Negeviruses isolated from mosquitoes in the Brazilian Amazon

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Abstract

Background: There are several groups of viruses including Insect Specific Viruses (ISV) such as the taxon Negevirus, a group of viruses phylogenetically related to plant viruses. Negeviruses replicate in mosquito cells, but not in vertebrate cells.

Methods: Pools of hematophagous arthropods were inoculated in Vero and C6/36 cells. The cells were observed to detect possible cytopathic effect. Then, indirect immunofluorescence, RT-PCR, and nucleotide sequencing were performed.

Results: Seven samples which presented negative results for flaviviruses, alphaviruses and bunyaviruses, but showed cytopathic effect in C6/36 cells were sequenced. We identified the occurrence of a variety of ISVs, most of them belonging to the taxon Negevirus: The Brejeira, Negev, Cordoba and Wallerfield viruses, including a new virus for science, tentatively named Feitosa virus.

Conclusions: We detected negeviruses in the Amazon region, including two viruses that were isolated for the first time in Brazil: Cordoba virus and the Negev virus and, a new virus for science: the Feitosa virus.

Keywords: Arboviruses, Insect-specific viruses, Negevirus

Introduction

Insect-specific viruses (ISVs) are viruses that naturally infect mosquitoes and replicate in mosquito cells in vitro, but they do not replicate in vertebrate cells. In recent years, there has been an increase in research on ISVs, and they are increasingly attracting the interest of the scientific community due to the evolution of molecular techniques. [1] Most ISVs are made up of RNA and are distributed in several virus families, such as the families Peribunyaviridae, Flaviviridae, Reoviridae, Rhabdoviridae, Togaviridae, Mesoniviridae, and the taxon Negevirus. [2]

The taxon Negevirus consists of positive-sense single-stranded RNA viruses that have been isolated in many regions around the world, including the Americas, Europe, Africa, and Asia [2–6]. This taxon is classified into two main clades, namely: Nelorovirus and Sandewavirus [3].

These viruses have a spherical particle size of 45–55 nm in diameter [7] Most of these viruses consist of three open reading frames (ORFs) flanked by untranslated regions (UTRs) at the 5′ and 3′ ends, while each ORF is separated by short intergenic regions. One large ORF
(ORF1) was found to be 233 to 7339 nt and encodes the viral polymerase protein. ORF 2 (medium) and ORF3 (small) encode glycoproteins and membrane proteins, respectively [7, 8]. The large ORF contains putative protein domains that correspond to non-structural proteins. In addition, four functional domains were also identified: (i) a methyltransferase domain at 522 to 1386 nt; (ii) a RNA ribosome methyltransferase domain at the position of 2511 to 3072 nt; (iii) a helicase domain from 4182 to 4908 nt; and (iv) a RNA-dependent RNA-polymerase (RdRp) domain from 5802 to 6927 nt [7].

The negeviruses that have been isolated so far belonging to the genus Nelorivirus are: Big Cypress virus (BCPV), Brejeira virus (BRJV), Corboda virus (CDBV), Loreto virus (LORV), Negev virus (NEGV), Ngewotan virus (NWTV), Piura virus (PIUV), and San Bernardo virus (SBDV). Regarding the Sandewavirus genus, the Biratnagar virus (BIRV), Dezidougou virus (DEZV), Goutanap virus (GANV), Santana virus (SANV), Tanay virus (TANAV), and Wallerfield virus (WALV) and Busto virus (BUSV) were identified [3, 8, 9].

In the current study we describe the genomic characterization of negeviruses isolated from mosquitoes resulted from arbovirus surveillance actions in the Brazilian Amazon, including viruses reported for the first time in Brazil and also a noval negevirus.

Materials and methods

Mosquitoes collection methods

The mosquitoes were collected in the areas of Marabá, Curionópolis and Canaã dos Carajás, in the state of Pará, Brazil, in 2014 and 2015. Hematophagous arthropods were collected on the ground and in the canopy of trees using two methods: human attraction protected and enlightened during the day, using hand nets (polyester net bag 30 cm in diameter, attached to a 30 cm aluminum handle) and an oral suction device, which stored captured mosquitoes. The other technique used was light attraction, using CDC light trap (John W. Hock Company, Gainesville, FL, USA) during the day (from 6 PM to 6 AM). After taxonomic identification, arthropods were organized in pools.

General methodological flow

All mosquitoes samples were inoculated in cells aiming for the virus isolation. Cytopathic effect (CPE) was investigated and registered, then, all the inoculated cells were submitted to indirect immunofluorescence test to detect Alphavirus, Flavivirus, Orthobunyavirus and Phlebovirus; furthermore, we also performed RT-PCR for detection of Alphavirus, Flavivirus and Orthobunyavirus. The culture supernatant presenting CPE had their genome sequenced.

