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First evidence of dengue virus infection in wild caught mosquitoes during an outbreak in Assam, Northeast India

Prafulla Dutta[1], Siraj Ahmed Khan[1], Sumi Chetry[1], Vas Dev[2], Chandra Kanta Sarmah[1] & Jagadish Mahanta[1]

[1]*Regional Medical Research Centre, NE Region, Dibrugarh;* [2]*National Institute of Malaria Research, Field Unit, Guwahati, India*

ABSTRACT

Background & objectives: Dengue is one of the major public health problems worldwide, transmitted mainly by *Aedes aegypti* and *Ae. albopictus* mosquitoes. Rapid urbanisation and industrialisation have led to an increase in vector population in Northeastern states of India. In 2013, Guwahati, the capital city of Assam, India experienced an outbreak of dengue. This study was undertaken with an objective to determine infection rates of dengue viruses (DENV) in both the established vectors present in this region.

Methods: During the outbreak (2013), adults and larvae of both the vector species were collected from different container habitats found in case reporting areas and container index was also recorded. The mosquitoes were first pooled, homogenised and processed for NS1-ELISA. This was followed by RT-PCR of the mosquito pools.

Results: Both *Ae. aegypti* and *Ae. albopictus* were found breeding in containers with container index in the range of 29.41 to 80%. Six pools of *Ae. aegypti* were found to be positive for NS1 antigen. RT-PCR assay revealed positivity in only the NS1-ELISA positive pools, exhibiting circulation of serotype DENV-2. Minimum infection rate of female and male *Ae. aegypti* was recorded as 10.87 and 11.03 respectively.

Interpretation & conclusion: This is the maiden report of detection of DENV in wild caught *Ae. aegypti* mosquitoes from Northeastern Region of India. The study also demonstrates the presence of transovarial transmission of dengue virus in this part of country. This information is useful in respect of both entomological as well as epidemiological point of view for taking appropriate vector control measures.

Key words *Aedes aegypti*; *Ae. albopictus;* dengue; NS1-ELISA; RT-PCR; TOT

INTRODUCTION

Dengue fever is an arboviral disease and a major public health problem worldwide. The incidence of dengue has been reported to increase 30-fold over the last 50 yr and approximately 50 million infections are estimated to occur annually with increasing geographic expansion and presently with a shift in setting from urban to rural[1]. There are five distinct serotypes of dengue viruses (DENV), *viz.* DENV-1, DENV-2, DENV-3, DENV-4 and DENV-5[2], belonging to the genus *Flavivirus* and family Flaviviridae. In most parts of South Asia both *Aedes aegypti* and *Ae. albopictus* are considered as potential vectors for dengue transmission[3]. However, *Ae. aegypti* is considered as the primary one in India[4-6]. These mosquitoes typically bite during the day, particularly in the early morning and in the evening but they are able to bite, and thus, spread infection at any time of day. These mosquitoes transmit the infection by acquiring the virus while feeding on the blood of an infected person or by transovarial transmission[7]. *Ae. aegypti* prefers to breed in stagnant fresh water in all forms of containers, particularly in artificial containers in and around human dwellings and often indoors; and such habitats are less sensitive to climatic variations that might increase the mosquitoes' endurance[8].

The outbreaks of dengue have been reported from several parts of India, namely Delhi, Kanpur (U.P.), Rajasthan, Haryana, Gujarat, Madhya Pradesh, *etc*[6]. The occurrence of dengue cases in Northeastern (NE) region of India has been observed in recent years, which may be due to the immigration of work force from dengue endemic areas and also increased urbanization and industrialization posing a favourable breeding ground for the vector. The transovarial transmission (TOT) of dengue is a well accepted phenomenon reported from different endemic areas around the world[9-11]. The knowledge of TOT that contributes for causing an outbreak of dengue is useful in surveillance of the infection to develop a proper early warning sign, since an early and advance warning is extremely significant for the control of dengue outbreak[7].

