

ORIGINAL ARTICLE

Zika virus replication in the mosquito *Culex quinquefasciatus* in Brazil

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Zika virus (ZIKV) is a flavivirus that has recently been associated with an increased incidence of neonatal microcephaly and other neurological disorders. The virus is primarily transmitted by mosquito bite, although other routes of infection have been implicated in some cases. The *Aedes aegypti* mosquito is considered to be the main vector to humans worldwide; however, there is evidence that other mosquito species, including *Culex quinquefasciatus*, transmit the virus. To test the potential of *Cx. quinquefasciatus* to transmit ZIKV, we experimentally compared the vector competence of laboratory-reared *Ae. aegypti* and *Cx. quinquefasciatus*. Interestingly, we were able to detect the presence of ZIKV in the midgut, salivary glands and saliva of artificially fed *Cx. quinquefasciatus*. In addition, we collected ZIKV-infected *Cx. quinquefasciatus* from urban areas with high microcephaly incidence in Recife, Brazil. Corroborating our experimental data from artificially fed mosquitoes, ZIKV was isolated from field-caught *Cx. quinquefasciatus*, and its genome was partially sequenced. Collectively, these findings indicate that there may be a wider range of ZIKV vectors than anticipated.

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INTRODUCTION

Zika is classically considered a mild disease whose symptoms include fever, joint pain, rash and, in some cases, conjunctivitis.¹ However, the recent Zika outbreak in Brazil has been associated with an increased incidence of neonatal microcephaly and neurological disorders.^{2,3} Zika virus (ZIKV) is a poorly understood, small, enveloped RNA virus with ssRNA (+) belonging to the family Flaviviridae. It was first isolated in April 1947 from a rhesus monkey and then, in January 1948, from the mosquito species *Aedes africanus*.⁴ Subsequently, several ZIKV strains have been isolated from *Aedes*, *Mansonia*, *Anopheles* and *Culex* mosquitoes.⁵

The first known Zika epidemic in an urban environment occurred in Micronesia in 2007, with ~73% of the human population on Yap Island becoming infected.⁶ Intriguingly, although many *Aedes* mosquitoes were collected in the field and evaluated for virus detection, no samples were found to be positive for ZIKV.⁶ In addition, it is important to highlight that *Ae. aegypti* is absent from most islands in the Micronesian archipelago and is rare on the islands where ZIKV is present.^{6,7}

There is a global consensus among scientists and health agencies that *Ae. aegypti* and *Ae. albopictus* are the main ZIKV vectors in urban areas (WHO, 2016). This belief is partly because vector competence experiments for ZIKV were conducted exclusively for species of this genus, mainly *Ae. aegypti*, until recently.^{8,9} In fact, previous laboratory studies^{8,10} suggested that *Ae. aegypti* is a ZIKV vector. Recently, high rates of dissemination and transmission of the ZIKV in *Ae. aegypti* have been observed under laboratory conditions.¹¹ Intriguingly, a few studies have demonstrated that *Ae. aegypti* and *Ae. albopictus* populations have low rates of ZIKV transmission^{12,13} or none,^{14,15} but the role of other vectors in the spread of ZIKV has been overlooked. Thus, other mosquito species, such as *Cx. quinquefasciatus*, that coexists with *Ae. aegypti* in urban areas, could contribute to ZIKV transmission.¹⁶ The literature is dichotomous regarding *Culex* vectors. Although there are recent reports showing that *Culex* is not a ZIKV vector,^{17–22} a recent paper demonstrated that *Cx. quinquefasciatus* collected in urban areas in China were able to be infected with a local ZIKV strain and then transmit it to mice.²³ These controversial results are expected and could be due to differences in the combination of mosquito

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and virus genotypes used in artificial blood-feeding assays, as previous studies have also shown negative results for the main vector *Ae. aegypti*.^{13,15} Diagne *et al.*¹⁵ reported that Dakar and Kedougou (Senegal) populations of the yellow fever mosquito did not transmit six different ZIKV strains, including the MR766 strain, which was first isolated from a sentinel monkey in Uganda. In an evident contrast, Wegner-Lucarelli *et al.*²⁴ found that *Ae. aegypti* from Poza Rica (Mexico) is a competent vector of the MR766 ZIKV strain. These are the first two studies that used the same strain of the virus *vis-à-vis* the same mosquito species. Therefore, it is reasonable to conclude that the genetic variability of mosquito species from different geographical origins might account for this apparent discrepancy in reported data.²⁵ Here, we report data that support the idea that in Recife, northeast Brazil, *Cx. quinquefasciatus*, the southern house mosquito in tropical and subtropical areas, is a potential ZIKV vector. We performed mosquito vector competence assays under laboratory conditions, comparing both *Ae. aegypti* and *Cx. quinquefasciatus* using different virus doses. ZIKV was detected in the salivary glands and in the saliva of artificially fed *Cx. quinquefasciatus* mosquitoes. In addition to these results, ZIKV was also detected in field-caught samples of *Cx. quinquefasciatus*, which had no signs of recent blood feeding. ZIKV was isolated from these samples, and its genome was sequenced, providing the first partial genome of ZIKV obtained from *Cx. quinquefasciatus* mosquitoes. Collectively, our results suggest that this species is likely a ZIKV vector in Brazil, which has several implications for vector control strategies as well as for the understanding of the epidemiology of ZIKV.

MATERIALS AND METHODS

Mosquitoes

The present study was conducted using two laboratory colonies of mosquitoes and field-collected specimens of *Ae. aegypti* (F₁–F₂) from the Archipelago of Fernando de Noronha (FN), a district of Pernambuco State (PE). *Cx. quinquefasciatus* (formerly known as *Cx. pipiens quinquefasciatus*) mosquitoes originated from eggs (rafts) collected in Peixinhos, a neighborhood in Recife, PE, Brazil, in 2009. The *Ae. aegypti* laboratory colony (Reclab) was established with ~1000 specimens collected in Graças, a neighborhood in the Recife Metropolitan Region, and has been maintained in the insectary of Aggeu Magalhães Institute (IAM)/FIOCRUZ since 1996 under standard conditions: 26 ± 2 °C, 65%–85% relative humidity, 12/12 light/dark cycle. More information regarding the two laboratory colonies has been described elsewhere.^{26,27} The mosquitoes were kept in the insectary of the Department of Entomology IAM/FIOCRUZ under standard conditions described above. Larvae were maintained in plastic trays filled with potable water and were fed solely on cat food (Friskies), while adults were given access to a 10% sucrose solution until they were administered defibrinated rabbit blood infected with ZIKV.

Virus strain

Experimental infections of mosquitoes with ZIKV were conducted using the ZIKV BRPE243/2015 strain derived from the serum of a patient with an acute maculopapular rash in Pernambuco State, Brazil, during the 2015 outbreak.²⁸ This strain was named the ZIKV/*H. sapiens*/Brazil/PE243/2015 strain, according to the nomenclature described by Scheuermann,²⁹ and was fully characterized (accession number KX197192.1). Following isolation, the virus was passed once on *Ae. albopictus* C6/36 cells. Viral stocks were then produced in VERO cells and stored at –80 °C until use. Prior to oral infection, the

viral titer of the stock was calculated via plaque assay on VERO cells and reached 10⁶ plaque-forming units per milliliter (PFU/mL).

