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Arbovirus Surveillance in Field-Collected Mosquitoes From Pernambuco-Brazil, During the Triple Dengue, Zika and Chikungunya Outbreak of 2015-2017

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The (re) emergence of arboviruses around the world is a public health concern once severe outbreaks are usually associated with these infections. The co-circulation of Dengue (DENV), Zika (ZIKV) and Chikungunya (CHIKV) viruses in the past few years has caused a unique epidemic situation in Brazil. The northeast region of the country was the most affected by clinical complications from such arboviruses' infections, including neurological disorders caused by ZIKV. In this particular region, *Aedes* mosquitoes are the main vectors of DENV, ZIKV and CHIKV, with *Culex quinquefasciatus* also considered as a potential vector of ZIKV. Therefore, virological surveillance in mosquitoes contributes to understanding the epidemiological profile of these diseases. Here, we report the circulation of DENV, ZIKV and CHIKV in *Aedes* spp. and *Cx. quinquefasciatus* female mosquitoes collected in areas with a high arbovirus circulation in humans in the Metropolitan Region of Recife, Pernambuco, Brazil, during the triple-epidemics of 2015-17. All the field-caught mosquitoes were sent to the laboratory for arbovirus screening after RNA extraction and RT-PCR/RT-qPCR. A total of 6,227 females were evaluated and, as a result, DENV, ZIKV and CHIKV were identified in *Ae. aegypti*, *Ae. taeniorhynchus* and *Cx. quinquefasciatus* mosquito pools. In addition, DENV and ZIKV were isolated in C6/36 cells. In conclusion, it is important to highlight that arbovirus surveillance performed in mosquitoes from DENV-ZIKV-CHIKV hotspots areas can serve as an early-warning system to target vector control actions more efficiently in each studied area.

Keywords: vector surveillance, arbovirus, RT-PCR, RT-qPCR, *Aedes*, *Culex*

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INTRODUCTION

Vector-borne diseases have expanded its geographical distribution in the past decades due to a myriad of factors, such as globalization, climate changes and the lack of specific therapeutics or vaccines (1, 2). Dengue virus (DENV), an arbovirus mainly transmitted to humans through the bite of *Aedes aegypti* and *Aedes albopictus* mosquitoes, was estimated to annually infect 80-100 million humans in 1998 (1, 3) and now is predicted to infect nearly 400 million people per year (4). Currently, Brazil is the country with the largest number of dengue fever cases in the world (5) with expected outbreak cycles occurring every three years (6). In 2015, Brazilian authorities reported a triple-arboviral epidemics of Dengue, Zika (ZIKV) and Chikungunya (CHIKV) viruses (7). With over 200,000 cases reported in 2016 (8), ZIKV infection was associated to neurological complications such as Guillain-Barré Syndrome in adults and Congenital Zika Syndrome (CZS) (9, 10). The Brazilian Northeast region was the most affected area by ZIKV and, the city of Recife (capital of Pernambuco state) reported the highest number of CZS cases in the country (9, 10). Recife displays favorable conditions for arboviruses outbreaks: poor sanitation structure, high densities of mosquito vectors, unsatisfactory healthcare structure, informal low-income housing, among others (11).

Regarding high densities of mosquito vectors, monitoring viruses in field-collected mosquito vectors is a key element in arboviruses surveillance programs, that can serve as a warning system to guide the appropriate measures prior to virus notification in humans (12–14). Molecular epidemiology studies can also be carried out based on entomological surveillance with virus detection, which allows the investigation of viral evolution or adaptation, as well as identifying new strains (15, 16). In addition, massive screening of arboviruses in mosquito species may also provide data over which vectors are participating in the local virus dynamics (14, 17). Since the public health emergency of international concern caused by the broad ZIKV circulation, several groups have reported a large dataset of diversified results of arbovirus detection in mosquitoes from around the world. In Brazil, for instance, *Ae. aegypti* mosquitoes from Rio de Janeiro were found naturally-infected with ZIKV during surveillance conducted in 2015-16, with no evidence of infection in *Ae. albopictus* and *Culex quinquefasciatus* (18). Arbovirus-surveillance from two independent datasets of field-collected mosquitoes from Recife (Northeast Brazil) and Vitória (Southeast Brazil) in 2016 showed the circulation of ZIKV not only in *Ae. aegypti*, but also in *Cx. quinquefasciatus* with no signs of recent bloodmeal (19, 20).

The proliferation of vectors, such as *Ae. aegypti* and *Cx. quinquefasciatus*, have been associated with environmental, ecological and socioeconomic factors, as arbovirus transmission has increased over the years in several parts of the world (21). In Brazil, regions with the highest risk of arbovirus transmission display all the above-mentioned factors, coupled with inadequate vector control strategies (22). In an attempt to reduce arbovirus circulation in the country, public health authorities usually rely on the massive use of chemical insecticides as the main strategy, which results in the selection of resistant mosquitoes (23, 24).

The main goal of the present study was to conduct an extensive arbovirus surveillance of DENV, ZIKV and CHIKV in natural populations of *Aedes* and *Culex* mosquitoes, collected in hotspots (areas previously known that have a higher probability of arbovirus circulation) from the Metropolitan Region of Recife (MRR), during the triple-epidemics reported from 2015 to 2017. In order to understand the dynamics of transmission, direct vector control and monitor the evolution of detected viruses.

