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Assessing natural infection of Zika virus in the southern house mosquito, *Culex quinquefasciatus,* during 2016 in Puerto Rico

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Abstract

The epidemic of Zika in the Western hemisphere has led to intense investigation of all species important in its transmission cycle, including the putative mosquito vectors. Although evidence points to *Aedes* (*Stegomyia*) mosquitoes as the primary vectors in nature among humans, there remains the possibility that other common mosquito species could be implicated in explaining the rapid spread of the virus. Herein we examined field caught *Culex quinquefasciatus* from different neighborhoods in San Juan, Puerto Rico collected during June 2016 for the presence of natural infection of Zika virus (ZIKV). *Aedes aegypti* from the same locations were also analyzed. None of the *Cx. quinquefasciatus* tested showed natural infection for ZIKV, whereas *Ae. aegypti* tested positive at 7 sites. Our results suggest that *Cx. quinquefasciatus* was not involved in the transmission of Zika virus in San Juan, Puerto Rico in 2016.

Keywords

Aedes aegypti; *Aedes albopictus*; arbovirus; San Juan; ZIKV

Introduction

During 2015 and 2016 many areas of South and Central America and the Caribbean saw the emergence of a new mosquito-borne pathogen, Zika virus (ZIKV). In some patients, exposure to ZIKV causes a series of symptoms including rash, joint pain, conjunctivitis, muscle pain, and headache. In addition, ZIKV infection during the early stage of pregnancy appears to lead to congenital microcephaly and neurological abnormalities in some newborn babies (Liang et al. 2016). Besides transmission via mosquitoes, ZIKV transmission between humans has been reported to occur by other methods, including sexual contact, and

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Conflict of Interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

infected mothers may pass the virus on to their fetus during pregnancy (Schuler-Faccini et al. 2015). Current evidence supports the view that Zika is transmitted to human and non-human primates by mosquitoes in the genus *Aedes*, subgenus *Stegomyia*, especially *Aedes* (*Stegomyia*) *aegypti* (L.). A recent report suggests that non-*Aedes* may be involved in transmission, most notably *Culex quinquefasciatus* (Say) from Brazil (Franca et al. 2016). Our goal was to test for natural infection of ZIKV in *Cx. quinquefasciatus* adults collected in an area of Puerto Rico that was experiencing high local transmission of ZIKV. As *Ae. aegypti* is the dominant urban mosquito in San Juan and the important vector in the spread of arboviruses like dengue and chikungunya (Sharp et al. 2014), we also collected *Ae. aegypti* adults and tested for ZIKV.

Materials and Methods

Adult sampling.

The island of Puerto Rico is a U.S. territory with approximately 3.4 million inhabitants concentrated near coastal regions and has a tropical to subtropical climate. Puerto Rico has experienced the greatest number of autochthonous human cases within all United States territories, with 35,397, compared to 997 in the U.S. Virgin Islands, 217 cases in Florida, and 6 in Texas as of the 12th of April 2017 (CDC 2017). This is likely a consequence of the dominant presence of *Ae. aegypti* in urban areas, high human densities, and favorable climatic conditions. We sampled adult mosquitoes around the capital, San Juan. Trapping took place in 13 locations at urban and suburban locations (Fig.1). Traps (BG Sentinel Traps, (Biogents AG, Regensburg, Germany)) were placed in a shaded location in homes or on porches of homes. All homes were occupied and free of window screens. Traps were baited with a human scent lure (BG-Lure ®) containing octenol. Trapping commenced on 7 June (6 sites) and 8 June 2016 (7 sites). Traps were left out for ~48 hrs at each location. Adults were collected and kept frozen for identification (using standard keys) and enumeration. Adults used for quantification of Zika were transferred to their respective labs for testing.

Zika quantification.