Artificial feeding

Two artificial-feeding assays were conducted using a viral stock concentration of 10⁶ PFU/mL as well as a 100-fold dilution from the viral stock in each experiment. Notably, in the first artificial-feeding assay, the frozen virus sample was mixed with defibrinated rabbit blood. In the second assay, ZIKV BRPE243/2015 was first grown in VERO cells at a multiplicity of infection of 0.5 for 4–5 days. Subsequently, the cell culture flasks were frozen at –80 °C, thawed at 37 °C twice and then mixed with defibrinated rabbit blood in a 1:1 proportion. Seven- to ten-day-old female mosquitoes were starved for 18 h prior to artificial feeding. The mosquitoes were exposed to an infectious blood meal for 90 min, as described in Salazar *et al.*,³⁰ with minor modifications. Briefly, infectious blood was provided in a membrane-feeding device placed on each mosquito cage. The blood feeding was maintained at 37 °C by using heat packs during the process. Fully engorged mosquito females were cold anesthetized, transferred to a new cage and maintained in the infection room for 15 days. Both assays included a control group fed on uninfected cultured cells mixed with defibrinated rabbit blood.

RNA extraction and virus detection

Four to fifteen mosquitoes were dissected in order to collect the midguts and salivary glands at three, seven and 15 days post infection (dpi). To prevent hemolymph contamination, all midguts and salivary glands were extensively washed with phosphate-buffered saline buffer immediately after dissection and prior to tissue extraction. Tissues were individually transferred to a 1.5-mL DNase/RNase-free micro-tube containing 300 µL of mosquito diluent³¹ and were stored at –80 °C until further usage. Each tissue was ground with sterile micropestles, and RNA extraction was performed with 100 µL of the homogenate. The TRIzol method (Invitrogen, Waltham, MA, USA) was performed according to the manufacturer's instructions with modifications as follows. Tissue homogenate (100 µL) was mixed with 200 µL of TRIzol, homogenized by vortexing for 15 s and incubated for 5 min at room temperature. Chloroform (100 µL) was added to the mixture, and homogenization was performed by shaking the tubes vigorously for 15 s by hand. The mixture was then incubated at room temperature for 2–3 min. The samples were centrifuged at 12 000g for 15 min at 4 °C. The aqueous phase of each sample was removed and transferred to a new tube containing 250 µL of 100% isopropanol. This mixture was incubated at room temperature for 10 min and then centrifuged at 12000g for 10 min at 4 °C. The supernatant was removed, and the RNA pellet was washed with 300 µL of 75% ethanol. The samples were homogenized briefly and then centrifuged at 7500g for 5 min at 4 °C. The supernatant was discarded, and the RNA was then air-dried for 15 min. The RNA pellet was resuspended in 30 µL of RNase-free water. After RNA resuspension, the samples were treated with DNase (Turbo DNase, Ambion, Foster City, CA, USA) according to the manufacturer's protocol.

Virus detection was performed by quantitative RT-PCR (RT-qPCR, also known as real-time RT-PCR) in an ABI Prism 7500 SDS Real-Time system (Applied BioSystems, Foster City, CA, USA) using the QuantiNova Probe RT-PCR kit (Qiagen, Hilden, Germany). RT-qPCR was performed in a 20-µL reaction volume containing 5 µL of extracted RNA, 2× QuantiNova Probe RT-PCR Master Mix, 0.2 µL of the QuantiNova Probe RT Mix, 0.1 µL of the ROX Reference Dye, 100 µM of each primer (stock) and 25 µM of the probe (stock). The primers, probe and PCR conditions were first described in Lanciotti

et al.,³² and each sample was tested in duplicate. RT-qPCR cycling included a single cycle of reverse transcription for 15 min at 45 °C, followed by 5 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation, and then 45 cycles of 5 s at 95 °C and 45 s at 60 °C (annealing-extension step). The amount of viral RNA in each sample was estimated by comparing the cycle threshold values (Ct) to the standard curve for every RT-qPCR assay. The standard curve consisted of different dilutions of previously titrated ZIKV BRPE243-/2015 RNA. Mosquitoes collected immediately after artificial feeding were used as positive controls, while control mosquitoes fed on uninfected blood and RT-PCR reactions containing no RNA represented negative controls. Fluorescence was analyzed at the end of the amplifications. Positive samples were used to calculate vector competence parameters, such as the infection rate (IR), which is the number of positive midguts divided by the total number of midguts tested, and the proportion of infected salivary glands (SR), which is the number of positive salivary glands divided by the total number of salivary glands tested.

Collection of virus-infected mosquito saliva

To confirm whether the viral RNA detected by RT-qPCR within the salivary glands was infective and could be released during blood-feeding meals, we assayed ZIKV from saliva samples. From the 8th to 14th dpi, mosquitoes from each group were exposed to honey-soaked Flinders Technology Associates (FTA) Classic Cards (Whatman, Maidstone, UK) placed on the top of the cages to collect mosquito saliva. Each FTA card was prepared in a sterilized Petri dish and soaked in ~10 g of anti-bacterial honey (Manuka Honey Blend, Arataki Honey Ltd, Havelock North, Hastings, New Zealand) and 1 mL of blue food dye (Soft Gel Mix) for 2 h. The blue food dye was used to determine whether the mosquitoes had fed on the FTA cards. After 24 h of exposure, each card was placed in a 15-mL falcon tube and stored at -80 °C until further use. To extract the RNA, the cards were placed individually in 2-mL microtubes with 600 µL of Ultra-PureDNase/RNase-Free Distilled Water (Invitrogen, Life Technologies, Grand Island, NY, USA). These eluted samples were kept on ice and vortexed five times for 10 s each. This process was repeated for 20 min. RNA was recovered from the FTA cards using the TRIzol method and was used to detect ZIKV as previously described.

Transmission electron microscopy

Salivary glands from *Cx. quinquefasciatus* were dissected at 7 dpi and fixed for 2 h in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer solution. After fixation, the samples were washed twice in the same buffer and post-fixed in a solution containing 1% osmium tetroxide, 2 mM calcium chloride and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone as previously reported,³³ and were then embedded using a Fluka Epoxy Embedding kit (Fluka Chemie AG, Buchs, Switzerland). Polymerization was performed at 60 °C for 24 h. Ultrathin sections (70 nm) were placed on 300-mesh nickel grids and then counterstained with 5% uranyl acetate and lead citrate, followed by examination using a transmission electron microscope (Tecnaï Spirit Biotwin, FEI Company, Hillsboro, OR, USA).

Statistical analysis

The IR and SR were calculated for each species at different time points. Logistic regression, χ^2 -test and Fisher's Exact tests were used to test for differences in both the IR and SR within the two species. The Cochran-Mantel-Haenszel and log linear regression tests were also performed to compare differences between the species. All statistical

tests were performed with R software (R DEVELOPMENT CORE TEAM, 2012). GraphPad Prism software v.5.02 (GraphPad, San Diego, CA, USA) was used to plot graphics and to compare viral genome quantification values between the different time points, tissues and samples using an unpaired *t*-test.

