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Natural infection by Culex flavivirus in Culex quinquefasciatus mosquitoes captured in Cuiabá, Mato Grosso Mid-Western Brazil

O. S. MORAES1, B. F. CARDOSO1, T. A. PACHECO1, A. Z. L. PINTO1, M. S. CARVALHO1, R. C. HAHN1, T. C. T. BURLAMAQUI2, L. F. OLIVEIRA2, R. S. OLIVEIRA2, J. M. VASCONCELOS2, P. S. LEMOS2, M. R. T. Nunes2 and R. D. SLHESSARENKO1

1Programa de Pós-Graduação em Ciências da Saúde, Faculdade de Medicina, Universidade Federal de Mato Grosso, Cuiabá, Brazil and 2Centro de Inovação Tecnológica, Instituto Evandro Chagas, Ministério da Saúde, Ananindeua, Brazil

Abstract. New species of insect-specific viruses (ISV) have been reported worldwide. In the present study, the complete genome of Culex flavivirus (CxFV) and partial sequences of other ISVs in Culex quinquefasciatus Say 1823 females (n = 3425) sampled in 200 urban areas census tracts of Cuiaba, state of Mato Grosso, were identified via reverse transcriptase-polymerase chain reaction for a NS5 region of flaviviruses, nucleotide and high-throughput sequencing, and viral isolation in C6/36 cells. CxFV was detected in 16 of 403 mosquito pools; sequences found in the study presented a high similarity with isolates from São Paulo, Brazil and other countries in Latin American that belong to genotype II, supporting the geographical influence on CxFV evolution. The monthly maximum likelihood estimation for CxFV ranged from 1.81 to 9.94 per 1000 mosquitoes. In addition to the CxFV complete genome, one pool contained an ORF1 sequence (756bp) that belongs to a novel Negevirus from the Sandewavirus supergroup most similar to the Santana virus (77.1%) and another pool presented an RNA-dependent RNA polymerase sequence (1081bp) of a novel Rhabdovirus most similar to Wuhan mosquito virus 9 (44%). After three passages in C6/36 cells, only CxFV was isolated from these co-infected pools. The importance of ISVs relies on their possible ability to interfere with arbovirus replication in competent vectors.

Key words. Culex flavivirus, high-throughput sequencing, Negevirus, Rhabdovirus.

Introduction

High-throughput sequencing (HTS) technologies have contributed to virus identification and to more accurate taxonomic and evolutionary classifications, leading to the discovery of a wide variety of novel viral species, including several insect-specific viruses (ISVs) (Bolling et al., 2015; Nouri et al., 2018).

ISVs replicate only in mosquito cells. Their pathogenesis in mosquitoes is largely unknown (Vasilakis et al., 2013; Nunes et al., 2015). Collectively, their discovery has opened new views on the extent of viral diversity and evolution because they appear to be ancient viruses compared with arboviruses classified within the same viral families (Vasilakis & Tesh, 2015). Their influence over vector competence to transmit human pathogens (e.g. arboviruses), as well as their potential development as...
biological control agents or novel vaccine platforms, has boosted studies involving ISV metagenomics (Bolling et al., 2015).

A growing number of ISVs have been described, with most of them being detected in arbovirus surveillance studies (Vasilakis & Tesh, 2015).

Culex flavivirus (CxFV) has been isolated from Culex pipiens Linnaeus 1758 complex in several countries (Hoshino et al., 2007; Moralles-Betoulle et al., 2008; Cook et al., 2009). In Brazil, this virus was previously identified in mosquitoes from the cities of São José do Rio Preto and São Paulo, both in the state of São Paulo (Machado et al., 2012; Fernandes et al., 2016).

An increasing number of non-flavivirus ISVs have been described in field-collected mosquitoes, indicating that these agents are surprisingly more abundant, ancient, diverse and widely distributed than previously predicted and implicated as a probable origin of some arboviruses (Vasilakis & Tesh, 2015).

The present study investigated the presence and phylogenetic relationships of CxFV and other ISVs in naturally infected adult females of Cx. quinquefasciatus Say 1823 captured in Cuiaba, capital of Mato Grosso (MT), Mid-Western Brazil.

Materials and methods

Study area and Culicidae capture

Cuiaba is located in the middle-south region of MT, with an estimated population of 575,480 inhabitants living in 173 neighbourhoods distributed into four administrative regions (midwestern, middle-eastern, southern and northern). These are further subdivided into 804 census tracts, each representing 250–350 residents (IBGE, 2011). Characterized by dry (April to September) and rainy (October to March) seasons and mean temperatures ranging from 21.4 to 32.8°C, the climate is tropical (i.e. hot and humid). For the collection period, an average maximum temperature of 32.45°C and an average humidity of 77.8% were recorded (IBGE, 2011; INMET, 2014).

