Chapter

Novel Single Hematophagous Insect RNA Detection Method Supports Its Use as Sentinels to Survey Flaviviruses Circulation

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Abstract

Anthropogenic actions, including deforestation, disorganized urbanization, and globalization, contribute to emergence and reemergence of arboviruses worldwide, where Flavivirus is the most prevalent, and its continuous monitoring can help in preventive control strategies. Thus, the aim of this study was to detect flavivirus RNA in single hematophagous insects, which are used as sentinels. Total RNA was extracted from six *Aedes aegypti* stored since 2003 and from 100 Culicidae and collected through CDC trap in a public park of a Brazilian Northwest city of São Paulo State. *Flavivirus* was detected through RT/PCR targeting 230–250 bp of the RNA polymerase coding sequence (NS5). PCR amplicons were sequenced by Sanger method, used in comparative analysis over Basic Local Alignment Search Tool (BLAST) in GenBank, and subjected to Neighbor-Joining phylogenetic analyses. Efficiency of *Flavivirus* diagnosis was confirmed by detection of Dengue virus serotype 2 in *Ae. aegypti*. From the 100 collected insects, 19 were positive for *Culex flavivirus* (CxFV). NS5 partial sequence phylogenetic analysis clustered all CxFV in one branch separated from vertebrate flaviviruses, being applicable to the identification of *Flavivirus* species. The dipteran RNA extraction methodology described in this work supports detection of flaviviruses in single insects maintained in 80% ethanol, which can be used to constant arbovirus surveillance.

Keywords: dipteran, *Flaviviruses*, hematophagous insect, molecular diagnosis, RNA extraction, single-insect virus diagnosis

1. Introduction

In recent years, due to anthropogenic actions, including deforestation, disorganized urbanization, and globalization, arboviruses have emerged as a major challenge to global health [1, 2]. The arboviruses (arthropod borne viruses) are transmitted to

humans through the bite of infected hematophagous insects, causing febrile diseases [3], with a broad variety of clinical manifestations, ranging from the absence of symptoms to the severe hemorrhagic and encephalitic disorders [4, 5]. The arbovirus vectors include different species of mosquitoes, flies, and ticks. The most worldwide prevalent arboviruses encompass Dengue Virus (DENV), Yellow Fever Virus (YFV), West Nile Virus (WNV), and Zika Virus (ZIKV) from Flaviviridae family and *Flavivirus* genus, and Chikungunya Virus (CHIKV) from family Togaviridae and genus *Alphavirus* [6], which are transmitted through mosquitoes belonging to the Culicidae family, mainly from *Culex* and *Aedes* genera [2].

Arbovirus transmissions occur principally in tropical and subtropical areas, since the presence of vector mosquitoes is associated, mainly, with hot and humid environments, fundamental requirements for their reproduction [7]. Recently, with the global warming and increase in international traveling, the dispersion of arthropod vectors is rising, especially mosquitoes of the genus *Aedes*, and the dissemination of arboviruses is reaching regions considered nonendemic [8–10]. Besides, mosquitoes' high genetic plasticity enables its adaptation to colder environments and international tourism to tropical and subtropical endemic areas, which contributes to arbovirus vectors' global dissemination [11, 12].

Dengue is the most prevalent arbovirus and is responsible for an estimate of 390 million annual cases worldwide [13], and 3.9 billion people, living in 128 countries, are on risk of infection [14]. Since 2010, dengue cases have been reported in non-endemic countries in Europe, including France, Croatia, and Portugal, where in 2012, an outbreak occurred with more than 2000 reported cases. In this period, 10 other European countries were affected by dengue fever. Further, among European travelers returning from low incoming endemic countries, dengue fever is the most diagnosed disease, after malaria [10, 15]. In 2016, more than 3.34 million cases of dengue were reported in American countries, Southeast Asia, and the Western Pacific. Only in the Americas, approximately 2.38 million people were affected, with 1032 deaths, including Brazil, responsible for almost 1.5 million of the reported cases [15]. In addition to Dengue, in Brazil, a South American country with high international touristic activity, according to the Ministry of Health, occurred 216,207 cases of Zika fever, another important emerging *Flavivirus* [16], and 691 cases of yellow fever with 220 confirmed deaths, only in 2016 [17]. This panel entails the flaviviruses negative impact on public health and also causes the economic burden, having direct and indirect consequences [18]. Direct costs include hospitalization, medications, diagnostic tests, vector control, training of professionals, and health surveillance. Among indirect costs are loss of worker productivity and profits, and interference in trade and tourism in affected areas.

There are no effective vaccines available for Dengue, Zika, and Chikungunya fevers [19], and the control of these arboviruses is exclusively implemented by chemical arthropod vector elimination [1]. Therefore, active searching for vectors is necessary to prevent the circulation of known arboviruses. Detection of arboviruses occurs only after detection of human cases, which causes delay in the disease and vector dissemination controls. Thus, to prevent emergency and reemergence of arboviruses, very early detection of vectors and arbovirus and the understanding of their diversity and infection cycle are of great importance. These strategies include also the identification of factors related to the dispersion and entrance of arboviruses in previous indene areas and the identification of wild animal natural reservoirs [20]. Considering the current difficulties in detecting silent circulation of arboviruses and also in obtaining samples from arthropod vectors, human and animal febrile cases, principally in the forests areas, the use of sentinels could be an alternative surveillance approach. Hematophagous insects are present in different wild natural and urban environments, being an excellent group of animal to be used

as sentinel. Thus, in this work, the single-insect nucleic acid extraction method [21] was evaluated in hematophagous dipterans collected in a Brazilian municipal public park using CDC traps, in order to detect RNA from flaviviruses.