ZIKV detection and viral isolation in field-collected mosquitoes

Mosquito samples were collected in the metropolitan region of Recife, from February to May 2016, in two distinct types of locations: at premises where zika cases were noted and at public Emergency Care Units. Pernambuco Secretary of Health personnel carried out collections at Emergency Care Units, and our own fieldwork team collected mosquitoes at premises with Zika infections. Both sets of collections were performed using a battery-operated aspirator (Horst Armadilhas Ltd, São Paulo, Brazil). Samples were sent alive to the IAM where they were anesthetized at 4 °C; morphologically identified; sorted by species, locality, sex and feeding status (engorged and not engorged); pooled (1–10 individuals/pool); and preserved at -80 °C until being assayed for RNA extraction and RT-qPCR as described above. The minimum IR (MIR) for ZIKV in adults captured in the field was calculated as (the number of positive pools for ZIKV/total number of mosquitoes tested) × 1000.³⁴

Positive samples were assayed for virus isolation in VERO cells as follows. In a tissue culture tube (Techno Plastic Products AG, Trasadingen, Schaffhausen, Switzerland), 1 mL of a 5×10^5 cells/mL suspension in Minimum Essential Media (MEM) was seeded for 24 h to form a monolayer. After that, the MEM medium was discarded, and 1 mL of the filter homogenate (100 µL of positive homogenate +900 µL of MEM medium) was inoculated in the cells. After a 1-h incubation for virus adsorption, 1 mL of fresh medium was added to the tissue culture tubes, and they were then incubated at 37 °C in 5% CO₂ atmosphere until the detection of cytopathic effects. After that, samples were frozen and thawed twice, followed by centrifugation for 10 min at 2000 r/min. The supernatants were then transferred to cryotubes and stocked at -80 °C until further usage.

Genome sequencing

Two positive field-collected samples of *Cx. quinquefasciatus* (Cx5 and Cx17) identified previously by RT-qPCR were inoculated in VERO cells for virus replication to obtain ZIKV first passage. For genome sequencing, total RNA from each sample was extracted from the VERO cell cultures using TRIzol reagent as described above. A MiSeq (Illumina, San Diego, CA, USA) sequencing library was prepared with a Nextera XT Library Prep Kit (Illumina) using 2 ng of input cDNA from multiplex PCR following the manufacturer's instructions. To obtain cDNA, RT-PCR reactions were carried out in a volume of 25 µL using a SuperScript III Platinum One-Step RT-qPCR Kit (Invitrogen, Life Technologies) with modifications. Briefly, the cDNA first strand was reverse-transcribed from previously extracted RNA (minimum 10 ng for reaction) at 50 °C for 30 min, and random hexamers (random sequence [d(N)6]) were used as the first-strand primer. After reverse transcription, a PCR program was performed to amplify the whole ZIKV genome. Primers for ZIKV genome amplification were described by the ZIBRA project.³⁵ A total of 35 cycles of 95 °C for 5 s and 65 °C for 15 min were performed on a Veriti Thermal Cycler (Invitrogen, Life Technologies). As described by the ZIBRA protocol, two different sets of primers were designed for a multiplex PCR, and amplification reactions from each primer set were carried out separately on single samples. After amplification, the PCR products were quantified using a Qubit dsDNA HS Assay Kit (Invitrogen, Life Technologies). A MiSeq Reagent Kit V3 of 150 cycles

Table 1 IR and proportion of infected SR of *Aedes aegypti* and *Culex quinquefasciatus* laboratory colonies after artificial blood feeding with ZIKV American strain (ZIKV BRPE243/2015)

Dose	Day	<i>Aedes aegypti</i> (RecLab)				<i>Culex quinquefasciatus</i>				Total				Among species; P-value
		N	%	N	%	N	%	N	%	N	%	N	%	
10 ⁶ PFU/mL IR	3	14	77.78	4	22.22			14	77.78	4	22.22			0.6196
	7	18	90.00	2	10.00	2	16.67	28	87.50	4	12.50			
	15	7	43.75	9	56.25	11	61.11	14	41.18	20	58.82			
	Total	39	72.22	15	27.78	13	43.33							
	P-value			0.0091			0.0256							
SR	3	4	20.00	16	80.00			4	20.00	16	80.00			0.7170
	7	12	60.00	8	40.00	0	0.00	24	75.00	8	25.00			
	15	6	37.50	10	62.50	13	72.22	11	32.35	23	67.65			
	Total	22	59.46	15	40.54	13	43.33							
	P-value			0.0388			<0.0001							
10 ⁴ PFU/mL IR	3	8	40.00	12	60.00	2	20.00	10	33.33	20	66.67			0.4190
	7	9	45.00	11	55.00	9	36.00	18	40.00	27	60.00			
	15	9	50.00	9	50.00	2	10.53	11	29.73	26	70.27			
	Total	26	44.83	32	55.17	13	24.07	41	75.93					
	P-value			0.9443			0.1762							
SR	3	11	55.00	9	45.00	1	10.00	9	90.00	18	60.00			0.0235
	7	2	10.00	18	90.00	2	8.00	23	92.00	41	91.11			
	15	4	22.22	14	77.78	0	0.00	19	100.00	33	89.19			
	Total	17	29.31	41	70.69	3	5.56	51	94.44					
	P-value			0.0353			0.6120							

Table 2 IR and SR of the *Aedes aegypti* field-caught (Fernando de Noronha—FN) colony after artificial blood feeding with ZIKV American strain (ZIKV BRPE243/2015)

Dose	Day	<i>Aedes aegypti</i> (FN)								P-value		Between dose					
		IR				SR				IR	SR	IR	SR				
		N	+	%	N	-	%	N	+					%	N	-	%
10 ⁶ PFU/mL	3	1	+	10.00	9	-	90.00	1	+	10.00	9	-	90.00	0.2967	0.0064	0.3485	0.0018
	7	4	+	40.00	6	-	60.00	6	+	60.00	4	-	40.00				
	15	4	+	40.00	6	-	60.00	0	+	0.00	10	-	100.00				
	Total	9	+	30.00	21	-	70.00	7	+	25.00	21	-	75.00				
10 ⁴ PFU/mL	3	2	+	20.00	8	-	80.00	1	+	10.00	9	-	90.00	0.4737	1.0000		
	7	0	+	0.00	10	-	100.00	0	+	0.00	10	-	100.00				
	15																
	Total	2	+	10.00	18	-	90.00	1	+	5.00	19	-	95.00				

Abbreviations: infection rate, IR; proportion of infected salivary gland, SR; Zika virus, ZIKV.

*N represents the number of analyzed individuals. Statistical analyses were performed using the R package (R DEVELOPMENT CORE TEAM, 2012). The significance level was set at $P < 0.05$.

