a homogenate of pooled viscera (heart, liver, kidney, and spleen) from a marsupial, *Philander opossum*, captured on September 11, 1969, in Serra do Navio, Amapá State, Brazil (1°N, 52°W). This virus was first listed as an ungrouped, unclassified, and possible arbovirus.1 Zeller and others proposed that Araguari virus be included as a member of the family Arenaviridae. Araguari virus was subsequently listed as an unassigned virus.3 To clarify the taxonomic status of Araguari virus, we conducted a series of studies and report their findings.

MATERIALS AND METHODS

Virus strain. Araguari virus prototype strain BE AN 174214 was obtained from the arbovirus collection of the Department of Arbovirology and Hemorrhagic Fevers of the Instituto Evandro Chagas (Belém, Brazil). It had had 25 previous passages in baby mice.

Complement fixation (CF) and hemagglutination inhibition (HI) tests. Cross CF and HI tests were used to demonstrate cross-reactions among Araguari and other selected orthomyxoviruses and conducted as previously described.4,5 For both serologic tests, titers were expressed as the highest dilution showing reactivity. For CF tests, titers ≥1:8 were considered positive, while for HI tests, titers ≥1:10 against four units of Araguari virus antigen were considered positive.

Cell culture. Virus was propagated in 25-cm² flask cultures of Vero cells in medium 199 (Sigma, St. Louis, MO) supplemented with 1% HEPES, 200 mM L-glutamine, 1% non-essential amino acids, 10% fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL), and amphotericin B (3 µg/mL). Cultures were maintained at 37°C in an incubator with atmosphere of 5% CO₂. After virus inoculation, cultures were incubated for 60 minutes at room temperature for adsorption. Afterwards, 10 mL of medium 199 supplemented with 2% FBS was added and incubated at 37°C. When cell cultures showed a cytopathic effect (CPE) of approximately 75%, they were harvested and supernatants were stored at −70°C.4

Transmission electron microscopy (TEM) and negative staining (NS). Two drops of supernatant from infected Vero cells were put over 400 mesh grids and covered with a formvar plastic membrane, and one drop of 2% phosphotungstic acid, pH 7.2, was added over the grid and incubated for 40 seconds. Afterwards, grids were examined by TEM and NS techniques with an electronic microscopy (Zeiss, Oberkochen, Germany).6

Immunoelectron microscopy. This technique was conducted as previously reported.7 Briefly, infected Vero cells showing a CPE of 75% and uninfected cells were harvested; Nine hundred microliters of each supernatant and 100 µL of specific mouse immune ascitic fluid (MIAF) for Araguari virus were diluted in distilled water (1:25) and mixed in 1.5-mL microtubes to obtain a final dilution of 1:250. The mixtures were incubated for 16 hours at 4°C. Subsequently, they were centrifuged at 12,000 rpm for 20 minutes at 4°C and the supernatants were discarded. The precipitates were resuspended in distilled water, and then placed on 400 mesh grids for examination by the NS technique.

Ultra-thin sections. Infected and uninfected Vero cells cultured after 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 60, and 72 hours were examined following the procedures described elsewhere.9–11 Ultra-thin sections were then obtained using an ultra-microtome (Zeiss) and placed on 300–400 mesh copper grids. Sectioned samples were stained and examined by the TEM technique as previously described.6

Ultrastructural cytochemical analysis. Infected and uninfected Vero cells were processed as described by Bullock and Petrusz.12 Briefly, cells were fixed in a solution of 0.1 M SCB, pH 7.2, 4% formaldehyde, 0.3% glutaraldehyde, and 1% picric acid for 30 minutes at 4°C and washed. Samples were then stained for one hour in 2.5% uranyl acetate and 25% methanol diluted in water at 4°C, followed by sequential ethanol dehydration incubations at 30%, 50%, 70%, and 90%, respectively. Infiltration was carried out in different mixtures (2:1, 1:1 and 1:2) of ethanol-lowicryl K4M (L-K4M) hydrophilic resin solution and absolute L-K4M resin. Samples were then embedded in absolute. After complete polymerization,
TABLE 1
Complement fixation tests between Araguari virus and other selected thogotoviruses