MATERIALS AND METHODS

Study Area

The study was conducted in the Metropolitan Region of Recife (MRR), in 236 collection sites distributed in six municipalities: Recife, Jaboatão dos Guararapes, Olinda, Camaragibe, Paulista and São Lourenço da Mata (**Figure 1**). Climate conditions in these municipalities are predominantly hot and humid, with temperatures ranging from 25°C to 32°C and humidity varies between 60-70%. Most of the collection sites were located in the state capital, Recife, which has approximately a population of 1,537,704 habitants and 218,435 km² of area (25). All the selected cities have large urban settings, except São Lourenço da Mata.

Viral Strains and *In Vitro* Transcription

Viral stocks used as positive controls for the assays were ZIKV BRPE243/2015 (KX197192), CHIKV BRPE408/2016, DENV-1 42735/BR-PE (EU259529), DENV-2 3808/BR-PE (EU259569), DENV-3 85469/BRPE (EU259607) and DENV-4 1385 (U18425) (were all kindly provided by Dr. Marli Tenório, LAVITE-AMI). DENV positive control for conventional RT-PCR was obtained by RNA extraction from the viral stocks, while the standard curve of ZIKV and CHIKV used in RT-qPCR was synthesized by *in vitro* transcription using MEGAscript T7 kit (Ambion, catalog #AM1334, Carlsbad, CA, USA). Each virus standard was quantified in Nanodrop 2000 and RNA concentration was converted in RNA copy number (26).

Mosquito Collection and Identification

Adult mosquitoes were obtained using battery-operated aspirators (Horst Armadilhas Ltd, São Paulo, Brazil) between January/2015 and December/2017, from houses with suspected arboviral human cases (either suspected or laboratory-confirmed) and public places, such as universities, public squares and government buildings. Adult mosquitoes were also collected from hospitals and Emergency Care Units (Unidades de Pronto Atendimento - UPA) which are places considered reference in clinical care and monitoring of arboviruses.

Field-caught mosquitoes were immediately sent in aspiration bags to the Entomology Laboratory – Aggeu Magalhães Institute (AMI), where individuals were kept at -20°C for 30 minutes and, morphologically identified on ice-chilled Petri dishes using taxonomic keys (27). Mosquito specimen were sorted in pools