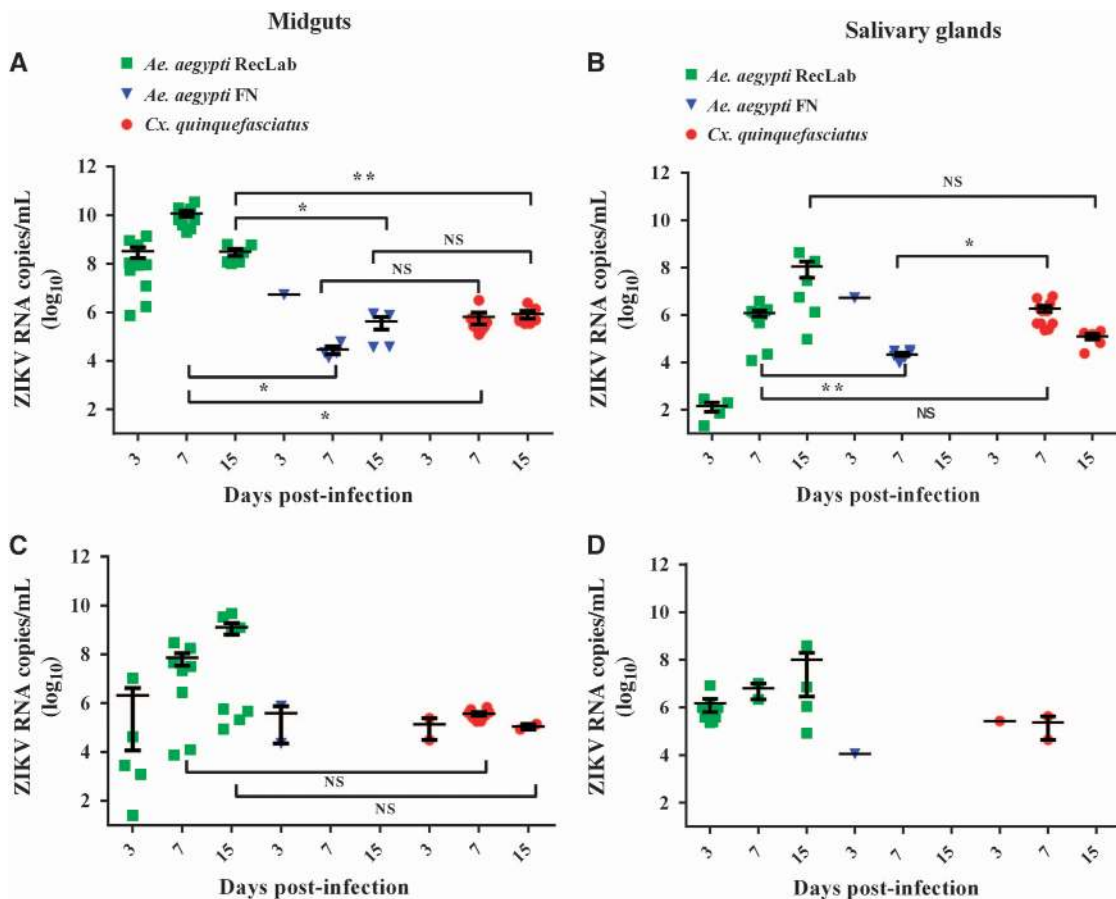


Figure 1 Quantification of RNA viral copy numbers in the midguts and salivary glands of *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes experimentally fed with blood containing ZIKV at 10⁶ PFU/mL (**A** and **B**) and 10⁴ PFU/mL (**C** and **D**). Green squares represent the *Ae. aegypti* (RecLab) population, blue inverted triangles represent the *Ae. aegypti* field population (FN) and red circles represent *Cx. quinquefasciatus*. Statistical analysis was performed using GraphPad Prism software (GraphPad) by unpaired *t*-test (* $P < 0.05$ and ** $P < 0.01$). Nonsignificant, NS; Zika virus, ZIKV.

Whatman) were placed on the top of each cage containing mosquitoes to feed upon from 8 to 14 dpi. At 9–12 dpi, ZIKV RNA was detected in the saliva of both *Ae. aegypti* and *Cx. quinquefasciatus* species (Figure 2). When a high viral dose (10⁶) was used, the amount of viral RNA copies expectorated during salivation in both *Ae. aegypti* and

Cx. quinquefasciatus were similar at all time points analyzed ($P > 0.05$). However, when the mosquitoes were challenged with a low viral dose (10⁴), *Ae. aegypti* expectorated more RNA viral copies than *Cx. quinquefasciatus* ($P = 0.0473$). It is important to highlight that we have no information regarding the number of mosquitoes that

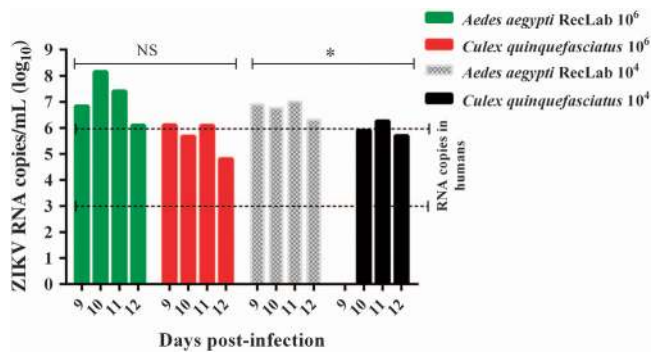


Figure 2 Quantification of ZIKV in *Ae. aegypti* and *Cx. quinquefasciatus* saliva expectorated onto FTA cards 9–12 days post infection (dpi). Green and red bars show the *Ae. aegypti* (RecLab) and *Cx. quinquefasciatus* populations blood-fed with ZIKV at 10⁶ PFU/mL, respectively. The hashed pattern gray and black bars show the *Ae. aegypti* (RecLab) and *Cx. quinquefasciatus* populations, respectively, blood-fed with ZIKV at 10⁴ PFU/mL. Parallel dashed lines indicate variations of ZIKV viremia in humans. Statistical analysis was performed using GraphPad Prism software (GraphPad) by unpaired *t*-test (**P* < 0.05). Nonsignificant, NS; Zika virus, ZIKV.

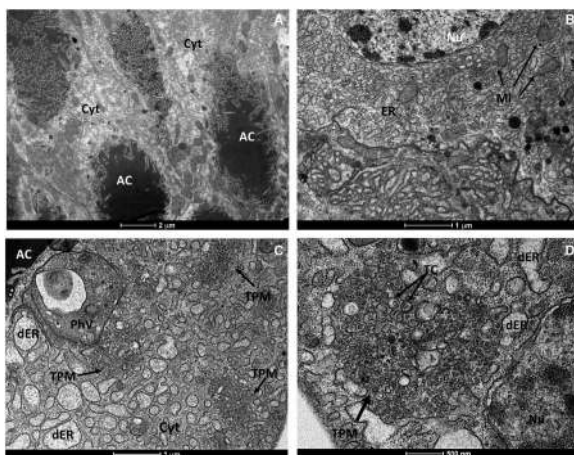


Figure 3 (A and B) Ultrathin sections of an uninfected *Cx. quinquefasciatus* salivary gland. (A) This micrograph shows the electron-dense content of the apical cavity (A and C) with membrane projections that extend from the wall. (B) Uninfected acinar salivary gland cell showing the Nu, ER and Mi. (C and D) Cytopathic effects of salivary glands cells infected with ZIKV showing several patches of TPM, dER and a PhV. Cell cytoplasm, Cyt; distended endoplasmic reticula, dER; endoplasmic reticulum, ER; mitochondria, Mi; nucleus, Nu; phagolysosome-like vacuole, PhV; thread-like center, TC; tubular proliferated membrane, TPM; Zika virus, ZIKV.

expectorated virus on the FTA cards per cage. The number of mosquitoes that salivated on the cards could affect the quantities of ZIKV particles detected by RT-qPCR.

