IDENTIFICATION OF BRAZILIAN FLAVIVIRUSES BY A SIMPLIFIED REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION METHOD USING FLAVIVIRUS UNIVERSAL PRIMERS

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Abstract. We report a simplified reverse transcription–polymerase chain reaction (RT-PCR) method for identification of Brazilian flaviviruses based on the patterns of electrophoretic separation of the amplicons. The RT-PCR was done on the culture fluids of Aedes albopictus C6/36 cells infected with Brazilian flaviviruses, without previous extraction of viral RNA, using Flavivirus universal primers that anneal to highly conserved sequences within the nonstructural protein 5 and 3′-nontranslated region of the virus genome. Genomes of 13 Brazilian Flavivirus isolates were amplified. It was not possible to amplify the genome of Bussuquara virus. Analysis of the RT-PCR products gave reproducible results and three distinct amplicon patterns were observed. Cacipacoré (800–850 bp) and yellow fever viruses (600 bp) yielded a single amplicon; dengue virus type 1 and 2 (650 and 550 bp), dengue virus type 4 (550 and 450 bp), Iguape (650–600 bp and 750–700 bp), St. Louis encephalitis (700 and 650–600 bp), and Rocío viruses (600 and 500–550 bp) yielded two amplicons; and Ilhéus virus yielded five amplicons, two larger than 1,000 bp, one 650–700 bp, one 550–600 bp, and one 450–500 bp. The analysis of amplicon DNA sequences of six viruses showed homology with the 3′-nontranslated region of Flavivirus genome. The use of the Flavivirus universal primers in this simple RT-PCR technique is suitable as a screening test for the genus Flavivirus, with the exception of Bussuquara virus, in Brazilian isolates in tissue culture fluid.

Mosquito-borne flaviviruses, which are the most important arboviral agents of disease, are a major public health problem in Brazil.1,2–4 These positive-sense, single-stranded RNA viruses belong to the genus Flavivirus of the family Flaviviridae.5 Several Brazilian flaviviruses, such as yellow fever virus, cause sylvatic zoonosis that occasionally infect humans, domestic animals, and wild birds. Monkeys (Callitrichidae and Cebidae) are hosts for this virus, which is transmitted by tree-top Haemagogus and Sabethes mosquitoes.1,2 Dozens of cases of yellow fever, many of them fatal, are reported yearly in the Brazilian Amazon rain forest, central plateau and Chaco plain.1 Other sylvatic Brazilian flaviviruses are Bussuquara, Cacipacoré, Iguape, Ilhéus, Rocío, and St. Louis encephalitis (SLE) (Coimbra TLM, Adolpho Lutz Institute, São Paulo, Brazil, unpublished data).2 Bussuquara and Ilhéus viruses cause febrile illnesses.6,7 Rocío virus caused an outbreak of more than 1,000 cases of encephalitis, approximately 100 deaths and more than 200 sequelae cases in the Ribeira Valley region in southeastern Brazil.2 One case of fever and jaundice caused by SLE virus has been reported in Brazil, but cases of encephalitis caused by this virus have not been reported.8 The SLE virus cycle has been reported to occur in the southeastern and Amazon regions of Brazil, with the mosquito Culex declarator as the vector and wild birds, monkeys, armadillos, sloths, and marsupials as hosts.9,10 Cacipacoré virus was isolated from a bird in the Amazon region and Iguape virus was isolated from a sentinel mouse in Southeast Brazil (Coimbra TLM, unpublished data), but they have not been described as causative agents of human disease.5

Urban Flavivirus transmission cycles with humans as hosts are also common in Brazil. Since the 1980s, dengue epidemics have occurred in all geographic areas of Brazil, producing millions of infections. An outbreak of dengue type 4 caused thousands of infections in Boa Vista City in the Brazilian Amazon region in 1981.2 Presently, dengue virus types 1 and 2 are endemic in Brazil and the Brazilian Ministry of Health reported 125,000 dengue cases in 1995 and 107,000 cases in the first six months of 1996.3 While the vast majority of cases were not life-threatening, hundreds of cases of dengue hemorrhagic fever/dengue shock syndrome have occurred in the States of Rio de Janeiro and Ceará, causing many deaths.11,12 The World Health Organization is concerned with the possibility of eruption of urban yellow fever outbreaks following sylvatic cases in many countries, including Brazil, where the vector Aedes aegypti infests many large cities.13

The identification of Brazilian Flavivirus isolates has been done mostly on the basis of antigenic cross-relations using hyperimmune mouse sera or ascitic fluids in serologic tests such as complement fixation, neutralization, hemagglutination inhibition, or immunofluorescence.14 The antigenic relationship between flaviviruses has separated dengue viruses and SLE viruses into two subgroups, the latter includes Japanese encephalitis virus.4 Bussuquara, Cacipacoré, Iguape, Ilhéus, Rocío, and yellow fever viruses are unassigned.4

Transcription of RNA to complementary DNA, followed by the polymerase chain reaction (PCR), has been used widely for rapid and specific identification of RNA viruses, including flaviviruses.15–18 We show here the application of a simplified reverse transcription–polymerase chain reaction (RT-PCR) method using Flavivirus universal primers for the presumptive identification of Brazilian flaviviruses based on the pattern of electrophoretic migration of the amplicons.