This study was undertaken in Guwahati, the capital city of Assam, NE Region of India following an outbreak

of dengue during September–October 2013. The objective of this study was to determine the existence of DENV in *Ae. aegypti* and *Ae. albopictus* as well as TOT in both these mosquitoes of this region. During the outbreak >4000 dengue cases were reported[12]. Virological surveillance in mosquitoes provides an early warning sign for the risk of transmission in an area. The identification of the specific predominant circulating serotype in the vector population further helps in effective clinical management of dengue[13]. In this study, the entomological findings during the outbreak along with the report of first evidence of DENV infection in *Ae. aegypti* and TOT of the virus in Northeast India have been presented.

MATERIAL & METHODS

Study site and mosquito collection

The study was carried out during September–October 2013 when Guwahati, Assam was experiencing a massive dengue outbreak. Samples comprising of adult mosquitoes and immature forms were collected from different localities of Guwahati reporting dengue cases (Fig. 1). Adult resting mosquitoes were collected using drop-nets from different breeding sites which included tyres, cement tanks, earthen pots, plastic containers, flower pots and battery containers[14]. The container index (CI) was calculated as per WHO guidelines[15].

The larvae were collected from all the water filled containers with the help of ladle and with Pasteur pipette for the areas inaccessible to spoon. Only a portion of water containing larvae was taken from large sized breeding sites like tyres and cement tanks while all the larvae were collected from small containers like plastic containers, earthen pots, *etc*. Larvae collected from different breeding habitats in 15 surveyed localities of Guwahati, namely Bamunimaidan, Bhaskarnagar, Narengi, Geetanagar, Lokhora, Athgaon, Islampur, Chandmari, Gandhibasti, Zoo Road area, Hatigarh, Sonapur, Jorabat, Azara and Ranigate were brought to the field laboratory. The immatures were reared in laboratory with utmost care until adult emergence. The emerged adults were then identified following standard mosquito identification keys[16] and pooled according to species, gender, habitat and place of collection in variable pool sizes (ranging from 1 to 50 mosquitoes/pool), and stored at –80°C till assayed for the presence of dengue virus.

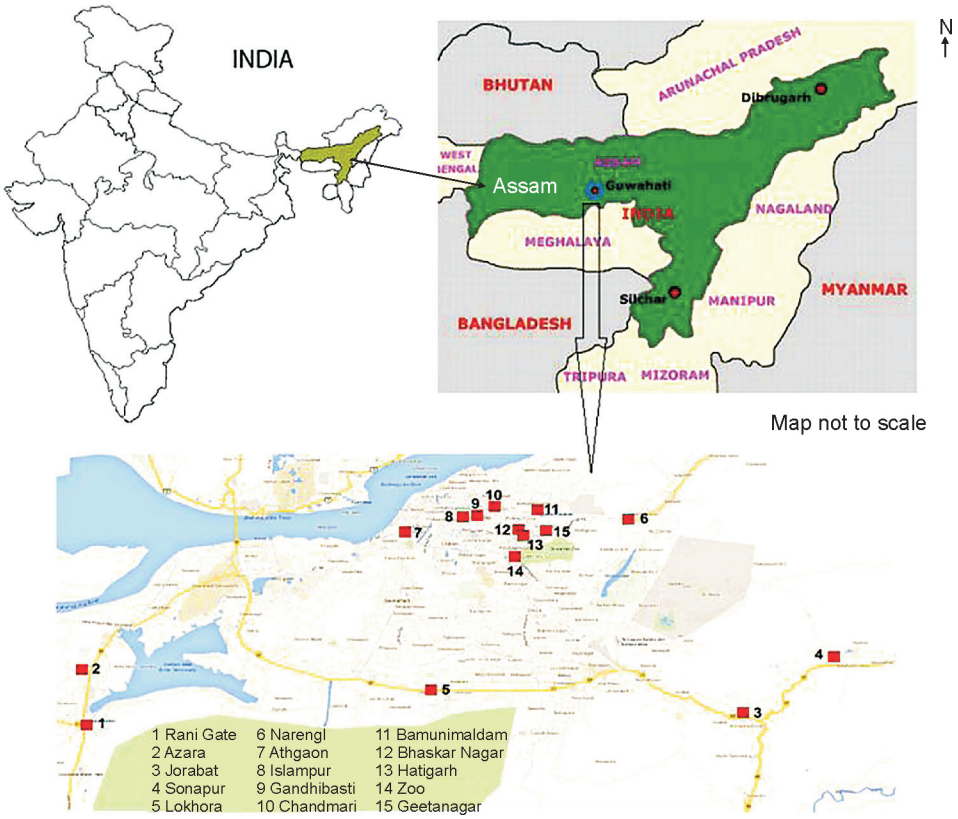

Fig. 1: Sample collection sites in Guwahati, the capital city of Assam, India.