Transmission electron microscopy

To further scrutinize our results from RT-qPCR, we performed transmission electron microscopy of salivary glands dissected from *Cx. quinquefasciatus* mosquitoes. The morphological organization of *Cx. quinquefasciatus* salivary glands showed an electron-dense apical cavity with membrane projections extending from the wall (Figure 3A) in addition to a basal nucleus, mitochondria and abundant endoplasmic reticulum (Figure 3B). ZIKV-infected salivary acinar cells of *Cx.*

quinquefasciatus showed signs of cytopathic disruptions, including cisternae distension of the endoplasmic reticulum and tubular proliferated membranes organized in several patches within a single cell, presenting thread-like centers (Figures 3C and 3D). Mature ZIKV particles of 40–50 nm in diameter, composed of a central electron-dense core (~30 nm in diameter) surrounded by a viral envelope, were observed inside the dilated endoplasmic reticulum (Figures 4A–4D). In some regions, viral envelope formation was shown to arise from the endoplasmic membrane (Figure 4B). Some ZIKV particles were observed proximal to the apical cavity of the salivary cell. Mitochondria also showed severe damage, including complete loss of cristae (Figure 4D). In summary, transmission electron microscopy analysis confirmed that *Cx. quinquefasciatus* mosquitoes are susceptible to ZIKV infection because viral particles were detected in the salivary glands of artificially fed mosquitoes.

ZIKV detection in field-caught *Cx. quinquefasciatus*

Lastly, we conducted ZIKV surveillance (February to May 2016) in mosquitoes collected from residences inhabited by individuals with clinical symptoms of Zika fever. A total of 1496 adult *Cx. quinquefasciatus* and 408 *Ae. aegypti* female mosquitoes were collected from different sites in the Metropolitan Region of Recife (Figure 5). From 270 pooled samples of adult female *Cx. quinquefasciatus* and 117 pools of *Ae. aegypti* mosquitoes assayed by RT-qPCR, three *Cx. quinquefasciatus* and two *Ae. aegypti* pools were positive for ZIKV. Interestingly, colorimetric analysis indicated no traces of undigested blood in two of the three positive *Cx. quinquefasciatus* samples and undigested blood in the two *Ae. aegypti*-positive pools. These findings suggest that, at least in the two ZIKV-positive *Cx. quinquefasciatus* samples, the virus was not derived from a recent blood meal. The Cts of the *Cx. quinquefasciatus*-positive pools when screened by RT-qPCR were 37.6 (sample 5), 38.0 (sample 17) and 38.15 (sample 163). For the *Ae. aegypti* pools, the Cts were 37.5 (sample 3) and 37.9 (sample 7). The MIR was calculated for both species; for *Cx. quinquefasciatus*, the MIR was 2.0‰ and for *Ae. aegypti* the MIR was 4.9‰. In an attempt to isolate ZIKV from the field-caught *Culex* mosquitoes, we inoculated African Green Monkey kidney cells (VERO cells) with samples from the two positive pools with the lowest Cts. ZIKV was isolated from these samples, thus unambiguously demonstrating that this species was carrying active ZIKV particles in Recife, Brazil.

ZIKV genome sequencing from field-caught *Cx. quinquefasciatus*

ZIKV was obtained from the first passage of two pools of field-caught *Cx. quinquefasciatus* in VERO cells. These viruses were named ZIKV/*C. quinquefasciatus*/Brazil/PE05/2016 (Cx5) and ZIKV/*C. quinquefasciatus*/Brazil/PE17/2016 (Cx17). We obtained two draft ZIKV genomes from these samples that were 5365 (Cx5) and 5380 (Cx17) nucleotides long, covering more than 50% of the virus genome at a coverage depth average of 13.32 and 12.18×, respectively. The two partial genomes were deposited in GenBank under accession numbers KX986760 and KX986761, respectively.

Sequence alignment of ZIKV PE243/2015 with the Cx5 partial genome detected a single-nucleotide polymorphism (SNP) in the ZIKV envelope region of the mosquito sample. A comparison between PE243 and Cx17 showed the presence of 10 different SNPs in the partial ZIKV genome from Cx17. Six and four SNPs were found in the NS3 and NS5 regions, respectively. The first five SNPs found in the NS3 region were supported by 11 reads, while the last one was supported by five reads. All four SNPs observed in the NS5 region were supported by seven reads.

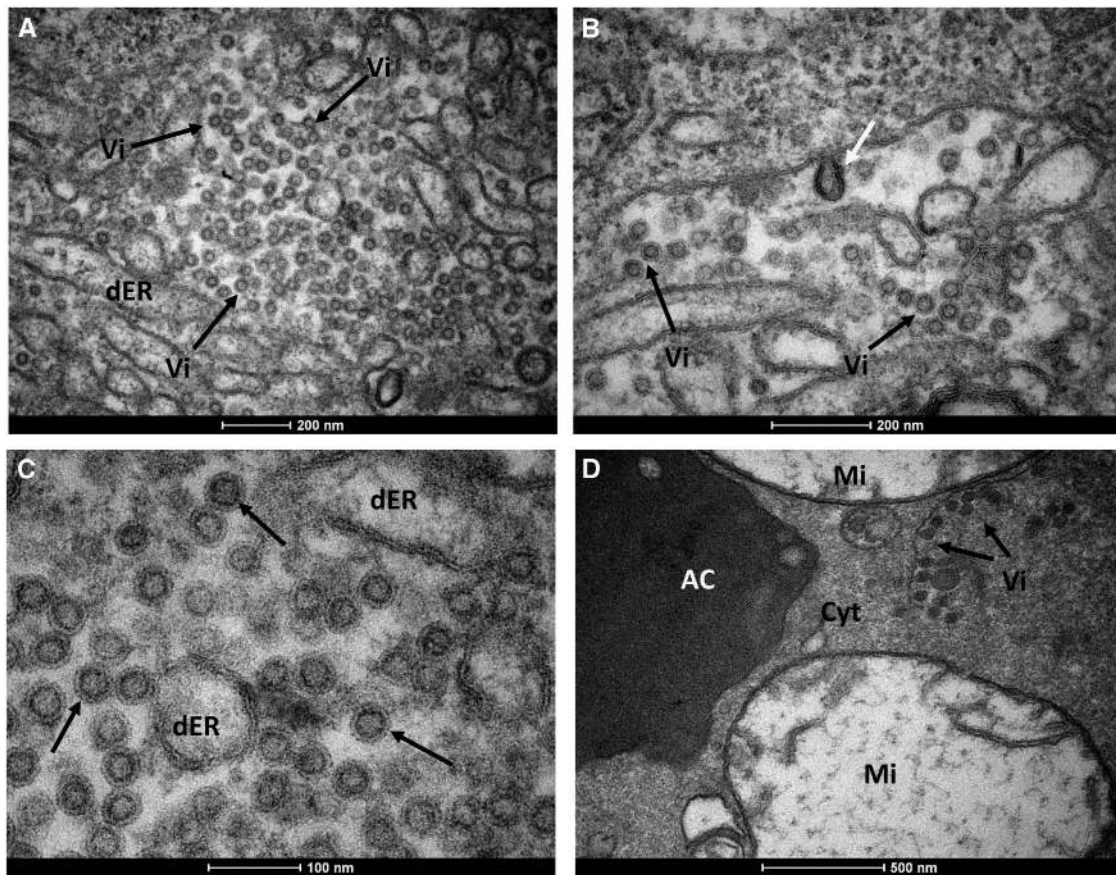


Figure 4 Mature ZIKV particles inside a *Cx. quinquefasciatus* salivary gland cell. (A) Numerous ZIKV particles (black arrows) within the dER. (B) Envelope formation from the endoplasmic membrane (white arrow). (C) Enveloped virus particles with electrodense cores. (D) Viral particles accumulated proximal to the acinar cavity (arrows); note the damaged mitochondria. Acinar cavity, AC; cell cytoplasm, Cyt; distended endoplasmic reticulum, dER; mitochondria, Mi; virion (s), Vi; Zika virus, ZIKV.