Virus isolation in cell cultures and indirect immunofluorescence test (IFA)

The Aedes albopictus clone C6/36 cell line (ATCC: CRL 1660) [10], and the Vero cell line, originating from African green monkey, Chlorocebus sabaeus (ATCC: CCL-81) [11], were maintained in laboratory through cell splitting on a weekly basis. For C6/36 cells (maintained at a temperature of 28 °C), the Leibowitz L-15 medium with L-glutamine (Gibco, MA, USA), supplemented with 5% fetal bovine serum (FBS) (Gibco, MA, USA), tryptose phosphate (HiMedia, Mumbai, India) (2.95%), antibiotics (penicillin 10,000 U/L and streptomycin 10,000 g/L) (Gibco, MA, USA) and non-essential amino acids (10 mL/L) (Baktron Microbiology, RJ, Brazil) was used. [12] The Vero cell line, maintained in an incubator at 37 °C and 5% CO2 (Thermo Scientific, MA, USA) was weekly splitted using a solution of trypsin (0.25%) (Difco, NJ, USA) with ethylenediaminetetraacetic acid (EDTA) (Invitrogen, MA, USA) for cell dissociation. The Vero cells were maintained in medium 199 (Gibco, MA, USA) supplemented with sodium bicarbonate 2.2 g/L (Sigma, NJ, USA), 5% of FBS and antibiotics (penicillin 10,000 U/L and streptomycin 10,000 g/L).

For processing the hematophagous arthropods, suspension of mosquitoes was prepared in 2 mL eppendorf-type tube (U-bottom) with 1 mL of D-PBS 1× diluent (Gibco, MA, USA) with 2% penicillin and streptomycin, 1% fungizone, and 5% FBS. Then, grinding of the mosquitoes was performed in the Tissuelyser equipment for 60 s using a 3 mm tungsten bead (frequency: 25 Hz). After grinding, the tubes with the arthropods were frozen in a freezer at −80 °C overnight, and, the next day or at the moment of inoculation, thawed and centrifuged in a refrigerated centrifuge at 4 °C at 11,200g for 10 min [13, 14].

Cells were inoculated from one to three days after cell splitting (confluence of approximately 90%). Immediately before inoculation, the growth medium (5% FBS) was discarded from the 24-well plates containing the cells. The C6/36 and Vero cell cultures were concomitantly inoculated with the specimen’s suspension. Each well of the plate was inoculated with 100 µL of the supernatant from a batch of arthropods, and each plate included positive controls, cells inoculated with arboviruses that replicate in these cells, such as Chikungunya virus (CHIKV), Dengue virus (DENV) and/or Oropouche virus (OROV), and a negative control ( uninoculated cells). After inoculation, the plates were incubated in an incubator for one hour at 28 °C (C6/36 cells) or 37 °C (Vero cells), and shaken gently every 15 min. Subsequently, 1.5 mL of L-15 maintenance medium was added to each well of the plate with C6/36 cells and 1.5 mL of 199 maintenance medium for Vero cells. The plates were then observed on a daily basis.
for seven days under an inverted microscope to visualize possible cytopathic effect and possible other abnormalities in the cell monolayer [15, 16].

To confirm cell infection and identify the viral agent, the indirect immunofluorescence test (IFA) was performed according to the protocol adapted from Gubler et al. [17]. The cell suspensions were tested using mouse polyclonal antibodies (produced in house) of group of arboviruses included at the genera *Alphavirus, Flavivirus, Orthobunyavirus* and *Phlebovirus*.

**RT-PCR for *Alphavirus, Flavivirus* and *Orthobunyavirus* detection**

RT-PCR was performed for *Flavivirus, Alphavirus* and *Orthobunyavirus* detection. RNA extraction of cell supernatant was performed using the Maxwell 16 equipment with the Maxwell 16 Total RNA purification kit (Promega, WI, USA) according to the manufacturer’s instructions.

After extraction, total RNA was reverse transcribed with 12.5 ng/µl random hexamers (Invitrogen, MA, USA), 0.5 mM dNTPs, ultrapure water and denatured at 65 °C for 5 min. 5× buffer, 5mMDTT, 40U RNAse inhibitor (RNAse OUT, Invitrogen, MA, USA) and 200U reverse transcriptase (SuperScript III, Invitrogen, MA, USA or M-MLv Invitrogen, MA, USA) were then added to the mix, which was then incubated at 25 °C for 5 min. For flaviviruses (220 bp), we used the ‘forward primer cFD2’ (GTG TCC CAG CCG GGG GTG TCA TCA GC) and the ‘reverse MA’ (CATGATGGAARAGRGRARRAG); for alphaviruses (434 bp), the ‘forward primer MAY 1’ (YAGACGTDTTTTCGCASTRGWCH) and ‘reverse MAY 2’ (ACATRANNKGNRGTTRACRAANC) were used; and for orthobunyavirus, (300 bp), the ‘forward primer BUN-S’ (AGTAGTTGCTCCAC) and ‘reverse BUN-C’ (AGTAGTATACTCCAC) were used.

