



Mosquito Based Zika Virus Surveillance in Florida, 2016

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Citation: Sylla AED, Zhou L, Rogers A, Donohue H, Karki P, et al. (2021) Mosquito Based Zika Virus Surveillance in Florida, 2016. Infect Dis Diag Treat 5: 171. DOI: 10.29011/2577-1515.100171

Received Date: December 22, 2020; **Accepted Date:** January 19, 2021; **Published Date:** January 27, 2021

Abstract

Following the first autochthonous reports of Zika Virus (ZIKV) in Florida during early 2016, mosquito surveillance was initiated to screen the ZIKV in *Aedes* and *Culex* mosquito population. From May to December 2016, 6,094 pools with a total of 78,610 individual female mosquito samples were collected from 33 Florida counties by using BG-Sentinel type 2 traps (Biogents AG, Regensburg, Germany), and tested for the presence of ZIKV by multiplex real-time RT-PCR using TaqMan[®] Zika Virus Triplex Kit (ZIKV/DENV/CHIKV). The majority of the mosquito species in the pools belonged to *Aedes aegypti* and *Aedes albopictus* with a small number belonging to *Culex quinquefasciatus*. ZIKV RNA was detected in eight of the mosquito pools. All eight positive pools were from *Aedes aegypti* and were collected in Miami-Dade County. Sequencing of ZIKV genome from seven of the eight positive pools, performed at the Scripps Institute in La Jolla, California, revealed similar but not identical sequences, further confirming the presence of ZIKV in *Aedes aegypti* mosquito populations. An attempt to isolate ZIKV from six of the eight positive mosquito pools using Vero 76 clone E6 cell line was unsuccessful. Our results highlight the importance of conducting routine surveillance of mosquito pools for the ZIKV in high-risk areas. Routine surveillance will assist human health professionals in virus mitigation strategies for the protection of Floridians and visitors to the Sunshine State and will provide a tool for other states that could be impacted by ZIKV.

Keywords: *Aedes aegypti*; *Aedes albopictus*; Florida; Mosquito surveillance; Zika virus

Introduction

Zika virus (family *Flaviviridae*, genus *Flavivirus*) is an arthropod-borne virus, mainly transmitted by mosquitoes belonging to the genus *Aedes*, similar to the flaviviruses that cause Dengue and Chikungunya virus infections. It has been identified in several countries in Central and South America, Mexico, and the Caribbean since 2015 [1-4]. The first case of sexually transmitted ZIKV infection was reported by the Florida Department of Health

on March 10, 2016 [5]. Since then, ZIKV outbreaks and cases of illness, including babies born with microcephaly to mild febrile illness caused by ZIKV, have been reported [6,7].

Outbreaks have previously been reported in Africa, Southeast Asia, and the Pacific Islands [8]. Local transmission has been reported in Puerto Rico and Florida [9]. Likewise, cases of Zika fever have been reported in travellers returning to the United States [9]. Introduction of ZIKV in the United States and a subsequent increase in cases of congenital microcephaly resulted in the activation of CDC's Emergency Operations Center on January 22, 2016. A coordinated response and timely dissemination of

information led the World Health Organization to declare a Public Health Emergency of International Concern on February 1, 2016. This declaration was intended to help protect pregnant women and their developing fetuses from the effects of ZIKV infection during pregnancy. The emergency declaration highlighted the fact that public health activities must focus on preventing mosquito-borne transmission through vector control efforts as threats from mosquito-borne infection are likely to continue until better vector control interventions are implemented.

The Florida Department of Agriculture and Consumer Services (FDACS), Bronson Animal Disease Diagnostic Laboratory (BADDL), anticipated the usefulness of surveillance testing in mosquitoes for ZIKV to better prepare for the upcoming mosquito season. Several communities in Florida appeared to be prime for local introductions due to abundant populations of the *Aedes* vectors and hot spots for travel to areas of the globe where ZIKV was circulating. Spatial and geographical surveillance data collected on ZIKV distribution can be beneficial in the early detection of the presence of the virus in mosquitoes up to weeks before there is a significant risk to human health. Therefore, the routine surveillance of mosquito populations can provide state and local authorities a means to target neighbourhoods and communities where virus introductions are expected and found to be reappearing. In this investigation, we have used the surveillance of mosquito populations from various Florida counties to determine the prevalence of ZIKV.