DISCUSSION

Our work has associated a second mosquito genus with the ZIKV transmission cycle in northeast Brazil. We showed that *Cx. quinquefasciatus*, also known as the southern house mosquito, which is the most common mosquito in urban areas in Brazil, is susceptible to infection with ZIKV during experimental blood feeding; moreover, we found that ZIKV has an active replication cycle in the salivary glands and is subsequently released in the saliva. In addition, we were able to detect ZIKV circulating in wild *Cx. quinquefasciatus* collected from Recife, and, for the first time, we sequenced a partial region of a ZIKV genome (50%) obtained from mosquito samples in Brazil.

Although *Ae. aegypti* is widely assumed to be the main ZIKV vector, previous vector competence studies are controversial. In the present study, a low dose of ZIKV (10^4 PFU/mL) was used for comparison with the higher doses used in previous studies.^{11,12,15} We found that both *Ae. aegypti* and *Cx. quinquefasciatus* can be experimentally infected by ZIKV even at low doses and can subsequently expectorate ZIKV in their saliva.

Cornet *et al.*¹⁰ concluded that part of a population of infected mosquitoes may not be able to transmit the virus, and that, even if they do, transmission occurs during a particular timeframe. This hypothesis differs from the idea that, once infected, a mosquito would transmit virus for its entire life. This finding has implications for arbovirus surveillance programs as a time window for vector-borne ZIKV transmission may exist. We found that after 11 dpi, most

samples were negative for ZIKV (apart from one positive salivary gland of *Ae. aegypti* fed 10^6 PFU/mL); thus, our maximum time point analysis was set to 15 dpi. However, Boorman and Porterfield⁸ reported that virus replication resumed at 15–20 dpi, and ZIKV remained present in *Aedes* mosquitoes for up to 60 days.

To confirm that the virus detected in the salivary glands by RT-qPCR was being released in saliva during consecutive blood meals, we followed the viral load from days 8 to 14 post infection using filter paper cards. FTA cards were always placed on the top of the cages to avoid excreta contamination as noted in previous studies.^{38,39} This strategy of viral RNA detection directly from FTA cards has been employed in previous studies for arbovirus surveillance.^{40,41} In the present study, we successfully detected ZIKV RNA copies in cards from *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes. This result demonstrates that, in addition to being susceptible to ZIKV infection and allowing virus replication in the salivary glands, both species are capable of effectively transmitting ZIKV.

RT-qPCR results were confirmed by transmission electron microscopy. The general mature ZIKV morphology observed for the salivary glands confirmed previous ultrastructural studies.^{42–44} In salivary gland cells, ZIKV replication causes cytopathic effects by 7 dpi, such as tubular membrane proliferation, similar to results shown elsewhere for West Nile virus.^{45,46} The fact that we found salivary glands positive for ZIKV when the midgut of the same mosquito was negative indicates that mosquitoes may be clearing the viral infection in the

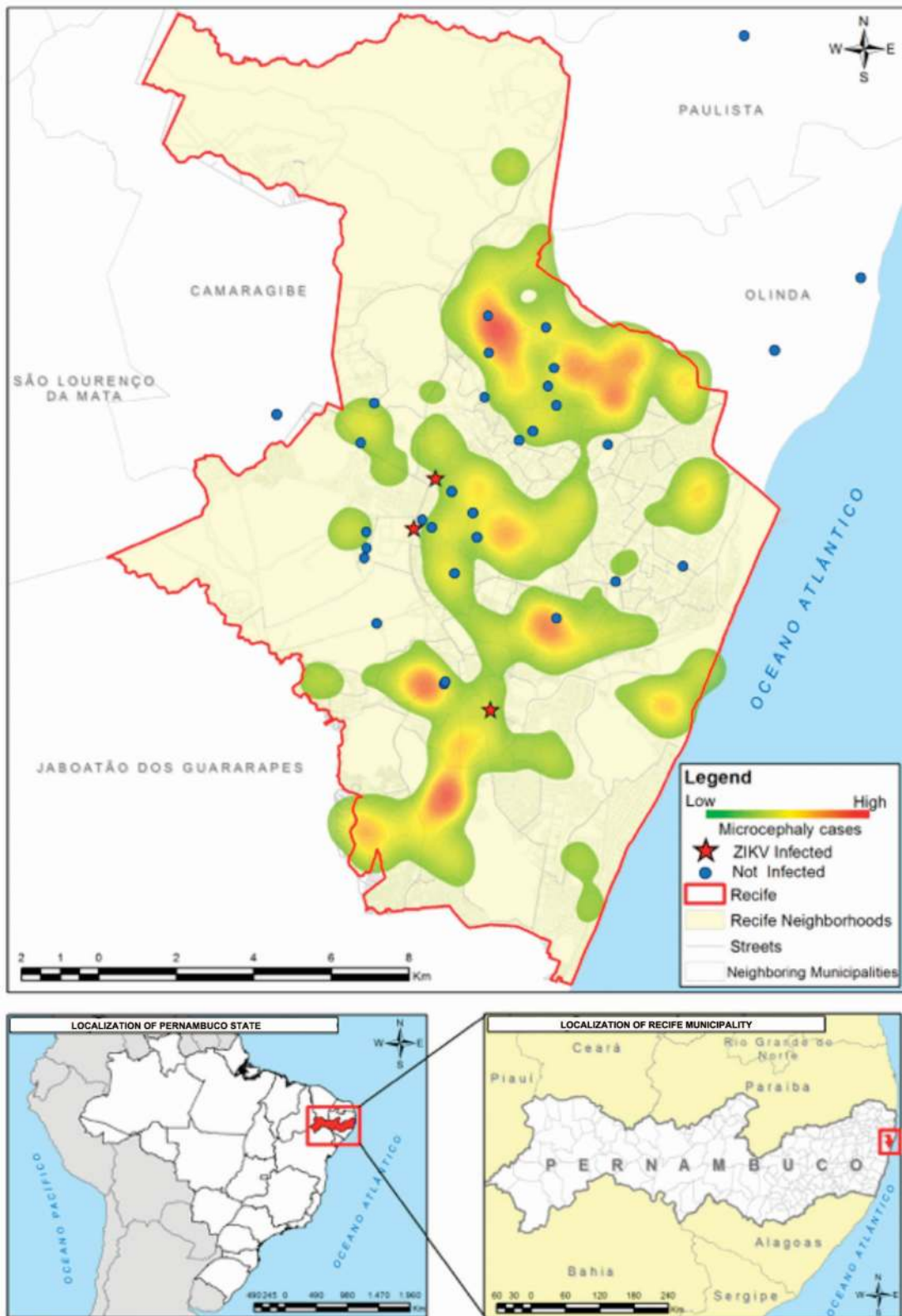


Figure 5 Kernel density map of reported cases of microcephaly versus a Point Map of the mosquito collection sites (with *Culex* samples positive and negative for the presence of ZIKV). Zika virus, ZIKV.

midgut while virus replication continues in the salivary glands. This finding has implications for the analytical methods employed in vector competence studies.

