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Research Articles

Patient-based dengue virus surveillance in *Aedes aegypti* from Recife, Brazil

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Abstract

Background & objectives: Dengue is currently one of the most important arthropod-borne diseases and may be caused by four different dengue virus serotypes (DENV-1 to DENV-4), transmitted mainly by *Aedes aegypti* (Diptera: Culicidae) mosquitoes. With the lack of a dengue vaccine, vector control strategies constitute a crucial mode to prevent or reduce disease transmission. In this context, DENV detection in natural *Ae. aegypti* populations may serve as a potential additional tool for early prediction systems of dengue outbreaks, leading to an intensification of vector control measures, aimed at reducing disease transmission. In Brazil, this type of surveillance has been performed sporadically by a few groups and has not been incorporated as a routine activity in control programs. This study aimed at detecting DENV in natural *Ae. aegypti* from Recife, Pernambuco, to check the circulating serotypes and the occurrence of transovarial transmission in local mosquito populations.

Methods: From January 2005 to June 2006, mosquitoes (adults and eggs) were collected in houses where people with clinical suspicion of dengue infection lived at. RNA was extracted from pooled mosquitoes and RT-PCR was performed in these samples for detection of the four DENV serotypes.

Results & conclusion: Out of 83 pools of adult mosquitoes collected in the field, nine were positive for DENV: five for DENV-1, two for DENV-2 and two for DENV-3. From 139 pools of adult mosquitoes reared from collected eggs, there were 17 positive pools: three for DENV-1, 10 for DENV-2, and four for DENV-3. These results are discussed in the paper in regard to the local dengue epidemiological data. The conclusions clearly point to the informative power and sensitivity of DENV entomological surveillance and to the importance of including mosquito immature forms in this strategy.

Key words Arbovirus; entomological surveillance; mosquito; RT-PCR; vertical transmission

Introduction

Dengue fever is one of the most important arthropod-borne diseases and may be caused by four dengue virus serotypes (DENV-1 to DENV-4), belonging to the genus *Flavivirus*, family *Flaviviridae*[1]. An estimated 2.5 billion people living in tropical and subtropical areas, distributed within 100 countries are at risk of epidemic dengue virus transmission. Annually, more than 100 million cases of classic dengue fever and around 450,000 cases of dengue hemorrhagic fever are notified[2].

In Brazil, since the introduction of DENV-1 in the state of Rio de Janeiro in 1986, more than 2.7 million dengue cases and nearly 2090 dengue hemorrhagic/dengue shock syndrome cases were reported in the country until 2002[3]. In the State of Pernambuco (north-east Brazil), over 30,000 cases of dengue disease were reported from 2005 to 2006[4–5]. In 2008, the Brazilian Ministry of Health reported >230,000 dengue cases in the country from January to April. Despite a decrease in approximately 11% of case numbers compared to the same period in 2007, the proportion of severe dengue cases increased[6], probably due to the circulation of multiple serotypes. With the lack of a commercial dengue vaccine, prevention/contention of dengue outbreaks depends on the surveillance of clinical cases, and very importantly on vector control.

Aedes aegypti (Linnaeus) is a widely distributed mosquito and the main urban vector involved in dengue virus transmission throughout the world, including Brazil. Demographic and social changes including unplanned urbanization, increasing population size, as well as ineffective mosquito control measures in most dengue endemic regions of the world have contributed to broaden the geographical distribution of this mosquito species[7]. Detection of dengue virus in natural *Aedes* populations has shown to be a potential additional tool for early prediction systems of dengue outbreaks[8–10]. In one of these studies, DENV were detected in field mosquitoes at 6–8 wk before dengue cases in the human population were apparent[8]. Early detection of infected mosquitoes could lead to intensification of vector control measures, aiming at reducing disease transmission[8,11]. In Brazil, monitoring of dengue virus serotypes circulating in *Aedes* spp has been performed sporadically by a few groups[11–14], and has not been incorporated as a routine activity in control programs. Moreover, only a few of these studies attempted to include *Ae. aegypti* immature forms in the surveillance.

The present study aimed at detecting, through RT-PCR, the presence of DENV serotypes circulating in *Ae. aegypti* collected in several districts of Recife, the capital of the state of Pernambuco. For that, mosquito collections were performed in places where patients with clinical diagnostic of dengue lived at. Besides adults, mosquito eggs were also collected, in order to check if natural vertical transmission was occurring in these populations. Results obtained in the study are discussed in regard to the local dengue epidemiological data.