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Antibody</th>
<th>Thogoto</th>
<th>Dhori</th>
<th>Araguari</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thogoto</td>
<td>32/≥8*</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dhori</td>
<td>0</td>
<td>64/≥4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Araguari</td>
<td>0</td>
<td>0</td>
<td>32/≥8</td>
<td></td>
</tr>
<tr>
<td>Batken†</td>
<td>–</td>
<td>64/≥8</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* Reciprocal of antisera titer/reciprocal of antigen titer, 0 = <1:8.
† Batken has been shown to be a variant of Dhori virus.

TABLE 2
Results obtained by hemagglutination-inhibition tests between Araguari virus and other thogotoviruses

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Thogoto</th>
<th>Dhori</th>
<th>Araguari</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thogoto</td>
<td>≥1:1280</td>
<td>1:80</td>
<td>1:10</td>
</tr>
<tr>
<td>Dhori</td>
<td>1:10</td>
<td>≥1:640</td>
<td>0</td>
</tr>
<tr>
<td>Araguari</td>
<td>1:10</td>
<td>0*</td>
<td>≥1:640</td>
</tr>
</tbody>
</table>

* <1:10.

Ultra-thin sections were made and put onto 400 mesh nickel grids. Araguari virus MIAF was then added to the grids, which were incubated. Mouse colloidal gold-marked antibody diluted 1:100 in 1% bovine serum albumin–phosphate-buffered saline (PBS), pH 8.0, was added. The grids were then stained and examined by the TEM technique.

**Scanning electron microscopy.** The procedure used was that described by Bozzola and Russell. Briefly, Vero cell cultures infected with Araguari virus and showing a CPE of 75% were used. Cells were fixed in a suspension of 2.5% glutaraldehyde (v/v) and 0.1 M SCB, pH 7.2, for two hours at room temperature and washed three times in PBS, pH 7.2. Cells were scraped from the flask surface and dehydrated using ethanol in concentrations of 30%, 50%, 70%, 90%, and 100% ethanol for five minutes, followed by three additional dehydration steps in anhydrous ethanol for 10 minutes each at room temperature. Cells were then dried and examined using a scanning electronic microscope (LEO model 1450 VP; Zeiss).

**Virus precipitation and purification.** This procedure was developed according to a method described by Rico-Hesse and others. Briefly, supernatants of Vero cell cultures infected with Araguari virus and showing a CPE of 75% were precipitated by adding an equal volume of 23.2 grams of NaCl and 70 grams of polyethylene glycol 8000 (Sigma). Suspensions were incubated at room temperature for 15 minutes and stirred for three hours. Precipitates were collected by centrifugation at 17,000 $\times$ g for 90 minutes, resuspended in 1 mL of TNE buffer (10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.2), and cleared by centrifugation at 1,500 $\times$ g for 10 minutes. Suspensions were loaded onto a 5–65% continuous sucrose gradient (ultra-pure sucrose; Gibco-BRL, Gaithersburg, MD) and centrifuged at 150,000 $\times$ g for 18 hours at 4°C. The intermediary band (Araguari virus) was collected, pelleted with 10 mL of a 50% polyethylene glycol, 23% NaCl suspension, rehydrated in 250 $\mu$L of sterile RNAse-free water or in 1× TNE buffer, pH 7.4, and stored at −70°C until used.