It is interesting to note that we were able not only to demonstrate ZIKV infection in *Cx. quinquefasciatus* in laboratory assays but also in field-caught mosquitoes. Viruses isolated from field-caught *Cx. quinquefasciatus* were passed in VERO cells and sequenced, and mutations in the NS3 and NS5 regions were detected. These particular regions encompass an RNA helicase (NS3-Hel) and a putative RNA-dependent RNA polymerase (NS5-RdRp), which are key components for genome replication and RNA synthesis.⁴⁷ In addition, more notably, these polymorphisms were not identified in the ZIKV strain circulating in Recife (PE243) that was obtained from human sera. Our findings are supported by Guo *et al.*,²³ but we are cognizant of previous studies that were unable to confirm ZIKV replication in *Cx. quinquefasciatus*.^{17,18,21,24,48}

Early studies have targeted only *Aedes* species, and only recent studies have compared the natural IRs of different species (including *Culex*). Notably, Diallo *et al.*⁵ observed a higher MIR for *Cx. perfuscus* (10× higher) than for *Ae. aegypti*. Positive *Ae. aegypti* field samples have always been reported at very low IRs, even in areas with high human ZIKV IRs, such as Malaysia.⁴⁹ Indeed, in Micronesia⁶ and French Polynesia, ZIKV was not detected in wild-caught *Aedes* spp. mosquitoes during outbreaks. It is interesting that in all of these areas, *Cx. quinquefasciatus* is an abundant mosquito species that may have also had an undetected role in ZIKV transmission. Indeed, Richard *et al.*¹³ demonstrated that two *Aedes* species from French Polynesia did not show enough vector competence to transmit ZIKV and suggested that another mosquito species could be contributing to the spread of the virus in that region.

Thus, our findings indicate that vector control strategies may need to be re-examined since reducing *Ae. aegypti* populations may not lead to an overall reduction in ZIKV transmission if *Culex* populations are not affected by *Aedes*-specific control measures. At the moment, there is no broad ongoing program for *Cx. quinquefasciatus* control in Brazil, although Recife, Olinda and Jaboatão dos Guararapes, three municipalities in the Recife Metropolitan Region, have undertaken specific control of *Cx. quinquefasciatus* to control lymphatic filariasis (LF) transmission locally,⁵⁰ as this species is the only LF vector.

Viral transmission via *Cx. quinquefasciatus* is not a new concept; in North America, this species is the major vector of West Nile virus⁵¹ and it is also a known vector of Japanese encephalitis virus⁵² and equine encephalitis virus.⁵³ Our data indicate that *Cx. quinquefasciatus* mosquitoes may be involved in ZIKV transmission in Recife. Thus, understanding the contributions of each species in the transmission of ZIKV is necessary to target each one properly. In conclusion, considering its high abundance in urban environments and its anthropophilic behavior in Brazil,^{54–56} *Cx. quinquefasciatus* may be a vector for ZIKV in this region.

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- Chang C, Ortiz K, Ansari A *et al*. The Zika outbreak of the 21st century. *J Autoimmun* 2016; **68**: 1–13.
- Cordeiro MT, Pena LJ, Brito CA *et al*. Positive IgM for Zika virus in the cerebrospinal fluid of 30 neonates with microcephaly in Brazil. *Lancet* 2016; **387**: 1811–1812.
- Moron AF, Cavalheiro S, Milani H *et al*. Microcephaly associated with maternal Zika virus infection. *BJOG* 2016; **123**: 1265–1269.
- Dick GW, Kitchen SF, Haddock AJ. Zika virus. I. Isolations and serological specificity. *Trans R Soc Trop Med Hyg* 1952; **46**: 509–520.
- Diallo D, Sall AA, Diagne CT *et al*. Zika virus emergence in mosquitoes in southeastern Senegal, 2011. *PLoS ONE* 2014; **9**: e109442.
- Duffy MR, Chen TH, Hancock WT *et al*. Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med* 2009; **360**: 2536–2543.
- Savage HM, Fritz CL, Rutstein D *et al*. Epidemic of dengue-4 virus in Yap State, Federated States of Micronesia, and implication of *Aedes hensilli* as an epidemic vector. *Am J Trop Med Hyg* 1998; **58**: 519–524.
- Boorman JP, Porterfield JS. A simple technique for infection of mosquitoes with viruses; transmission of Zika virus. *Trans R Soc Trop Med Hyg* 1956; **50**: 238–242.
- Li MI, Wong PS, Ng LC *et al*. Oral susceptibility of Singapore *Aedes (Stegomyia) aegypti* (Linnaeus) to Zika virus. *PLoS Negl Trop Dis* 2012; **6**: e1792.
- Cornet M, Robin Y, Adam C *et al*. Comparison between experimental transmission of yellow fever and Zika viruses in *Aedes aegypti*. *Cah Orstom* 1979; **17**: 7.
- Dutra HL, Rocha MN, Dias FB *et al*. Wolbachia blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes. *Cell Host Microbe* 2016; **19**: 771–774.
- Chouin-Carneiro T, Vega-Rua A, Vazeille M *et al*. Differential susceptibilities of *Aedes aegypti* and *Aedes albopictus* from the Americas to Zika virus. *PLoS Negl Trop Dis* 2016; **10**: e0004543.
- Richard V, Paoaafaite T, Cao-Lormeau VM. Vector competence of French Polynesian *Aedes aegypti* and *Aedes polynesiensis* for Zika virus. *PLoS Negl Trop Dis* 2016; **10**: e0005024.
- Bearcroft WG. Zika virus infection experimentally induced in a human volunteer. *Trans R Soc Trop Med Hyg* 1956; **50**: 442–448.
- Diagne CT, Diallo D, Faye O *et al*. Potential of selected Senegalese *Aedes* spp. mosquitoes (Diptera: Culicidae) to transmit Zika virus. *BMC Infect Dis* 2015; **15**: 492.
- Ayres CF. Identification of Zika virus vectors and implications for control. *Lancet Infect Dis* 2016; **16**: 278–279.
- Aliota MT, Peinado SA, Osorio JE *et al*. *Culex pipiens* and *Aedes triseriatus* mosquito susceptibility to Zika virus. *Emerg Infect Dis* 2016; **22**: 1857–1859.
- Fernandes RS, Campos SS, Ferreira-de-Brito A *et al*. *Culex quinquefasciatus* from Rio de Janeiro is not competent to transmit the local Zika virus. *PLoS Negl Trop Dis* 2016; **10**: e0004993.
- Hall-Mendelin S, Pyke AT, Moore PR *et al*. Assessment of local mosquito species incriminates *Aedes aegypti* as the potential vector of Zika virus in Australia. *PLoS Negl Trop Dis* 2016; **10**: e0004959.
- Huang YJ, Ayers VB, Lyons AC *et al*. *Culex* species mosquitoes and Zika virus. *Vector Borne Zoonotic Dis* 2016; **16**: 673–676.
- Amraoui F, Atyame-Nten C, Vega-Rua A *et al*. *Culex* mosquitoes are experimentally unable to transmit Zika virus. *Euro Surveill* 2016; **21**.
- Ferreira-de-Brito A, Ribeiro IP, Miranda RM *et al*. First detection of natural infection of *Aedes aegypti* with Zika virus in Brazil and throughout South America. *Mem Inst Oswaldo Cruz* 2016; **111**: 655–658.
- Guo XX, Li CX, Deng YQ *et al*. *Culex pipiens quinquefasciatus*: a potential vector to transmit Zika virus. *Emerg Microbes Infect* 2016; **5**: e102.
- Weger-Lucarelli J, Ruckert C, Chotiwan N *et al*. Vector competence of American mosquitoes for three strains of Zika virus. *PLoS Negl Trop Dis* 2016; **10**: e0005101.
- Leal W. Zika mosquito vectors: the jury is still out [version 2; referees: 5 approved]. *F1000Research* 2016; **5**: 2546–2556.
- Amorim LB, Helvecio E, de Oliveira CM *et al*. Susceptibility status of *Culex quinquefasciatus* (Diptera: Culicidae) populations to the chemical insecticide temephos in Pernambuco, Brazil. *Pest Manag Sci* 2013; **69**: 1307–1314.
- Araujo AP, Melo-Santos MA, Carlos SO *et al*. Evaluation of an experimental product based on *Bacillus thuringiensis* sorovar. israelensis against *Aedes aegypti* larvae (Diptera: Culicidae). *Biol Control* 2017; **41**: 339–347.
- Donald CL, Brennan B, Cumberworth SL *et al*. Full genome sequence and sRNA interferon antagonist activity of Zika VIRUS from Recife, Brazil. *PLoS Negl Trop Dis* 2016; **10**: e0005048.
- Scheuermann RH. Zika virus: designate standardized names. *Nature* 2016; **531**: 173.
- Salazar MI, Richardson JH, Sanchez-Vargas I *et al*. Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes. *BMC Microbiol* 2007; **7**: 9.
- Lambrechts L, Chevillon C, Albright RG *et al*. Genetic specificity and potential for local adaptation between dengue viruses and mosquito vectors. *BMC Evol Biol* 2009; **9**: 160.