The cDNA synthesis was carried out at 55 °C for one hour, followed by 70 °C for 15 min and kept at 4 °C until the PCR step. PCR amplification was performed with 5 µL of cDNA mixed with 10× buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, 2U Taq DNA polymerase (Invitrogen, MA, USA) and ultrapure water to 50 µL reaction volume. The cycle conditions are described in the Table 1.

PCR products were revealed by using 3% agarose gel electrophoresis (Ultra pure gel—Invitrogen, MA, USA) in 1X T.A.E. buffer (10 mM Tris; 0.1 M Acetate; 1 mM EDTA pH 7.2) stained with SYBR® Safe DNA gel stain (Invitrogen, MA, USA), in a 50-min run using a transiluminator with an ultraviolet light source [18].

**Nucleotide sequencing**

For RNA extraction, the commercial QIAamp®Viral RNA Mini Kit was used, following the manufacturer’s recommendations. Sequencing first occurred with reverse transcription and the second cDNA strand was obtained with the cDNA Synthesis System kit (Roche Diagnostics, Basel, Switzerland) by using random primers (400 µM Roche “random” Primer). The product of this reaction was purified by using the magnetic beads from the Agencourt AMPure XP Reagent kit (Beckman Coulter, CA, USA). The transcribed and purified product served as input for genomic library preparation by applying the methodology described in the Nextera XT DNA Library Preparation Kit.

The library was evaluated in terms of quantity through the Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay Kits, and in terms of the size of the fragments produced, by using the High Sensitivity DNA Analysis Kits (Agilent Technologies, CA, USA) and the Bioanalyzer 2100 (Agilent Technologies, CA, USA). After the library checking steps, sequencing was performed by using the synthesis methodology via the MiniSeq platform (Illumina, CA, USA) using the MiniSeq High Output kit (300 cycles), according to the manufacturer’s instructions.

The generated files were treated and used for assembly by using the same methodology using SPAdes v.3.13.0 [19] and IDBA-UD v.1.1.3 [20]. The k-mer values for SPAdes of 21, 33, 55, 77 and for IDBA-UD the program’s default k-mer were used. Initially, a Multiple Sequencing Alignment (MSA), using the entire ORFs of the Brazilian strain and sequences available on NCBI, was performed using Mafft v7.310 software [21]. The aligned data was

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**Table 1** Thermostating programs used for each pair of genera universal primers

| Genera      | No. of cycles | Denaturation | Hybridization/increment | Elongation       | Final elongation |
|-------------|---------------|--------------|-------------------------|------------------|------------------|
| *Flavivirus* | 35            | 94 °C por 2′ | 94 °C por 30′ 95 °C por 30′ | 72 °C por 2,5′   | 72 °C por 5′    |
| *Alphavirus*| 45            | 95 °C por 5′ | 95 °C por 30′ 95 °C por 30′ | 68 °C por 1′     | 68 °C por 5′    |
| *Orthobunyavirus* | 35   | 94 °C por 5′ | 94 °C por 1′ 95 °C por 1′ | 72 °C por 2′    | 72 °C por 7′    |
statistically evaluated to identify the best amino acid replacement model to be applied in the phylogenetic analysis by using the ProtTest v.3 software [22]. Subsequently, the Maximum Likelihood methodology was employed through the RaxML (Randomized Axelerated Maximum Likelihood) software, which is used to build the phylogenetic tree [23]. The bootstrap test was also applied along with the maximum-likelihood estimation (MLE) method by fixing 1000 replicates to provide reliability to the values of the clusters [24].

**Results**

The samples studied were negative for *Flavivirus*, *Alphavirus*, *Orthobunyavirus* and *Phlebovirus* through IFA and also negative by RT-PCR for three viral genera (*Flavivirus*, *Alphavirus* and *Orthobunyavirus*). Although, seven pools of mosquitoes (BE AR 805503, BE AR 805511, BE AR 805514, BE AR 805520, BE AR 805525, BE AR 820396, BE AR 805529) presented CPE in these cells, but not in Vero cells. The isolates presented lytic-type CPE in C6/36 cells, characterized by the presence of dead cells as well as refringent cell, clumps and syncytial formation until complete destruction of the single layer between day four and day six after inoculation (Fig. 1).

No arbovirus genome was detected through sequencing, while sequences of 14 strains of ISVs belonging to the taxon *Negevirus* were obtained: BRJV (6 strains), Like-Biratnagar, herein tentatively named Feitosa virus (FEITV) (3 strains), CDBV (1 strain), NEGV (2 strains), WALV (2 strains). Many of these viruses have been detected in the same pool of hematophagous diptera (Table 2).

Descriptive genome analysis shows that 5′ and 3′ non-coding regions (NCR) were found, which varied in size among the detected viruses from 17 to 562 nt and 143 nt to 534 nt, respectively. In addition, coding regions (ORFs) were also observed, with most viruses showing three ORFs (ORF 1, ORF 2 and ORF 3), except for CDBV, which showed a single ORF (ORF 1) 7023 nt in size. For the aforementioned viruses obtained, ORF 1 ranged in size from 6723 nt to 7107 nt, ORF 2 from 1203 nt to 1269 nt, and ORF 3 from 585 to 627 nt. As for the total nucleotide count of the samples, it ranged from 7574 nt to 9855 nt. Genome coverage ranged from 61.1 to 26,329 nt.