Among 804 census tracts, 200 were randomly selected for adult culicidae capture between January and April 2013 using Nascia aspirators and hand nets (13.00 to 17.00 hours). For census tract definition, the first sector and fourth sectors were selected, leaving out the middle three sectors (Fig. 1). Three locations of the central street were randomly selected and sampled for at least 30 min in intra- and peri-residential areas of each census tract.

Specimens (n = 11,090) (Fig. 1) were transported under controlled temperature and humidity conditions, with 10% sucrose feeding, and were identified alive when in a dormant state (4 min at 4°C), with determination of species level using a dichotomous key (Forattini, 2002). Mosquitoes were pooled (1–20 mosquitoes) according to collection date, local, species and sex. Although morphological identification of mosquitoes by dichotomous key is a useful method, the identification is inconsistent for Cx. pipiens complex species. Thus, pools initially identified as Cx. p. p. pipiens and Culex sp. Linnaeus 1758 (n = 180) were additionally confirmed by an
acetylcholinesterase gene region polymerase chain reaction (PCR) of *Culex quinquefasciatus* Say 1823 (Smith & Fonseca, 2014).

**Total RNA extraction and reverse transcriptase (RT)-PCR for flaviviruses**

In total, 610 pools of culicidae females were obtained, comprising 14 species of eight genera (Serra et al., 2016). Among these 610 pools, 403 *Cx. quinquefasciatus* pools were macerated and eluted into 800 μL of RNase-free phosphate-buffered saline and centrifuged at 5500 g for 4 min at 4 °C; 400 μL of the supernatant was subject to total RNA extraction with Trizol LS reagent (Invitrogen, Carlsbad, CA, U.S.A.). RNA was immediately subjected to a RT-PCR protocol for a NS5 gene region (958 bp) of flavivirus genus (Bronzoni et al. 2005; Serra et al., 2016). These products were visualized via 1.5% agarose gel electrophoresis; positive samples were purified with 20% polyethylene glycol 8000 (Promega, Madison, WI, U.S.A.), quantified with specific kits (Quantus Fluorometer; Promega) and subjected to nucleotide sequencing (3500 Genetic Analyzer; Applied Biosystems, Foster City, CA, U.S.A.).

**Virus isolation**

Supernatant of macerated pools positive for CxFV (*n = 16* pools), *Rhabdovirus* (pool #549) and *Negevirus* (pool #1256) were inoculated at a dilution of 1:10 in L-15 medium into C6/36 cells (ATCC CLR-1660). These monolayers, cultivated in L-15 medium supplemented with 5% fetal bovine serum and incubated at 28 °C with 5% CO₂, were monitored daily for 7 days. Three passages were performed and the supernatant was harvested and stored at −80 °C. The monolayer of passages 2 and 3 was subjected to total RNA extraction with Trizol LS (Invitrogen) and followed by a second round of HTS.

**Sequence analysis and genome assembly**

Nucleotide sequences of flavivirus NS5 region (*n = 16*) were analysed, aligned and compared with reference sequences available at BLASTn and BLASTp (Nucleotide Basic Local Alignment Search Tool; NCBI, PubMed) using geneious r10 (https://www.geneious.com).

Contigs obtained after HTS from total RNA of nine *Cx. quinquefasciatus* pools and from two isolates (#549 and #1256) were compared with viral sequences in the NCBI database using the BLASTx algorithm after de novo assembly using MIRA 4.0.2 (http://www.chevreux.org/projects_mira.html) and geneious r10.

Nucleotide sequences obtained in the present study were deposited in GenBank database (accession numbers: KP764769-KP764781, KY349933, KY401153, KY361743, KY435948 and MG682011). The alignment of our sequences and reference sequences available on GenBank (NCBI) was generated with MAFFT (Geneious R10). Subsequently, phylogenetic analysis of the CxFV complete genome, RNA-dependent RNA polymerase (RdRp) region of *Rhabdovirus* and ORF1 region of *Negevirus* was inferred via Bayesian methodology (MrBayes, version 3.2.6; geneious r10) (Huelsenbeck & Ronquist, 2001). This method is justified given that the posterior probability of a given node on inferred topology has an explicit interpretation that is proportional to its presence in the true phylogeny. Phylogenetic trees and genomic maps were edited using figtree, version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree) and ILLUSTRATOR CC (Adobe Master Collection CS5; Adobe Systems Incorporated). Conserved protein domains, cysteine residues and N potential glycosylation sites were also assessed using the web CD-search tool (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi), geneious r10 and netNGlyc, version 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc).

