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Real time molecular detection of Chikungunya and dengue virus in the female Aedes mosquitoes collected in Chennai, Tamilnadu, India

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Abstract. Monsoon favors the breeding sites of many mosquito borne viral vectors resulting in increased outbreaks of vector borne infections, such as Chikungunya and dengue infections in the post monsoon period. Hence a pre-monsoon surveillance study of competent vectors carrying Chikungunya and dengue virus would help in better management of vectors and infections. Thus a surveillance study was carried out in Chennai before the onset of monsoon to study the distribution of competent vectors and detection of Chikungunya and dengue virus in female *Aedes* mosquitoes. Mosquitoes were collected from north, south, east and west zone of Chennai during March to July 2014. Chikungunya (CHIKV) and Dengue virus (DENV) were detected from the collected mosquitoes by Reverse Transcription Real time PCR (RT-PCR) followed by virus isolation in Vero cells. The most predominant species was *Culex quinquefasciatis* followed by *Culex pipiens* in the premonsoon period in our study. Viral infection rate during March to July (Premonsoon period) with respect to CHIKV and DENV virus were 0.456. The central zones of Chennai were highly found to inhabit the *Aedes* species in comparison to other zones surveyed during the premonsoon period. Viral infection rate of CHIKV and DENV were very low in female Aedes mosquitoes during the Pre-Monsoon period in Chennai.

INTRODUCTION

Aedes aegypti and *Aedes albopictus* are the primary vectors in the transmission of Chikungunya and Dengue in urban areas. India encounters annual incidence of Chikungunya and Dengue since 2006 causing high morbidity. Also both the viruses share the common vectors,hence they can co circulate and are transmitted together (Harendra, 2009).

Chikungunya virus belongs to the alphaviridae and exists in three lineages such as Asian lineage, ECSA Lineage and West lineage (Powers, 2002). The disease onsets with fever, rash and arthralgia. After 2006 epidemics, a few virulent strains had caused ocular, neurological and hepatological complications (Ganesan, 2008; Rose, 2010). Also the disability adjusted life years (DALYs) in 2006 epidemic was 25,588 and the average burden was 45.26 DALYs per million ranged from 0.01 to 265.62 per million in different states and 69% cases of total DALY were of persistent atrophy cases that had resulted in the overall productivity loss of foregone income of 39.1 crore (Krishnamoorthy, 2009).

Dengue virus belongs to Flaviviridae and the disease onsets with rapid onset of fever, viremia, headache, pain and rash. Dengue Haemorrhagic fever and Dengue Shock Syndrome results in increased edema, hemorrhage, thrombocytopenia and shock. Severe disease results in 5–30% mortality. Dengue virus has been classified into four serotypes (types 1–4) and infection by one serotype predisposes individuals to more

severe disease following subsequent infection by a different dengue serotype (Gupta, 2012).

During the past four decades, increased number of infected cases by mosquito borne viruses are reported due to the geographical expansion, rapid urbanization and increase in transportation facilities in several parts of India (John Victor, 2009). Despite the vigorous preventive and control measures taken in an integrated approach, annual infection of Chikungunya and Dengue cases are reported in India and the number of cases reported in 2013 was about 74 454 for dengue with 167 deaths and 18639 for chikungunya (Dayaraj Cecilia, 2014).

Hence the identification of viruses in wild caught mosquitoes only during outbreaks will not be of much help to prevent any future outbreaks. Mapping of areas with prevalence of viruses is essential to develop a forecasting system (John Victor, 2009). Thus a surveillance study was carried out in Chennai from the month of March to July before the onset of monsoon to study the distribution of Competent vectors and viruses.

METHODOLOGY

Study area

The study comprised the north, south, east and west zone of Chennai and mosquitoes were collected from March to July 2014. The study area comprised of Guindy, Alandur, Saidapet, Triplicane, Mylapore, Santhome, Royapettah, Royapuram, Padi, Avadi, Kodungaiyur, Mugappair, Koyambedu, Maduraivayal, Vadapalani, K.K. Nagar, Saligramam, Virugambakam, Pallikaranai, Tambaram, Medavakkam, Madipakam, Sirusery, Punagar, Kovalam, Sengundram, Parry's, Central, Kundrathur, Thiruvallur (Fig. 1). Sweep nets were adopted for the collection of mosquitoes at dawn between 6.00–7.00 am and Dusk between 6.30–7.30 pm.