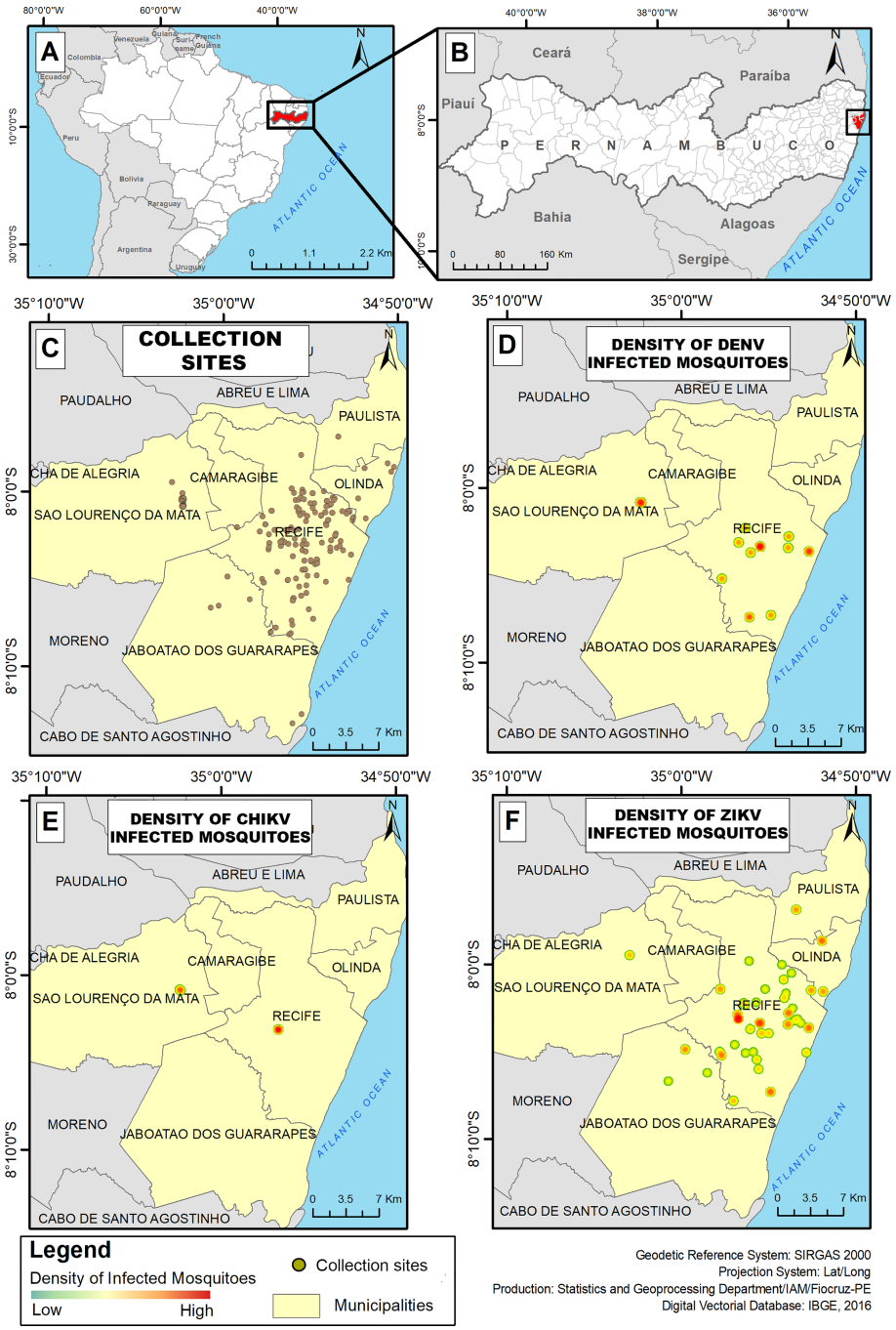

FIGURE 1 | Maps of the Metropolitan Region of Recife with collection sites and densities of infected mosquitoes. **(A)** Map of Brazil with Pernambuco state in red; **(B)** Map of Pernambuco state with MRR in red; **(C)** Study area with collection sites; **(D)** Density of DENV infected; **(E)** Density of CHIKV infected mosquitoes; **(F)** Density of ZIKV infected mosquitoes.

(up to 10 mosquitoes) by locality, species, sex and feeding status (blood-engorged and non-engorged). And then they were transferred to 1.5 ml DNAse/RNAse free microtubes and stored at -80°C until further usage. Prior to RNA extraction, each mosquito pool was homogenized as described in Barbosa et al. (28).

Molecular Detection of DENV, ZIKV, and CHIKV

RNA extraction protocol was carried out as described in Guedes et al. (20). Two approaches were used for arbovirus detection, conventional RT-PCR for DENV and multiplex RT-qPCR for ZIKV and CHIKV. DENV molecular detection was performed

using a single-tube Multiplex RT-PCR, as described in Barbosa et al. (28). Each PCR run was performed using a positive control and two negative controls (one comprising all RT- PCR reagents except RNA template and the other one, the negative control from RNA extraction). PCR products were run for 50 minutes at 120V on a 1.5% agarose gel stained with ethidium bromide and visualized on a UV transilluminator. Due to the recent introduction of both ZIKV and CHIKV in the northeastern region in 2015, laboratory diagnosis protocols were adapted to increase the sensitivity of the reaction and to simultaneous detection of arbovirus in the same sample. A quantitative RT-PCR for both ZIKV and CHIKV (Duplex) was assayed in a QuantiStudio 5 system (Applied BioSystems, Foster City, CA, USA). Reactions were performed using the QuantiNova Probe RT-PCR kit (Qiagen, catalog #208354, Hilden, Germany) in a 15 µl final volume, with primers of both viruses in a 0.8 µM final concentration and probes in a 0.1 µM final concentration. Primers and probes are described in **Table 1**. All samples were tested in duplicates, using a standard curve consisting of a serial dilution of previously quantified RNA transcript and negative controls (all reagents except RNA and negative control from RNA extraction). RT-qPCR results were analyzed using QuantStudio Design and Analysis Software 1.3.1 (Thermo Fisher Scientifc) with automatic threshold and baseline. Samples that produced Cq values of ≤ 38.5 in both duplicates were considered positive.

Minimum Infection Rate

After DENV, ZIKV and CHIKV detection in mosquito pools, we calculated the Minimum Infection Rate (MIR) as the following formula: (the number of positive pools/total number of mosquitoes tested) × 1,000, for each virus and species, according to Chow et al. (32).

Molecular Confirmation of Positive Mosquito's Feeding Status

To confirm the feeding status in each pool of mosquitoes identified as positive for any arbovirus tested, we use a well-stablished PCR protocol based on mitochondrial DNA identification (33). DNA was extracted from a 50 µl aliquot of the stored homogenates using an alcohol precipitation protocol described by Ayres et al. (34). Each PCR run contained a positive control (DNA from a human blood sample) and negative controls (all reagents except DNA and negative control from DNA extraction). Confirmation of the amplified fragments was performed on 1.5% agarose gel for 50 minutes at 120V, stained with ethidium bromide and visualized in U.V. After confirmation, PCR products were sequenced on ABI 3500xL Genetic Analyzer sequencer (Applied Biosystems), using BigDye® (Applied Biosystems Foster City, CA, USA). The sequencing was carried out at the AMI/FIOCRUZ Technological Platforms Nucleus. Sequences were then edited, contigs assembled and aligned with CodonCode Analyzer program (v.3.7.1) and compared with the sequences previously archived in GenBank, through the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/blast.cgi).

Viral Isolation and Indirect Immunofluorescence Assays

For virus isolation from positive mosquito pools, twenty microliters of arbovirus-confirmed homogenates (samples with Cq values that ranged from 13.2 to 38.3 are described in **Table 2**) were inoculated into confluent monolayers C6/36 cells for 1 hour, in 10 cm^2 cell culture tubes (TTP®, Techno Plastic Products AG, Trasadingen, Switzerland) with Leibovitz Medium (L-15, Gibco, catalog #41300-039, Carlsbad, CA, USA) supplemented with 2% of fetal bovine serum and 1% of antifungal (Fungizone) (Gibco, catalog # 15290-018, Carlsbad, CA, USA) and antibiotic (Penicillin/Streptomycin) (Gibco, catalog #15140-122, Carlsbad, CA, USA). Cell culture flasks were incubated at 27°C, during seven days for cytopathic effects (CPE) visualization. After CPE visualization, samples were frozen and stored at −80°C for further usage. This procedure was repeated for four passages. Viral isolation was monitored by RT-qPCR in each passage (**Table 2**), as previously described for both ZIKV and CHIKV. DENV reactions were

TABLE 1 | Primers and probes sequences used in DENV, CHIKV and ZIKV with field-caught mosquito collected from 2015 to 2017 in Pernambuco, Brazil.

