

Short Communication

Evidence of Zika Virus RNA Fragments in *Aedes albopictus* (Diptera: Culicidae) Field-Collected Eggs From Camaçari, Bahia, Brazil

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Abstract

A major mosquito-borne viral disease outbreak caused by Zika virus (ZIKV) occurred in Bahia, Brazil, in 2015, largely due to transmission by the mosquito, *Aedes aegypti* (L.). Detecting ZIKV in field samples of *Ae. aegypti* has proven problematic in some locations, suggesting other mosquito species might be contributing to the spread of ZIKV. In this study, several (five) adult *Aedes albopictus* (Skuse) mosquitoes that emerged from a 2015 field collection of eggs from Camaçari, Bahia, Brazil, were positive for ZIKV RNA; however, attempts to isolate live virus were not successful. Results from this study suggest that field-collected *Ae. albopictus* eggs may contain ZIKV RNA that require further tests for infectious ZIKV. There is a need to investigate the role of *Ae. albopictus* in the ZIKV infection process in Brazil and to study the potential presence of vertical and sexual transmission of ZIKV in this species.

Key words: Zika virus, *Aedes albopictus*, vertical transmission

In early 2015, an outbreak of mosquito-borne Zika virus (ZIKV, *Flaviviridae*; *Flavivirus*) occurred in Bahia, Brazil. Zika virus, a single-stranded RNA arbovirus, can cycle between mosquitoes and humans in an urban environment (Duffy et al. 2009, Haddow et al. 2012) and is transmitted to humans primarily by *Aedes aegypti* (L.) (Centers for Disease Control and Prevention [CDC], Guerbois et al. 2016, Ferreira-de-Brito et al. 2016). Other *Aedes* spp. are thought to be secondary vectors (Diallo et al. 2014, Ios et al. 2014). *Aedes albopictus* (Skuse) has been shown to be a natural vector for ZIKV (Grard et al. 2014, Pan American Health Organization/World Health Organization, [PAHO/WHO] 2016) and is capable of transmitting ZIKV in the laboratory (Chouin-Carneiro et al. 2016). Multiple *Aedes* species and *Culex perfuscus* Edwards were found infected with live ZIKV in field studies in Senegal (Diallo et al. 2014). During a ZIKV outbreak in Micronesia, 73% of the human population were infected with ZIKV, and *Aedes hensilli* Farner was the most abundant mosquito, suggesting it was the primary vector (Duffy et al. 2009, Ledermann et al. 2014).

The Zika outbreak in Bahia resulted in ~110,000 human cases (PAHO/WHO 2016). The first cases were detected in Camaçari,

Bahia, Brazil (Campos et al. 2015), and subsequently, Zika could be found throughout Brazil (PAHO/WHO). Zika virus has rapidly spread to most of the southern hemisphere of the Americas and has made inroads into the southern United States, primarily in local *Ae. aegypti* populations. *Aedes aegypti* in Rio de Janeiro, Brazil, were the primary ZIKV vector while collections of *Ae. albopictus* and *Culex quinquefasciatus* Say were negative for ZIKV (Ferreira-de-Brito et al. 2016). This study suggested vertical and sexual transmission of ZIKV because one *Ae. aegypti* male was positive for ZIKV. Recent studies in Brazil and China suggested that *Cx. quinquefasciatus* might have a role in ZIKV transmission (Ayres 2016, Guo et al. 2016).

Here, we report the detection of ZIKV RNA in adult *Ae. albopictus* collected as eggs during 2015 from Camaçari, Bahia, Brazil.

Materials and Methods

In August 2015, mosquito eggs were collected to establish a laboratory colony from neighborhoods where Zika cases were identified (7,391 suspected cases of exanthematous illness like Zika were reported in Camaçari with 2,626 cases/100,000 inhabitants

Table 1. Primers and probes used to verify presence of ZIKV in mosquito samples

Primers and probe name	Sequences	Position	Amplicon (Base pairs)
ZIKV NS2B set 1 F	5'-GTTACGTGGTCTCAGGAAAGAG-3'	4274–4295	191
ZIKV NS2B set 1 R	5'-CATCAGGACCACCTTGAGTATG-3'	4464–4443	
ZIKV NS2B F	5'-GTTACGTGGTCTCAGGAAAGAG-3'	4274–4295	135
ZIKV NS2B R	5'-GGGAGAAATCACCACTCTCATC-3'	4408–4387	
ZIKV NS2B probe	FAM/TGCGGAAGT/ZEN/CACTGGAACAGTCC-3IABkFQ-3'	4344–4367	