The protein domain analysis for ORF 1 of the isolated virus of the taxon *Negevirus* (FEITV, CDBV, WALV, BRJV and NEGV) performed through the InterProScan software using the databases (PFAM, PROSITE, PROFILE...)

![Fig. 1 Cytopathic effect (CPE) observed in infected C6/36 cells (red arrow).](image)

- **a** Cells inoculated with the sample BE AR 805503 (CPE at 6th day post-infection), showing destruction of cell's monolayer; **b** cells inoculated with the sample BE AR 805529 (CPE at 4th day post-infection), showing destruction of cell's monolayer and formation of clumps; **c** cells inoculated with the sample BE AR 805511 (CPE at 6th day post-infection), showing destruction of cell's monolayer and large cells (larger than normal cells); **d**-**f** cells inoculated with the sample BE AR 805514 (CPE at 6th day post-infection), BE AR 820396 (CPE at 6th dpi) and BE AR 805520 (CPE at 6th day post-infection), respectively, showing destruction of the cell's monolayer; **g** cells inoculated with the sample BE AR 805525 (CPE at 6th day post-infection), highlighting destruction of the cell's monolayer and large cells (larger than normal cells); **h** negative control (C6/36 cells) showing no CPE (100X).
and PANTHER) showed the recognition of conserved domains for alphavirus MT, VMethyltransf, FtsJ, PSRV_Helicase (Viral Helicase 1), Ribosomal RNAm and RdRp. Regarding to viruses presenting ORF 2, the PFAM database identified only the protein domain for DiSB, solely in the BRJV and NEGV strains. With regard to ORF 3, the PFAM database recognized protein domain for SP24 in all strains of the isolated negevirus that have ORF 3 (FEITV, WALV, BRJV and NEGV).

For phylogenetic analysis, a tree of the polymerase domain of ORF 1 of the sequenced negeviruses, BRJV, NEGV, CDBV, WALV and FEITV was first constructed, since all the viruses obtained have ORF 1. The isolated negeviruses clustered within the main groups previously described for these viruses: *Nelorpivirus* (BRJV, NEGV, CDBV) and *Sandewavirus* (WALV and FEITV). The isolated BRJV strains (BE AR 805511, BE AR 805514, BE AR 805520, BE AR 805525, BE AR 805396, BE AR 805503, BE AR 805503) formed a group with the other previously described Brazilian BRJV strains, this group being more closely related to that of the PIUV. The NEGV strains obtained (BE AR 805514 and BE AR 805503) formed a single clade with the previously identified strains of this virus from the United States and Europe, with the NEGV group being more closely related to the NWTV. The CDBV strain, BE AR 805503, clustered with the other strains of this virus. The negeviruses of the *Nelorpivirus* genus have common ancestry with some plant viruses, such as the Citrus leprosis virus group C (CiLV-C), Hibiscus green spot virus (HGSV), and Blueberry necrotic ring blotch (BNRB). Regarding the negeviruses belonging to the *Sandewavirus* genus, the detected WALV strains—BE AR 805520 and BE AR 805503—formed a clade with the other American strains (Brazil, Panama, Trinidad and Tobago, USA and Colombia), with the WALV clade being more closely related to that of GANV. In turn, the FEITV relates more to the BIRV and BUSV (Fig. 2).

The phylogeny of BRJV showed the formation of three clades, group I corresponding to clades of Brazilian strains isolated in 2013, 2014 and 2015, including the strains isolated in the herein study. Group II is made up of isolates from Brazil in 2005 and 2010. On the other hand, group III is made up of the isolates from Colombia in 2013. All six BRJV strains that were isolated (Canaã dos Carajás and Curionópolis areas), in relation to the three concatenated ORFs, were shown to be more genetically related to the 2013 strains also from Canaã dos Carajás described by Nunes et al. [17], while the other Brazilian samples taken in 2005 and 2010 (group II) were shown

### Table 2 Accession number of the isolate’s sequences deposited in the Genbank according to the mosquito specie and place of collection

| Virus  | Lab. record | Mosquito specie | Place of collection | Accession number |
|--------|-------------|-----------------|---------------------|------------------|
| BRJV   | BE AR 805511 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615324 |
|        | BE AR 805514 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615325 |
|        | BE AR 805520 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615328 |
|        | BE AR 805525 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615330 |
|        | BE AR 803936 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615333 |
|        | BE AR 805503 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615334 |
|        | BE AR 805530 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615335 |
|        | BE AR 805520 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615320 |
| CDBV   | BE AR 805503 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615321 |
|        | BE AR 805514 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615326 |
|        | BE AR 805503 | Cx. (Cux.) coronator | Curionópolis, PA, BR | MZ615331 |
|        | BE AR 805503 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615322 |
|        | BE AR 805514 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615327 |
|        | BE AR 805503 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615323 |
|        | BE AR 805520 | Cx. (Cux.) species | Canaã dos Carajás, PA, BR | MZ615329 |