Reaction	Virus	Primers and probes	Size (bp)	Sequence (5' 3')	Reference
Multiplex RT-PCR	DENV	Dcon1	–	TCAATATGCTGAAACGCGCGAGAAACCG	(29)
	DENV1	TS1	419	CGTCTCAGTGATCCGGGGG	
	DENV2	TS2	119	CGCCACAAGGGCCATGAACAG	
	DENV3	TS3	290	TAACATCATCATGAGACAGAGC	
	DENV4	TS4	392	CTCTGTTGTCTTAAACAAGAGA	
Duplex RT-qPCR	CHIKV	6856	126	TCACTCCCTGTTGGACTTGATAGA	(30)
		6981		TTGACGAGAGTTAGGAACATACC	
		6919 VIC		AGGTACGCGCTTCAAGTTCGGCG	
	ZIKV	1087	77	CCGCTGCCCAACACAAG	(31)
		1163c		CCACTAACGTTCTTTTGCAGACAT	
		1108 FAM		AGCCTACCTTGACAAGCAGTCAGACACTCAA	
RT-qPCR	DENV	NS5F	104	GGAAGGAGAAGGACTGCACA	(26)
	DENV	NS5R		ATTCTTGTGTCCCATCCTGCT	
In vitro transcription	CHIKV	6856 IVT	–	TAATACGACTCACTATAGGGTCACTCCCTGTTGGACTTGATAGA	(30)
	ZIKV	1087 IVT	–	TAATACGACTCACTATAGGGCCGCTGCCCAACACAAG	(31)

VIC, VIC dye (2'-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein); FAM, FAM dye (Fluorescein amidites); IV, In vitro transcription primer.

TABLE 2 | Description of 32 positive pools for DENV, ZIKV and CHIKV inoculated in C6/36 cell culture during arbovirus surveillance in Pernambuco, Brazil from 2015 to 2017.

N°	ID	Year of collection	Local	City	Virus	Specie	Total of females	Engorged	Reaction or Cq	CPE (P1)	Cq	CPE (P2)	Cq	CPE (P3)	Cq	CPE (P4)	Cq	IFA
1	13BCD	2015	Domicile	São Lourenço da Mata	DENV 2	*Ae. aegypti*	4	No	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	No
2	13DE	2015	Domicile	São Lourenço da Mata	DENV 2	*Ae. aegypti*	4	Yes	RT-PCR	No	N.D.	No	N.D.	No	25.7	Yes	19.0	Yes
3	17C	2015	Hospital	Recife	DENV 2	*Ae. aegypti*	10	Yes	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	No
4	17F	2015	Hospital	Recife	DENV 2/ZIKV	*Ae. aegypti*	10	Yes	RT-PCR/ 34.5	No	N.D.	No	35.0	No	21.7	Yes	23.0	–
5	18	2015	Hospital	Recife	DENV 2/ZIKV	*Ae. aegypti*	1	Yes	RT-PCR./ 35.5	No	N.D.	No	34.5	Yes	20.9	Yes	23.0	–
6	20 A	2015	Hospital	Recife	DENV 2/ZIKV	*Ae. aegypti*	10	Yes	RT-PCR/ 33.5	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
7	21 E	2015	Hospital	Recife	DENV 2/ZIKV	*Ae. aegypti*	10	Yes	RT-PCR/ 34.0	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
8	22 A	2015	Hospital	Recife	DENV 2	*Ae. aegypti*	6	Yes	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
9	22K	2015	Hospital	Recife	DENV 2	*Ae. aegypti*	10	Yes	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
10	23	2015	Hospital	Recife	DENV 2	*Ae. aegypti*	1	Yes	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
11	1055	2016	Public place	Recife	DENV2/ DENV3	*Cx. quiquefasciatus*	10	No	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
12	1059	2016	Public place	Recife	DENV 2/ZIKV	*Cx. quiquefasciatus*	1	Yes	RT-PCR/ 34.4	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
13	1060	2016	Public place	Recife	DENV 2/ZIKV	*Cx. quiquefasciatus*	5	No	RT-PCR./ 35.0	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
14	1067	2016	Domicile	Recife	DENV2	*Cx. quiquefasciatus*	2	Yes	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
15	1068	2016	Domicile	Recife	DENV 2/ZIKV	*Cx. quiquefasciatus*	1	No	RT-PCR./ 35.0	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
16	1084	2016	Emergency Care Units	Recife	DENV2	*Ae. aegypti*	1	Yes	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
17	1172	2016	Hospital	Recife	DENV 2/ZIKV	*Cx. quiquefasciatus*	1	No	RT-PCR/ 37.0	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
18	1229	2016	Hospital	Recife	DENV 2/ denv4/ZIKV	*Ae. aegypti*	10	Yes	RT-PCR/ 33.0	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
19	1249	2017	Emergency Care Units	Recife	DENV2/ DENV4	*Ae. aegypti*	4	Yes	RT-PCR	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
20	1246	2017	Emergency Care Units	Recife	ZIKV	*Ae. aegypti*	6	No	33.5	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
21	1296	2017	Public place	Recife	ZIKV	*Cx. quiquefasciatus*	7	Yes	35.0	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
22	1349	2017	Hospital	Recife	ZIKV	*Ae. aegypti*	10	Yes	34.7	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
23	1317	2017	Hospital	Recife	CHIKV	*Ae. aegypti*	10	Yes	35.1	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
24	1318	2017	Hospital	Recife	CHIKV	*Ae. aegypti*	10	Yes	36.9	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
25	1323	2017	Hospital	Recife	CHIKV	*Ae. aegypti*	10	Yes	37.7	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
26	1324	2017	Hospital	Recife	CHIKV	*Ae. aegypti*	10	Yes	35.9	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
27	1A (2015)	2015	Hospital	Recife	ZIKV	*Ae. aegypti*	9	Yes	13.2	Yes	27.0	Yes	17.3	–	–	–	–	Yes
28	04 (2015)	2015	Hospital	Recife	ZIKV	*Ae. taeniorhynchus*	1	No	34.0	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–