In all cases heads from individual females were analyzed so as to assess disseminated virus rather than viruses that may be in a blood meal in the midgut. Female *Culex* were caught in conjunction with a wider project investigating Zika and *Ae. aegypti* in San Juan, and thus because of funding constraints we partnered with another researcher (F.F. Hunter) who uses a different published protocol for the analysis of *Culex* mosquitoes. For *Cx. quinquefasciatus*, heads were placed individually in 2 mL flat-cap tubes containing a single copper bead and 1mL sDMEM (88% Dulbecco's Modified Eagle Medium, 10% FBS, 2% penicillin/streptomycin/glutamine mix, Sigma Aldrich). Tubes were shaken for 2 min at 30Hz/s in a mixer mill and centrifuged to settle mosquito debris. To isolate RNA, 100μL of supernatant was added to 250μL Buffer RLT (Qiagen RNeasy mini kit) and the remaining manufacturer's instructions were followed. Zika viral RNA was detected by RT-qPCR using an iCycler (BioRad, Hercules, CA, USA). Protocols were based on the two primer and probe sets of Lanciotti et al. (2008), namely, ZIKV Set 1: 835 fwd 5'-TTGGTCATGATACTGCTGATTGC-3'; 911c rev 5'-

CCTTCCACAAAGTCCCTATTGC-3'; 860 FAM probe 5'-CGGCATACAGCATCAGGTGCATAGGAG-3' and ZIKV Set 2: 1086 fwd 5'-CCGCTGCCCAACACAAG-3'; 1162c rev 5'-CCACTAACGTTCTTTTGCAGACAT-3'; 1107 FAM probe 5'-AGCCTACCTTGACAAGCAGTCAGACACTCAA-3' using iTaq Universal Probe One-step Master Mix (Bio-Rad) and iScript advanced reverse transcriptase (Bio-Rad). Amplification was performed in 25mL reaction volumes consisting of 12.5μL iTaq Universal Probe One-step Master Mix (Bio-Rad), 0.5μL iScript-RT, 1.0μL 10μM forward primer, 1.0μL 10μM reverse primer, 0.5μL 10μM FAM-probe, 4.5μL nuclease free water, and 5.0μL template. The thermal cycler was programmed for 30 min at 50°C for the reverse transcription reaction, 15 min at 95°C for polymerase activation and initial DNA denaturation, followed by 40 cycles of amplification with 15s at 94°C for denaturation and 1 min at 60°C for annealing and extension.

For *Ae. aegypti*, total RNA in mosquito heads was isolated using TRI-reagent (Molecular Research Center). The complementary DNA (cDNA) was synthesized using the iSCRIPT cDNA synthesis kit (Bio-Rad) and RT-qPCR assays was performed in a CFX96 Real-Time system (Bio-Rad) using iTaq universal probe supermix (Bio-Rad). We repeated the RT-qPCR procedure two times for each sample. Primers and probe specific to the Envelope gene of ZIKV were designed and synthesized according to previous work (Acharya et al. 2016).

Results

One trap malfunctioned (not shown in Fig. 1) and therefore did not capture any adults, whereas another trap (site 5, Fig. 1) appeared to be functioning normally but also failed to collect adult mosquitoes. Of the remaining 11 traps, four yielded female *Cx. quinquefasciatus* that were subsequently tested (Fig. 1, site 4, n = 2 tested, site 8, n = 6, site 11, n = 74, site 12, n = 22). Of the 104 adults tested, none was positive for ZIKV. However, *Ae. aegypti* females were collected at all 11 traps and adults were tested from 10 of those. Overall, seven of the 10 sites produced females that were positive for the presence of Zika virus (Fig. 1). The positive sites were not spatially clustered, occurring throughout our sampling area. For the four sites with adult *Cx. quinquefasciatus*, only site 11 had *Ae. aegypti* specimens that were positive for Zika, whereas the other three sites (i.e., 4, 8, 12) were also negative (Fig. 1). However, in most cases, sites with negative *Cx. quinquefasciatus* were within a short distance of sites with positive *Ae. aegypti* (e.g., sites 6 and 8, Fig. 1). Finally, relative abundance of the two species among trapping locations was variable (site = *Ae. albopictus*%:*Cx. quinquefasciatus*%; 1 = 23.5%:76.5%, 2 = 71.4%:28.6%, 3 = 85.7%:14.3%, 4 = 21.3%:78.7%, 5 = 0%:0%, 6, 80.0%:20.0%, 7 = 100.0%:0%, 8 = 25.5%:74.5%, 9 = 100.0%:0%, 10 = 100.0%:0%, 11 = 16.9%:83.1%, 12 = 8.9%:91.1%)