2. Materials and methods

2.1 Specimens and ethical aspects

A total of 106 insects of the order Diptera were analyzed, of which 100 were collected in the Municipal Park of the city of Marília—São Paulo, and six were specimens of *Aedes aegypti*, RED strain, of which five were experimentally infected with DENV2. More detailed information on the specimens used in this study is shown in **Table 1**. The protocol of collection and transport of arthropods in the Municipal Park of the city of Marília-São Paulo was authorized by the Biodiversity Authorization and Information System (SISBIO), of the Chico Mendes Institute for Biodiversity Conservation (ICMBio), Brazilian Ministry of Environment (MMA), under number 64603-1 (10/18/2018). *Ae. aegypti* strain RED specimens infected with DENV2, maintained in a biosafety level 2 (BSL-2) insectary facility in Institute of Biomedical Sciences from University of São Paulo, were gently donated by Margareth de Lara Capurro Guimarães at the Department of Parasitology of the Institute of Biomedical Sciences of the University of São Paulo (USP), of which five were submitted to infection by DENV-2, while one sample was not submitted to infection and was used as a negative control for DENV. These insects were previously used for purposes that are not related to this study and had approval and permissions needed in their own respective study.

This work did not involve collection of human samples, total or partial, and specimens or tissue samples from vertebrate animals and/or embryos. In addition, no threatened or protected species were collected.

2.2 Single-insect nondestructive RNA extraction

For RNA extraction, each dipteran was digested for 16 h at 56°C, inserted in 200 μL of a lysis buffer composed of 200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA (pH 8.0), 0.5% of SDS, and 400 μg/mL of proteinase K as described for DNA extraction [21]. Before digestion, ethanol from insects stored in 80% ethanol was removed after washing twice with 1 mL of 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). Following the incubation period,

Taxon	Storage method	Collection local, data	Number of specimens
Anopheles (Culicidae)	Ethanol 80%	Marília-SP, December 2017	3
Phlebotomus (Psychodidae)	Ethanol 80%	Marília-SP, December 2017	2
Ceratopogonidae	Ethanol 80%	Marília-SP, November 2017	3
Cecidomyiidae	Ethanol 80%	Marília-SP, December 2017	1
Culicinae (Culicidae)	Ethanol 80%	Marília-SP, June 2017	91
Aedes aegypti (Culicidae)	Frozen at −20 °C	USP-SP, March 2003	6

Table 1.
Specimens of dipteran included in the study.

the digestion buffer was transferred to 1.5 mL RNase free tube and submitted to RNA purification. One milliliter of 80% ethanol was added to the insect specimen, which was stored in freezer at −20°C. Total RNA purification for each insect from the obtained digestion solution was performed through Qiagen® RNA Mini Kit and PureLink® RNA Mini Kit according to the manufacturer's instructions.

2.3 RNA quality and *Flavivirus* nucleic acid detection

The quality and quantity of extracted RNA from each insect were done through agarose gel electrophoresis and by reverse transcription and polymerase chain reaction (RT/PCR), with oligonucleotides that amplify a 464 bp fragment encoding the 28S ribosomal RNA fraction (28S rRNA) of dipteran (**Table 2**). RNA from each insect was diluted into a final volume of 60 μL of RNase free water, and 4 μL was subjected to 1.5% agarose gel electrophoresis in 0.5× TBE buffer and stained with SafeBlue® according to manufacturer's instructions. Ten insect samples were used to verify quality by RT/PCR, and before RT reaction, 40 μL of the total RNA was treated with DNase I by means of the Biometra Kit from Analytik Jena, following manufacturer's instructions, being diluted in 40 μL of RNase free water. Subsequently, three complementary DNA (cDNA) syntheses were performed, each with a different oligonucleotide, detailed in **Table 2**, being 0.5 μM of oligonucleotide 28SD7r, six bases random primers from Promega, or Oligo $dT_{18.}$ For each cDNA synthesis, 5.5 μL of one insect total extracted RNA, treated with DNase I, was submitted to RT reaction with 200 units of MMLV Reverse Transcriptase (Invitrogen) following fabricator's instructions, in a total volume of 20 μL. Two microliter of the obtained cDNAs was used in PCR to amplify a 464 bp fragment corresponding to 28S rRNA of Dipteran (**Table 2**) through Brazil Platinum Taq DNA polymerase (Invitrogen) according to producer's instructions. PCR condition was one cycle of 94°C for 3 min; 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and 72°C for 7 min. To control DNase I treatment, 5.5 μL of untreated RNA was used directly on PCR. Reaction products were subjected to 1.5% agarose gel electrophoresis in 0.5× TBE buffer, after staining with SafeBlue®.

For *Flavivirus* and DENV-specific detection, 5.5 μL of total RNA was used in RT/PCR with MMLV reverse transcriptase from Invitrogen, to amplify a 250 bp nonstructural protein 5 (NS5) fragment, with the oligonucleotides cFD2 and MAMD (**Table 2**), and a 511 bp fragment, corresponding the junction of the capsid protein (C) and the premembrane protein (prM) of DENV encoding region, through primers D1 and D2 (**Table 2**). The reaction conditions for RT reactions were incubation at 42°C for 1 h, followed by reverse transcriptase inactivation at 80°C for 5 min. PCR conditions for cFD2 and MAMD were 94°C for 3 min; 40 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min; and 72°C for 7 min. The same conditions were used with oligonucleotides D1 and D2, except for the association temperature with 50°C. As a positive control of *Flavivirus* amplification, RNA from the Zika Virus and from the vaccine strain of Yellow Fever Virus was used in RT/PCR. PCR products were subjected to 1.5% agarose gel electrophoresis in 0.5× TBE buffer, after staining with SafeBlue®.