(Continued)

TABLE 2 | Continued

N°	ID	Year of collection	Local	City	Virus	Specie	Total of females	Engorged	Reaction or Cq	CPE (P1)	Cq	CPE (P2)	Cq	CPE (P3)	Cq	CPE (P4)	Cq	IFA
29	962	2016	Domicile	Recife	ZIKV	*Cx. quiquefasciatus*	5	No	34.2	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
30	1001	2016	Domicile	Recife	ZIKV	*Cx. quiquefasciatus*	4	Yes	33.1	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
31	15 A	2015	Domicile	São Lourenço da Mata	CHIKV	*Ae. aegypti*	1	Yes	38.3	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–
32	16 ABCD	2015	Domicile	São Lourenço da Mata	CHIKV	*Ae. aegypti*	5	Yes	36.0	No	N.D.	No	N.D.	No	N.D.	No	N.D.	–

Cq, Cycle quantification; CPE, Cytopathic effects; P1, Passage 1; P2, Passage 2; P3, Passage 3; P4, Passage 4; IFA, Immunofluorescence Assays; ND, Non-detected.

assayed using the QuantiTect SYBR® Green RT-PCR Kit (Qiagen, catalog # 204243, German) in a 20 µl final volume with primers described by Kong et al. (26) in a 0.2 µM final concentration.

For confirmation of viral isolation, samples that resulted in CPE or increased viral load on the RT-qPCR assay were submitted to an indirect immunofluorescence assay (IFA). At the 4th (CHIKV) and 6th day (DENV and ZIKV) after inoculation in cell culture flasks, an aliquot of 10 µl of each cell suspension, obtained from cell scraping the vials, was deposited on IFA slides. IFA slides were dry at room temperature, and then fixed with acetone P.A for 30 minutes at - 20°C. The primary antibody (Anti-DENV 2, Anti-4G2 for ZIKV and Anti-CHIKV) was added to IFA slides and then incubated for one hour in a humid chamber at 37°C. Slides were then washed twice with 1x PBS and the secondary antibody (Anti-mouse) was added, following incubation of one-hour in a humid 37°C chamber. After incubation, slides were again washed twice with 1x PBS and dried for 5 minutes. After drying, slides were mounted in VECTASHIELD® Medium with DAPI (Vector Laboratories, California, USA) under the coverslip. Slides were visualized in a Fluorescence microscope (Leica Microsystems, Germany) using the green filter (FIT C) for virus fluorescence and the blue filter (DAPI) for cell nuclei fluorescence. The images were captured by the microscope software on the 400x objective.

RESULTS

From 2015 to 2017, 9,834 adult mosquitoes were obtained from which 6,227 were identified as females. All the females were pooled in a total of 1,049 pools (497 of *Ae. aegypti* and 549 of *Cx. quinquefasciatus*) that were assayed for conventional and quantitative RT-PCRs. *Ae. albopictus, Aedes taeniorhynchus* and *Aedes scapularis* specimens were also collected and assayed as one pool each. After molecular analysis, a total of 219 pools (20.8%) were positive for at least one of the tested arboviruses. In summary, DENV was detected in 19 pools (89.5% DENV2 and 10.5% DENV4), ZIKV was detected in 194 pools (Cq values described in **Figure 2**) and CHIKV in six pools. Cq values found ranged from 13.2 to 38.3 (equivalent to 5,22E+19 and 1,89E+07 RNA copy number). Out of the 219 positive-pools, five pools of *Ae. aegypti* and four of *Cx. quinquefasciatus* resulted in double infection of DENV2 and ZIKV. In addition, a simultaneous DENV2 and DENV4 infection was detected in a single pool of *Cx. quinquefasciatus*. No virus was detected in *Ae. scapularis* and *Ae. albopictus* samples. The distribution of the positive samples is mapped in **Figure 1**. From the total of positive samples, 150 were identified as blood-fed females after PCR confirmation (**Supplementary Figure 1**). The sequencing analysis of the pools that contained engorged females revealed the presence of human blood in all samples.