Materials and Methods

Mosquito sample collection, handling, and transportation

Mosquitoes were collected from 35 Florida counties using BG-Sentinel type 2 traps (Biogents AG, Regensburg, Germany). The mosquitoes were sorted by date and location of collection and identified to a species level based on morphological characteristics by the local Mosquito Control Program [10]. Any unidentified mosquito pools received at BADDL were identified as *Aedes* species using Stereo microscopy. Following identification, mosquitoes were placed in 6 mL (12 mm X 75 mm) disposable tubes and were separated into pools of 50 or less.

The local Mosquito Control Program used two approaches to preserve mosquito pool specimens and send them to BADDL for testing including cold packs and RNAlater. The integrity of the mosquito samples for viral RNA detection and virus isolation depended on proper sample collection, storage temperature, and transportation to the testing laboratory [11]. Cold packs were utilized, and when possible, throughput from collection to transportation. For instances in which cold packs could not be utilized, RNA stabilizer, RNAlater (Invitrogen ThermoFisher) was used for mosquito collection and transportation to BADDL. In brief, 1 mL of RNAlater™ was used for a pool of >10 to 50 mosquitoes and 0.5 mL of RNAlater™ was used for a pool of

less than 10 mosquitoes. The samples were mixed gently and transported to the laboratory.

RNA Isolation

For samples submitted without RNAlater™, RNAs were extracted by adding copperhead BBs (copper-clad airgun shot, caliber 0.177) to each tube with a homogenization buffer (1X Phosphate Buffered Saline (PBS), pH 7.4 with 1% Fetal Bovine Serum (FBS) and MS2 RNA (*Escherichia coli* MS2 Phage RNA, ATCC® 15597B1) as an Internal Positive Control (IPC). The samples were homogenized using a Qiagen Tissuelyser (Retsch Mixer Mill 300 modified to hold microcentrifuge tubes with 2 x 24 adapter plates for 2 mL micro centrifuge tubes) set at 25 cycles/second for four minutes and clarified by centrifugation at 500 RCF for 4 minutes at 4°C. The supernatant was extracted using the MagMax™ Viral Isolation Kit (ThermoFisher, Waltham, MA), following the manufacturer's standard extraction procedures.

For samples submitted with RNAlater™, RNAs were extracted by removing the RNAlater™, washing once using refrigerated 1X PBS, pH 7.4, then homogenized and clarified as stated above. The supernatant was extracted using the MagMax™-96 for Microarrays Total RNA Isolation Kit (ThermoFisher, Waltham, MA), following the manufacturers spin procedures.

Reverse transcription and quantitative real-time PCR Amplification (RT-qPCR): RT-qPCR was performed immediately on all the extracted RNA samples and were tested using the TaqMan® Zika Virus Triplex Kit (ZIKV/DENV/CHIKV) (Thermo Fisher, Waltham, MA) on the Applied Biosystems® 7500 Real-time PCR System (ThermoFisher, Waltham, MA). The cycling conditions were as follows: 20 minutes at 50°C for reverse transcription, 2 minutes at 95°C for activation of the Taq enzyme; and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Any positive or suspect positive samples were confirmed with Zika1086/Zika1162c/Zika1107FAM assay, which detects all known genotypes of the Zika virus [12], as well as Zika4481/Zika4552c/Zika4507cFAM assay, which is specific for the Asian genotypes currently circulating in the Western Hemisphere [13]. Total reaction volumes for both assays are 25 µL, containing 1X of QuantiTect Probe One-step RT-PCR master mix (Qiagen, Hilden, Germany), 5 µL RNA template, 1 µM of each primer, and 0.15 µM probe. The cycling conditions were as follows: 30 minutes at 50°C for reverse transcription, 15 minutes at 95°C for activation of the HotStarTaq enzyme; and 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. Samples testing positive on these assays were forwarded to CDC for confirmation and to Florida Gulf Coast University (FGCU) for sequencing.