![Fig. 2](image-url) Phyllogenetic tree based on the amino acid sequences of viral polymerase (RdRp) from ORF 1 of insect-specific viruses belonging to the taxon Negevirus. Phylogeny generated with the Maximum Likelihood method and LG model. The virus strains obtained in the study are in blue font and arranged in a cartoon format, while the virus groups related to the isolated viruses are collapsed. Viruses with all three ORFs are highlighted in yellow; those with two ORFs are highlighted in blue; and those with only one ORF are highlighted in green. The host that originated each isolate is identified with an image of the host (mosquito, bee, plant), identified in the legend. The bottom bar represents the rate of amino acid replacement.
Fig. 2 (See legend on previous page.)
Fig. 3  Phylogenetic tree of the concatenated nucleotide sequences of ORF1, ORF2 and ORF3 of the isolated strains (in red font) and other strains available in the Genbank, using the Maximum Likelihood method and GTR model. The bottom bar represents the rate of nucleotide replacement. a BRJV, b NEGV, c CDBV, d WALV, e FEITV
to be more genetically distant from group I, with genetic
distances ranging from 10.5 to 11.4% (Fig. 3a).

The isolated NEGV strains clustered in a different clade
from that previously identified strains from the USA,
Portugal, Italy, and Israel. Four groups were formed,
differentiated by geographic region (Europe, Asia, Bra-
zil, and USA, respectively). Group I was made up of the
strains isolated in Israel, Italy, and Portugal; group II con-
sisted only in the strain isolated in the Philippines; group
III included the NEGV samples isolated in this study,
and group IV and V included the viruses from the USA.
Although the BE AR 805503 and BE AR 805514 strains
are the most genetically distant from the other NEGV
strains, with short nucleotide distances ranging from 4.2
to 6.6%, all strains from all groups, including the strains
in this study, were shown to be strains of the same virus
with nucleotide distances in a range of 0.1–6.6%. It is
important to note that NEGV had not yet been isolated
in Brazil (Fig. 3b).

The CDBV strain isolated from sample BE AR 805503,
from Culex species mosquitoes collected in 2015, is the
first isolation of this virus in Brazil. The Phylogenetic
analysis of ORF 1 (the only identified ORF of this virus)
showed the formation of three distinct clades (groups
I, II, and III) involving strains isolated in several places
around the world, such as the USA, Colombia, Brazil,
and Nepal. The sample isolated hereby was shown to be
related to strains isolated in Colombia and the United
States in 2013, which formed group I, being more geneti-
cally distant from groups II and III viruses and made
up by viruses detected in the United States and Nepal,
respectively, with nucleotide distances ranging from 15.8
to 25.1% (Fig. 3c).

The WALV isolated from samples BE AR 805520 and
BE AR 805503, from Culex (Cux) species, collected in
2014 in Canaã dos Carajás, were grouped in the same
group as the strains identified in the previous year, 2013,
also collected in Canaã dos Carajás area, which formed
group I, being even more genetically distant, around
4–4.3%, in nucleotide terms from the strains isolated in
2005 (group VI) in the same state (Pará), but in a different
city, called Trairão. Besides, other groups were formed
based on geographic distribution, with groups II, III, IV
and V consisting of viruses from Colombia, Panama, the
USA and Trinidad and Tobago, respectively (Fig. 3d).

The FEITV, a new virus to science, was isolated in three
out of the eight sequenced samples (BE AR 805529, BE
AR 805503 and BE AR 805514), two of which from Culex
(Cux) species and one from Culex coronator, all collected
in 2014 in Canaã de Carajás area. These samples grouped
into a distinct clade, being most closely related to the
BUSV and BIRV isolated in the Philippines and Nepal,
respectively. Despite this comparison, it is a different

Discussion

This study aimed to investigate the circulation of arbo-
viruses and ISVs from hematophagous arthropods col-
clected near to areas of mining in the southeastern Pará
state, Brazil. Investigations like this of entomosurveil-
lance, which analyze how anthropological impacts could
affect the natural balance of biodiversity in the region, are
very important.

The sequenced ISVs strains showed CPE only in C6/36
cells. This result corroborates the study of Vasilakis et al.
[7], which showed that six ISVs (NEGV, PIUV, DEZV,
NWTV, LORV and SANV), caused CPE only in C6/36
cells and inoculated mice did not become ill, thus show-
ing the replication restriction of ISVs only to mosquitoes
and their cells.