Material & Methods

Mosquito collections: Ae. aegypti mosquitoes were collected in 70 residences inhabited by individuals with clinical diagnostic of dengue from January 2005 to June 2006. These houses were scattered throughout 25 out of 94 neighbourhoods of Recife (Fig. 1), a city located at 8.05S, 34.88W. Diagnostic of den-

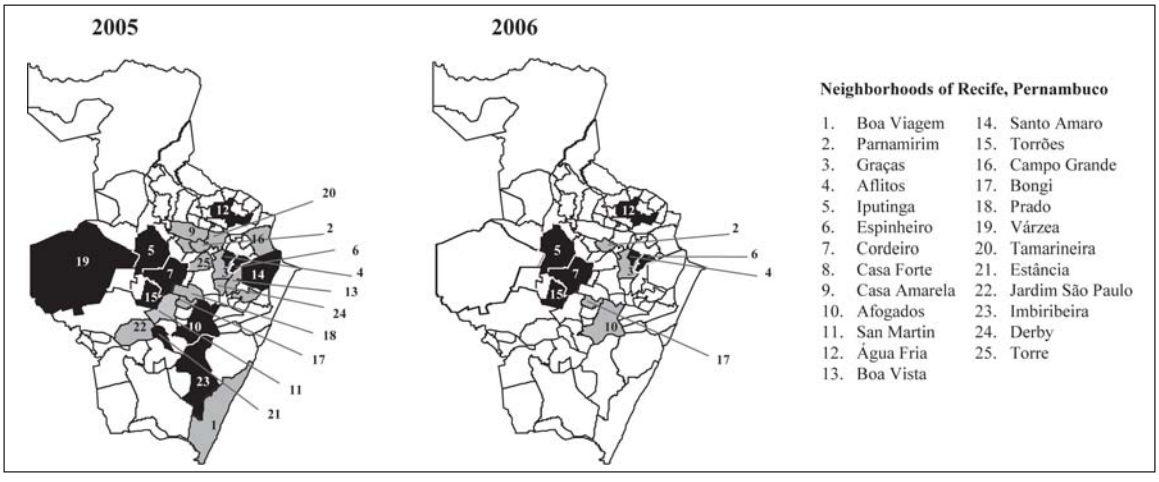

Fig. 1: Map of Recife showing areas where mosquitoes were collected (grey and black) and where positive pools originated from (black) in 2005 and 2006

gue cases were confirmed by RT-PCR, viral isolation and serology at the Laboratório de Virologia e Terapia Experimental (LAVITE) at Centro de Pesquisas Aggeu Magalhães/FIOCRUZ[15]. Only those who agreed to participate in this study were included, after signing a proper Informed Consent Form, approved by the Brazilian Ethics Committee (CAAE 25000.119007/2002–03). Mosquitoes were collected between 1 and 30 days after notification of a suspected dengue case.

Adult mosquitoes were captured indoors in resting places, using a battery-operated backpack aspirator (modified CDC backpack aspirator, Model 1412). Live adult mosquitoes were anaesthetized at 4°C, morphologically identified, sorted by species, locality and sex, pooled (1–9 individuals/pool) and preserved at –80°C until tested.

Eggs were collected through ovitraps based on Fay and Perry[16] model. Traps were placed outdoors for seven days in the same residences where adults were collected. Eggs were counted and each batch was placed in individual containers with water for larval eclosion, which were identified according to place and date of collection. Newly hatched larvae were reared to adults under laboratory conditions (temperature of 26 ± 2°C; relative humidity 60–80%; 12/12 h L/D). Emerged adult mosquitoes were maintained on water and 10% sugar solution. Seven days after emergence, adults were identified to species and pooled (15–28 individuals/pool, except for one pool with 2 mosquitoes only) by date and place of collection. These pools were composed of males and females. Samples were kept at –80°C until used in the experiments.