[Secretaria de Saúde do Estado da Bahia, 2016]). Standard oviposition cups, containing germination paper, were placed at three sites in Camaçari (two cups per site; two collections 7 d apart). Field-collected eggs (≥ 50) were hatched, aquatic stages reared, and the adults maintained under standard conditions (Alto et al. 2014). The field-collected eggs provided 20 female and 19 male adult *Ae. albopictus* and one adult *Ae. aegypti* that was discarded. The adults were mated with one another; females were blood-fed by providing chicken blood to produce F1 eggs to establish a colony following approved IACUC procedures (IACUC number 201507682). The same adult female and male mosquito bodies were placed individually in 1.5-ml tubes and stored at -80°C until processed to extract RNA.

The adult mosquito bodies (not including the F1 mosquitoes) were analyzed to detect ZIKV RNA using reverse transcription and quantitative PCR (RT-qPCR). The legs from each mosquito were placed in individual tubes and stored at -80°C for later processing to detect live virus. The RNA was extracted from each *Ae. albopictus* body using Trizol reagent (Thermo Fisher, Waltham, MA). Primer sequences specific to ZIKV were designed (IDT, Coralville, IA) to the NS2B gene of a ZIKV isolate from human blood collected in 2015 in Salvador, Brazil (GenBank KX520666, Table 1). RNA was treated with DNase (Fisher) and reverse transcribed using Enhanced Avian Reverse Transcriptase (42°C for 50 min, Sigma Aldrich, St. Louis, MO), and qPCR reactions were performed using SsoAdvanced SYBR green Supermix on a BioRad CFX96 Real-Time PCR Detection System following standard protocols (BioRad, Hercules, CA). The qPCR conditions were 95°C for 30 s followed by 39 amplification cycles of 95°C for 5 s and 60°C for 30 s. The positive control used in all qPCR reactions was a ZIKV isolate from French Polynesia (strain H/PF/2013, GenBank KJ776791.1) provided by the CDC in January 2016. The titer of ZIKV for each positive *Ae. albopictus* body was calculated as described elsewhere (Shin et al. 2014), but with ZIKV stock virus to generate the standard curve. Specifically, ZIKV titration in samples was performed using the iTaq Universal SYBR Green One-Step Kit (BioRad, Hercules, CA) on the Bio-Rad CFX96 Real-Time PCR Detection System with the same ZIKV specific primers as mentioned. The standard curves for ZIKV titer were obtained by serial dilution of ZIKV stock ($7.2 \log_{10}$ plaque forming unit). The standard curve was defined as the regression line of the logarithm of standard copy number versus Cq (quantification cycle) value.

Quantitative PCR reactions showing ZIKV RNA were analyzed by gel electrophoresis, and DNA was isolated by following the protocols using the GenElute Gel Extraction Kit (Sigma Aldrich, Shin et al. 2014). PCR products (191 nucleotides) were sequenced at Eurofins MWG Operon LLC (Louisville, KY) in both directions using the same primers from the qPCR reaction. A mosquito was considered positive for ZIKV RNA if RT-qPCR showed a Cq ≤ 36 and the PCR product sequence matched ZIKV sequence ($\geq 98\%$ to GenBank KX520666) by Basic Local Alignment Search Tool analysis (Blastn, GenBank). Samples with ZIKV RNA were further screened with a nested qRT-PCR reaction using the iTaq Universal

Table 2. Determination of Cq value and associated titer of ZIKV in adult *Ae. albopictus* collected in Camaçari

Individual mosquito (ID no.)	Sex	Avg Cq ^a	Titer (pfue/ml)
9	F	35.8	154.2
18	F	34.9	273
20	F	33.0	793.8
4	M	34.9	265.5
11	M	35.3	213.4

^a Avg Cq-Average of the Cq values from the technical replicates for each body.