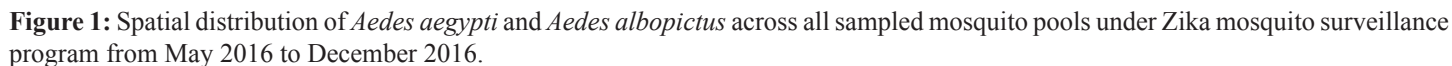
Viral Culture

Attempts were made to isolate the ZIKV from 6 pools of PCR-positive ZIKV mosquito homogenates using cell culture.

Results

Only 33 Florida county mosquito pools were tested for the presence of ZIKV. Two counties submitted mosquitoes that were not *Aedes aegypti* nor *Aedes albopictus* and subsequently, those pools were not tested. All remaining mosquitoes submitted to the laboratory were placed in separate tubes based on the collection date, site and species, including no more than 50 mosquitoes per tube. A total of 6,094 pools of mosquitoes, representing 78,610 individual female mosquitoes were tested between May and December of 2016 (Table 1, Figure 1) including 46,182 of individual *Aedes aegypti*, 32,363 of individual *Aedes albopictus*, 12 individual mosquitoes from mixed *Aedes* spp. and 53 of individual *Culex quinquefasciatus*.

Species	No. of Mosquitoes Tested
<i>Aedes aegypti</i>	46,182
<i>Aedes albopictus</i>	32,363
Mixed <i>Aedes</i> spp.	12
<i>Culex quinquefasciatus</i>	53
Total	78,610



The proportion of *Aedes aegypti* and *Aedes albopictus* is represented as a ‘pie chart, and the pie chart sizes correspond to the total mosquitoes collected with Log10 transformation.

Counties in Florida conducting mosquito surveillance for *Aedes aegypti* and *Aedes albopictus*, along with the numbers of mosquitoes collected by each county, are listed in Table 2. Of these, 58.7% were *Aedes aegypti* and 41.3% were *Aedes albopictus*. Lee and Miami-Dade are the two counties in Florida that submitted most of the mosquito samples, with 28.4% from Lee County and 20.9% of mosquitoes from Miami-Dade, respectively.

Spatial distribution of *Aedes aegypti* and *Aedes albopictus* across all sampled mosquitoes is presented on the Florida county map (Figure 1). Different mosquito species can be observed on the pie chart on the same map. Mosquito samples with more than 99% of *Aedes aegypti* were submitted by counties in the south and west coast of Florida, including Broward, Monroe, Pinellas, Miami-Dade, and Pasco counties. The numbers of *Aedes aegypti* gradually reduced toward the northwest, while mosquito samples with more than 99% of *Aedes albopictus* were submitted by counties from north to northwest of Florida, including Citrus, Calhoun, Franklin, Leon and Liberty counties.

Table 2: Mosquito surveillance (*Aedes aegypti* and *Aedes albopictus*) from May to December 2016 in different counties of Florida.

Counties	No. of Mosquito Pools	No. of Mosquitoes	<i>Aedes aegypti</i>		<i>Aedes albopictus</i>	
			Total No.	%	Total No.	%
Bay County	10	173	10	5.8	163	94.2
Brevard	271	2487	819	32.9	1668	67.1
Broward	56	742	742	100	0	0
Calhoun	33	1520	0	0	1520	100
Citrus	58	816	1	0.1	815	99.9
Clay	1	12	0	0	12	100
Duval	60	1299	472	36.3	827	63.7
Franklin	11	323	0	0	323	100
Hamilton	2	4	0	0	4	100
Hardee	9	62	9	14.5	53	85.5
Highlands	1	3	3	100	0	0
Hillsborough	293	7455	6315	84.7	1140	15.3
Lake	22	211	110	52.1	101	47.9
Lee	669	22291	7942	35.6	14349	64.4
Leon	195	3531	0	0	3531	100
Levy	6	31	0	0	31	100
Liberty	18	141	0	0	141	100
Marion	23	267	42	15.7	225	84.3
Miami-Dade	1890	16344	16249	99.4	95	0.6
Monroe	353	2109	2109	100	0	0
Okeechobee	8	54	40	74.1	14	25.9
Orange	455	6431	3341	52.0	3090	48.0
Osceola	441	1743	525	30.1	1218	69.9
Palm Beach	147	1609	1553	96.5	56	3.5
Pasco	35	855	855	100	0	0
Pinellas	111	1020	1012	99.2	8	0.8