MATERIALS AND METHODS

Virus strains. Fourteen isolates of 10 known Brazilian flaviviruses (listed in Table 1), were analyzed. Dengue, Ilhéus, Rocío, and SLE BeH 355964 viruses were originally isolated from patients.2,3,8,19,20 The other viruses were isolated
from wild animals. The *Bunyavirus* Oropouche TRVL 9760 was used as a negative control.\(^{21}\)

The viruses were grown in *Ae. albopictus* C6/36 cells. Culture supernatants were collected for the RT-PCR after seven days and virus infection was confirmed by an indirect immunofluorescent test using hyperimmune mouse ascitic fluids.\(^{22}\) The infected cell culture supernatants were stored at \(-70^\circ\)C until use.

**Primers.** The mosquito-borne *Flavivirus* universal primer pair GGT CTC CTC TAA CCT CTA G and GAG TGG ATG ACC ACG GAA GAC ATG C, selected by Tanaka\(^{25}\) for maximum homology with six species of non-American flaviviruses, was based on the original sequence of the yellow fever virus (17D vaccine) reported by Rice and others.\(^{26}\) This primer pair is located in the highly conserved nonstructural protein 5 (NS5) and 3'- non translated region of the genome (nucleotides 10709 to 10052 in the yellow fever virus genome).\(^{25}\) The primers were obtained from Gibco-BRL (Gaithersburg, MD).

**Reverse transcription–polymerase chain reaction.** This test was done on 0.5 \(\mu\)l of cell culture fluid diluted 1:10 with 4.5 \(\mu\)l of distilled, deionized water containing 5 units of RNase inhibitor (Pharmacia, Piscataway, NJ) and incubated for 3 min at room temperature. The RT mixture contained 50 pmol of *Flavivirus* universal primers, 100 \(\mu\)M of each dNTP, 7.5 units of Moloney murine leukemia virus reverse transcriptase (Pharmacia), one unit of Taq DNA polymerase (Pharmacia), and 5 \(\mu\)l of a buffer solution containing 5 mM Tris (pH 9.0), 750 mM MgCl\(_2\), and 25 mM KCl. The volume was adjusted to 50 \(\mu\)l by adding distilled, deionized water. The reaction mixture covered with two drops of mineral oil and incubated for 3 min at 37°C, followed by 30 cycles of a two-step PCR amplification (92°C for 60 sec and 53°C for 60 sec) using a thermal cycler (Techne, Cambridge, United Kingdom). The temperature increment was slow, taking 120 sec from 53°C to 92°C, and 15 sec between 70°C and 75°C. The total time for the test was 150 min.

**Analysis of the amplified DNA.** Eight microliters of each PCR product was analyzed by electrophoresis in 3% agarose gels and visualized at with UV light after staining with ethidium bromide. The sizes of the amplified DNA fragments were determined by comparison with a 50-basepair (bp) molecular weight marker (Pharmacia).

Amplions of dengue type 1 RIB 830 (550 bp), dengue type 1 RIO 28973 (650 bp), dengue type 2 CEA 2462 (550 bp), dengue type 2 TOC 213 (550 bp), SLE SPAN 11916 (600 bp), and yellow fever BeAn 131 viruses were directly sequenced after recovery from an agarose gel. Aliquots of 100 \(\mu\)l of each amplicon were purified using a DNA purification system (Promega, Madison, WI) and resuspended in 50 \(\mu\)l of sterile, double-distilled water. The purified DNAs were sequenced using a Thermocycler sequence kit (Amersham, Buckinghamshire, United Kingdom) with fluorescein-labeled *Flavivirus* universal primers. The products were subjected to electrophoresis in a denaturing 6% polyacrylamide sequencing gel in an ALF DNA sequencer (Pharmacia, Uppsala, Sweden) at 1,200V for 4–6 hr.