**Data analysis**

The digital mesh of the census tracts of Cuiabá provided by the IBGE was plotted using arcmap (ESri ArcGIS; http://www.esri.com/software/arcgis). Data on mosquito distribution and infection were plotted in maps of spatial distribution. Data from mosquito collections were analysed according to census tract, species, sex, number of specimens and ISV positivity. Sizes of the pools varied (1–20 mosquitoes).

Maximum likelihood estimation (MLE) was calculated using the PooledInfRate v4 EXCEL (Microsoft Corp., Redmond, WA, U.S.A.) add-in with a confidence interval of 95% (Biggerstaff, 2007). The positivity of these pools for ISVs was compared with their positivity for Alphavirus, Flavivirus and Orthobunyavirus as identified in previous studies (Cardoso et al., 2015; Serra et al., 2016).
Results

*Culex flavivirus natural infection in adult Cx. quinquefasciatus females*

Among 403 Cx *quinquefasciatus* pools, 16 (4%) were positive for CxFV (Table 1). Two pools, #549 and #1256, presented the envelope sequence and the complete genome of CxFV, respectively. CxFV was isolated during the second passage in C6/36 cells (pool #1256), which contained two non-engorged females respectively. CxFV was isolated during the second passage in C6/36 cells.

CxFV sequences obtained in the present study presented 98.1–99.5% nucleotide identity with those of the virus isolated from *Culex* spp. in São José do Rio Preto, SP, Brazil (BR_SJRJ_01_CxFV_BR_MT) and in Argentina (Chupali 323), as well as with those from *Cx. quinquefasciatus* in Guatemala (TR3116) and Trinidad and Tobago (TR3115).

In total, 3465 *Cx. quinquefasciatus* females were captured and grouped in 403 pools out of which 57 (n = 642 females) were obtained in January, 86 (n = 405) in February, 127 (n = 1292) in March and 133 (n = 1126) in April. The MLE values and respective confidence intervals of CxFV, expressed as the number of infected mosquitoes per 1000 *Cx. quinquefasciatus* females along the collection months, were 7.90 (3.00–16.74) in January, 9.94 (3.27–23.13) in February, 3.98 (1.49–8.64) in March and 1.81 (0.32–5.85) in April. As a result of the large variation in confidence intervals, these results were not statistically significant.

CxFV complete genome sequences detected in Cuiabá formed a cluster with genotype II, which includes other Latin American isolates (Fig. 2B).

In addition, eight of 16 (50%) CxFV-positive *Cx. quinquefasciatus* pools, comprising 1–16 mosquitoes, were also positive for Dengue 4 virus (DENV-4) and two of 16 (12.5%) for the segment S of Oropouche virus (OROV), identified in previous studies (Table 1) (Cardoso et al., 2015; Serra et al., 2016).

The CxFV complete genome exhibited 97–99% identity at amino acid and nucleotide levels with other sequences deposited in GenBank and 97–100% identity among those included in the same cluster (Table 2). The CxFV complete genome presents 10,640 nucleotides in length with an open reading frame flanked by a 5′ and a 3′ UTR (29 and 152 amino acids, respectively). The gene CDS spans a region of 10,092 nucleotides and the deduced polyprotein contains 3,363 amino acids, along the structural and non-structural proteins can be identified (Fig. 2A).

The anchored capsid protein C has 137 amino acids, the capsid protein alone has 117 amino acids, the pr protein presents 83 amino acids and the membrane glycoprotein M has 59 amino acids.

Protein domains were identified in three non-structural viral proteins of CxFV genome. The NS5 protein presented two conserved domains: the Flavi_NS5 superfamily (PSSM-ID 279336), which is the flavivirus RNA dependent RNA polymerase that possesses a number of short regions and motifs homologous to other RNA polymerases, and the FtsJ-like methyltransferase (PSSM-ID 307718) in the N terminus of
Fig. 2. Genomic map conserved protein domains (A) and phylogenetic tree (B) via Bayesian estimation method for Culex flavivirus (CxFV) complete genome (CxFV_BR_MT_CbaArt1256p2/2013) identified in Culex quinquefasciatus in Cuiaba, Mato Grosso. CxFV sequences obtained from Culex pipiens are indicated in green, Cx. quinquefasciatus in yellow, Culex tritaeniorhynchus in green, Culex restuans in pink, Cx. interrogator in red, Culex spp. in grey and Anopheles sinensis in purple. (C) Potential N-glycosylation sites. [Colour figure can be viewed at wileyonlinelibrary.com].