DENV detection in mosquito pool

Mosquito homogenisation: Each pool prepared from the emerged adults was homogenised in commercially available MagNA lyser beads (Roche Diagnostic, Germany) using 1 ml of 2% foetal calf serum (FCS) in minimum essential medium (MEM) containing 50 µg gentamicin, 50 IU penicillin, 50 µg streptomycin and 50 µg amphotericin B per ml, respectively. After grinding the mosquitoes in MagNA Lyser the supernatant was transferred to a new 1.5 ml centrifuge tube and centrifuged at 1100 g for 15 min at 4°C[7, 17]. The supernatant was used for further processing.

NS1 ELISA and RNA extraction

A total of 73 pools were processed for detecting the presence of dengue virus antigen, NS1 by using enzyme linked immunosorbent assay (ELISA). About 75 µl of the mosquito homogenate from each pool was diluted with equal amount of sample diluents from Dengue early ELISA kit (Panbio, Australia) and 100 µl of this preparation was used to detect the presence of NS1 antigen according to the procedure provided with the kit. For confirmation of the result with RT-PCR and to detect the infecting serotype, viral RNA was extracted from 140 µl of the remaining mosquito homogenate using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) accord-

Table 1. Entomological survey of *Aedes* in Guwahati, Assam during September–October 2013

Location	Habitat type	Total breeding site searched	Positive breeding site (CI)	No. of emerged *Aedes* adults pooled	No. of breeding sites positive for *Ae. aegypti* (CI)	No. of breeding sites positive for *Ae. albopictus* (CI)	Species and gender wise *Aedes* pooled (Total No.)			
							Ae. aegypti		*Ae. albopictus*	
							Female	Male	Female	Male
Bamunimaidan	Cement tank	4	2 (50)	26	2 (50)	2 (50)	2	4	4	16
	Tyre	3	1 (33.3)	23	1 (33.3)	NA	13	10	ND	ND
Bhaskarnagar	Bamboo stamp	11	6 (54.54)	7	NA	6 (54.54)	ND	ND	4	3
	Tyre	7	3 (42.86)	60	3 (42.86)	1 (14.28)	34	15	8	3
	Plastic container	5	4 (80)	11	4 (80)	NA	8	3	ND	ND
Narengi	Plastic container	4	3 (75)	30	2 (50)	2 (50)	5	18	3	4
	Battery container	4	1 (25)	6	NA	1 (25)	ND	ND	4	2
	Cement tank	3	1 (33.3)	58	1 (33.3)	1 (33.3)	18	31	0	9
	Tyre	20	7 (35)	67	6 (30)	3 (15)	39	21	5	2
Geetanagar	Tyre	17	5 (29.41)	78	4 (23.53)	3 (17.65)	9	26	18	25
	Cement tank	3	2 (66.67)	14	NA	2 (66.67)	ND	ND	3	11
	Plastic container	5	4 (80)	7	NA	4 (80)	ND	ND	2	5
	Earthen pot	2	1 (50)	1	NA	1 (50)	ND	ND	0	1
Lokhora	Tyre	11	8 (72.73)	26	6 (54.55)	4 (36.36)	9	1	6	10
	Battery container	2	1 (50)	50	1 (50)	NA	21	29	ND	ND
Athgaon	Tyre	20	11 (55)	18	9 (45)	4 (20)	4	6	5	3
Islampur	Tyre	10	4 (40)	14	4 (40)	NA	3	11	ND	ND
	Plastic container	5	2 (40)	5	2 (40)	NA	3	2	ND	ND
Chandmari	Tyre	10	3 (30)	30	3 (30)	NA	11	19	ND	ND
	Cement tank	2	1 (50)	6	1 (50)	NA	2	4	ND	ND
Gandhibasti	Tyre	9	3 (33.33)	18	3 (33.33)	NA	7	11	ND	ND
	Plastic container	30	6 (20)	5	NA	6 (20)	ND	ND	1	4
Zoo Road area	Plastic container	17	9 (52.94)	12	NA	9 (52.94)	ND	ND	3	9
Hatigarh	Cement tank	2	1 (50)	24	1 (50)	NA	20	4	ND	ND
Sonapur	Tyre	9	4 (44.44)	23	NA	4 (44.44)	ND	ND	10	13
Jorabat	Tyre	15	7 (46.67)	100	7 (46.67)	NA	50	50	ND	ND
Azara	Tyre	10	8 (80)	8	8 (80)	NA	5	3	ND	ND
Ranigate	Tyre	5	3 (60)	7	NA	3 (60)	ND	ND	7	0