Dengue virus detection through reverse-transcription PCR (RT-PCR): Mosquito pools were homogenized with a pestle in Leibowitz medium (L-15) supplemented with Fetal Bovine Serum 2% (FBS), Penicillin-Streptomycin (1%) and Fungizone (1%). Viral RNA was extracted from these samples using a silica method described previously[17], with minor modifications. Before carrying out reverse transcription and semi-nested PCR reactions, RNAs were treated with recombinant DNAse I (Invitrogen) to ensure total elimination of genomic DNA. RT-PCR for detecting and classifying DENV in mosquito pools was performed according to Lanciotti *et al*[18]. Briefly, the first step of RT-PCR consisted of a reverse transcription reaction to synthesize cDNA from RNA templates, followed by amplification of a 511 bp region between genes C and PrM of DENV with primers D1 (forward) and D2 (reverse). The second step of the semi-nested PCR was carried out with D1 and TS1, TS2, TS3 and TS4 as reverse primers, which amplify regions of 482, 119, 290 and 392 bp of DENV-1, DENV-2, DENV-3 and DENV-4, respectively. Primers sequences used were:

D1– 5'TCAATATGCTGAAACGCGCGAGAAACCG-3';
D2– 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3';
TS1 – 5'-CGTCTCAGTGATCCGGGGG-3';
TS2 – 5'-CGCCACAAGGGCCATGAACAG-3';
TS3 – 5'-TAACATCATCATGAGACAGAGC-3';
TS4 – 5'-CTCTGTTGTCTTAAACAAGAGA-3'.

Minimum infection rate (MIR): The MIR of DENV in captured adults and adults reared from collected eggs was calculated as: (number of positive pools for DENV/total number of mosquitoes tested) x 1000[8].

Results

Mosquito collections: Mosquito collections using an aspirator revealed the presence of *Culex quinquefasciatus*, *Ae. aegypti* and *Aedes albopictus*, where the first two species were more abundant than the latter. Only *Ae. aegypti* and *Ae. albopictus* were collected in ovitraps (placed outdoors), with a higher frequency of the first over the second.

The number of captured adults was substantially lower than that of adults emerged from collected eggs. A total of 83 pools (301 specimens) of field-caught *Ae. aegypti* adults and 139 pools (2972 specimens) of adults reared from eggs were assayed by RT-PCR for DENV detection (Table 1). In houses where adult mosquitoes were captured, the number of specimens varied from 1 to 60, whereas the number

Table 1. Collection of *Aedes aegypti* in houses of patients with suspicion of dengue, and dengue virus (DENV) serotypes detected in mosquito pools through RT-PCR

Locality[a]	Number of houses visited for mosquito collection (confirmed human dengue cases)[b]	Adult *Ae. aegypti* captured in the field (pools assayed for DENV detection)[c]		DENV detected in mosquito pools (DENV in patient)[d]		Adult *Ae. aegypti* reared from collected eggs (pools assayed for DENV detection)[e]	DENV detected in mosquito pools (DENV in patient)[f]	
		M	F	2005	2006	(M & F)	2005	2006
Boa Viagem	8 (3)	5 (3)	2 (1)	–	–	390 (16)	–	–
Parnamirim	6 (4)	26 (8)	20 (4)	–	–	110 (6)	–	–
Graças	6 (4)	11 (4)	3 (2)	–	–	88 (5)	–	–
Iputinga	4 (1)	4 (1)	–	–	D1 (Neg)	77 (5)	–	D2 (D3)
Espinheiro	4 (1)	11 (5)	7 (2)	D1 (Neg)	–	156 (9)	–	D2, D2, D2, D2 (D3)
Boa Vista	2 (1)	14 (2)	2 (1)	–	–	113 (5)	–	–
Cordeiro	4 (1)	4 (2)	1 (1)	–	D1 (Neg)	259 (10)	D1 (Neg)	D2, D2 (Neg)
Casa Amarela	3 (1)	5 (2)	1 (1)	–	–	25 (3)	–	–
Várzea	1 (1)	–	–	–	–	228 (8)	D1 (D3)	
Imbiribeira	1 (0)	5 (1)	3 (1)	–	–	70 (4)	–	–
Tamarineira	1 (1)	3 (2)	–	–	–	71 (3)	D3	(D3)
Aflitos	4 (2)	57 (12)	12 (4)	D1 (Neg)	D3 (D3)/ D1, D2 (D3)	02 (1)	–	–
Santo Amaro	2 (1)	–	–	–	–	170 (8)	D3 (D3)	–
Afogados	3 (1)	1 (1)	2 (2)	D3 (D3)	–	– –	–	
Estância	1 (0)	2 (1)	–	–	–	290 (17)	D1, D3 (Neg)	
Jardim São Paulo	1 (0)	–	–	–	–	68 (3)	–	–
Bongi	2 (0)	7 (1)	–	–	–	76 (3)	–	–
San Martin	3 (0)	7 (2)	4 (1)	–	–	40 (2)	–	–
Torrões	2 (0)	1 (1)	–	–	–	106 (5)	D3 (Neg)	D2 (Neg)
Campo Grande	2 (0)	6 (2)	1 (1)	–	–	123 (4)	–	–
Casa Forte	4 (0)	25 (3)	6 (3)	–	–	93 (5)	–	–
Água Fria	2 (2)	1 (1)	–	–	D2 (D3)	58 (3)	–	D2 (D3)/D2 (D3)
Derby	1 (0)	–	–	–	–	40 (2)	–	–
Torre	1 (0)	–	–	–	–	135 (5)	–	–
Novo Prado	2 (0)	18 (2)	24 (3)	–	–	184 (7)	–	–
Total	70	213 (56)	88 (27)	3	6	2972 (139)	7	10