Polk	12	279	24	8.6	255	91.4
Sarasota	8	68	66	97.1	2	2.9
Seminole	471	3312	1671	50.5	1641	49.5
St. Johns County	30	718	631	87.9	87	12.1
St. Lucie	237	2238	1373	61.3	865	38.7
Volusia	135	318	263	82.7	55	17.3
Wakulla	14	79	5	6.3	74	93.7
Total	6085	78545	46182	58.7	32363	41.3
Counties submitted more than 100 mosquitoes with >99% <i>Aedes aegypti</i> or >99% <i>Aedes albopictus</i> were highlighted in bold.						

Detection and confirmation of ZIKV in mosquitoes

A total of 6,094 single-species pools were tested for ZIKV, Dengue virus (DENV), and Chikungunya virus (CHIKV) in Bronson Animal Disease Diagnostic Laboratory (BADDL) using TaqMan® Zika Virus Triplex Kit (ZDC Assay). Escherichia coli MS2 Phage RNA was used as an Internal Positive Control (IPC) for monitoring the RNA extraction and PCR inhibitors in the sample. Dengue virus or Chikungunya virus were not detected in any pool sample; however, Zika virus was detected from 8 mosquito pools with a Cycle threshold (Ct) value from 19.22 to 27.63 (Table 3) and was confirmed to be of Asiatic origin using the secondary assay to detect ZIKV Asian-specific genotype. The Ct value of Positive Amplification Control (PAC) and IPC in all runs were in an expected range, and no amplification was observed from negative controls, indicating the test was valid. Eight positive mosquito pools were all *Aedes aegypti* collected from Zika active transmission areas within Miami-Dade County in 2016.

ZIKV in 8 mosquito pools was confirmed by the CDC and sequenced at FGCU. Only 7 positive pools yielded enough material for amplicon sequencing or hybrid capture sequencing. The sequence data from these 7 pools have been deposited in NCBI GenBank [15,16].

Table 3: Positive sample results from *Aedes aegypti* collected from Miami-Dade County.

Sample ID	Date of collection	No. of mosquitoes per pool	Zika ZDC Ct.	Zika Universal Ct.	Zika Asian Ct.	GenBank ID
1	8/22/2016	39	22.94	27.97	27.36	KX838904.2*, KY014324.2 [#]
2	8/23/2016	25	24.88	30.00	29.30	KX838905.2*, KY014323.2 [#]
3	8/23/2016	15	19.22	25.17	24.95	KX838906.2*, KY014322.2 [#]
4	9/4/2016	50	21.25	30.49	29.77	KX922708.1*, KY014299.2 [#]
5	9/9/2016	21	23.56	31.16	30.55	KY075937.1*, KY785422.1 [#]
6	9/20/2016	23	22.29	27.15	26.70	KY075938.1*, KY785472.1 [#]
7	9/23/2016	1	27.63	31.79	31.88	Insufficient material
8	10/5/2016	13	27.09	31.61	30.91	KY075939.2*, KY785468.1 [#]

*Sequences were deposited in NCBI GenBank by Scripps Research Institute at La Jolla, CA for FGCU [15].

[#]Sequences were deposited in NCBI GenBank by Infectious Disease Program at Cambridge, MA [22].

ZIKA Virus Isolation

The positive control showed CPE with rounding and clumping of cells on 4-5 days, whereas the homogenate samples showed the toxic effect on the first passage. No CPE was observed on the second passage. Hence the results for all the six pools were concluded as “No virus isolated.”

Effect of RNAlater on the Zika virus Isolation

The positive control, without RNAlater propagated on the cell line. All the 6 PCR positive ZIKV mosquito pools with RNAlater did not show CPE. An additional effort was made to determine if there is any effect of RNAlater on the ZIKV. In this experiment, the ZIKV positive control was serially diluted (10^{-1} - 10^{-10}) in serum diluent. 300 μ L of each dilution was added to 700 μ L of RNAlater and 1 mL of known-negative mosquito homogenates. Samples were homogenized, centrifuged and adsorbed onto VeroE6 cell line by adsorption method for Virus isolation [14]. Attempts were made up to 4 passages. All the flasks were observed daily for CPE. First passage all the flasks exhibited toxic effect, but consecutive passages did not show any toxic effects. All 4 passages did not show any CPE, hence, on the fourth passage, the virus isolation was stopped and all the tissue culture fluids from the first passage and the 4th passage were tested for ZIKV by RT-qPCR. ZIKV RNA was detected only on the 10^{-1} (Ct 34.53) and 10^{-2} (Ct 37.68) tissue culture fluid on the first passage.

