Scientific Note

Detection of *Culex flavivirus* (*Flaviviridae*) from a natural *Culex (Culex) chidesteri* Dyar, 1921 population, Caatinga Biome, Semi-arid Scrubland, Brazil

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Abstract. The first detection of *Culex flavivirus* (CxFV) in mosquitoes was described in 2007 in Japan and subsequently in different areas of the Americas, Africa, and Asia. In this study, we report the identification of CxFV in *Culex (Culex) chidesteri* Dyar, 1921 mosquitoes collected in the Açu National Forest - FLONA/ Açú-RN, a preserved area of Biome Caatinga, State of Rio Grande do Norte, Brazil. We performed nucleotide-sequencing analysis in order to compare with other previously identified CxFV collected from around the world. A total of 129 samples (pools of ≤30 female mosquitoes) were processed for generic reverse transcriptase PCR assay. CxFV infection was identified in only two pools from *Culex chidesteri*. Our phylogenetic analysis revealed that the AssuCxFV identified in this study belongs to the Africa/Caribbean/South America Subtype. Our study represents the first report of the CxFV from a natural *Culex chidesteri* population.

Keywords: Arbovirus; *Flaviviridae*; Mosquitoes, Açu National Forest, Culicidae.

According to the IXth Report of the International Committee on Taxonomy of Viruses, the genus *Flavirus* (family *Flaviviridae*) contains 53 species of which 40 are known to cause human disease. *Flaviviruses* are small (~50 nm), enveloped, single-strand positive-sense RNA viruses. Phylogenetic analysis has classified the genus *Flavirus* into mosquito-borne, tick-borne and viruses that are insect specific and do not infect vertebrates (Kuno & Chang 2005; Goenaga et al. 2014). This final group includes cell fusion agent virus (Stollar & Thomas 1975), *Culex flavivirus* (Hoshino et al. 2007), *Aedes flavivirus* (Hoshino et al. 2009), *Nakiwogo virus* (Cook et al. 2009), *Chaoyang virus* (Wang et al. 2009) and others.

*CxFV* was described and isolated for the first time from *Culex pipientis* s.l. Linnaeus, 1758 in 2007 in Japan and subsequently in different areas of the Americas (Hoshino et al. 2007; Kim et al. 2009; Machado et al. 2012; Goenaga et al. 2014; Moraes et al. 2019), Africa (Cook et al. 2009), and Asia (Chen et al. 2013). In this study, we report the identification of CxFV from *Culex (Culex) chidesteri* Dyar, 1921 mosquitoes collected in the Açu Nacional Forest - FLONA/ Açú-RN, a semiarid scrubland preserved area of Caatinga Biome, State of Rio Grande do Norte, Brazil. Additionally, we perform the nucleotide sequencing analysis in order to compare with other previously identified CxFV collected worldwide.

This work was performed in FLONA/ Açú-RN, located in the southwest of the urban site of Assu (05°34′20″S, 36°54′33″W), in the central region of the state of Rio Grande do Norte), an area of 215.25 hectares, with a perimeter of 6766.30 meters (Fig. 1). Mosquitoes were sampled monthly in the twilight and in the beginning of the night periods (17h-20h) between September 2011 to August 2013 using a Shannon trap, which consisted of a large central compartment and two smaller lateral ones with a central light. Mosquitoes were killed by CO2, freezing, transferred to collection tubes, and stored at -70°C until identification and pooling. Adult mosquitoes were identified by their morphological characteristics and pooled according to species, sex, location, and date. The identifications were carried out with the morphological keys of Forattini (2002) and the classification adopted for Aedini tribe was proposed by Wilkerson et al. (2015). Pools of ≤30 female mosquitoes (for each species) were macerated by using plastic pistils in 500 μL of Leibowitz L15 medium (GIBCO-BRL, Gaithersburg, MD, USA) containing 2% fetal bovine serum, and centrifuged at 2500×g for 20 min at 4°C, to pellet the carcasses. The supernatant was split into 2 aliquots and stored at -70°C until use. Viral RNA was extracted from 140 μL original suspension of mosquitoes by the QiAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA), in accordance with the manufacturer’s suggested protocol. RNA was eluted in 60 μL of buffer AVE.