[a]Districts of Recife PE, Brazil, where mosquitoes were collected based on patients with suspicion of dengue, as informed by LAVITE; [b]Number of patient's houses where mosquito collection was performed in the study and in parentheses the number of confirmed dengue positive patients, according to LAVITE; [c]Number of adult *Ae. aegypti* collected in the houses and in parentheses number of assayed pools for DENV detection; [d]Dengue virus (DENV) serotypes detected in mosquito pools from adults collected in the field and in parentheses DENV detected in patient's sample corresponding to the house where the mosquito pool was originated from (Neg= Patient's sample negative for DENV; D1= DENV-1; D2= DENV-2; D3=DENV-3); [e]Number of adult *Ae. aegypti* obtained from eggs collected in the houses and in parentheses number of assayed pools for DENV detection; [f]DENV serotypes detected in mosquito pools from eggs collected in the field and in parentheses DENV detected in patient's sample corresponding to the house where the mosquito pool was originated from. In d and f, samples separated by "/" were collected in different houses. M–Male; F–Female.

of eggs in houses where these samples were collected varied from 2 to 600.

Dengue virus detection in mosquito samples: From 83 pools of adults collected directly in nature, nine were positive for dengue virus and three serotypes were detected: DENV-1 was found in five of them, DENV-2 in two of them, and DENV-3 in two pools (Table 1). In the latter case, DENV serotype matched with that of the patient's sample. All positive pools were composed of adult females.

Regarding adults reared from eggs collected in ovitraps, the same DENV serotypes were detected, in a total of 17 positive pools (out of 139): three for DENV-1, 10 for DENV-2, and four for DENV-3 (Table 1). In this case, two DENV-3 positive pools matched with the patient's dengue serotype. In all the cases, mosquito and patient serotype matching did not occur with DENV-1 and DENV-2. Figure 1 shows the areas where positive mosquito samples were collected from.

MIR: DENV infection rate in wild *Ae. aegypti* was expressed as MIR. In the case of captured adults, MIR was 29.9 (Table 2), however, if we consider only pools composed of females (since DENV was detected in those, only), MIR rises to 42.2. MIR in mosquitoes reared from collected eggs was 6.0.

Discussion

The co-circulation of three DENV serotypes has been recorded in the state of Pernambuco since the introduction of DENV-3 in 2002, when DENV-1 and DENV-2 were already present. DENV-3 became highly predominant in 2005 and 2006, and a low percentage of human samples were found infected with DENV-1 and DENV-2[5,19]. Results presented here on DENV detection in wild *Ae. aegypti* samples provide important information when considering the local dengue epidemiology. In adult mosquitoes collected in the field, as much as nine positive pools were found among a relatively low number of assayed mosquitoes (301 specimens). Moreover, DENV-1, DENV-2 and DENV-3 were detected in these samples, i.e. besides the predominant serotype circulating in human population at the time (DENV-3), the two serotypes circulating in a lower frequency were also found. In the case of adults reared from eggs collected in the field, the same three serotypes were detected, showing that transovarial transmission is occurring in these populations with these serotypes, and indicating surveillance of immature forms can be as informative as monitoring adults in regard to detecting DENV serotypes circulating in mosquito populations.

When the data presented here are separated by year, only DENV-1 and DENV-3 were found in mosquitoes collected in 2005, corresponding to the same serotypes found in human samples in that year, where 95% of reported dengue cases were DENV-3 and 5% were DENV-1[19]. In 2006, the 3 serotypes were detected in mosquitoes, while in human samples, 94% of reported dengue cases were DENV-3 and 6% were DENV-2[19]. The results presented here show that although DENV-1 was not detected in human samples in 2006, it was still present in mosquito populations from Recife. Another observation was that the number of positive mosquito pools found in 2006 was twice of those found in 2005, even though the number of mosquito samples assayed in 2006 was much lower than those in 2005 and surveillance was performed in the first six months only. Interestingly, the number of dengue cases reported in 2006 (18,595) was higher than that of in 2005 (12,990), with a concentration of cases in the same period our study was conducted[19].