Discussion

Florida's first case of sexually transmitted ZIKV was reported in March 2016. By the end of March 2016, there were 108 travel related ZIKV cases and that number increased to 1,112 by the end of December 31, 2016 (Figure 2). In addition to these cases, there were 125 confirmed locally transmitted cases identified. On July 29, 2016, the first locally transmitted case was reported in Miami and the number of the locally acquired infections increased to 285 by the end of December 31, 2016. These increasing number of cases, and the severity and complications of the diseases caused by ZIKV, such as microcephaly in new-borns and Guillain Barre Syndrome, prompted FDACS to initiate a mosquito-based surveillance plan. The surveillance consisted of the systematic collection of mosquito samples, species identification, and transportation to the lab for Zika, Dengue, and Chikungunya virus screening.

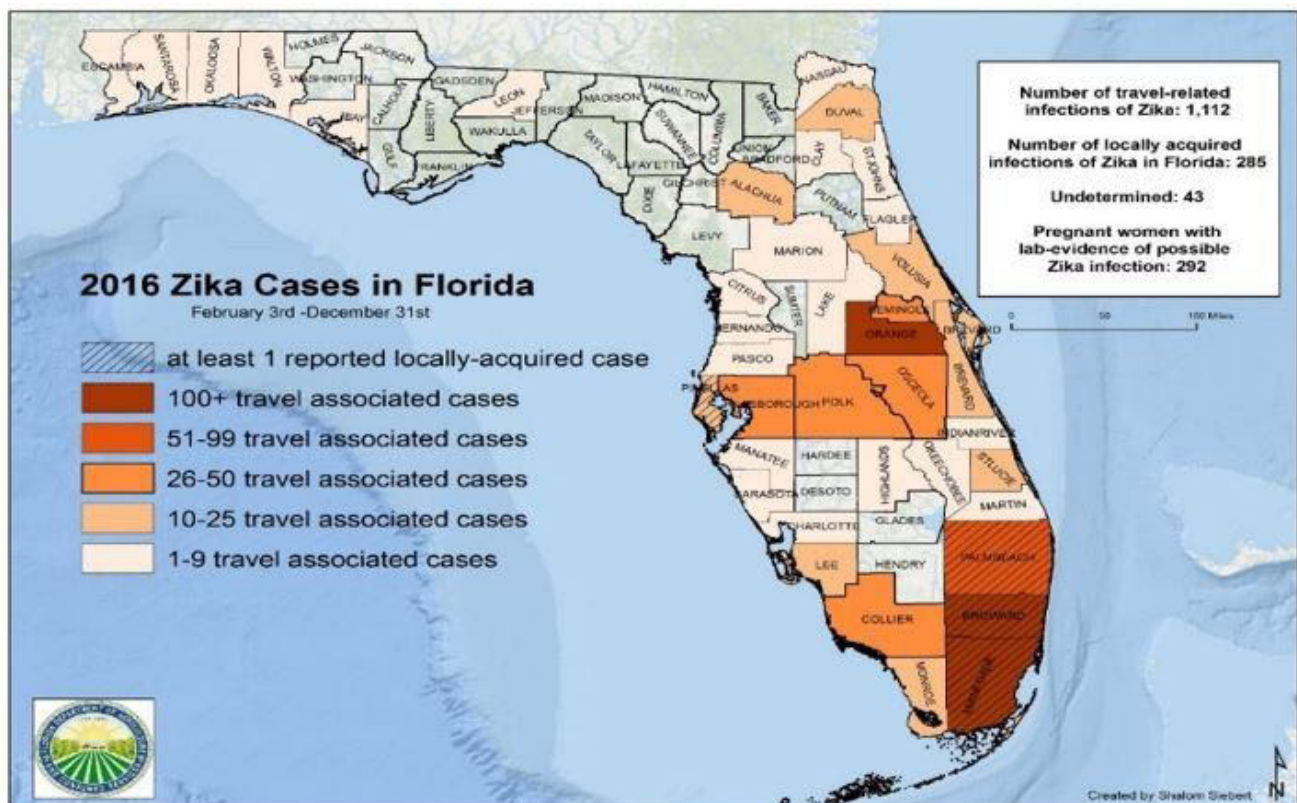


Figure 2: Distribution of Confirmed Zika Cases in Florida for 2016.

ZIKV has been detected in more than 20 mosquito species [17-19] but primarily transmitted by *Aedes* mosquitoes, mainly *Aedes aegypti* [17,19]. From August to October, ZIKV was detected from 8 *Aedes aegypti* mosquito pools and they were all collected from Miami-Dade County, Florida. Like many other arboviruses, ZIKV local transmission was identified close to the end of the rainy season, which peaked between August and October [20,21]. In the areas of the state with local transmission, *Aedes aegypti* (58.7%) was the more prevalent mosquito species collected and tested than *Aedes albopictus* (41.3%). Another observation from the surveillance data is some counties have either *Aedes aegypti* or *Aedes albopictus*, but not both species together (Table 2). These observations are also supported by CDC and other labs that actual mosquito populations will vary by state and county [22,23]. In this study, 99.4% of mosquitoes collected from Miami Dade county were *Aedes aegypti*, and none of the *Aedes albopictus* collected throughout Florida were found to be positive for ZIKV. A possible reason could be that *Aedes aegypti* is mainly considered an anthropophilic, day-time indoor feeder [17]. They prefer to feed on humans in the tropical urban areas of Asia and the Americas [24,25], and susceptible to contract and transmit the ZIKV [26], whereas *Aedes albopictus* is more opportunistic in its host preference and can be found in more suburban to rural areas. 