Concerning DENV surveillance in field-caught mosquitoes, pools were tested by conventional RT-PCR. In 2015, DENV was detected in 10 pools of *Ae. aegypti* samples. In 2016, four pools of *Ae. aegypti* and four pools of *Cx. quinquefasciatus* were positive for DENV. In 2017 only one pool was positive for DENV. Minimum

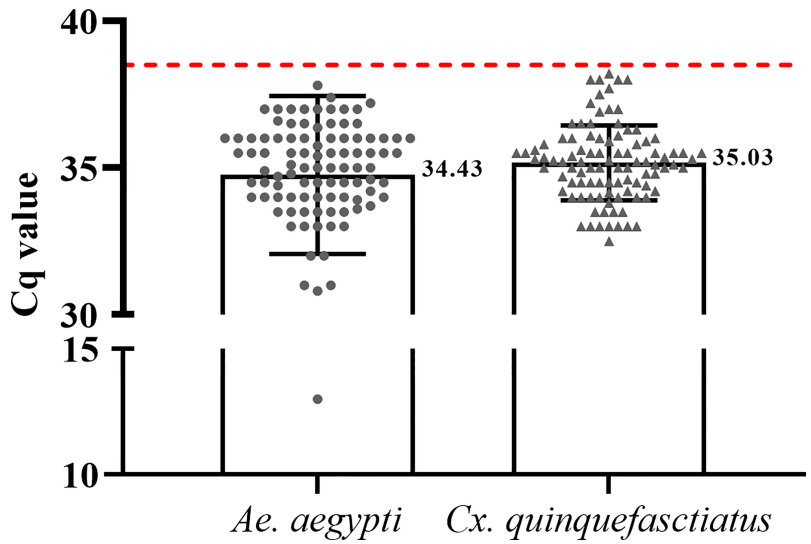

FIGURE 2 | Cq values of positive *Ae. aegypti* and *Cx. quinquefasciatus* pools for ZIKV plotted using GraphPad Prism 9 software. The red traced line represents the threshold used for positive samples (Cq = 38.5).

infection rate (MIR) for DENV ranged from 0.68 to 13.77 (**Table 3**). With regards to ZIKV in 2015, 30 pools of *Ae. aegypti* and one pool of *Ae. taeniorhynchus* from a hospital were positive. In 2016, 40 pools of *Ae. aegypti* and 69 pools of *Cx. quinquefasciatus* were ZIKV positive. In 2017, 27 pools of *Ae. aegypti* and 27 pools of *Cx. quinquefasciatus* were ZIKV-positive, with a MIR ranging from 18.59 to 41.32 (**Table 3**). CHIKV was detected in two pools of *Ae. aegypti* in 2015 and in four pools of *Ae. aegypti* in 2017. The MIR observed for both years was 2.75 (**Table 3**). For virus isolation, all positive samples for DENV and CHIKV and samples with the lowest Cq values for ZIKV were selected (**Table 1**). A total of 32 samples out of 219 positive sample were inoculated in a C6/36 cells monolayer. After inoculation, virus isolation was confirmed by cytopathic effects (CPE) visualization in three positive samples for DENV (13DE/2015RA, 17F/2015HA and 18/2015HA) and in one sample for ZIKV (1A/2015HA). RT-qPCR and indirect immunofluorescence assays also confirmed the isolation of DENV and ZIKV (**Figure 3**).

DISCUSSION

Environmental and ecological settings, combined with areas of poor living conditions such as: overcrowding, inadequate sewage system and discontinuing water supply forge the Metropolitan Region of Recife (MRR) as a year-long hotspot for arbovirus transmission, resulting in a permanent vector control issue. In our current surveillance study, we collected, during a triple epidemic of DENV-ZIKV-CHIKV from 2015 to 2017, adult mosquitoes in different sites located at the Metropolitan Region of Recife (MRR), Pernambuco State, Brazil where arbovirus-suspected cases were reported in the human population (hotspots). From over 1,049 pools screened (over 6,000 females), 219 were RT-PCR/RT-qPCR positive for at least one of the viruses (DENV, ZIKV and CHIKV). The spatial distribution of these positive samples showed a higher risk of DENV, ZIKV and CHIKV transmission in low and medium low class neighborhoods of MRR. Our findings for ZIKV corroborate with the study by Souza and collaborators (2018) (11), which demonstrated a higher prevalence of ZIKV and microcephaly in environments of Recife with a high level of poverty. This critical scenario indicates the need to put even more effort into environmental health improvements.

Considering DENV data, while our results showed the circulation of DENV4 in those mosquitoes, not a single infection with this particular serotype was confirmed in 2016-17 in the human population from Recife (35). DENV2 and DENV3 were the only serotypes confirmed in the human population during 2016 unlike what we found in mosquito's population (35). Such results acted as an early warning as DENV2 became the most frequent serotype circulating in Brazil in 2019 (two years after our study) (36). In addition to DENV, CHIKV was also detected in *Ae. aegypti* collected in the different sites from the MRR. Our results of CHIKV MIR are similar to those reported by Cevallos and collaborators (2018) (37), who conducted an arbovirus surveillance in *Ae. aegypti* from active epidemic urban areas. However, mosquitoes were collected during the epidemics, the MIR value of CHIKV was lower than those reported in *Ae. aegypti* samples from Maranhão and Aracaju (northeast Brazil) and, Yucatan (Mexico) (38–40). We were unable to detect CHIKV in pools from mosquito collections performed in 2016 and, CHIKV positive samples from 2015 and 2017 were all from engorged pools and, did not result in any CPE in C6/36 cells. We did not detect the

TABLE 3 | Description of results of arbovirus infection obtained from mosquito samples collected in the metropolitan region of recife in three distinct years.