Pinheiro *et al*[11], while surveying DENV in *Ae. aegypti* from Manaus-Amazonas, Brazil, found a high infection rate, however, they only detected the local predominant serotype (DENV-3). It was suggested that DENV-3 had replaced the other previously circulating serotypes, since it had been recently introduced in the area and probably found in a non-immune population, facilitating its dispersion. Some studies mention that the prevalent serotype may persist for one to two years until it is replaced by a new one[8,20]. The study by

Table 2. Minimum infection rate (MIR) for dengue virus in *Aedes aegypti* collected in the field found in different studies

Stage of *Ae. aegypti* collected in the field	Stage of *Ae. aegypti* assayed for DENV	MIR	DENV	Country	Period	Includes dengue epidemic/ outbreak period	Reference
Adult females	Adult females	56.2	1, 2, 3	Singapore	Apr 1995–Jul 1996	Yes	Chow *et al* 1998
		69	1, 2, 3, 4	Singapore	Apr 1997–Dec 2000	Yes	Chung and Pang 2002
		8.52	2	Brazil	Jul 2000–Jun 2001	Yes	Lourenco-de-Oliveira *et al* 2002
		15.9	1, 3, 4	Venezuela	Nov 2000–Dec 2001	Yes	Urdaneta *et al* 2005
		18	1, 2, 3	Mexico	Mar 2007–Feb 2008	Yes	Garcia-Rejon *et al* 2008
Adult males and females	Adult males and females[a]	20.7	3	Brazil	Feb–Jun 2003	Yes	Pinheiro *et al* 2005
		29.9	**1, 2, 3**	**Brazil**	**Jan 2005–Jun 2006**	**Yes**	**This study**
Adult males	Adult males	13.3	1, 2, 3, 4	Singapore	Sep 1997–Aug 1998	Yes	Chung *et al* 2001
		2.7	2, 3[b]	India	Mar 2003–Dec 2004	NI	Arunachalam *et al* 2008
Larvae	Larvae	0.48	2	Burma	Sep 1978–Jul 1980	NI	Khin and Than 1983
		0	–	Singapore	Apr 1995–Jul 1996	Yes	Chow *et al* 1998
		0	–	Brazil	Feb–Jun 2003	Yes	Pinheiro *et al* 2005
		0	–	Mexico	Jan–Dec 2005	Yes	Gunther *et al* 2007
Eggs	Larvae	0	–	Brazil	Nov 2006–May 2007	Yes	Zeidler *et al* 2008
		18.3	2[c]	Brazil	May 2003	Yes	Cecilio *et al* 2009
Larvae	Adult males and females	0.12	2	Burma	Sep 1978–Jul 1980	NI	Khin and Than 1983
Eggs, larvae, adults	Adult males and females	0.36	4	French Guiana	Oct 1993–Sep 1995	No	Fouque *et al* 2004
Larvae	Adult females	4.6	2, 3, 4	Mexico	Jan–Dec 2005	Yes	Gunther *et al* 2007

[a] Positive pools found only in females; [b]In this study, ELISA was the test to detect DENV, thus in many samples the serotype was not identified – serotypes 2 and 3 represent only a small fraction of samples; [c]The authors do not make clear if DENV-1 was found in *Ae. aegypti*, but mention it was detected in *Ae. albopictus*. NI= Not informed in the paper; MIR = Number of positive samples/total number of specimens assayed x 1000.

Urdaneta *et al*[10] in Venezuela serves as a good example of correlation on the serotypes circulating in the human population and the ones detected in mosquito samples. The authors were able to detect DENV-1 and DENV-4 in mosquitoes when four serotypes were simultaneously circulating in the human population, however, after the apparent displacement of the other serotypes by DENV-3, only the latter was detected in mosquitoes, with a relative high infection rate. Here, detection of DENV-1 and DENV-2 in mosquito samples may indicate that in Pernambuco these serotypes were starting to submerge in 2005 and 2006. In fact, in 2008 the predominant serotypes isolated from human samples were DENV-1 and DENV-2[21].