2.4 Sequencing and phylogenetic analysis

PCR positive fragments were purified by using the Thermo Scientific GeneJET PCR Purification Kit and sequenced with BigDye 3.1 (Applied Biosystems®) and PCR fragment-specific oligonucleotides, according to manufacturer's instructions. Sequence cycle conditions were 96°C for 1 min; 39 cycles at 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min. After DNA precipitation

Primers	Sequence 5′-3′	Amplicon size	Genomic region	Reference
28SD7forw	AGAGAGAGTTCAAGAGTACGTG	464 bp	28S rRNA (Diptera)	[44]
28SD7rev	TTGGTCCGTGTTTCAAGACGGG			
D1 forw	TCAATATGCTGAAACGCGCGAGAAACC	511 bp	C/prM (DENV)	[45]
D2 rev	TTGCACCAACAGTCAATGTCTTCAGGTTC			
cFD2 rev	GTGTCCCAGCCGGCGGTGTCATCAGC	220–250 bp	NS5 (Flavivirus)	[46]
MAMD forw	AACATGATGGGRAARAGRGARAA			
Oligo dT18	TTTTTTTTTTTTTTTTTT		Poly A tail	

Table 2.
Description of the oligonucleotides used in this study.

with 10% of NaOAc (3 M, pH 5.2), 10% of 1.5 μL of glycogen (1 mg/mL), and two volumes of ethanol, the reactions were pelleted by centrifugation and were washed with 70% of ethanol. The reactions were loaded in an ABI PRISM® 3130XL Genetic Analyzer /HITACHI (16 capillaries). The source and specificity of the obtained sequences were evaluated by BLAST in GenBank [22].

The nucleotide and amino acid partial 226 bp sequence corresponding to the NS5 protein of *Flavivirus* were used to infer the evolutionary history by the Neighbor-Joining method [23]. Phylogenetic trees of confidence value of phylogenetic trees was determined by bootstrap analysis with 1000 replicates, for individual branches [24]. The length of branches was calculated by using the Tamura-Nei method [25] for nucleotides and the Poisson corrected method for amino acid sequences [26]. The coding (first, second, and third codon) and non-coding regions were included in the analysis, and all positions containing gaps and lack of data were discarded only after pairwise comparisons. Phylogenetic analyses were conducted using MEGA4 software [27].

In analysis, partial 16 NS5 sequences of CxFV generated in this study from *Culicidae* mosquitoes from Marilia, São Paulo, were aligned with the corresponding sequences from YFV vaccine strain, Zika Virus, and DENV from serotype 2 and with the sequences deposited in GenBank from CxFV isolated from China in Shanghai (MG602497), Argentina (GenBank KU726615), Africa in Uganda (GenBank GQ165808), and the Brazilian States of Mato Grosso (GenBank KY349933) and São Paulo, and the city of São José do Rio Preto (GenBank KT726939), using the CLUSTAL X [28]. Two alignments were generated to Neighbor-Joining analysis: alignment 1, consisting of nucleotide sequences (226 characters) (S1), and alignment 2, containing amino acid sequences (75 residues) (S2).

3. Results

Agarose gel electrophoresis analysis of the total RNA extracted from 10 specimens of dipteran maintained in 80% ethanol by the nondestructive nucleic acid extraction method described before [21] showed integrity, and an approximately amount of 1 μg/mL, per specimen (**Figure 1**). The presence of RNA was also confirmed by RT/PCR to amplify a 464 bp corresponding to the 28S rRNA from dipteran, after treatment of RNA with DNase I (**Figure 2**). The presence of DNA was observed in all samples (**Figure 2**; DNA). After DNase I treatment, DNA was present in samples 1, 2, 6, and 8, corresponding to PCR products obtained from

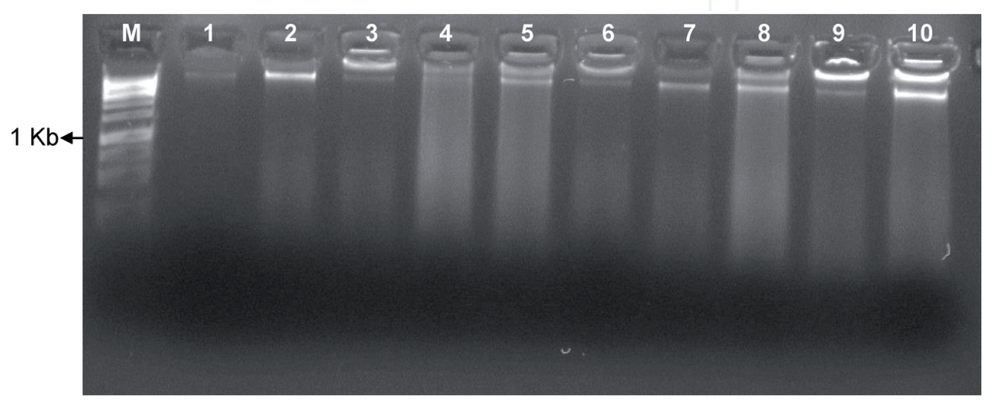

Figure 1.
1.5% Agarose gel electrophoresis containing 4 μL of extracted total RNA from a single dipteran. M, molecular size marker (O'GeneRuler DNA Ladder—1 kb). Lanes 1–10 correspond to different hematophagous dipterans collected in the Municipal Park of the city of Marília—São Paulo.