Mosquito collection and identification

Mosquitoes were collected using sweep nets and then aspirated through mechanical aspirator (Clark, USA). "Cold-chain" was maintained throughout the procedure. Live adult mosquitoes were anaesthetised by placing mosquito collection bag in a -20°

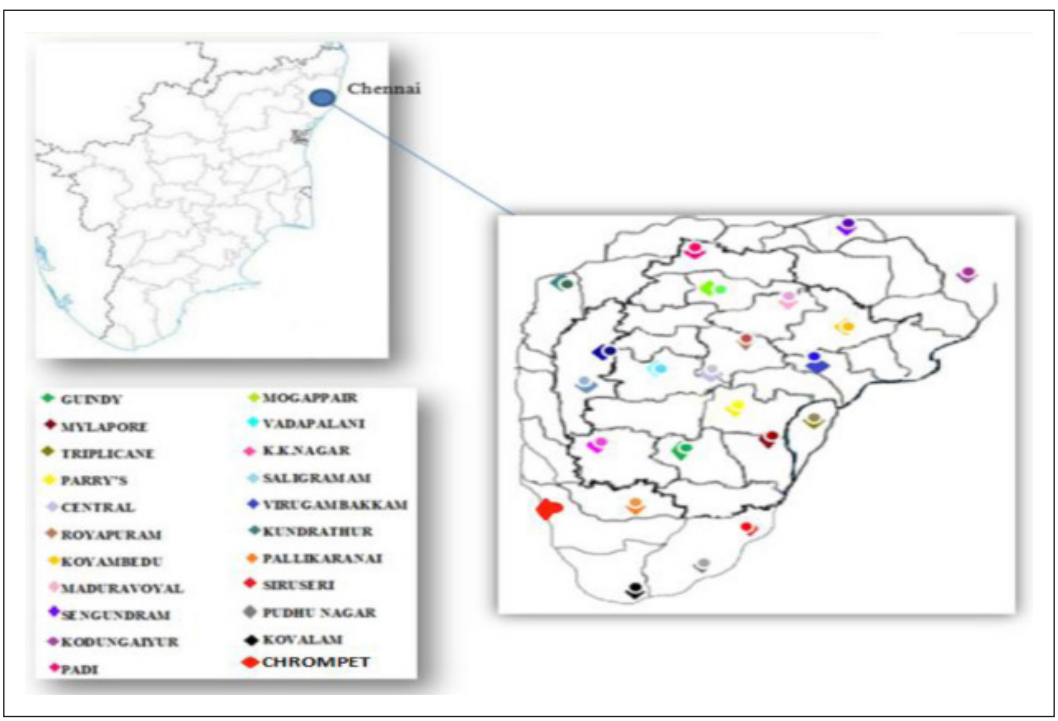

Figure 1. Study areas Covered in Chennai for the Mosquito collection.

freezer for 5 minutes and the collection bag containing mosquitoes. Then the collection bag was transferred to an insulated container containing dry ice. Mosquitoes were exposed to carbon-dioxide for 1–3 minutes to ensure complete knock-down. Individual female mosquitoes were separated and identified using descriptive taxonomic keys based on external mosquito morphology with the aid of a stereo dissecting microscope (10–60X zoom) (Barraud *et al.*, 1934; Banerjee *et al.*, 1979; Aynsley *et al.*, 2007).

Identified mosquito species were made into a pool of ≤50 individuals in 2 mL snap-cap vials containing a copper BB. Vials were placed in labelled bags and stored at -80ºC until virus detection.