ND: Not detected; NA: Not applicable; CI: Container index.

ing to the manufacturer's protocol, and stored at –80°C until use.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The total RNA, extracted from the mosquito pools were converted to cDNA which was followed by a PCR using serotype specific primers for dengue virus capsid region[18-19]. One positive control (DENV-2 strain "P23085") as well as one negative control (nuclease free water) was used during the experiments. The positivity of a pool was confirmed by the amplification products of molecular weight 482, 119, 290 and 392 bp for DENV-1, DENV-2, DENV-3 and DENV-4, respectively. A 5 µl aliquot of each amplified product was electrophoresed in 1.5% agarose gel containing ethidium bromide. Bands were visualised under the gel documentation system (GelDoc system, BioRad, USA).

Estimation of minimum infection rate (MIR)

Minimum infection rate (MIR) per 1000 mosquitoes was calculated as the ratio of the total number of positive pools to the number of tested mosquitoes, multiplied by 1000 to estimate dengue virus infection rate[20]. MIR assumes that there is only one infected mosquito present in a positive pool[21].

RESULTS

During the outbreak, both *Ae. aegypti* and *Ae. albopictus* were collected from the studied locations. Only a few numbers of resting adults (19 nos.) from tyres could be collected in two locations out of 15 locations surveyed. Out of the 19 field collected adults, 17 were *Ae. aegypti* (13 females and four males) and two were *Ae. albopictus* females. A total of 734 adults were found to emerge from immatures collected from 227 different breeding habitats of 15 locations in surveyed areas of the capital town (Fig. 1). The CI value was found in the range of 29.41 to 80%. The highest CI was recorded for tyre and plastic containers followed by cement tanks (Table 1). *Aedes aegypti* was found to be more abundant than *Ae. albopictus* in the study area, as the emergence record of *Ae. aegypti* was higher (n=531) against *Ae. albopictus* (n=203). The emerged mosquitoes were identified and pooled on the basis of species, gender, breeding habitat and locality. The total emerged adults constituted 69 pools which included 531 *Ae. aegypti* (263 females and 268 males) and 203 *Ae. albopictus* (83 females and 120 males). Thus, a total of 73 pools (four pools of adults from resting collection and 69 pools of adults emerged from larvae) were tested for the presence of NS1 antigen.

Out of 73 pools, six pools were found to be positive for the presence of NS1 antigen by NS1 ELISA (Table 2). The positive pools included the adults emerged from the field collected larvae and consisted of both male and female pools of *Ae. aegypti*. No pool of adult mosquitoes collected was found to be positive. Also, none of the *Ae. albopictus* pools was found positive for DENV infection. The mosquitoes in the positive pools were collected from Narengi, Bamunimaidan, Bhaskarnagar and Geetanagar areas of Guwahati. Upon PCR, only the NS1 ELISA positive pools were found to be positive for DENV RNA and the infective serotype was determined as DENV-2 (Fig 2). The MIR of both male and female

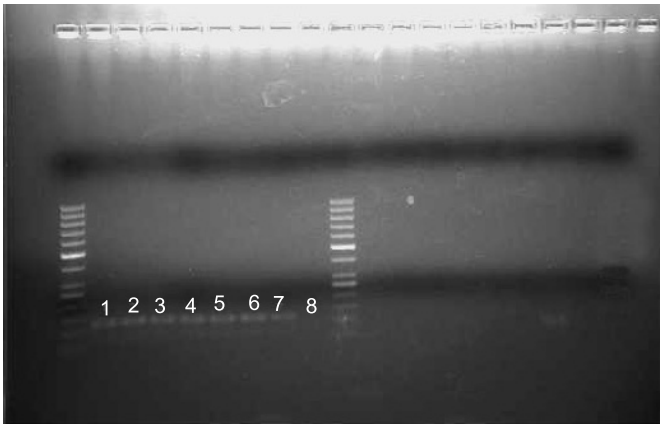

Fig. 2: PCR based detection of DENV in the mosquito samples. Lane 1: Positive control, Lanes 2–7: Mosquito pools, Lane 8: Negative control. The presence of bands at the position of 119bp confirms the serotype of the circulating virus during the outbreak as DENV-2.