The DNASIS software, version 2.1, (Hitachi, Yokohama, Japan) was used for DNA sequence homology analysis and sequence alignment of the six Brazilian *Flavivirus* DNA sequences, including the previously known genomes of the dengue type 2 Jamaica strain (nucleotides 10311–10410), and the yellow fever 17D vaccine strain (nucleotides 10501–10600).\(^{26,27}\)

**RESULTS**

Thirteen Brazilian flaviviruses tested had their genomes amplified by RT-PCR using the *Flavivirus* universal primers. The genome of Bussuquara BeAn 4073 virus was not amplified with the conditions used. Analysis of the RT-PCR products from at least three tests with each virus allowed the detection of patterns of RT-PCR products with one, two, and three or more amplicons, as shown in Figures 1, 2, and 3. Cacipacoré BeAn 327600 and yellow fever BeAn 131 virus amplicons produced bands of 800–850 bp and approximately 600 bp, respectively (Figures 1 and 3). Dengue type I RIB 830 and RIO 28973 and dengue type 2 CEA 2462, SP 125367, and TOC 213 virus amplicons produced products of approximately 650 and 550 bp (Figure 2). Dengue type 4 BV virus amplicons produced products of approximately 550 bp (weak band) and 450 bp (Figure 2). Iguape SPAN 71686 virus amplicons produced bands of 700–750 bp and 600–650 bp (Figure 1). The SLE viruses BeH 355964 and SPAN 11916 virus amplicons produced bands of approximately 700 bp and 600–650 bp (Figure 1). Rocio SPH 34675 virus amplicons produced two bands of approximately 600 bp and 500–550 bp (Figure 3). Ilhéus BeH 7445 virus amplicons yielded five bands, two of them larger than 1,000 bp, one 650–700 bp, one 550–600 bp, and one 450–500 bp (Figure 1).

Sequence alignment of 100 nucleotides from the small amplicon of SLE SPAN 11916 and YF BeAn 131 viruses with nucleotides 10501 to 10600 of the yellow fever virus 17D vaccine strain showed homologies of 56% and 80%, respectively (Figure 4A). Sequence alignment of approximately 100 nucleotides from the small amplicon of dengue type I virus RIB 830, dengue type 2 viruses CEA 2462 and TOC 213, and the large amplicon of dengue type 1 RIO 29733 virus with nucleotides 10311–10410 of the dengue type 2 virus Jamaica strain produced homologies of 92%, 93%, 99%, and 86%, respectively (Figure 4B).

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**TABLE 1**

*Flavivirus* analyzed by reverse transcription–polymerase chain reaction, including place (Brazil) and year of isolation

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Place of isolation*</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bussuquara BeAn 4073</td>
<td>Belém(^{b})</td>
<td>1956</td>
</tr>
<tr>
<td>Cacipacoré BeAn 327600</td>
<td>Belém(^{a})</td>
<td>1977</td>
</tr>
<tr>
<td>DEN 1 RibBB80</td>
<td>Ribeirão Preto(^{21})</td>
<td>1991</td>
</tr>
<tr>
<td>DEN 1 RioH 28973</td>
<td>Rio de Janeiro(^{10})</td>
<td>1986</td>
</tr>
<tr>
<td>DEN 2 CEAH 2462</td>
<td>Fortaleza(^{2})</td>
<td>1994</td>
</tr>
<tr>
<td>DEN 2 SPH 125367</td>
<td>Rio de Janeiro(^{7})</td>
<td>1979</td>
</tr>
<tr>
<td>DEN 2 TOCH 213</td>
<td>Araguaina(^{24})</td>
<td>1991</td>
</tr>
<tr>
<td>DEN4BV</td>
<td>Boa Vista(^{29})</td>
<td>1981</td>
</tr>
<tr>
<td>Iguape SPAN 71686</td>
<td>São Paulo(^{21})</td>
<td>1979</td>
</tr>
<tr>
<td>Ilhéus BeH 7445</td>
<td>Belém(^{a})</td>
<td>1978</td>
</tr>
<tr>
<td>Rocio SPH 34675</td>
<td>São Paulo(^{2})</td>
<td>1975</td>
</tr>
<tr>
<td>SLE BeH 355964</td>
<td>Belém(^{b})</td>
<td>1969</td>
</tr>
<tr>
<td>SLE SPAN 11916</td>
<td>São Paulo(^{b})</td>
<td>1955</td>
</tr>
</tbody>
</table>

* DEN = dengue; SLE = St. Louis encephalitis; YF = yellow fever.
\(^{1}\) TLM Cotrim, Adolpho Lutz Institute, São Paulo, Brazil, unpublished data.
FIGURE 1. Agarose gel stained with ethidium bromide showing two amplicons obtained from St. Louis encephalitis (SLE) BeH 355964 (BE), SLE SPAN 11916 (SP), and Iguape (IGU) SPAN 71686 viruses. The gel also shows one band obtained from Cacipacoré (CAC) BeAn 327600 virus and five bands obtained with Ilhêus (ILH) BeH 7445 virus. Oropouche (ORO) TRVL 9760 virus was used as a negative control. MW = molecular weight marker; bp = basepairs.