It is noteworthy that some of the houses showed high infestation of *Ae. aegypti*, with over 50 adult females collected in a single house. This fact points out to the efficiency of indoor aspiration to collect mosquito specimens, as shown in other studies[22]. Because adult females are the transmitters of DENV, we believe indoor aspiration of adults could serve as an additional control activity[23]. Garcia-Rejon *et al*[22] performed mosquito collection in houses of people with suspicion of dengue in Mexico and showed that whenever there was concomitant DENV detection in human and mosquito samples, DENV serotype matched in all the cases. In this study, DENV serotype matching between mosquito and human samples occurred in a few cases and only with DENV-3. However, there were cases where DENV-1 and DENV-2 were found in mosquitoes and DENV-3 in patient samples, possibly reflecting the persistence of infected eggs in that environment for a long period or the active circulation of infected mosquitoes.

The lower minimum infection rate (MIR) observed in adults reared from eggs compared to adults collected straight from the field was expected, once transovarial transmission occurs in a much lower rate than horizontal transmission. A variety of studies have reported vertical transmission in *Aedes* spp collected in the field[12,24–28]. These data, combined with the results presented here, demonstrate the relevance of including immature forms in DENV detection system in natural mosquito populations. Vertical transmission is one of the hypotheses on how the virus persists in nature in the absence of viremic vertebrate hosts[9,29]. Quiescent *Ae. aegypti* eggs can remain viable in the environment for many months and when hatched may be responsible for a burst of infections, especially if immunity to the serotype present in mosquitoes is low in the local human population. Moreover, passive transportation of eggs (e.g. transport of tyres containing eggs) is the main mode of dispersion for mosquitoes and infected eggs may introduce dengue in areas previously free of the virus. The fact that adult mosquitoes infected through transovarial transmission may not need to feed in a viremic vertebrate host to infect a naïve host, thus, eliminating the extrinsic incubation period, is also epidemiologically relevant.

In this study, MIR in collected adults was high. Interestingly, only pools composed of females were DENV-positive, and if MIR is calculated only for females, it increases by 1.5-fold. Infection rates in field-collected mosquitoes vary a lot among studies, going from half to higher values than the one obtained here (Table 2). In immature forms, MIR also varies among studies (Table 2). However, to compare infection rates among studies is extremely complex, since a variety of factors must be considered. For instance, the collection period (e.g. epidemic versus inter-epidemic), sample pool sizes, and number of processed samples have an influence on MIR[30].

In case of immature forms, the stage processed for DENV detection assays (e.g. larvae *vs* adults reared from eggs) seem to influence the results, since a few studies show that when assays were performed with larvae DENV was not detected, while if adults reared from these larvae were assayed, DENV was detected[9,26]. Additionally, the type of assay (e.g. PCR *vs* ELISA) and PCR primers used to detect DENV may also affect results. Thus, all these factors combined with the local epidemiology, must be considered when analyzing MIR.

If DENV surveillance in mosquitoes is to be incorporated in control programs, and we believe it should,

it must follow some criteria such as the one utilized here (mosquito collection in houses related to potentially infected people) or others such as vector density data. Although we have not collected mosquitoes in all neighborhoods of Recife, sample collection was reasonably distributed throughout the city, as shown in Fig. 1. It is interesting to note that a few districts where mosquito samples were positive in 2005 were also positive in 2006. Regis *et al*[31] recently showed that *Ae. aegypti* in Recife is distributed throughout the city, however, some points, called hot spots, presented a higher density of mosquito populations. These areas remained as the most concentrated for *Ae. aegypti*, although in variable levels, through a period of at least one year, showing a certain stability of mosquito population distribution in a highly urbanized area. Such data could also be used for selecting areas for insect collection to perform DENV detection assays.

To conclude, the results presented here on DENV detection in mosquitoes corroborated with the local epidemiological data. First, the higher number of positive mosquito pools in 2006, in comparison to 2005, was also seen in human cases. Second, DENV-1 and DENV-2 were found in mosquito samples in 2005 and 2006, when these serotypes were found either in a very low frequency (5–6%) or not found at all in the human population, however, epidemiological data from 2008 show that these serotypes were seen in a higher frequency in human samples than DENV-3, which suggest that mosquitoes may serve as a maintenance mode for the virus in the environment and that when levels of population immunity to a certain serotype start to decrease, they will submerge. These results point out the informative power and high sensitivity of vector infection surveillance, with inclusion of immature forms.

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