Species	Year of collection	Total of females	Number of pools tested	DENV +	Engorged*	MIR	ZIKV +	Engorged*	MIR	CHIKV +	Engorged*	MIR
Ae. aegypti	2015	726	99	10	9	13.77	30	22	41.32	2	2	2.75
	2016	1,045	233	2	2	1.91	40	34	38.27	–	–	–
	2017	1,452	165	1	1	0.68	27	26	18.59	4	4	2.75
Ae. albopictus	2015	–	–	–	–	–	–	–	–	–	–	–
	2016	2	1	–	–	–	–	–	–	–	–	–
	2017	–	–	–	–	–	–	–	–	–	–	–
Ae. scapularis	2015	11	1	–	–	–	–	–	–	–	–	–
	2016	–	–	–	–	–	–	–	–	–	–	–
	2017	–	–	–	–	–	–	–	–	–	–	–
Ae. taeniorhynchus	2015	1	1	–	–	–	1	0	N/A	–	–	–
	2016	–	–	–	–	–	–	–	–	–	–	–
	2017	–	–	–	–	–	–	–	–	–	–	–
Cx. quinquefasciatus	2015	121	22	–	–	–	–	–	–	–	–	–
	2016	2,344	435	6	3	2.55	69	35	29.43	–	–	–
	2017	525	92	–	–	–	27	12	51.42	–	–	–
Total		6,227	1,049	19	15		194	129		6	6	

**Classification of engorged pools performed after human blood detection in mosquito samples by conventional PCR. N/A, Not applicable.*

circulation of CHIKV in *Cx. quinquefasciatus* from the MRR, although two independent studies reported the virus circulation in this species during outbreaks of CHIKV in the Amazon region (Brazil) and Bangkok (Thailand) (41, 42). Regarding ZIKV data, we found higher ZIKV MIR values in *Ae. aegypti* than those reported in studies conducted during the same period in Rio de Janeiro (Brazil) (18, 43), as well as in Chiapas and Jalisco states (Mexico) (44, 45). However, these studies were performed with random collections, where MIR values are expected to be lower. Similar to our results, ZIKV was also detected in *Ae. taeniorhynchus* collected from the city of Vitória (southeast Brazil) during 2016 (19), but the role of this species in transmitting ZIKV still needs elucidation (46). We identified 96 ZIKV-positive pools of *Cx. quinquefasciatus* from the MRR, but even years after the 2015 ZIKV epidemics, the role of this species in transmitting this particular virus is still pondered (47–50). Yet, natural ZIKV infection has also been reported in salivary glands from wild-caught *Cx. coronator* and *Cx. tarsalis* from Guadalajara (Mexico) (44), in whole-body field-collected *Cx. quinquefasciatus* females from Recife, Vitória (Brazil) and Medellin (Colombia) (19, 20, 51).

Parameters such as vector competence, mosquito populational density and blood feeding status added to arboviral detection in field mosquitoes are important aspects taken into consideration when studying the transmission cycle in field mosquitoes (52, 53). Once an outbreak occurs in an urban area, the species with the highest density in the region should be included in the investigation, in order to assess the epidemiological situation of each location (52). In the present study, we detected DENV in *Cx. quinquefasciatus* samples. Among 549 *Cx. quinquefasciatus* pools from the MRR screened, DENV was detected in six samples. Studies conducted in Cuiaba (Western Brazil) and São Paulo (southern Brazil) have also reported DENV circulation in field-caught *Culex*. spp (54, 55). Although *Culex* mosquitoes are not considered competent in transmitting DENV (56), the data obtained from this species may provide valuable information over the transmission of pathogens in areas where *Cx. quinquefasciatus* is found in high densities. Therefore, it can be considered an epidemiological indicator of which arbovirus are circulating in humans during outbreaks and epidemics. We reinforce here the importance of implement arbovirus surveillance in *Culex* species, which is not commonly performed (57). Characteristics such as high population density and high anthropophilic behavior of *Cx. quinquefasciatus* in Brazil can increase the chance of collecting specimens that contain blood in your organism. In this way, increase the probability of identifying circulating arboviruses in the vector population (13, 58, 59).