Isolation of Viral RNA

Twenty female mosquitoes of same species were pooled and to that 1ml of MEM was added and homogenised at 5000 rotations per minute in a Homogeniser (Bertin Technologies). About 200 µl of the homogenised sample were added with 400 µl of Lysis buffer and 5 µl of carrier RNA .20 µl of proteinase K and about 5 µl of Internal control template was added and mixed immediately by inverting and incubated at 56ºC for 15 min. 300 µl of 100% ethanol was added and mixed well by vortex for 30 seconds. Spinned down few seconds to bring down drops to bottom of the tube. Pipetted entire sample into the Pure Fast^{R} Spin column. Centrifuged at 12000 rpm for 1 min. Discarded the flow –through and placed the column back into the same collection tube. 500 µl of 70% ethanol was added to the Pure Fast^{R} Spin column. Centrifuged at 12000 rpm for 1 min. The flow –through was discarded and placed the column back into the same collection tube. Repeated 70% ethanol wash once. Centrifuged the empty spin column attached with collection tube at 12000 rpm for an additional 2 min and the collection tube was discarded. Transferred the spin column into a fresh 1.5 ml microcentrifuge tube. 50 µl of Elution buffer was added to the center of spin column membrane and incubated for 2 min at room temperature. Centrifuged at 12000 rpm for 1 min and discarded the purefast spin column. The eluted nucleic acid was stored at -80ºC for further analysis.

Molecular detection of Chikungunya virus

A Ready to use HELINI Chikungunya virus real time PCR kit was used for the detection of CHIKV targeting the NSP2 gene of 105 bp amplicon as it was the highly conserved region for Chikungunya virus. Real time Reverse transcriptase reaction was carried out in Roto gene 2 plex PCR machine using the Green and Yellow channel.

Positive Control was set up by adding 12.75 µl of Probe RT-PCR master mix; 0.25 µl of enzyme mix; 7 µl of CHIKV/IC Primer probe mix; 5 µl of QS2 to a total reaction volume of 25 µl. Negative Control was set up by adding 12.75 µl of Probe RT-PCR master mix; 0.25 µl of enzyme mix; 7 µl of CHIKV/IC Primer probe mix; 5 µl of nuclease free water to a total reaction volume of 25 µl. Sample preparation was carried out by adding 12.75 µl of Probe RT-PCR master mix; 0.25 µl of enzyme mix; 7 µl of CHIKV/IC Primer probe mix; 5 µl of sample to a total reaction volume of 25 µl. The holding process was performed by setting up Reverse transcription reaction for 15 min at 50ºC followed by activation of Taq enzyme for 5 min at 95ºC. Then the PCR amplification cycle was carried for 40 cycles with the denaturation at 95ºC for 20 sec; Annealing at 55ºC for 20 sec and extension at 72ºC for 20 sec. CHIKV was detected through FAM channel and internal control through JOE channel.

Molecular detection of Dengue virus

A Ready to use HELINI Dengue virus real time PCR kit was used for the detection of Dengue targeting the 3'UTR which is highly conserved. The holding process was performed by setting up Reverse transcription reaction for 15 min at 50ºC and activation by Taq enzyme for 5 min at 95ºC. Then the PCR amplification cycle was carried for 40 cycles with the denaturation at 95ºC for 20 sec; Annealing at 55ºC for 20 sec and extension at 72ºC for 20 sec.

Isolation of virus

Homogenised samples were filtered using 0.22 µm syringe filter (Millipore sartorious) and 100 µl of filtered sample were added to the confluent Vero cell line. The inoculated sample was allowed to adsorb into the cell for 1 hour and incubated at 37ºC for 7 days to observe the remarkable Cytopathogenic effect.

Minimum Infection rate

Virus infection rate in Mosquitoes was expressed as Minimum infection rate (MIR) per 1000 females tested.

$$MIR/100 = \frac{\text{Number of mosquito pools positive}}{\text{Total number of mosquitoes tested}} \times 1000$$

RESULTS

A total of 1520 mosquitoes comprising both male and female were collected from North, South, East and west zones of Chennai. The collected mosquitoes were identified as *Aedes*, *Anopheles*, *Culex*, *Toxorhynchites*, *Armigerous* species.

Culex quinquefasciatus was the abundant and predominant species observed during March to July in Chennai. *Culex quinquefasciatus* was 26.08% Male and 21.81% Female mosquitoes. *Culex quinquefasciatus* were identified in Guindy, Padi, Mogappair, Koyambedu, Vadapalani, K.K. Nagar, Saligramam, Virugambakkam, Pudunagar, Sirusery, Kovalam, Central, Mylapore, Maduravoyal, Kodungaiyur and Triplicane.