The generic RT-PCR protocol for *Flavivirus* detection was used for testing these RNA extracts as described previously by Sánchez-Seco et al. (2005). Degenerated primers were designed based on conserved motifs in a region of the NS5 gene. Positive controls tested for dengue virus serotypes 1, 2, 3 and 4 (obtained from Laboratory of Molecular Biology for Infectious Diseases and Cancer, UFRN), and yellow fever virus (17D) (obtained from Biomanguinhos, Fiocruz, Brazil). Products from the second round of generic amplification were purified by using the PCR purification kit or gel extraction kit (Qiagen, US). The quantification of the purified DNA was performed by electrophoresis on a 2% agarose gel, using the kit “low mass DNA” (Invitrogen, Carlsbad, CA). Sequencing reactions on both strands were performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, US), and analyzed by using an ABI Prism 3730 Sequencer (Applied Biosystems, US). Electropherograms were visualized by Chromas...
The identities of nucleic acid and deduced amino acid (in bold) sequences of CxFV partial NS5 gene.

|   | Africa/Caribbean/Latin America | Asia/U.S. |
|---|-------------------------------|-----------|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | R33 CxFVAssu RN Brazil 2013 | - | 100 | 91.8 | 91.8 | 91.8 | 91.8 | 91.8 | 91.8 | 83.6 |
| 2 | R34 CxFVAssu RN Brazil 2013 | 100 | - | 91.8 | 91.8 | 91.8 | 91.8 | 91.8 | 91.8 | 83.6 |
| 3 | GQ165808 CxFVUganda 2008 | 91 | 91 | - | 100 | 100 | 100 | 100 | 100 | 89.7 |
| 4 | EU879060 CxFVMexico 2007 | 91 | 91 | 98.6 | - | 100 | 100 | 100 | 100 | 89.7 |
| 5 | JX979704 CxFV Taiwan 2010 | 91 | 91 | 95.8 | 95.8 | - | 100 | 100 | 100 | 89.7 |
| 6 | HQ634596 CxFV US 2009 | 89.7 | 89.7 | 94.5 | 94.5 | 95.8 | - | 100 | 100 | 89.7 |
| 7 | AB377213 CxFV Japan, 2003 | 89.7 | 89.7 | 94.5 | 94.5 | 95.8 | 100 | - | 100 | 89.7 |
| 8 | AB701766 CxFVToyama 2004 | 88.3 | 88.3 | 93.1 | 93.1 | 95.8 | 98.6 | - | 89.7 |
| 9 | GQ165809 Nakiwogovirus Uganda2008 | 76 | 76 | 80.8 | 80.8 | 78.7 | 80.8 | 80.8 | 81.5 | - |

Other insect-specific flaviviruses have been identified in Brazil. Kenney et al. (2014) isolated a novel flavivirus (designated ‘Nhumirim virus’; NHUV) that represents an example of a unique subset of apparently insect-specific viruses that phylogenetically affiliate with dual-host mosquito-borne flaviviruses despite appearing to be limited to replication in mosquito cells. In another study, Pauvold-Corrêa et al. (2015) describe the isolation of NHUV, isolated from a pool of mosquitoes identified as Culex (Culex) chidesteri collected in 2010 from the Pantanal region of central west Brazil.

In this study, the phylogenetic tree based on partial NS5 gene sequences revealed that the AssuCxFV belongs to Africa/Caribbean/Latin America subtype (also called genotype 2), closely related to Uganda, Mexico, and Taiwan isolates. In the same way, Chen et al. (2013) show that the Taiwan isolates are closely related to the Africa/Caribbean/Latin America subtype, although they form an independent
cluster, based on full-length, E gene, NS3 gene, or NS5 gene sequences. Must flaviviruses can be classified into multiple genotypes/subtypes/lineage using the criteria of 6% nucleotide variation between genotypes (Kuno et al. 1998; Chen et al. 2013). In our study, the nucleotide distance between Africa/Caribbean/Latin America and Asia/U.S. subtypes range from 4.2% to 11.7%. Additional studies including those isolated from other parts of the world are essential for a better understanding of the evolutionary history of CxFV.

Figure 2. Maximum-likelihood (ML) phylogenetic tree of 37 flavivirus sequences (36 CxFV and 1 Nakiwogo virus - outgroup) using 143 nucleotides from the NS5 region. New insect flavivirus isolated from Culex flavivirus (Diptera: Culicidae) in Brazil. Characterization of a novel insect-specific flavivirus from Brazil: potential for inhibition of infection of arthropod cells with medically important flaviviruses. Journal of General Virology, 95: 2796-2808. doi: 10.1099/vir.0.068031-0

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Authors’ Contributions

Conceived and designed the project: RLC, RAG, JMGA. Collected field samples: RAG, RCMF, MSDB. Performed the lab work: JMGA, RCMB, DMPC, DMCS. Conceived and designed the project: RLC, RAG, JMGA. Collected field samples: RAG, RCMF, MSDB. All authors read and approved the final version of the manuscript.

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