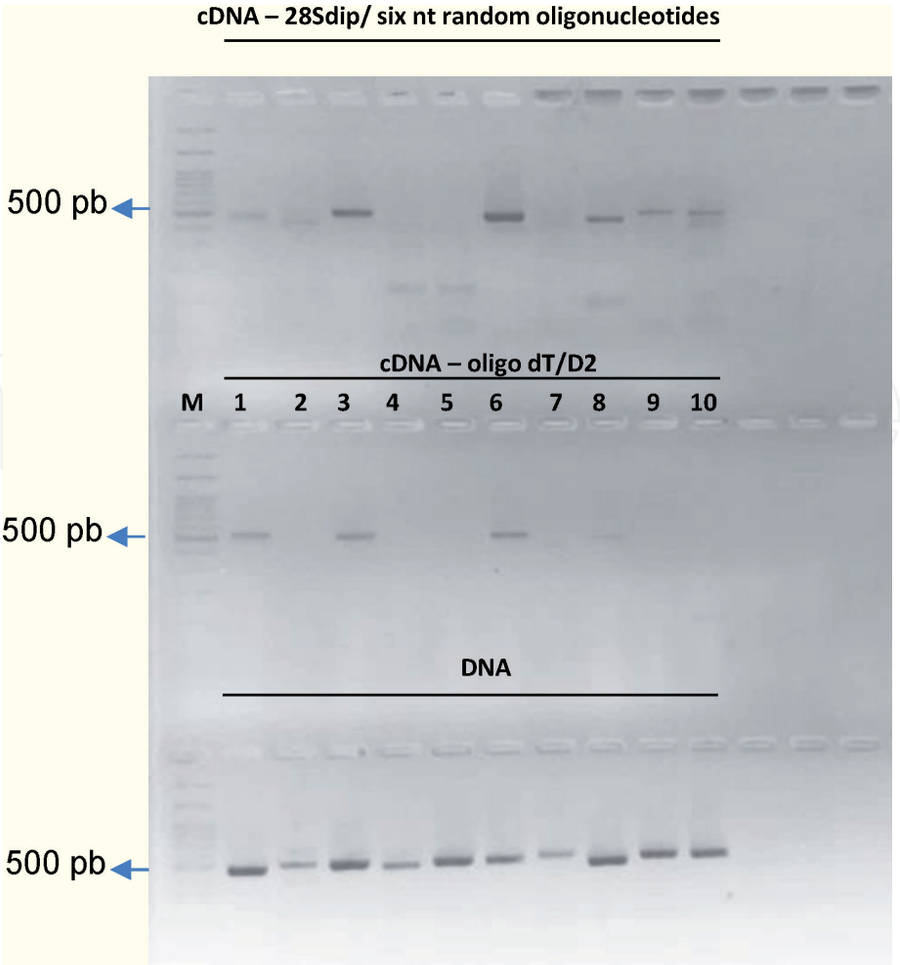

Figure 2.
*1.5% Agarose gel electrophoresis containing 10 μL of PCR performed on cDNA and DNA of the dipteran samples. 28Sdip, 6 nt random oligonucleotides, Oligo (dT), and D2 correspond, respectively, to the gene coding for the 28S fraction of the dipteran rRNA, to six nucleotide random oligonucleotides, to a string of 18 deoxythymidylic acid residues that hybridize, and to the gene encoding the dengue virus prM protein, used in cDNA synthesis by reverse transcription reaction. **DNA**, DNA samples from insects; M, molecular size marker (Sinapse Biotecnologia DNA 100 pb Ladder).*

cDNA synthesized with oligo (dT), which should amplify mRNA and not the 28S rRNA. Specific RNA amplification was observed in samples, 3, 4, 5, 7, 9, and 10, after 28S rRNA PCR performed on cDNA synthesized with random six nucleotides and 28S rRNA-specific oligonucleotides (**Figure 2**).

To verify the feasibility to use the dipteran extracted RNA for *Flavivirus* molecular diagnosis, initially, specimens of *Ae. aegypti* infected with DENV2 and frozen at −20°C, since 2003, were submitted to *Flavivirus* and to DENV-specific diagnostic methods by RT/PCR. **Figure 3** shows that from five *Ae. aegypti* infected with DENV2, four specimens were positive for NS5-based *Flavivirus* detection method. The specificity of the obtained PCR fragments, obtained from each *Ae. aegypti* specimen, was confirmed through Sanger sequencing. The DENV-specific diagnostic based on C/prM encoding gene was negative for all *Ae. aegypti* specimens tested (data not shown).

After *Flavivirus* molecular diagnosis standardization, the NS5-based detection method was applied to the total RNA extracted of the remaining Culicidae specimens included in the study (**Figure 4**), and 19 were positive for a 250 bp PCR fragment, which were sequenced by Sanger method. BLAST evaluation of the 19 amplicons showed 94–96% similarity to CxFV described in Africa, Argentina, China, and Brazil (Cuiabá in the State of Mato Grosso; São José do Rio Preto in the State of Sao Paulo).