NS5 protein, which is involved in viral RNA capping. The P-loop containing nucleoside triphosphate hydrolase (PSSM-ID 328724) is a member of the P-loop NTPase domain superfamily involved in diverse cellular functions, divided into KG (kinase GTase), ASCE ATPases and ATPases associated with a wide variety of activities (AAA) (Fig. 2A).

The conserved flavivirus domain flavi_NS1 superfamily (PSSM-ID 279316; 360 amino acids) presents 12 cysteine residues in the NS1 protein and undergoes glycosylation in a manner similar to other NS proteins.

The peptidase S7 superfamily domain (PSSM-ID 307204; 130 amino acids) was found on the NS3 serine protease gene. This protein, with NS2B as a cofactor, is responsible for processing the polyprotein precursor into mature proteins.

The helicase superfamily c-terminal (HELICc) domain (PSSM-ID 238034; 131 amino acids) was found between NS3 and NS4A and is characteristic of helicases and helicase related proteins (Fig. 2A).

Prediction of GC contents was estimated at 53%. Moreover, the presence of TGT/TGC codons predicts the existence of 176 cysteine residues in CxFV genome. In addition, 12 potential NGlyc sites were also identified (Fig. 2C).

Other ISVs partial sequences identified in Cx. quinquefasciatus pools

After HTS, pool #1256 presented a region (756 bp) of ORF1 encoding the 3' end of the viral helicase (VHEL) gene and the S' end of the RdRp gene of a Negevirus (Fig. 3) 77.1% similar to Santana virus, obtained from Culex sp. in Amapá, Brazil.

Phylogenetic analysis demonstrated a relatively low amino acid similarity (75.3–80.72%) between the Negevirus identified in the present study and the other six negeviruses clustering in the same branch, supported by high bootstrap values. The Sandewavirus clade includes the Wallerfield, Goutanap, Dezidouguo,
Table 2. Nucleotide identities of the Culex flavivirus complete genome and envelope gene sequences of genotype II isolates.

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Biratnagar, Tanay and Santana viruses and the negevirus identified in the present study, named Siriri virus. Using BLASTp, the identity with the closest viruses varied between 52% Tanay virus and 58% with Dezidougou virus, with these data confirming that this sequence belongs to a new negevirus (Fig. 3).

HTS also provided a Rhabdovirus RdRp partial sequence (1081 bp) from pool #549, with 32.9% identity and 53.3% amino acid similarity with Wuhan mosquito virus 9, obtained from *C. tritaeniorhynchus* Giles 1901 in China. Phylogenetic analysis demonstrated an evolutionary relationship with other rhabdoviruses isolated from insects not assigned to any of the existent genera. Identity (19–32.9%) and amino acid similarity (48.6–53.3%) with other rhabdoviruses included in the same branch, such as Iye green virus, *Drosophila busckii* rhabdovirus, jingshan fly virus, shuangao bebug virus and Wuhan mosquito virus, were relatively low and therefore, support the hypothesis that this also represents a novel virus, named Cururu virus (Fig. 4).

After viral isolation, only the CxFV complete genome was recovered by HTS from pool #1256p2, although these pools were co-infected with a new Negevirus. Further attempts to achieve the complete genome sequences of these viruses were unsuccessful.

**Discussion**

ISVs comprise a large and diverse group of viruses that evolved parallel to their hosts. The present study reports the first identification of the CxFV complete genome in naturally infected *C. quinquefasciatus* females in Mato Grosso, Middle-West Brazil, during the rainy period, when arbovirus outbreaks are common in the area.

CxFV-positive pools were also positive for DENV-4 and OROV (Table 1) and two pools presented a putative novel Rhabdovirus and a Negevirus, named Cururu and Siriri viruses, respectively (Table 1). Although the biological importance of dual infections by ISVs is largely unknown, superinfection and co-infection by arboviruses or arboviruses and ISVs is relatively common in their hosts.

*Culex quinquefasciatus* was identified as naturally infected and is a competent vector for arboviruses, especially to Flavivirus (Hoch et al., 1987; Segura & Castro, 2007; Heinen et al., 2015; Serra et al., 2016).

Mosquito-borne, tick-borne and unknown vector flaviruses are placed in a phylogenetic sister group to the classical ISVs within the *Flavivirus* genus, which includes CxFV (Fernandes et al., 2016). Studies have shown that prior infection by CxFV can suppress the replication of dual-flavivirus infections in mosquito cell lines (Kenney et al., 2014; Bolling et al., 2015). This observation implies that CxFV and other ISVs could potentially inhibit the replication of medically important arboviruses in arthropods.