Descriptive genome analysis of the viruses sequenced
through this study shows that 5’ and 3’ non-coding
regions (NCRs) and coding regions (ORFs) described for
negeviruses were found according to Nunes et al. [8]. The
protein domains recognized by the Interproscan software
in ORF 1, ORF 2 and ORF 3 of the negeviruses isolated in
this study were also recognized in studies performed by
Vasilakis et al. [7] and Nunes et al. [8], who demonstrated
the presence of these domains for most negeviruses
(GANV, BIRV, DEZV, BREV, NEGV, PIUV, San Bernardo
virus (SBNV), TANV, WALV, NWTV, SANV and BCPV),
except the conserved domain for Alphavirus MT, found
in ORF 1 and ORF 2 of WALV, which had not yet been
described for negeviruses; also the conserved protein
domain for DiSB was not recognized in the FEITV [8].

The phylogenetic analysis of the polymerase domain of
ORF 1 of the detected negeviruses (BRJV, NEGV, CDBV,
WALV and FEITV) showed that the strains obtained
hereby clustered with the strains of these viruses and
within groups (genera) previously established by Kallies
et al. [3] and also described by Nunes et al. [8], Nelorpivi-
rus (BRJV, NEGV, CDBV) and Sandewavirus (WALV
and FEITV), highlighting the inclusion of a new negevirus
obtained herein, FEITV, within the genus Sandewavirus.
Furthermore, the arrangement of the virus groups in the
phylogenetic tree and the genetic relationship between
them agreed what was described by Nunes et al. [8].

BRV, belonging to the genus Nelorivirus, was iso-
lated in Brazil in 2005 and 2013, in the North region, in
the state of Pará; it was also isolated in Colombia in the
Cordoba region in 2013, and in Mato Grosso do Sul state
(Pantanal) in 2010. In most studies, the virus was isolated
from Culex sp. [25]; this study also isolated six strains of 
BRJV from a pool of Culex (Cax.) species collected in the 
Canaã de Carajás and Curionópolis areas in 2014 and 
2015, respectivamente.

NEG is part of the Negevirus genus and has been 
isolated from several mosquito species such as Culex 
quinquefasciatus, Culex univittatus, Anopheles constant, 
Culex coronator, and Ochlerotatus caspius. This virus 
has been identified in the USA, Portugal, Italy, and Israel 
[6-9]. In the herein study, two strains of the NEGV were 
identified, and this is the first description of the virus in 
Brazil, specifically in the state of Pará. The strains were 
also isolated from arthropods of the Culex (Cax.) species 
collected in the Canaã dos Carajás area in 2014.

The first isolation of CDBV in Brazil was obtained in 
our study, from Culex (Cax.) species collected in 2015. 
Phylogenetic analysis of ORF 1 showed the formation of 
three distinct clades (groups I, II and III), whereas, Nunes 
et al. [8] described the formation of two groups, group I 
including the same strains used herein from Colombia 
and the USA in 2013 and group II, including viruses from 
Nepal.

WALV has already been isolated in several regions in 
the world such as Brazil, Trinidad and Tobago [4], the 
USA, Colombia and Panama between 2005 and 2014. 
These samples were isolated from several species of 
hematophagous diptera, such as Deinocerites sp., Anophe-
les atropos, Anopheles punctipennis, Anopheles crucians, 
Culex iolambdis, Anopheles crucians, Aedes taeniorhyn-
chus, Culex sp. and Culex declarator. We isolated WALV 
strains from Culex species mosquitoes which were more 
genetically related to strains also from Brazil.

This study isolated a new negevirus for science, tenta-
ively named FEITV, related to negeviruses of the genus 
Sandewavirus, BIRV and BUSV, due to the fact that it was 
obtained from hematophagous diptera captured in Vila 
Feitosa area, in Canaã dos Carajás municipality, in 2014.
Despite being most closely related to these two viruses, 
the three FEITV strains that were isolated grouped into 
a distinct clade and showed nucleotide difference rang-
ing from 39.3 to 39.6%, thus showing that it is a differ-
ent virus and a new member of the taxon Negevirus. In 
fact, FEITV demonstrated to have genomic organization 
compatible with the organization of negeviruses of the 
Sandewavirus genus, with the presence of the three ORFs 
with sizes similar to those described for the other viruses 
of the taxon, and the recognition of conserved protein 
domains was observed in this virus as well as in the other 
gevieviruses.

The importance of ISVs and their relationship with 
arboviruses has been investigated, for example the 
Culex mosquitoes infected by the insect-specific flavivi-
irus, Culex flavivirus (CxFV), were less susceptible to 
secondary infection by West Nile virus (WNV). Fur-
ther studies indicated that other insect-specific flavivi-
rus, Nhumirim virus (NHUV), significantly reduced 
the replication of arboviruses such as WNV, Japanese 
Encephalitis virus (JEV) and Saint Louis Encephalitís 
Virus (SLEV) in co-infected C6/36 cells. [26] Previous 
studies are also verifying the antiviral potential of other 
insect-specific flaviviruses, such as the Parramatta 
River virus (PaRV) [27]. Recently, a study conducted by 
Patterson et al. [28] demonstrated that certain negevi-
ruses reduce alphavirus replication during in vitro 
co-infection.