**Extraction of RNA and amplification of cDNA.** For genomic RNA extraction, TRIZOL reagent (Gibco-BRL) was used according to the manufacturer’s instructions. After extraction, the genomic RNA of Araguari virus was stored at −70°C until used or used directly to obtain cDNA. Amplifications of viral sequences by reverse transcription–polymerase chain reaction (RT-PCR) were as previously described, using the following degenerated primer pairs: for segment 4 (glycoprotein) forward primer OS4F 5′-gc(c/a) tac ca(c/t) a(c/t)g tgc aac-3′ and reverse primer OS4R 5′-agt ct(I) tc(a/g) tc(I) atc tgg gc-3′; for segment 5 (nucleoprotein) forward primer OS5F 5′-gat (g/a)mL atc ttg tgg cag-3′ and reverse primer OS5R 5′- a(a/g)a act cc(a/c) ag(a/g) tct tc-3′. The PCR products were visualized by gel electrophoresis and extracted using a gel extraction kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. Direct sequencing was done with a Perkin-Elmer 377 sequencer (Applied Biosystems, Foster City, CA).

**FIGURE 2.** Electron microsopy of Araguari virus. Immunocytochemical analysis of virus using colloidal gold without (A) and with (B) antibodies to Araguari virus. Arrows in A show virus particles. Arrows in B show virus particles labeled with colloidal gold. C, Negative staining showing a single virus particle. D, Viral immunocomplexes that exhibit spherical (arrowheads) and pleomorphic (arrow) virus particles.
Figure 3. Partial nucleotide sequence alignments of Araguari virus glycoprotein (a) and nucleoprotein (b) with homologous regions of other viruses of the genus Thogotoivirus, family Orthomyxoviridae. Stars indicate conserved nucleotides among the sequences and dashes indicate gaps.
Partial amplification of the glycoprotein and nucleoprotein genes. For the amplification of the partial nucleotide sequences of the glycoprotein and nucleoprotein genes of Araguarvi virus, a standard two-step RT-PCR protocol was used. For first-strand amplification, we used a 20-pL reaction mixture that consisted of 5 pL of viral RNA (1 ng–5 pL) and 15 pL of reverse transcription master mixture that included 1× first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 0.1 M dithiothreitol), 20 units of RNasin RNase inhibitor (Invitrogen, Carlsbad, CA), 200 μM of dNTPs, and 50–250 ng of random hexamer primers (Invitrogen). The reactions were reverse-transcribed for 60 minutes at 42°C. The PCR was performed in a final volume of 50 μL containing 2 ng of the reverse transcription products and a PCR mixture containing 1× PCR buffer, 2.5 mM MgCl₂, 200 μM of dNTPs, 10 pmol of degenerated primers (OS4F, OS4R, OS5F,
Nucleotide sequencing. Nucleotide sequences of the cDNAs obtained for Araguari virus were determined by automated sequencing using the ABI PRISM Dye Terminator kit (Applied Biosystems) and an ABI 377 DNA sequencer. The same primer pairs used in the PCR amplifications were applied for direct sequencing.

Phylogenetic analysis. The partial nucleotide sequences obtained for Araguari virus were aligned with the available homologous sequences of other viruses belonging to the genus *Thogotovirus* (Thogoto: NC006506, NC006507; Dhori: M17435, M34002; Batken: X97340, X97341) using ClustalX software (Heidelberg, Germany). Phylogenetic trees were constructed using both neighbor-joining (NJ) and maximum parsimony (MP) methods implemented with the PAUP version 4.0 and Mega 2.1 software packages. For NJ and MP analysis, a distance matrix was calculated from the aligned sequences using the Kimura two-parameter formula. Weights of four for transitions and one for transversions were applied for direct sequencing.

### RESULTS

**Complement fixation and hemagglutination inhibition tests.** The results of cross CF tests between Araguari and three other thogotoviruses (Thogoto, Dhori, and Batken) are shown in Table 1. Dhori and Batken viruses were indistinguishable by the CF test, but Batken is considered a variant of Dhori. The MIAF for Araguari virus showed a weak reaction with Thogoto viral antigen in the HI test, but did not react with Dhori antigen (Table 2).