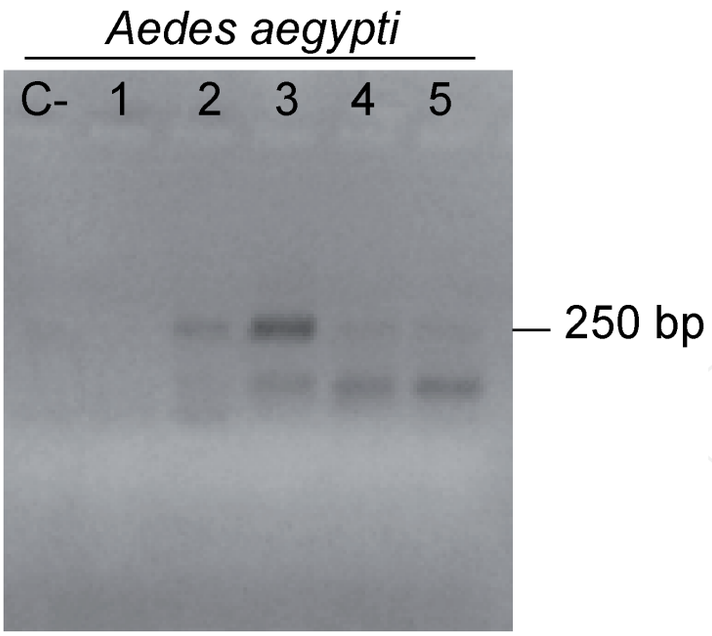

Figure 3.
1.5% Agarose gel electrophoresis stained with SafeBlue® for analysis of the RT/PCR products obtained from Ae. aegypti *infected with DENV-2. C–, PCR negative control performed with water; 1,* Ae. aegypti *uninfected; 2–5, samples of* Ae. aegypti *infected with DENV-2. The PCR reaction was done with primers cFD2 and MAMD, which amplify a 250 bp fragment corresponding to* Flavivirus *NS5 encoding gene.*

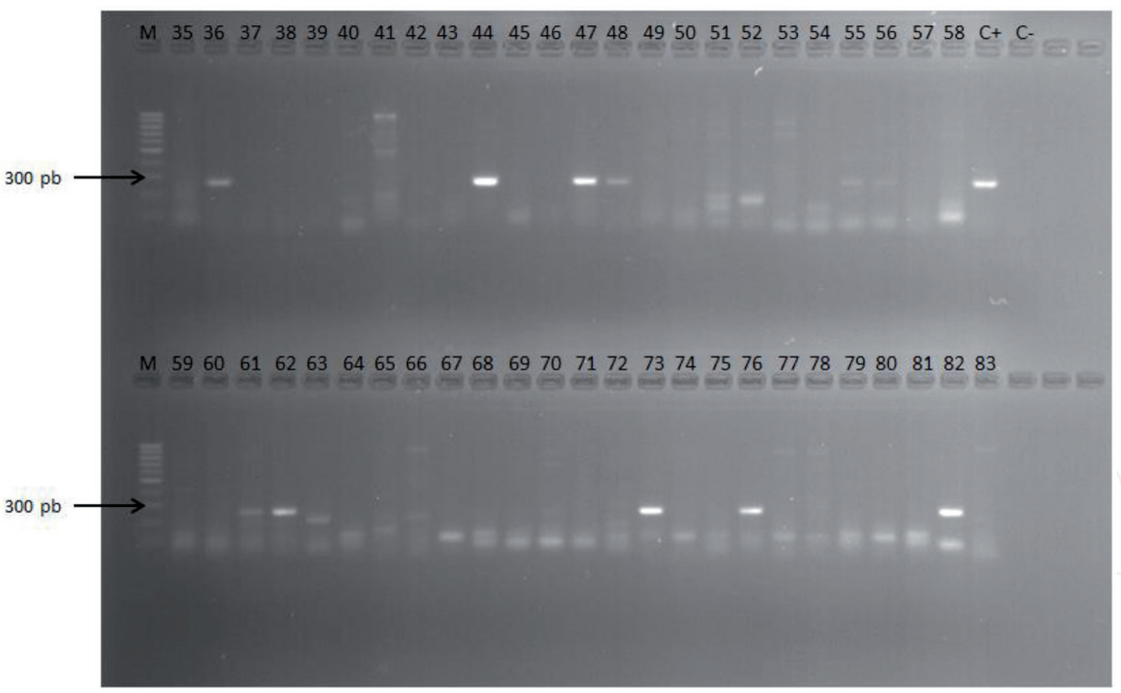

Figure 4.
2% Agarose gel electrophoresis for analysis of Flavivirus *molecular diagnosis based on the 250 bp PCR fragment corresponding to NS5 encoding gene. M, molecular size marker (Sinapse Biotecnologia 100 pb DNA Ladder); C–, negative control (water); C+, positive control (YFV); 35–83, samples of Culicidae mosquitoes collected in the Municipal Park of the city of Marília—São Paulo. Positive samples: 36, 44, 47, 48, 55, 56, 61, 62, 73, 76, and 82.*

Sixteen CxFV nucleotide (226 bp in length) and translated partial NS5 amino acid (75 residues) sequences, with good quality, were used for phylogenetic reconstruction by the Neighbor-Joining method (**Figures 5** and **6**). NS5 nucleotide and amino acidic partial sequence of CxFV obtained from culicids of Marilia