Discussion

Data from wild caught *Cx. quinquefasciatus* in June 2016 suggests no natural infection with ZIKV in locations around San Juan. The fact that many sites had *Ae. aegypti* females positive for ZIKV seems to rule out spatial variation in infection as an explanation for the lack of positive *Cx. quinquefasciatus* females. The best evidence for discounting *Cx. quinquefasciatus*' role are the data from site 11, where both species were collected, but only

Ae. aegypti was positive. This evidence would be even stronger had *Ae. aegypti* also tested positive at sites 4, 8, and 12 (from which negative *Cx. quinquefasciatus* were collected). However, sites 4 and 8 were adjacent to sites with positive *Ae. aegypti*, and were likely within the dispersal range of females of both species. There has been some controversy as to the importance of *Culex* in Zika transmission, with some unpublished data pointing to a part to play for *Cx. quinquefasciatus* in Brazil (Franca et al. 2016). However, various other authors have failed to find evidence that *Culex* (including *Cx. quinquefasciatus*) can become infected under laboratory conditions. For instance, Aliota et al (2016) challenged *Cx. pipiens* and *Ae. triseriatus* with ZIKV and found no mosquitoes that were positive for the virus. Both Boccolini et al. (2016) (*Cx. pipiens*) and Amraoui et al. (2016) (*Cx. pipiens* and *Cx. quinquefasciatus*) found that adults were unable to become experimentally infected or transmit ZIKV. An assessment of populations from California (*Cx. pipiens*), New Jersey (*Cx. pipiens*), and Florida (*Cx. quinquefasciatus*) also failed to produce any females who were infected or showed disseminated Zika virus (Haung et al. 2016). Furthermore, Fernandes et al. (2016) tested *Cx. quinquefasciatus* from Rio de Janerio, Brazil, and showed that adults who were given a blood meal containing locally acquired strains of ZIKV were unable to become infected, and there was no evidence of dissemination or transmission. In all these studies, authors were able to successfully infect *Ae. aegypti* or *Ae. albopictus*. However, Guo et al. (2016) were able to produce infection and transmission of Zika in *Cx. pipiens quinquefasciatus* based on a virus strain originally collected from a Chinese patient returning from Samoa in 2016. It is not clear why there should be such differences between the study by Guo et al. (2016) and the others mentioned previously, but differences in the strain of virus used or specific populations of mosquitoes may be one explanation.

Outside of laboratory experiments, these findings from the field further bolster previous work from other locations where Zika was circulating that species in the *Cx. pipiens* complex, including *Cx. quinquefasciatus*, are not naturally infected with Zika (Grard et al. 2014, Guerbois et al. 2015). The weight of evidence, including the results of our own study, seems to point to a limited or population specific role of *Culex* in the transmission of Zika. Given this, further testing of other species within genus *Aedes*, and a focus on their surveillance and control seems a more prudent approach (Amaroui et al. 2016). For instance, it has been shown that *Ae. mediovitattus* is a competent vector of dengue alongside *Ae. aegypti* in San Juan (Poole-Smith et al. 2015), and thus efforts should be made to understand if this species is also important in the transmission of Zika.