**Cell culture.** Araguari virus replicated in Vero cells after the inoculation of a 10% suspension of infected mouse liver. A CPE was detected seven days post-inoculation on the initial cell passage, and at 72 hours post-infection in subsequent passages (Figure 1B). The CPE was first observed as focal areas of cell deformation. Some hours later, CPE spread to the entire cell monolayer, completely destroying all cells. Araguari virus was confirmed by detection of its antigen by an immunofluorescent assay and the CF test.

**Scanning electron microscopy.** This procedure showed many Araguari virus particles budding from the surface of infected Vero cells after 72 hours. Virus particles had a diameter of approximately 105 nm (Figure 1D). Viral particles were not observed in uninfected Vero cells (Figure 1C).

**Ultra-thin section analysis.** Ultra-thin sections of Vero cells infected with Araguari virus showed completely enveloped virus particles as well as budding virus particles on the cell surface membrane (Figure 1F). Virus particles had a diameter of approximately 105 nm. Intracellular particles were not observed.

**Ultrastructural immunocytochemical analysis.** When Araguari virus was incubated with its MIAF (diluted 1:20) in the presence of a colloidal gold-labeled anti-mouse antibody, viral particles marked with the gold particles (10 nm in diameter) were observed outside the plasma membrane of Vero cells (Figure 2B).

**Negative staining analysis.** Araguari virus was recovered from infected Vero cell supernatants and observed by TEM using the NS procedure. Viral particles were spherical in shape, again showing a mean diameter of 105 nm (Figure 2C).

**Electron immunomicroscopic analysis.** Araguari virus MIAF reacted with infected Vero cells. Immunocomplexes were observed in Araguari virus–infected Vero cells, using a specific MIAF diluted 1:100, 1:250, 1:300, and 1:500. Viral particles were observed in groups showing spherical and pleomorphic morphologies surrounded by antibodies (Figure 2D).

**Partial nucleotide sequence determination of the glycoprotein and nucleoprotein genes of Araguari virus.** Partial nucleotide sequences obtained for Araguari virus were 575 and 526 nucleotides in length and showed considerable similarity to nucleotide sequences of the glycoprotein and nucleoprotein genes of other thogotoviruses, which are encoded in the segments 4 and 5, respectively (Figure 3 and Table 3). The 575 nucleotide sequence obtained for Araguari virus using primer set OS4F-OS4R corresponded to segment 4 nucleotides 460–1057 of Thogoto virus and nucleotides 480–1101 of Dhori virus. The 526 nucleotide sequence obtained for Araguari virus using primer combination OS5F-OS5R corresponded to segment 5 nucleotides 448–960 of Thogoto virus and 425–949 of Dhori virus. Comparisons of partial nucleotide sequences of glycoprotein and nucleoprotein between Araguari virus and other thogotoviruses are summarized in Figure 3.

**Phylogenetic analysis.** To establish genetic relationships between Araguari virus and other orthomyxoviruses of the genus *Thogotovirus*, phylogenetic trees were constructed using the partial nucleotide sequences obtained for the nucleoprotein (526 nucleotides) and glycoprotein (575 nucleotides) genes using NJ and MP methods. Representative members of the genus *Influenzavirus* (influenza viruses A, B, and C) were used as outgroups to root the tree and place confidence values on grouping.
clade (II), while Dhori and Batken viruses were grouped in a different clade (I) (Figure 4a). Segment 5 (nucleoprotein gene) of Araguari virus, although genetically related to segment 5 of the other orthomyxoviruses, fell into a single clade (II) separate from Thogoto virus, which fell into clade III. Dhori and Batken viruses still shared clade I (Figure 4b).

DISCUSSION

We propose that Araguari virus should be classified as member of the family Orthomyxoviridae, genus Thogotovirus together with Dhori and Thogoto viruses. Dias demonstrated that suckling mice inoculated with Araguari virus showed focal necrosis in the liver. The hepatic tropism observed with Araguari virus in suckling mice, which is characterized by high titers of viral antigen in the liver, is similar that shown with Dhori and Thogoto viruses. The CPE produced by Araguari virus in Vero cells (Figure 1B) is also similar to that caused by Thogoto virus. Moreover, Araguari virus did not replicate in mosquito (C636) cells, as observed with Thogoto virus.