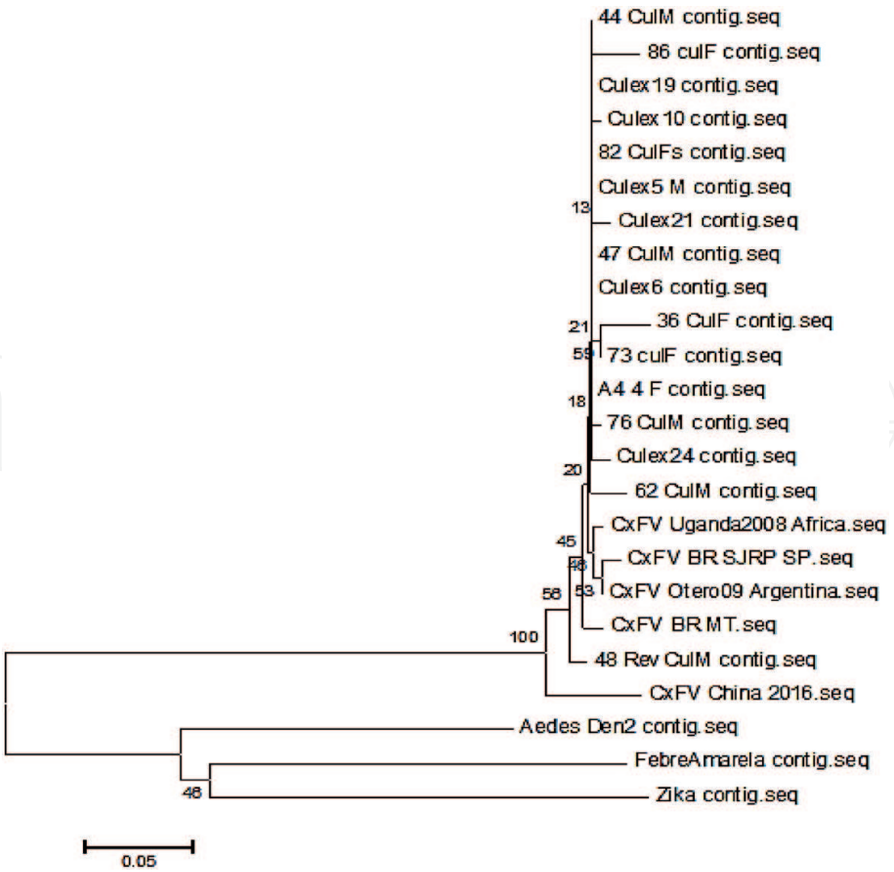

Figure 5.
Phylogenetic analysis based on partial nucleotide sequence of NS5 encoding gene from C. flavivirus*. The figure shows the best tree obtained after Neighbor-Joining phylogenetic analysis with the sum of branch lengths of 1.11310318. The tree was constructed after alignment of 226 nucleotides of 16 translated NS5 partial sequences of* C. flavivirus *obtained from Culicidae mosquitoes from Marilia—São Paulo, YFV vaccine strain, Zika Virus, and DENV2 and sequences recovered from GenBank of* C. flavivirus *isolated from China in Shanghai (MG602497), Argentina (KU726615), Africa in Uganda (GQ165808), and the Brazilian states of Mato Grosso (KY349933) and São Paulo, and the city of São José do Rio Preto (KT726939).*

city varied from 0 to 3% among the same virus species of other Brazilian regions, Africa, Argentina, and China.

The morphological characteristics of 19 CxFV infected dipterans were observed by optical microscopy in order to confirm the taxonomic position and to identify their gender. Nineteen specimens confirmed to belong to the genus *Culex* (Diptera: Culicidae); 9 specimens were females (47.37%), and 10 (52.63%) were males (**Figure 7**). The images were made after RNA extraction, showing the efficiency of the technique in preserving the specimen chitinous skeleton. Blood cells in ingurgitated females are also visible before and after RNA extraction (**Figure 8**).

4. Discussion

The technique of RNA extraction from a single mosquito preserving their chitinous cytoskeleton is described for the first time, and its use can contribute not only to the detection of infectious agents with RNA genome but also to the evolutionary and morphological studies of Diptera since the RNA molecule is an important tool to understand the physiology and evolutionary relationships among organisms [29], and the physical structure of the insect is maintained [21]. Also, vectorial capability of arthropods to several infectious agents can be investigated using specific molecules, with conditional expression profiles, as biomarkers. Molecular identification