Our results demonstrated the phylogenetic relation-
ship between negeviruses and certain plant viruses, rein-
force the necessity of further studies including analysis by 
molecular clock to better understand the aspects related 
to the evolution of both group of viruses. Hypotheses are 
raised as to evolutionary relationship between ISVs and 
plant viruses, standing out the ISVs of the taxon Negevi-
rus and those of the family Tymoviridae. It is possible 
that because of the insect food habit of feeding on plant 
nectar and plant aquatic material (initial life cycles) [2], 
plant viruses may have evolved to become ISVs, passing 
to insect hosts; otherwise, it is also possible that ISVs 
evolve to become plant viruses, which in turn can now 
infecct plants [1].

In the herein study it was not possible to evaluate 
the relationship between ISVs and arboviruses, but it 
is important to emphasize the importance of conducting 
replication studies that seek to evaluate their relationship with 
arboviruses in vitro and in vivo, even as potential 
control strategies for arboviruses, which is one of the pos-
bile applications of ISVs [1]. Furthermore, ISVs are still 
being studied with regard to the possibility of being used 
as platforms for safe diagnostic and vaccine development 
[1].

**Conclusions**

Therefore, the study ascertained the occurrence of a vari-
ety of ISVs of the taxon Negevirus (BRJV, NEG, CDBV, 
WALV and FEITV) in the Canaã dos Carajás area, in Pará 
state, Brazil. The BRJV has also been detected in Curio-
nopolis, Pará state. It should be noted that two negevi-
ruses were isolated for the first time in Brazil, namely the 
NEG and the CDBV. C6/36 cells have proven to be a 
good system for isolation of ISVs, especially those of the 
taxon Negevirus. In the Canaã dos Carajás area, in Pará 
state, a new virus was detected for science and tentatively 
named Feitosa negevirus. No arboviruses of the Flavi-
irus, Alphavirus, Orthobunyavirus and Phlebovirus genera 
detected in this study.
Abbreviations
ISVs: Insect-specific viruses; dpi: Days post infection; CPE: Cytopathic effect; BRV: Brequela virus; CDV: Cordoba virus; LORV: Loreto virus; NEGV: Negev virus; NWTV: Ngewotan virus; PIJV: Piura virus; SBVD: San Bernardo virus; BİR: Biratnagar virus; DEZV: Dezidougou virus; GANV: Goutanap virus; SANV: Santana virus; WALV: Wallerfield virus; BUSV: Bustos virus; CHIKV: Chikungunya virus; DENV: Dengue virus; OROV: Oropouche virus.

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Authors’ contributions
All the authors herein contributed significantly for this research. The conceptualization, design, writing and revision were carried out by the authors ACSR, VLC, SGR, DBAM, PFCV and LCM. The authors KKPM, ACSR, SPS, JPNN, HAOM, BLSN, JWRJ and ACRC performed experiments of the study and analyzed the data. All authors have read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
The necessary licenses to carry out this study have already been acquired for Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) number 02-2013 and 16-2014, obtaining the following registration: LO 1096/2012—Constraint 2.8: Annual monitoring of arthropods, birds and small mammals related to the life cycle of arboviruses found in the project LO 1096/2012—Constraint 2.8: Annual monitoring of arthropods, birds and small mammals related to the life cycle of arboviruses found in the project. This was also approved by the Ethics Committee on the use of animals of the Evandro Chagas Institute under registration number 006/2014.

Consent for publication
Not applicable.

Competing interests
The authors do hereby declare no conflict of interest.