RT-PCR and RT-qPCR are ideal techniques for massive screening of mosquitoes during virus surveillance, reducing diagnosis time in relation to viral isolation and decrease the possibility of cross-reaction when compared to serological methods (60, 61). RT-qPCR is considered the gold standard method for arboviruses detection in humans and mosquito samples and produces high quality results (31, 60) and new nucleic acid tests can be used to detect arboviruses, such as Loop-mediated isothermal amplification (LAMP), a point-of-care

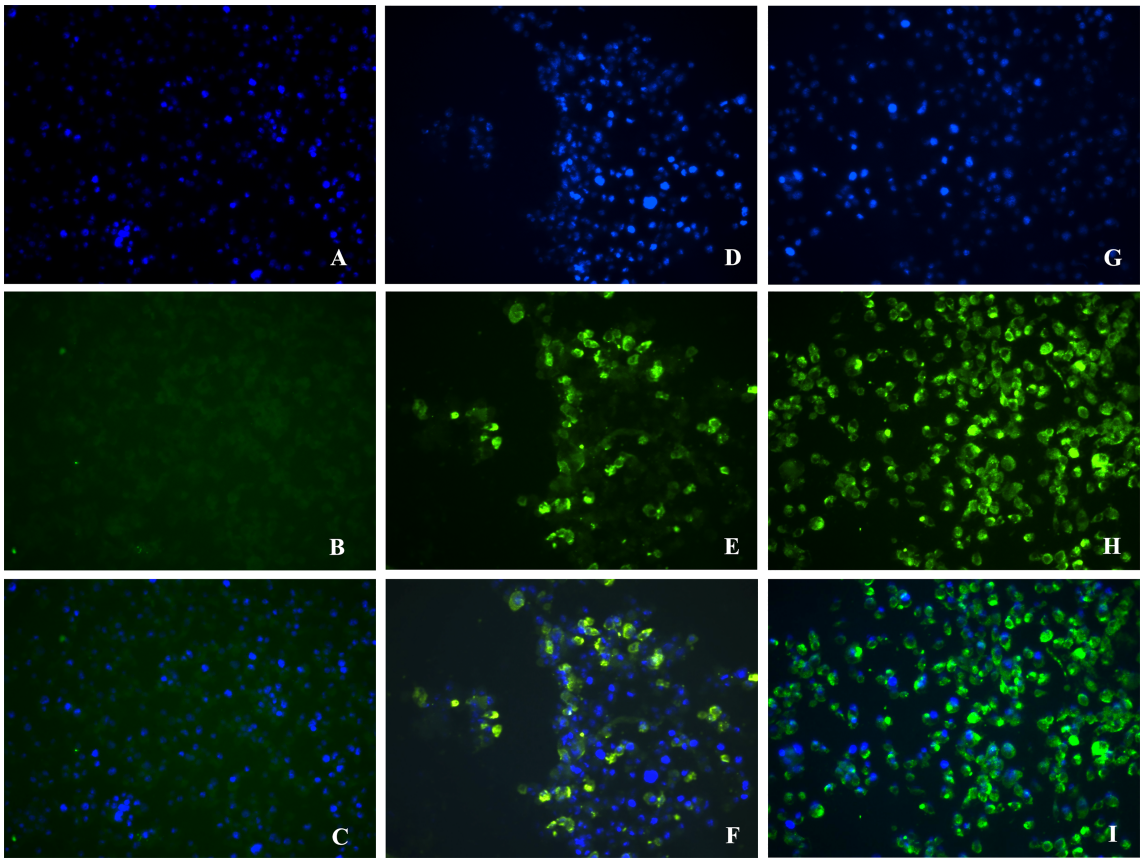

FIGURE 3 | ZIKV visualization in C6/36 cells by immunofluorescence assay and isolated from field-caught mosquitoes. Image captured with 400x objective. **(A–C)** Immunofluorescence of non-infected C6/36 cells; **(D–F)** Immunofluorescence of C6/36 cells inoculated with the sample 1A / 2015HA and **(G–I)** Immunofluorescence of infected C6/36 cells with the positive control of ZIKV. ZIKV is labelled in green (FIT C) and the nucleus is labelled in blue (DAPI).

assay, which has already been used to detect DENV, ZIKV and CHIKV in patient and mosquito samples (62–64). In our study, from a total of 32 RT-PCR/RT-qPCR positive samples inoculated in C6/36 cells, CPE was observed in three samples containing pools of *Ae. aegypti* mosquitoes with DENV2 and in one sample comprised of *Ae. aegypti* positive mosquitoes for ZIKV. The difficulty in isolating arboviruses in C6/36 cells observed here could be related to samples that are improperly transported to the lab, thawed more than once or have a low viral titer. In addition, these factors also directly influence the detection of a low number RNA copies number in RT-qPCR (38, 39).

In conclusion, since strategies implemented by control programs in the MRR have historically struggled in preventing arbovirus transmission, the implementation of a specific mosquito collection protocol for arbovirus surveillance in epidemic and inter-epidemic periods serves as an early-warning system to implement control actions before the surge of human cases. These studies must be conducted in accordance with the needs and peculiarities of each location, as well as having focus on precarious areas. The implementation of such surveillance in mosquitoes from the Metropolitan Region of Recife resulted in the identification of DENV, ZIKV and CHIKV circulating in *Ae. aegypti*, *Ae. taeniorhynchus* and *Cx. quinquefasciatus* collected from 2015-17.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study was performed based on caught-field mosquitoes in houses and was approved by the Research Ethics Committees of the Aggeu Magalhães Institute (FIOCRUZ-PE) under the registration number CAAE 51012015.9.0000.5190 and PlatBr 1.547.598.

AUTHOR CONTRIBUTIONS

LK, MP, and DG wrote the manuscript. CA, MP, DG, and LK conceived and planned the study. LK and DG performed

laboratory experiments. LK, DG, MP, and AO performed data analysis. RB, DA, and CP planned and super-vised the mosquito collections. CA, MP, and DG supervised and administered the study. CA, MP, and RB financed the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2022.875031/full#supplementary-material

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