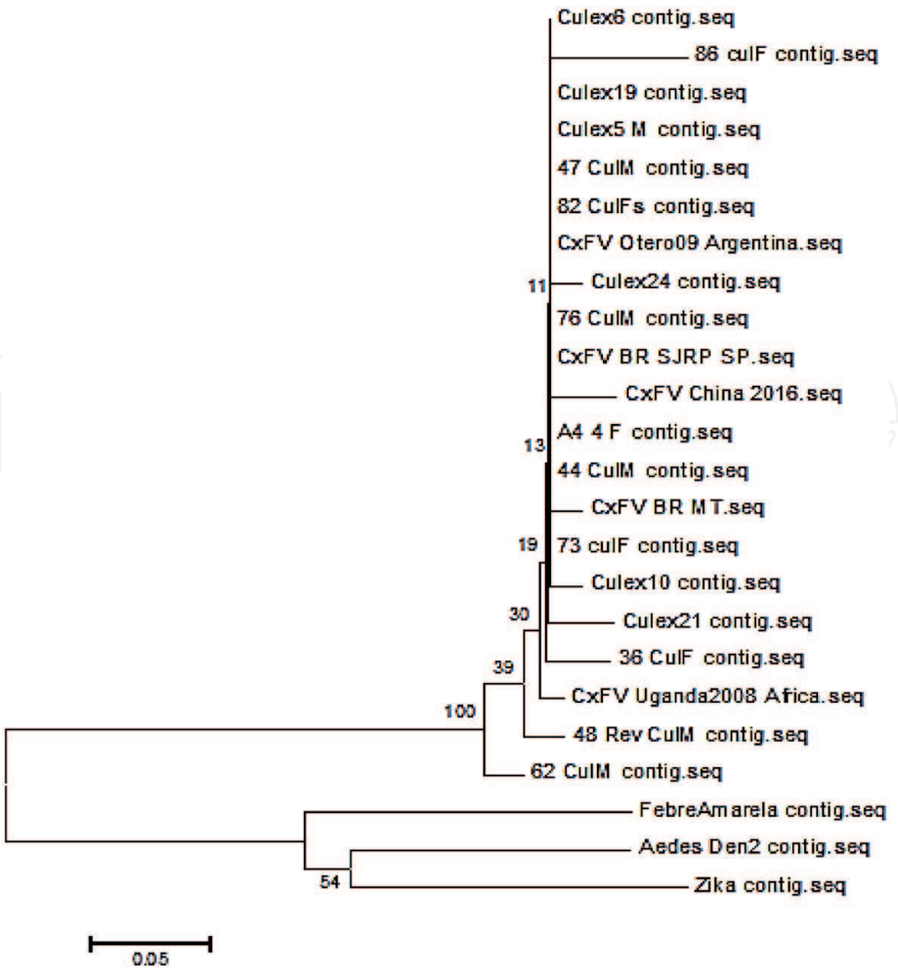

Figure 6.
Phylogenetic analysis based on partial amino acid sequence of NS5 from C. flavivirus*. The figure shows the best tree obtained after Neighbor-Joining phylogenetic analysis with the sum of branch lengths of 0.98879448. The tree was constructed after alignment of 75 amino acids of 16 translated NS5 partial sequences of* C. flavivirus *obtained from Culicidae mosquitoes from Marilia—São Paulo, YFV vaccine strain, Zika Virus, and DENV2 sequences recovered from GenBank of* C. flavivirus *isolated from China in Shanghai (MG602497), Argentina (KU726615), Africa in Uganda (GQ165808), the Brazilian states of Mato Grosso (KY349933) and São Paulo, and the city of São José do Rio Preto (KT726939).*

of the arthropod specimen can be performed by RT/PCR on RNA using oligonucleotides complementary to barcode genes. Also, if DNA is necessary, after proteinase K solution treatment, an aliquot of the arthropod nucleic acid solution can be used for DNA extraction.

Genome of the most important medical arboviruses is composed of RNA, and research works on the detection of these viruses in hematophagous mosquitoes are accomplished on pools of 20–50 specimens, which are macerated and destroyed [30–33]. In this work, a good quality of RNA was obtained from a single dipteran specimen, which was confirmed by electrophoresis in agarose gels (**Figure 1**) and by a specific amplification of a 464 bp fragment of the 28S fraction of diptera rRNA, after DNase I treatment (**Figure 2**). The RNA obtained from a single insect was appropriately to detect DENV-2 in frozen *in vitro* infected *Ae. aegypti* stored for approximately 15 years (**Figure 3**). Probably, the extraction of nucleic acid without maceration of mosquito cells circumvents liberation of high amounts of proteases, which when associated with slow tissue proteinase K digestion preserves the quality of the RNA. These results point toward the possibility to use the presented methodology to investigate undisclosed arthropod vectors through analysis of specific pathogen molecules expressing in the arthropod host. Moreover, since hematophagous dipterans are broadly disseminated, it can also be used as sentinels

Figure 7.
Morphology of sexual dimorphic structures of Diptera from Culicidae mosquitoes infected with C. flavivirus *after RNA extraction. Head (a) and posterior segment of the abdomen (b) of a female; head (c) and posterior segment of the abdomen (d) of a male.*

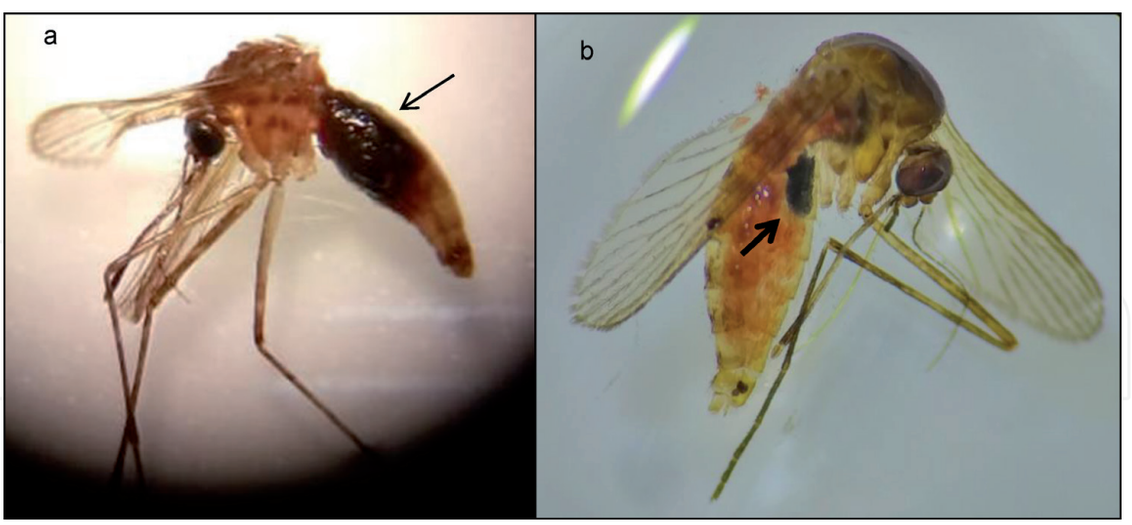

Figure 8.
Evidence of blood in Culicidae ingurgitated female. Two different Culicidae ingurgitated females, before (a) and after (b) RNA extraction. The arrow shows evidence of blood in the abdomen.

of infectious agent presenting RNA genome, contributing directly to epidemiological surveillance of arboviruses.