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References
1. Bolling BG, Weaver SC, Tesh RB, Vasilakis N. Insect-specific virus discovery: significance for the arbovirus community. Viruses. 2015;7:4911–28. https://doi.org/10.3390/v7092851.
2. Vasilakis N, Tesh RB. Insect-specific viruses and their potential impact on Arbovirus transmission. Curr Opin Virol. 2015;15:69–74. https://doi.org/10.1016/j.coabi.2015.08.007.
3. Kallies R, Kopp A, Zirkel F, Estrada A, Gillespie TR, Drosten C, Junglen S. Genetic characterization of Goutanap virus, a novel virus related to Negevirus, cilevirus and higrevirus. Viruses. 2014;6:4346–57. https://doi.org/10.3390/v6114346.
4. Auguste AJ, Carrington CVF, Forrester NL, Popov VL, Guzman H, Widen SG, Wood TG, Weaver SC. Tesh RBCharacterization of a novel Negevirus and a novel Bunyaviruses isolated from Culex (Culex) declarator mosquitoes in Trinidad. J Gen Virol. 2014;95:481–5. https://doi.org/10.1099/ijvi.0.069412-0.
5. Nabeshima T, et al. Tanay virus, a new species of virus isolated from mosquitoes in the Philippines. J Gen Virol. 1945;9:130–5. https://doi.org/10.1099/0.016887-0.
6. Carapeta S, Bern B, Guiness JM, Esteves A, Abecasis A, Lopes A, Matos AP, Priedade J, Almeida APC, Parreira R. Negevirus found in multiple species of mosquitoes from southern Portugal: Isolation, genetic diversity, and replication in insect cell culture. Virology. 2015;483:318–28. https://doi.org/10.1016/j.virol.2015.04.021.
7. Vasilakis N, et al. Negevirus: a proposed new taxon of insect-specific viruses with wide geographic distribution. J Virol. 2013;87:2475–88. https://doi.org/10.1128/VIROL.00776-12.
8. Nunes MRF, et al. Genetic characterization, molecular epidemiology, and phylogenetic relationships of insect-specific viruses in the taxon Negevirus. Virology. 2017;504:152–67. https://doi.org/10.1016/j.virol.2017.01.022.
9. Fujita R, Kuwara R, Kobayashi D, Bertuso AG, Isawa H, Sawabe K. Bustos virus, a new member of the negevirus group isolated from a Mansonia mosquito in the Philippines. Arch Virol. 2017;162:79–89. https://doi.org/10.1007/s00705-016-2068-4.
10. Igarashi A. Isolation of a Singh's Aedes albopictus cell clone sensitive to Dengue and Chikungunya viruses. J Gen Virol. 1978;40:531–4. https://doi.org/10.1099/0022-1317-40-3-531.
11. Rhim JS, Schell K, Creasy B, Case W. Biological characteristics and viral susceptibility of an African Green Monkey Kidney cell line (Vero). Proc Soc Exp Biol Med. 1962;126:670–8. https://doi.org/10.3181/0037727-132-34285.
12. Barbosa ML, Rocco IM, Felippe JMM, Cruz AS. Growth and maintenance of Aedes Albopictus cell line, clone C6/36, in different media. Revista do Instituto Adolfo Lutz. 1993;53(1):63–70.
13. Auguste AJ, et al. Isolation and phylogenetic analysis of Mucambo Virus (Venezuelan Equine Encephalitis Complex Subtype IIIA) in Trinidad. Virol J. 2009;6(2):123–30. https://doi.org/10.1186/1743-5889-6-282.
14. Thangamani S, Huang J, Hart CE, Guzman H, Tesh RB. Vertical transmission of Zika virus in Aedes aegypti mosquitoes. Am J Trop Med Hyg. 2016;95(5):1169–73. https://doi.org/10.4269/ajtmh.16-0448.
15. Tesh RB. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am J Trop Med Hyg. 1979;28:1033–9. https://doi.org/10.4269/ajtmh.1979.28.1053.
16. Beatty BJ, Calisher CH, Shope RE. Arboviruses. In: Lennette EH, Lunette EL, editors. Diagnostic procedures for viral rickettsial and chlamydial infections. 7th ed. Washington: American Public Health Association; 1995. p. 189–212.
17. Gubler DJ, Kuno G, Sather GE, Oliver VLA. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am J Trop Med Hyg. 1984;33(1):158–65. https://doi.org/10.4269/ajtmh.1984.33.158.
18. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB. Phylogeny of the genus Flavivirus. J Virol. 1998;72(1):75–83.
19. Bankevich A, et al. Spades: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19(5):455–77. https://doi.org/10.1089/cmb.2012.0021.
20. Peng Y, Leung HCM, Yiu SM, Chin FYL. Idba-Ud: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics. 2012;28:1420–8. https://doi.org/10.1093/bioinformatics/bts174.
21. Katah K, Standley DM. MAFFT. Multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30(4):772–80.
22. Darriba D, Guillermo LT, Ramón D, David P. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods Nat Res. 2013;10:2013-302.
23. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies—4. Bioinformatics. 2014;30(9):1312–3. https://doi.org/10.1093/bioinformatics/btu333.
24. Felsenstein J. Phylogenies and the comparative method. Am Nat. 1985;125(1):1–15.
25. Nunes MRT, et al. Emergence of new insect-restrictive viruses in the Amazon region. Genome Announc. 2015;3(2):e00131-e215. https://doi.org/10.1128/genomeA.00131-15.

26. Kenney JL, Solberg OJ, Langevin SA, Bauta AAC. Characterization of a novel insect-specific flavivirus from Brazil: potential for inhibition of infection of arthropod cells with medically important flaviviruses. J Gen Virol. 2014;95(12):2796–808. https://doi.org/10.1099/vir.0.068031-0.

27. Hall Roy A, et al. Commensal viruses of mosquitoes: host restriction, transmission, and interaction with arboviral pathogens. Evol Bioinforma. 2016;12(2):35–44. https://doi.org/10.4137/EBO.S40740.

28. Patterson E, Kautz TF, Contreras-Gutierrez MA, Guzman H, Tesh RB, Hughes GL, Forrester NL. Negeviruses reduce replication of alphaviruses during co-infection. J Res. 2020. https://doi.org/10.1101/2020.09.01.27751733.

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