Table 2. NS1 ELISA and RT-PCR results of the mosquito pools tested

Mosquito species	Gender	No. of pools assayed	Total No. of mosquitoes	No. of positive pools (NS1)	DENV serotype detected (PCR)	MIR/1000
Ae. aegypti	Female	21	276	3	DENV-2	10.87
	Male	20	272	3	DENV-2	11.03
Ae. albopictus	Female	16	85	ND	NA	NA
	Male	16	120	ND	NA	NA

ND: Not detected; NA: Not applicable; MIR: Minimum infection rate.

Ae. aegypti were also calculated (Table 2) and found to be 11.03 and 10.87 respectively.

DISCUSSION

Aedes aegypti and *Ae. albopictus* are prevalent in Assam and were detected in the present as well as earlier studies[22-23]. As Guwahati, the capital city of Assam, is the Gateway of all the northeastern states, it is presumed that potential vectors of dengue have invaded the major townships of all the northeastern states through this route. Solid wastes are frequently found dumped in industrial areas and major townships which not only contribute to soil pollution but also become potential health hazard. Rainwater accumulates in waste materials and these become the breeding habitats of *Aedes* mosquitoes. These man-made situations favour the proliferation of container breeder *Aedes* mosquito population[24].

In our present study, we detected DENV antigen in wild caught *Ae. aegypti* mosquitoes by ELISA as this technique is useful in DENV surveillance for monitoring DENV activity in endemic area to develop an early warning sign to plan vector control strategy[3]. The NS1-ELISA is an easy and less labour intensive method to detect the presence of dengue infection in mosquitoes and can be used in field during an epidemic for the vector surveillance[25-26]. No DENV in *Ae. albopictus* was detected in this study; this result is consistent with earlier studies done in Tezpur Military area of Assam[27]. During present study, the circulating DENV was identified as DENV-2. Circulation of all the four serotypes in this region has already been established in dengue cases[28]. The detection of DENV was done previously from *Aedes* mosquitoes in different countries including India[13]. The identification and typing of DENV in both the field collected *Aedes* mosquitoes as well as in clinical specimens are important for epidemiological and clinical management of cases. There was no previous report of detection of DENV in *Aedes* mosquitoes from the north-eastern region of India. This is the maiden report of detection of DENV in mosquitoes from this part of the country.

In the study, the detection of DENV in the mosquitoes that were emerged from the larvae collected and specifically in some male mosquitoes is a direct evidence of natural TOT of this virus in this region. Transovarial transmission of dengue by *Aedes* plays an important role in the maintenance of these viruses in nature. The MIR value found in the study is slightly higher in males than females. This may have an epidemiological implication in regards to transmission of DENV from males because the infection can be transmitted to females through venereal transmission which is experimentally observed in case of *Aedes* mosquitoes[29]. Sometime, a low MIR might also be sufficient to maintain the virus during unfavourable seasons or during periods when the numbers of mosquitoes present are scarce to maintain the circulation of DENV in susceptible host[30].

CONCLUSION

This study demonstrates the presence of DENV in wild caught mosquitoes for the first time in this region. Also the TOT of DENV was observed in this study. As there is high abundance of dengue vectors in this region, the appropriate vector control measures is the only prophylactic measure when no safe and effective vaccine is available. Virological surveillance in mosquitoes provides an early warning sign for the risk of transmission in an area. This will further help in controlling the infection, before it culminates into an outbreak.

Conflict of interest: None

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Correspondence to: Dr Prafulla Dutta, Scientist 'G' & Director Incharge, Regional Medical Research Centre (ICMR), NE Region, Post Box No. 105, Dibrugarh–786 001, Assam, India.
E-mail: duttaprafulla@yahoo.com

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