It is possible that the coevolution between those insect-specific flaviruses, such as CxFV, and their mosquito hosts could favour immune evasion or suppression that would otherwise interfere with the replication of ISV or other subsequently-infecting viruses (Kent et al., 2010), CxFV
suppress West Nile virus replication in mosquitoes up to 7 days post-infection and this potential defence mechanism could include an activated mosquito innate immune response or competition for essential cellular factors (Bolling et al., 2012).

Genetically, CxFV represents a stable group that evolved over many years, in a habitat-dependent manner, with evident trans-mission between various mosquito species (Liang et al., 2015). This virus has been described in several Culex spp. (Newman et al., 2011; Machado et al., 2012), Anopheles sinensis Chow 1950 and Aedes vexans Meigen 1830 (Liang et al., 2015) in several countries, which suggests this virus may infect different sympatric species and is widely distributed.

Culex quinquefasciatus MLE values ranged from 1.81 to 9.94. The month with highest MLE was February and the lowest MLE occurred in April, 2013. Studies have shown a high CxFV annual infection rate in Culex spp. in several countries, although, in certain areas, CxFV activity is seasonal (Blitvich et al., 2009; Kim et al., 2009; Farfan-ale et al., 2010). The similarity of the CxFV genotype II identified in the present study with others obtained from Culex mosquitoes in Brazil and in other American countries confirms the hypothesis that this virus is disseminated across North and Latin America. Furthermore, studies support the concept that geographical location plays a role in evolution of CxFV (Machado et al., 2012) because this genotype is primarily composed of isolates obtained from Cx. quinquefasciatus in tropical regions (Bittar et al., 2016). Moreover, the cysteine residues and potential glycosylation sites found in CxFV sequences in the present study are associated with genomic secondary structure maintenance, pathogenicity and viral infectivity in their hosts (Murrell et al., 2004; Nunes et al., 2017).

Negevirus ORF1 (756 bp) phylogenetic analysis revealed a major split in two groups, named Neloripivirus and Sandewavirus, with a different ancestral origin, corroborating previous observations indicating that geography may also play a role in Negevirus evolution and sub-speciation (Nunes et al., 2017). Santana and Siriri viruses formed a cluster inside Sandewavirus subgroup, very close to other negeviruses identified in mosquitoes worldwide (Vasilakis et al., 2013; Kallies et al., 2014).

Relatively high infection rates in biting Diptera indicate that negeviruses are more diverse and abundant than previously known. These viruses are more related to some plant viruses (Fig. 3) and were described in mosquitoes and sand-flies, with identification in Aedes larvae demonstrating that these
viruses may be acquired by transovarian route (Nunes et al., 2017).

Cururu virus clustered with other unclassified Rhabdovirus, such as Wuhan mosquito virus 9, Shuangao bedbug virus 2, Sanxia water strider virus 5, Jingshan fly virus 2, Drosophila buzzatii rhabdovirus and Lyce green virus, all isolated from insects. These isolates represent a clade sister to the Varicosavirus, Cytorhabdovirus and Nucleorhabdovirus clades that comprise plant isolates. RdRp or L protein of the Rhabdovirus represent a conserved region useful for distant evolutionary relationships of this family because few complete genome sequences of rhabdoviruses are available to date. These sequences could be very divergent among rhabdoviruses, although there is sufficient similarity to make a phylogenetic analysis (Bourhy et al., 2005). Viral sequences from arthropods, vertebrates and plant form distinct clusters in the phylogeny (Bourhy et al., 2005; Vasilakis et al., 2014; Charles et al., 2016).

Several closely related ISVs belonging to Rhabdoviridae comprise a phylogenetically divergent group and a major split between those infecting arthropods and other hosts (Bourhy et al., 2005; Vasilakis et al., 2014; Charles et al., 2016). This major phylogenetic division between Rhabdoviridae genera indicates that their biology is strongly influenced by mode of transmission and host species, as observed for other RNA viruses (Bourhy et al., 2005).

Studies have suggested that ISV infections could result in changes in the immune response or in the silencing of mosquito genes involved in the regulation of reproduction, longevity and viral pathogenesis, resulting in a selective pressure favouring the coevolution of mosquitoes and pathogens (Newman et al., 2011; Kenney et al., 2014). Thus, these agents might be studied as potential mosquito population control strategies that interfere with the replication and dissemination of arboviruses (Bolling et al., 2015).

The findings of the present study emphasize the diversity of ISVs, highlighting the importance of metagenomic studies for the discovery of new viruses and viral variants, as well as for obtaining more accurate and complete genomic sequences via evolution and phylogenetic characterization.

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