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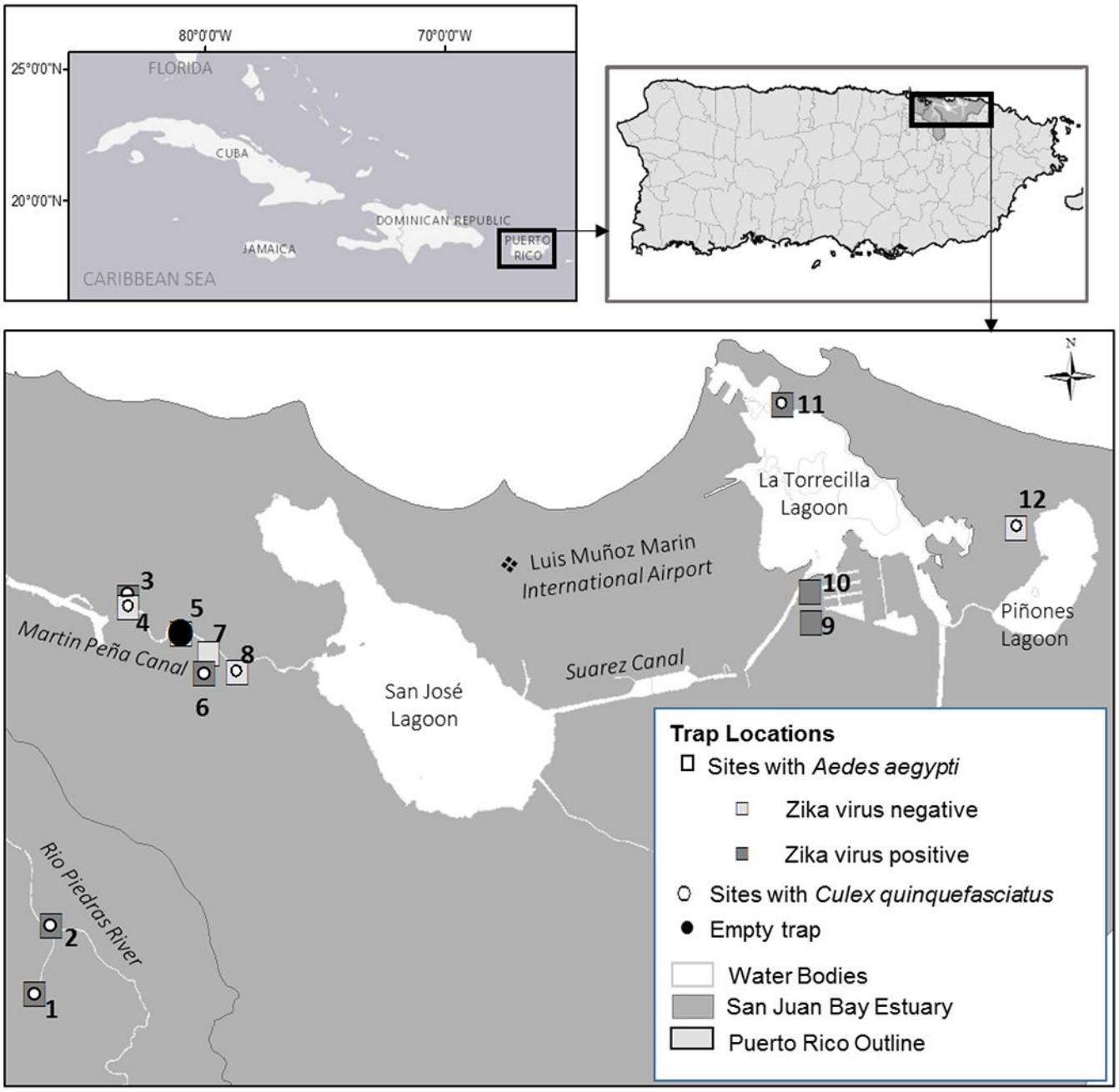

Figure 1.

Location of mosquito traps within the San Juan Bay Estuary watershed, in the Caribbean island of Puerto Rico. Sources: Puerto Rico outline (United States Postal Service); Caribbean Map (DeLorme, MapmyIndia, © OpenStreetMap contributors, and the GIS community); Water Bodies (Puerto Rico GIS http://www2.pr.gov/agencias/gis/Pages/DatosCensales.aspx)