FIGURE 2. Agarose gel stained with ethidium bromide showing two amplicons obtained from dengue type I RibH830 (RIB), dengue type I RioH 28973 (RIO), dengue type 2 CEA 2462 (CEA), dengue type 2 TOC 213 (TOC), dengue type 2 SPH 125367 (SP), and dengue type 4 (BV) viruses. MW = molecular weight marker; bp = basepairs.

DISCUSSION

Isolates of all known Brazilian flaviviruses, with the exception of Bussuquara virus, were amplified by RT-PCR using the Flavivirus universal primers. The results show that these viruses, most of which have not had their genome sequences determined, possess conserved sequences in part of the NS5 gene and the 3′-untranslated region. Our data confirm results published by Tanaka for dengue virus, SLE, and other flaviviruses from Asia.25 The absence of DNA amplification with the uninfected cell culture supernatant negative controls and the Bunyavirus Oropouche indicates that our assay is specific. This specificity is further suggested by the high homology between amplicons of Brazilian dengue types 1 and 2, yellow fever, and SLE viruses with previously known sequences of the 3′-nontranslated region of the yellow fever and dengue type 2 virus genomes (Figure 4).26–28 A study including a more extensive analysis of Brazilian Flavivirus genome sequences is ongoing.

The amplified DNA products from Brazilian flaviviruses obtained with the Flavivirus universal primers under the stringency conditions of our RT-PCR permitted a preliminary differentiation of species based on the number of amplicons: one amplicon, Cacipacoré and yellow fever viruses; two amplicons, dengue types 1, 2, and 4, Iguape, SLE, and Rocio viruses; three or more amplicons, Ilhêus virus. Two RT-PCR products obtained with the same primers used in our study have been previously reported for dengue viruses types 2, 3, and 4, Japanese encephalitis, and West Nile viruses since the primer GGT CTC CTC TAA CCT CTA G is expected to anneal with two conserved sequences near the 3′-end of these viruses.25 The specific origin of the larger amplicon observed with dengue viruses was also confirmed by the high homology match (86%) and clear sequence alignment with the dengue virus type 2 Jamaica strain (Figure 4B).

The mosquito-borne Flavivirus universal primers used in the RT-PCR amplify sequences located in the highly conserved NS5 region, which is believed to code for the viral RNA-dependent RNA polymerase and about 400 bases (yellow fever virus genome) of the 3′-nontranslated region of Flavivirus RNA, including the conserved sequences called CS I and CS2, which are complementary to regions close to 5′ end of the genome. These conserved sequences may be responsible for genome cyclization, a possible regulatory mechanism of virus translation, replication, or packaging.25, 26, 29, 30 The presence of repeated conserved sequences in the genome of Brazilian flaviviruses may explain the differences in amplicon size in some of these viruses and the multiplicity of amplicons observed with Ilhêus virus.25, 29, 30 The extension of this observation to other unsequenced flaviviruses awaits confirmation. The failure after exhaustive attempts to amplify Bussuquara virus RNA by our RT-PCR could result from sequence differences in the Flavivirus universal primer regions. Further studies on the genome sequence of Bussuquara virus are necessary to elucidate this point.

Two-step PCR cycles have been reported as generating as much DNA as three-step cycles, especially for short frag-
FIGURE 3. Agarose gels stained with ethidium bromide showing A, one amplicon obtained from Caciparocó (CAC) BeAn 327600 and yellow fever (YF) BeAn 131 viruses, and the absence of amplicons from Bussuquara (BUS) BeAn 4073 and Oropouche (ORO) TRVL 9760 viruses; and B, two amplicons obtained from Rocio (RO) SPH 34675 virus and the absence of amplicons from Bussuquara (BUS) BeAn 4073 virus. MW = molecular weight marker; bp = basepairs.

A suitable extension of DNA in a two-step PCR is achieved by the high activity of Taq DNA polymerase that occurs during the temperature transition from 53°C to 92°C. The optimal action-range temperature of Taq DNA polymerase at 70–75°C took 15 sec in the extension time of the RT-PCR. Compared with other reported assays, this single-tube RT-PCR is less laborious, has a shorter turn around time, a reduced risk of contamination, and uses smaller amounts of reagents, resulting in an economy that may be significant for third-world countries.

In summary, the use of the Flavivirus universal primers in this simplified RT-PCR assay is suitable as a fast screen-
ing test for Brazilian flaviviruses in infected tissue culture fluids, with the possible exception of Bussuquara virus. For previously sequenced viruses such as dengue, yellow fever, and SLE, following the screening RT-PCR with Flavivirus universal primers, species-specific primers could be used for an accurate identification of these viruses. Further studies on the sequences of the genomes of Cacipacore, Iguape, Ilhéus, Rocío and SLE viruses are necessary to identify specific primers for species identification of these viruses.

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