Probes One-Step kit (BioRad). Primers and a probe specific to ZIKV were designed to the NS2B gene of the same ZIKV isolate mentioned previously (Table 1). The amplification was performed as follows: 50°C for 10 min; 95°C for 2 min; 39 cycles of 95°C for 15 s and 60°C for 30 s on the previously mentioned PCR detection system. Quantitative RT-PCR products were 135 nucleotides and Cq values were ≤ 35 . To rule out contamination during processing, four replicates were performed for each sample. Standard no template controls and uninfected mosquito samples were tested in all ZIKV amplification assays.

Results and Discussion

Adult *Ae. albopictus* mosquito bodies from Camaçari were screened for ZIKV. Quantitative PCR found three female (infection rate [IR] = 15%) and two male (IR = 10%) mosquito bodies with ZIKV RNA with a Cq ≤ 36 . The titer of ZIKV in each body was $\sim 2 \log_{10}$ ZIKV plaque-forming unit equivalents (pfue)/ml, consistent with little to no virus replication in these *Ae. albopictus* (Table 2). Sequence analysis of five PCR products revealed matches ($\geq 98\%$, Blastn GenBank) to several ZIKV isolates, including ZIKV from human blood collected in 2015 from Salvador, Brazil, and a human urine sample collected in 2016 from Florida (GenBank KX520666 and KX922707, respectively). Phylogenetic analysis rooted with Spondweni virus showed that sequences from the NS2B gene from the *Ae. albopictus* adults belonged to ZIKV Asian lineage (Zhu et al. 2016, Supp. Figure S1 [online only]).

The detection of ZIKV RNA from five adult *Ae. albopictus* reared from eggs collected during the 2015 outbreak in Camaçari, Bahia, Brazil, is consistent with the potential for vertical or sexual transmission of ZIKV by *Ae. albopictus*; however, evidence supporting this was not conclusive. Vertical transmission has been observed for dengue, yellow fever, West Nile, Japanese encephalitis, and St. Louis encephalitis viruses in several species of mosquitoes (Lequime et al. 2016). Natural vertical transmission of ZIKV was observed in a pool of male *Aedes furcifer* (Edwards) collected in Senegal (Diallo et al. 2014). Vertical and sexual transmission of ZIKV was observed for *Ae. aegypti* collected in Rio de Janeiro, Brazil (Ferreira-de-Brito et al. 2016), and vertical transmission of ZIKV was shown in

Ae. aegypti after intrathoracic inoculation of ZIKV but not for *Ae. albopictus* (Thangamani et al. 2016).

Two questions require answers to assess the significance of this observation. 1) Was the ZIKV RNA owing to contamination during the processing of these mosquitoes? 2) Does the ZIKV RNA represent infectious ZIKV? We believe contamination is unlikely. The RNA extractions were all performed prior to us having ZIKV in our laboratory. Additionally, sequences from the PCR products of the *Ae. albopictus* samples differed sufficiently from our laboratory ZIKV to exhibit separate phylogenetic clustering. Nonspecific amplification of reverse-transcribed mosquito RNA during qPCR reactions could be a source of false positives; however, sequence analysis of positive products did not support this. The low titer of the ZIKV RNA in the adult samples suggests ZIKV did not replicate in these mosquitoes. Absence of replication could mean that complete ZIKV genome or live ZIKV was not introduced into the eggs, or that *Ae. albopictus* is not a competent vector for ZIKV. We were unable to isolate live ZIKV from the Camaçari *Ae. albopictus* samples using cell culture isolation. This and the low ZIKV RNA titer is the cause for uncertainty that vertical or sexual transmission of live ZIKV was responsible for the ZIKV RNA in these field-collected *Ae. albopictus* eggs, although this does not preclude viral replication under different conditions. Future work is needed to characterize the mechanism responsible for transfer of ZIKV RNA to *Ae. albopictus* eggs and whether live ZIKV can accompany this under various yet unknown conditions.

Finding *Ae. albopictus* from Brazil with ZIKV RNA adds further cause for considering *Ae. albopictus*' role in ZIKV epidemiology in Bahia, Brazil, in 2015.

Zika virus RNA in field-collected eggs from mosquitoes where there is current ZIKV transmission is concerning. Samples of mosquitoes, including those resulting from field-collected eggs that are returned to the laboratory from regions with ZIKV, must be treated with the potential that resulting adult mosquitoes or their offspring might be positive for ZIKV RNA. These mosquitoes must be characterized for live ZIKV to ensure they are uninfected or they must be treated as if they did contain infectious ZIKV and maintained under the appropriate required safety and containment practices.

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