Aedes albopictus were identified in Kundrathur, Red hills, Central, Royapuram, Triplicane. The next predominant species were *Ae. albopictus* (Male 8.45% and Female 8.81%) followed by the distribution of *Ae. aegypti* (6.69% female and 8.81% male); *Ae. vitttatus* (3.9% female and 1.49% male) *Ar. subalbatus* (6.39%), *Cx. vishnui* (4.76%), *Cx. pipiens* (2.34%). Aedes species were identified in Royapuram, Parrys, Central, Sirusery, Mylapore, Pallikaranai, Chrompet, Red hills, Kundrathur, Triplicane.

Anopheles quadrimaculatus, *An. stephensi* were observed from Guindy and other identified species includes *Culex pipiens*, *Cx. pilosus*, *Cx. vishnui*, *Cx. tritaeniorhynchus*, *Cx. pseudovishnui*, *Ar. subalbatus*, *Toxorhynchitis*. The detailed distribution of mosquito species are given in Table 1.

The collected and identified female mosquitoes of *Aedes aegypti* and *Aedes albopictus* were subjected for the detection of Chikungunya and Dengue virus. Chikungunya Virus were detected in the *Aedes aegypti* mosquitoes collected at Royapuram, Chennai and the Dengue virus were detected from Chrompet Region of Chennai by Realtime RT PCR. (Table 2).

Table 1. Distribution of Mosquito species in Chennai

Place	Genus	Species	Sex
Guindy	Anopheles	*An. quadrimaculatus*	Male-17
		An. stephensi	Male-07
	Culex	*Cx. quinquefasciatus*	Male-57
		Cx. pilosus	Female-01
		Cx. pipens	Male-03
Kundrathur	Aedes	*Ae. albopictus*	Male-07; Female-17
Padi	Culex	*Cx. quinquefasciatus*	Male-20; Female-19
Mogappair	Culex	*Cx. quinquefasciatus*	Male-43
		Cx. pipens	Male-04; Female-23
Koyambedu	Culex	*Cx. quinquefasciatus*	Male-32; Female-32
		Cx. pipens	Female-03

Vadapalani	Culex	*Cx. quinquefasciatus* *Cx. vishnui*	Male-10; Female-07 Male-17; Female-05
K.K. Nagar	Culex	*Cx. quinquefasciatus* *Cx. vishnui*	Male-10; Female-14 Male-07; Female-20
Saligramam	Culex	*Cx. quinquefasciatus* *Cx. pseudovishnui*	Male-07; Female-05 Male-05; Female-04
Virugambakkam	Culex	*Cx. quinquefasciatus*	Male-19; Female-04
Pallikaranai	Culex	*Cx. vishnui* *Cx. pseudovishnui* *Cx. quinquefaciatus* *Cx. tritaeniorhynchus* *Cx. pipiens*	Female-03 Female-03 Male-39; Female-49 Female-05 Female-07
	Aedes	*Ae. aegypti*	Female-08
	Armigeres	*Ar. subalbatus*	Male-15
Pudunagar	Culex	*Cx. quinquefasciatus*	Male-01; Female-29
	Armigerous	*Ar. subalbatus*	Male-30
Sirusery	Aedes	*Cx. vishnui*	Female-15
	Culex	*Cx. quinquefasciatus*	Male-27; Female-42
	Toxorhynchites	Toxorhynchites	Male-01
Kovalam	Culex	*Cx. pseudovishnui*	Female-03
	Aedes	*Ae. aegypti*	Female-06
	Culex	*Cx. quinquefasciatus*	Male-17; Female-72
	Toxorhynchites	Toxorhynchites	Male-29
Sengundram (Redhills)	Aedes	*Ae. albopictus*	Male-66; Female-77
Central	Aedes	*Ae. vexan* *Ae. aegypti* *Ae. vittatus* *Ae. albopictus*	Male-05 Female-29 Female-17 Male-05; Female-03
	Culex	*Cx. quinquefasciatus*	Male-64; Female-03
Royapuram	Aedes	*Ae. aegypti* *Ae. albopictus* *Ae. vexan* *Ae. vittatus*	Male-111; Female-05 Male-41; Female-12 Female-18 Female-28
Mylapore	Aedes	*Ae. vittatus* *Ae. aegypti*	Malae-04; Femlae-10 Female-01
	Culex	*Cx. quinquefasciatus*	Male-12; Female-20
Parry's	Armigerous	*Ar. subalbatus*	Male-25; Female-20
	Aedes	*Ae. vittatus*	Male-17
Triplicane	Aedes	*Ae. aegypti* *Ae. albopictus*	Male-13; Female-17 Female-15
	Culex	*Cx. quinquefasciatus*	Male-09; Female-11
Chrompet	Aedes	*Ae. aegypti*	Male-04; Female-18
Maduravoyal	Culex	*Cx. quinquefasciatus* *Cx. pipens*	Male-42; Female-23 Female-03
Kodungaiyur	Culex	*Cx. quinquefasciatus*	Male-11; Female-12