6 pools with a total of 53 *Culex quinquefasciatus* were tested in this study and ZIKV were all negative. Research findings from Stenn, et al. [27] suggested that *Aedes aegypti* and *Aedes albopictus* are the most likely vectors of ZIKV and that *Culex quinquefasciatus* would likely play a lesser role in ZIKV transmission in Florida. However, the relative importance of the three species in ZIKV transmission is likely location and population specific.

ZIKV RNA was detected from 8 mosquito pools with *Aedes aegypti* collected from Miami-Dade County using TaqMan Zika Virus Triplex Kit. This sensitive and easy to use multiplex Arbovirus screening kit has played an important role in mosquito-borne arbovirus surveillance tests in Florida during the time of the Zika outbreak in 2016 by providing a rapid, sensitive, and specific method for early detection of ZIKV. No mosquito pool was found positive for Dengue and Chikungunya virus. It has been reported by Lanciotti and Nasci [11,28] that nucleic acid detection assays are the most sensitive assays for virus detection and confirmation of WNV in mosquito pools. Similar findings were observed in our study for ZIKV in mosquito pools.

The attempt to grow the virus in our hands was not successful despite PCR positive pools with Ct value less than 28. It has been observed that there will be a good chance to isolate the virus if the Ct value is less than 28 (Wuze Ren, NYC Department of Health & Mental Hygiene, personal communication, 2019). The key is that a high titer of virus is needed for promising isolation. The reason for not being able to grow the virus could be (1) Inadequate sample for virus isolation (as per standard virus isolation protocol, 1 mL-3 mL

of sample is needed), (2) some viruses may stop growing due to interferon response which is true in case of Vero cells or (3) RNA later may inhibit the virus propagation.

Based on our small study and the findings, we believe that RNAlater will preserve the RNA, however, it may affect the infectivity of ZIKV. Mutebi, et al. [29] reported that they could grow the virus from one mosquito pool of *Aedes aegypti*. The pool was collected from the Miami-Dade County, adjacent to a site where ZIKV has been detected by our lab. In their study, the mosquitoes captured were frozen and shipped on dry ice to their laboratory without RNAlater, unlike in our study, mosquito pools were collected in RNAlater and shipped to the laboratory at room temperature. Further study is required to conclude the role of RNAlater in ZIKV isolation.