The Orthomyxoviridae is a family of RNA viruses with a single-stranded segmented RNA ranging from six to eight fragments with three major genera, Influenzavirus, Isavirus, and Thogotovirus. The first genus includes some of the most important human viral pathogens. Influenza viruses A (eight RNA segments), B (eight RNA segments), and C (seven RNA segments) are responsible worldwide for most respiratory disease epidemics and are associated with thousands of deaths annually. The genus Isavirus is represented by a single virus, the infectious salmon anemia virus. Until now, the genus Thogotovirus consisted of only two viruses, Thogoto virus (six RNA segments) and Dhori virus (seven RNA segments), with a variant known as Batken virus. Thogoto virus has been associated with febrile encephalitis disease in humans, including encephalitis and meningoencephalitis.

The immunocytochemical findings with Araguari virus, which is characterized by budding of virus particles directly from the plasma membrane, size of virus particles, and the similarity of envelope pattern with that of Dhori and Thogoto viruses, are additional supporting evidence that Araguari virus is a member of Orthomyxoviridae, genus Thogotovirus.

To confirm that Araguari virus is a member of the genus Thogotovirus and to compare it with other known members of the genus, electrophoresis with a 1.4% agarose formaldehyde gel was performed using medium from cultures.
of Vero cells infected with Araguari, Thogoto or Dhori viruses. Media from an uninfected Vero cell culture and from Vero cells infected with Pichinde virus (Arenaviridae) were used as negative controls. Six clear RNA bands of decreasing molecular weight were observed for Araguari virus and had estimated sizes of 9.4, 4.4, 2.3, 2.0, 1.2, and 0.8 kb, respectively. The Thogoto and Dhori virus samples also had six segments of comparable size. In contrast, Pichinde virus had only two bands. The uninfected Vero cell medium had no bands.

The molecular alignment (Figure 3) and nucleotide sequencing (Figure 4) of Araguari virus provide the definitive proof that it is an orthomyxovirus. Electrophoresis of Araguari virus RNA showed that it has six segments, similar to Thogoto virus. The sequencing of cDNA obtained from RNA segments 4 and 5, using the same protocol developed for sequencing Thogoto virus, showed that Araguari virus aligns with members of the genus *Thogotovirus* and is more related to Thogoto virus than to Dhori virus, and that it is more distant from Batken and the influenza viruses. However, the individual analyses of each RNA segment show interesting differences. Fragment 4 of Araguari virus formed a single clade with Thogoto, Dhori, Batken, and influenza C viruses, but not with influenza A and B viruses (Figure 4a). A different clade pattern was observed for fragment 5. For this fragment, only two clades were easily differentiated, one for the influenza viruses and another for the other (Thogoto-related viruses) orthomyxoviruses (Figure 4b).

In summary, analyses of nucleotide sequencing support the data obtained by serologic tests, namely that Araguari virus is related to Thogoto virus. Additionally, the sequencing showed that at least for segment 4, Araguari and influenza C viruses probably had a common ancestor. Based on the results presented, we proposed that Araguari virus be designated a member of the genus *Thogotovirus* in the family *Orthomyxoviridae*.

Received May 20, 2005. Accepted for publication August 19, 2005.

Acknowledgments: We thank Basílio S. Buna and Helena B. Vasconcelos for their technical assistance. The GenBank accession numbers of the sequences reported in this paper are: DQ 23096–97.

Financial support: This work was supported in part by contract N01-AI30027 from the U.S. National Institute of Health, by CNPq grants 302770/02-0 and 550275/01-0, and by CAPES (Eliana V. Pinto da Silva).

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