- 32 Lanciotti RS, Kosoy OL, Laven JJ *et al*. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis* 2008; **14**: 1232–1239.
- 33 Donato MA, Ribeiro EL, Torres Dde O *et al*. Chronic treatment with Sildenafil has no effect on folliculogenesis or fertility in C57BL/6 and C57BL/6 knockout for iNOS mice. *Tissue Cell* 2015; **47**: 515–525.
- 34 Chow VT, Chan YC, Yong R *et al*. Monitoring of dengue viruses in field-caught *Aedes aegypti* and *Aedes albopictus* mosquitoes by a type-specific polymerase chain reaction and cycle sequencing. *Am J Trop Med Hyg* 1998; **58**: 578–586.
- 35 Faria NR, Sabino EC, Nunes MR *et al*. Mobile real-time surveillance of Zika virus in Brazil. *Genome Med* 2016; **8**: 97.
- 36 Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; **30**: 2114–2120.
- 37 Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 2012; **9**: 357–359.
- 38 Fontaine A, Jiolle D, Moltini-Conclois I *et al*. Excretion of dengue virus RNA by *Aedes aegypti* allows non-destructive monitoring of viral dissemination in individual mosquitoes. *Sci Rep* 2016; **6**: 24885.
- 39 Pilotte N, Zaky WI, Abrams BP *et al*. A novel xenomonitoring technique using mosquito excreta/feces for the detection of filarial parasites and malaria. *PLoS Negl Trop Dis* 2016; **10**: e0004641.
- 40 Flies EJ, Toi C, Weinstein P *et al*. Converting mosquito surveillance to arbovirus surveillance with honey-baited nucleic acid preservation cards. *Vector Borne Zoonotic Dis* 2015; **15**: 397–403.
- 41 Hall-Mendelin S, Ritchie SA, Johansen CA *et al*. Exploiting mosquito sugar feeding to detect mosquito-borne pathogens. *Proc Natl Acad Sci USA* 2010; **107**: 11255–11259.
- 42 Bell TM, Field EJ, Narang HK. Zika virus infection of the central nervous system of mice. *Arch Gesamte Virusforsch* 1971; **35**: 183–193.
- 43 Mlakar J, Korva M, Tul N *et al*. Zika virus associated with microcephaly. *N Engl J Med* 2016; **374**: 951–958.
- 44 Rosen L. Overwintering mechanisms of mosquito-borne arboviruses in temperate climates. *Am J Trop Med Hyg* 1987; **37**: 69S–76S.
- 45 Girard YA, Popov V, Wen J *et al*. Ultrastructural study of West Nile virus pathogenesis in *Culex pipiens quinquefasciatus* (Diptera: Culicidae). *J Med Entomol* 2005; **42**: 429–444.
- 46 Girard YA, Schneider BS, McGee CE *et al*. Salivary gland morphology and virus transmission during long-term cytopathologic West Nile virus infection in *Culex* mosquitoes. *Am J Trop Med Hyg* 2007; **76**: 118–128.
- 47 Zhu Z, Chan JF, Tee KM *et al*. Comparative genomic analysis of pre-epidemic and epidemic Zika virus strains for virological factors potentially associated with the rapidly expanding epidemic. *Emerg Microbes Infect* 2016; **5**: e22.
- 48 Boccolini D, Toma L, Di Luca M *et al*. Experimental investigation of the susceptibility of Italian *Culex pipiens* mosquitoes to Zika virus infection. *Euro Surveill* 2016; **21**: e30328.
- 49 Marchette NJ, Garcia R, Rudnick A. Isolation of Zika virus from *Aedes aegypti* mosquitoes in Malaysia. *Am J Trop Med Hyg* 1969; **18**: 411–415.
- 50 Chalegre KD, Romao TP, Amorim LB *et al*. Detection of an allele conferring resistance to *Bacillus sphaericus* binary toxin in *Culex quinquefasciatus* populations by molecular screening. *Appl Environ Microbiol* 2009; **75**: 1044–1049.
- 51 Nasci RS, White DJ, Stirling H *et al*. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. *Emerg Infect Dis* 2001; **7**: 626–630.
- 52 Sucharit S, Surathin K, Shrestha SR. Vectors of Japanese encephalitis virus (JEV): species complexes of the vectors. *Southeast Asian J Trop Med Public Health* 1989; **20**: 611–621.
- 53 Wang Z, Zhang X, Li C *et al*. Vector competence of five common mosquito species in the People's Republic of China for Western equine encephalitis virus. *Vector Borne Zoonotic Dis* 2012; **12**: 605–608.
- 54 Nunes AT, Brito NF, Oliveira DS *et al*. Comparative proteome analysis reveals that blood and sugar meals induce differential protein expression in *Aedes aegypti* female heads. *Proteomics* 2016; **16**: 2582–2586.
- 55 Goertz GP, Fros JJ, Miesen P *et al*. Non-coding subgenomic flavivirus RNA is processed by the mosquito RNAi machinery and determines West Nile virus transmission by *Culex pipiens* mosquitoes. *J Virol* 2016; **90**: 10145–10159.
- 56 Regis L, Silva-Filha MH, de Oliveira CM *et al*. Integrated control measures against *Culex quinquefasciatus*, the vector of filariasis in Recife. *Mem Inst Oswaldo Cruz* 1995; **90**: 115–119.



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