Mosquito-based surveillance is an integral component of an integrated vector management program [30] and is a vital tool for quantifying Zika virus transmission and human risk. The goal of early detection was to enhance mosquito control efforts in high-risk areas to stop the virus amplification before it could cause a significant impact on human health, which is a principal function of a mosquito-based surveillance program.

The genomic sequences from seven positive mosquito pools were similar but not identical. *In silico* sequence analysis showed many Single Nucleotide Polymorphisms (SNPs) among the ZIKV isolates in mosquitoes. These results highlight the presence of various introductions of ZIKV in *Aedes aegypti* mosquito populations in Florida. Phylogenetic analysis for the ZIKV genomes from clinical and mosquito samples from different countries and territories in the Americas indicated the main source of virus circulated in Miami-Dade County, Florida was from the Caribbean islands [15,16,29].

The phylogenetic study [16,31] for 110 ZIKV genomes revealed that locally transmitted cases were confirmed several months after the ZIKV has been circulated but undetected in multiple areas. This finding also highlights the importance of surveillance for mosquito-borne diseases.

The importance of detailed surveillance in this study can provide a road map for future studies by organizations like CDC or other labs. Because of the local transmission in the Miami area, Miami-Dade County could be used as a resource for targeted surveillance in the future. As a matter of fact, CDC has already done targeted surveillance of Miami-Dade County and reported an isolate of ZIKV, strain MB 16-23 from a pool of 50 *Aedes aegypti* mosquitoes collected in Miami Beach, Florida [29]. Local transmission of ZIKV in Florida prompted the expansion of sentinel surveillance to enhanced passive surveillance starting in August 2016 by NYC-DOHMH [32]. Though the previous report indicated that ZIKV can be transmitted by *Aedes aegypti*

and *Aedes albopictus* [33-37], the present study found ZIKV was transmitted principally by *Aedes aegypti* mosquitoes in Florida. The predominance of *Aedes aegypti* mosquitoes trapped during this outbreak suggests the important role this species plays in ZIKV transmission. Enhanced monitoring through surveillance for the early detection of this virus or other arboviruses outside of the affected area will be crucial to guide vector control measures in the future [37-39].

Our results revealed one hot spot, Miami-Dade County, with *Aedes aegypti* harbouring ZIKV. These results highlight that proactive surveillance of mosquito pools and early detection of the virus can be key to mosquito control activities in high-risk areas in order to interrupt the amplification of the virus before it can impact human health. Further surveillance studies are needed to determine the role of ZIKV in animal populations and transmission of the virus from animal to human or vice versa as observed in other arboviruses.

Acknowledgements

We thank the timely help from CDC for confirming positive Zika pools and FGCU for the sequencing of positive ZIKV pools. Dr. Danielle Stanek for providing the FL-DOH positive travel related and locally transmitted numbers of ZIKV cases. Dr. Sudheesh Ponnerassery for reviewing and many helpful suggestions for the manuscript. Anne Vuxton for final proofreading and David Simon, Alexander Nemethy for initial proofreading of the manuscript. Shalom Siebert for analyzing the spatial data and creation of the Figure 2 map.

This publication was supported by funding from Florida Department of Agriculture and Consumer Services' (FDACS). A.E.D. Sylla, L. Zhou, H. Donohue and P. Karki coordinated with mosquito control program, performed the experiments, provided the data and materials and methods for the manuscript. L. Zhou, in addition has made a great contribution for editing the manuscript. A. Rogers Coordinated and organized targeted *Aedes aegypti* and *Aedes albopictus* surveillance activities and species identification throughout Florida by providing equipment, training, and guidance to local mosquito control programs and counties. Additionally, assisted local mosquito control programs in establishing and fine-tuning integrated mosquito management programs designed to combat these vector species. B. Zale, A. Winslow and A. Yan helped to do the experimental work. Remy Powell was responsible for effect of RNA later experiment. Y. R. Bommineni, E. Field, M. Short, L. Conti† conceived, planning and critically reviewed the manuscript. S. D. Mohan facilitated, designed the study, interpreted data, and wrote the manuscript.

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