Different geographical regions present divergences in arthropod infectious diseases vectors diversity and distribution. In Brazil, the main urban vector for YFV is *Ae. aegypti*, whereas in the wild, it is the *Haemagogus janthinomys* [18, 34]. Moreover, a Brazilian study carried out in the Recife city of Pernambuco State revealed the transmission of ZIKV not only by *Culex quinquefasciatus* and but also by *Aedes*

mosquitoes, as traditionally accepted [35]. The single insect RNA extraction method facilitates the identification of arboviruses and also the blood source ingurgitated by a dipteran through molecular techniques, since each individual insect feeds on few vertebrates [32] (**Figure 8**). Furthermore, even after nucleic acid extraction, the taxonomic position of the dipteran can be reevaluated (**Figure 7**).

Flavivirus diagnosis of 100 culicids collected in the city of Marilia, from São Paulo Brazilian State, based on NS5, revealed the occurrence of 19 positive specimens (19%) to CxFV. These viruses are part of the insect-specific flavivirus (ISF) group and have a wide geographic distribution, encompassing tropical and temperate regions, in various insect groups [36]. There is evidence that ISF infection may suppress or raise the rate of *in vitro* and/or *in vivo* replication of medical important flaviviruses [37]. A study conducted with Palm Creek virus (PCV), another ISF, originally found in Northern Australia, demonstrated that previously infected insect cells were suppressed for WNV and Murray Valley Encephalitis Virus replication, two important human arboviruses [38]. Virus replication suppression by ISF is also associated with mosquito strains [39], as demonstrated for WNV infection in two different lineages of *C. quinquefasciatus*. The mosquitoes isolated in Honduras presented increased WNV transmission rate when infected with CxFV. The ISF Nhumirim virus (NHUV), first isolated in the Pantanal region of Brazil, was evaluated in the growth and replication rates of ZIKV in insect cells [40]. They found that both in the previously inoculated cells and in cells coinoculated with NHUV, growth, and replication rates of ZIKV were significantly reduced. Also, in this work, the rates of ZIKV infection in *Ae. aegypti* infected with NHUV were significantly reduced, but the transmission rate was maintained. According to Romo (2018), ISFs can be used as models to understand the mechanisms involved in virus interference infection process, which may be used to reduce or suppress infections of important human pathogens in arthropod virus vectors. The methodology described in this work enables investigations of interactions among medical important flavivirus and ISFs at natural mosquito's population level through the use of field collected specimens.

It is believed that *Culex flaviviruses* may be specific to the culicid species and also to the region from which it is obtained [41]. In order to investigate the specificity of CxFV from Marilia, a phylogenetic reconstruction analysis was performed using homologous partial NS5 sequences of CxFV isolates from China, Africa, Argentina, and two Brazilian isolates (one in Mato Grosso and the other in São José of Rio Preto, São Paulo). The nucleotide and amino acid sequences corresponding to the *Flavivirus* partial NS5 protein varied little among then (about 0–3%). Two Neighbor-Joining-based phylogenetic trees constructed with partial NS5 nucleotide (**Figure 5**) and amino acid (**Figure 6**) homologous sequences showed the clustering of flaviviruses in two main branches, one formed by human pathogens (DENV, YFV, and ZIKV), and the other formed by the CxFV. The first branch is formed by taxa that share the same capacity to infect mammalian vertebrate cells, including humans, and present the *Ae. aegypti* as the main vector [42, 43]. The second branch is formed by organisms that differ in origin and time of collection. However, in the tree obtained with the nucleotide sequences, it is possible to observe a clade formed by all the records obtained from Marília, except for the specimen identified by number 48, which was closest to the CxFV of China. This result was different when the tree was constructed with amino acid sequences, where there was no geographical clustering of CxFV. Perhaps, the molecular marker employed in this work is not appropriate to investigate *CxFV* geographical specificity, and more studies are needed to explore the association of their genetic variability with geographical distribution and/or with *Culex* species and strains.

5. Conclusions

The single dipteran RNA extraction technique described in this work permits the use of hematophagous insects as sentinels to detect arboviruses, preserving the chitinous skeleton of the insect and guaranteeing the subsequent morphological studies. The possibility to obtain RNA from a single dipteran also makes possible the investigation of infectious agent's vector capability and the identification of the ingurgitated blood meal source, enabling the description of arthropod alimentary habit and an indication of which vertebrates may be implicated in a virus life cycle. The method also opens the possibility for constant arbovirus surveillance, which can be used to prevent and control epidemics that affect millions of people each year. The presence of CxFV in *C. flavivirus* vectors may interfere with replication, transmission, and infection rates of arboviruses of medical importance, and the method described facilitates natural population studies.

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Conflict of interest

The authors declare no conflict of interest.

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