The load of Chikungunya virus in the positive mosquito pool was 40 copies which was quantified by Real time PCR. Subsequent inoculation of the confirmed isolate in Vero cell line had shown remarkable CPE in the detected Chikungunya virus. However subsequent viral isolation from mosquito sample that was Positive for Dengue virus by Realtime RT PCR failed to produce CPE in Vero cell line.

DISCUSSION

Culex quinquefasciatus was the predominant species observed in our studies during March to July which was in accordance with the reports of Banerjee *et al*., 1977; Mourya *et al*., 1989; Ruben *et al.*, 1992.

As *Culex quinquefasciatus* has 63.5% transmission rate of Japanese encephalitis virus and has the blood feeding pattern of human to about 53–63% (Reuben, 1992; Victor 2000). Other *Culex* species transmitting Japanese encephalitis transmitting that were earlier reported to transmit in Tamilnadu were *Culex vishnui* and *Culex pseudovishnui* (Lalitha 2004).

Positive JE cases from humans were mostly reported from northern districts of Tamil Nadu and a total of 28 positives cases of Japanese encephalitis patients in the three years (2009 to 2012) were reported in Tamilnadu (Gunasekaran, 2012). Of the 28 JEV positives during the three years, 16 were from Chennai followed six from Thiruvallore district, one from Kancheepuram, two each from Vellore and from Thiruvannamali district. Also the cases are found to appear in the month of April - May and peak during late August to early September and start to decline from October (Gunasekaran, 2012).

Hence earlier initiation of control and monitoring of JE vectors during the Pre Monsoon period in the prevalent areas in Chennai could possibly reduce the transmission rate and subsequently the infection rate of Japanese Encephalitis virus. However inoculation of the homogenates of *Culex* species has not resulted in the subsequent isolation of virus in Vero cells.

Molecular detection of Chikungunya and Dengue virus had resulted in the identification of Infection rate during March to July (Premonsoon period) with respect to Chikungunya and Dengue virus from the subjected female Aedes species as 0.456. The infection rate was found to be significantly low in Chennai during Premonsoon Period in female Mosquitoes. Although the surveillance included both the male and female species of mosquitoes however we subjected exclusively the female mosquitoes for virus isolation and molecular detection to signify the direct human infection rate during premonsoon period. The low MIR in our studies could be attributed to the fact and observations of Arunachalam *et al*., 2007 that the infection rate in males (28.0/1000) of *Ae. aegypti* was found to be higher in the summer months (June and July) than female and it can be concluded that vertical transmission of dengue viruses by *Ae. aegypti* helps the maintenance of viruses in nature especially during inter epidemic period when the vector density was low. It was interesting to note that *Aedes albopictus* subjected in our studies showed negative for both Chikungunya and Dengue virus by Real time Reverse Transcription PCR .

Among the 19 places that were studied for the mosquito species Royapuram, Parrys, Central, Sirusery, Mylapore, Pallikaranai, Chrompet, Red hills, Kundrathur, Triplicane had shown significant *Aedes aegypti* and *Aedes albopictus* distribution. Hence these places can be given an additional care during mosquito control especially for *Aedes* species as they may serve as the initial carriers for breeding during monsoon season. This forms the first hand comprehensive report in the distribution of mosquito species in Chennai during the premonsoon period ie March to July in 2014.

CONCLUSION

The central zones of Chennai were highly found to inhabit the *Aedes* species in comparison to other zones surveyed during the premonsoon period. Viral infection rate

of Chikungunya and Dengue were very low during the Pre-Monsoon period in Chennai.

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