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Detection of Zika virus in *Anopheles stephensi* Liston, 1901 (Diptera: Culicidae) in India - First report

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ABSTRACT: Zika virus (ZIKV) a mosquito-borne, causing acute febrile illness associated with rash, arthralgia and conjunctivitis in the patient, was reported from Thiruvananthapuram, Kerala, as an outbreak with 83 cases. Entomological surveillance revealed the presence of aedine mosquitoes *viz., Aedes aegypti* (Linnaeus, 1762), *Ae. albopictus* (Skuse, 1894) and *Ae. vittatus* (Bigot, 1861) and non-aedine mosquitoes *viz., Anopheles stephensi* Liston,1901, *Mansonia uniformis* (Theobald, 1901), *Culex tritaeniorhynchus* Giles, 1901 and *Cx. gelidus* Theobald,1901. *Aedes* (*Ae. aegypti, Ae. vittatus and Ae. Albopictus*) mosquito larvae were high in the Zika affected areas. Moreover ZIKV was detected in *An. stephensi* mosquitoes collected from Parassala, Thiruvananthapuram (the native place of the first ZIKV confirmed case in the present outbreak in Kerala). Molecular diagnostics of *Ae. Aegypti, Ae. vittatu* and *An. stephensi* mosquitoes revealed that the species were loaded with ZIKV. Significantly this is the first ever report of ZIKV detecting in *An. stephensi* in the world. *Aedes* adults (male and female) and *An. stephensi* emerged from fourth instar larvae and pupae were found to have ZIKV, indicating transovarial transmission of the virus.

KEY WORDS: Flavivirus, entomological surveillance, vector incrimination, RTPCR, Kerala

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne *flavivirus* closely related to human pathogenic viruses such as dengue, chikungunya, yellow fever, Japanese encephalitis and West Nile viruses, and is a public health concern all over the world. ZIKV was first detected in *Aedes* (*Stegomyia*) *africanus* (Theobald, 1901) mosquitoes from Zika forest of Uganda (Dick *et al*., 1952). ZIKV originally circulated in enzootic cycles between forest dwelling canopy-feeder mosquitoes and non-human primates (Weinbren and Williams, 1958). The virus caused outbreaks in different Pacific regions during the period from 2007 to 2015 and began extending throughout the Americas in 2015 (Musso and Gubler, 2016). The ability of ZIKV to cause congenital defects in newborn, as evidenced by the

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microcephaly epidemic in Brazil, has been an unprecedented characteristic in a mosquito-borne viral infection (de Oliveira *et al*., 2017). Although transmission of ZIKV has declined in the Americas, Zika fever outbreaks continue to occur in South East Asia including India (Grubaugh *et al.,* 2019). It was first reported in Asia during 1966. India reported ZIKV infections from 2016 onwards from Gujarat, Tamil Nadu, Rajasthan, Madhya Pradesh and Kerala (Sasi *et al*., 2021). The first confirmed ZIKV case from Uttar Pradesh was detected in Kanpur on 24th October 2021 as confirmed by National Institute of Virology, Pune. In Rajasthan, ZIKV was detected in *Aedes aegypti* (Linnaeus, 1762) mosquitoes collected from Jaipur (Singh *et al*., 2019). As of latest, Sasi *et al.* (2021) reported ZIKV in *Ae. aegypti, Ae. albopictus* (Skuse, 1894) and *Ae. vittatus* (Bigot, 1861) from Thiruvananthapuram, Kerala.

The virus caused a perceptible Pan-American epidemic after its first advent in Brazil in 2015 (Brasil *et al*., 2016). Though the virus can scarcely be transmitted between humans, transmission by mosquito bite is considered to be the most common way of virus dissemination in disease outbreak zones (Musso and Gubler, 2016). ZIKV was first reported in East Africa in 1947 and expanded from lineal enzootic cycle in Africa to Asia. At the beginning of the 21st century, the virus expanded into the South Pacific and Americas, triggering a pandemic that led to 87 countries or territories reporting active ZIKV transmission by 2021. The first documentation of inter-human urban transmission of ZIKV through *Aedes* (*Stegomyia*) *aegypti* came from Malaysia in 1966. *Aedes aegypti* is a known vector of dengue, chikungunya and yellow fever (Rajendran *et al*., 2021). Many investigators attempted to detect the virus during outbreaks, but the virus could be detected in few instances (Akoua-Koffi *et al*., 2001). Chouin-Carneiro *et al*. (2016) established *Ae. aegypti* as the vector in spreading the virus in laboratory assays. Natural infections of ZIKV have been detected in several mosquito species during outbreaks in Africa. However, viral detection in mosquitoes in urban outbreak areas has been scarce to non-existent (Musso and Gubler, 2016). Vector incrimination study is crucial in ZIKV transmission dynamics (Gutierrez- Bugallo *et al.,* 2019).

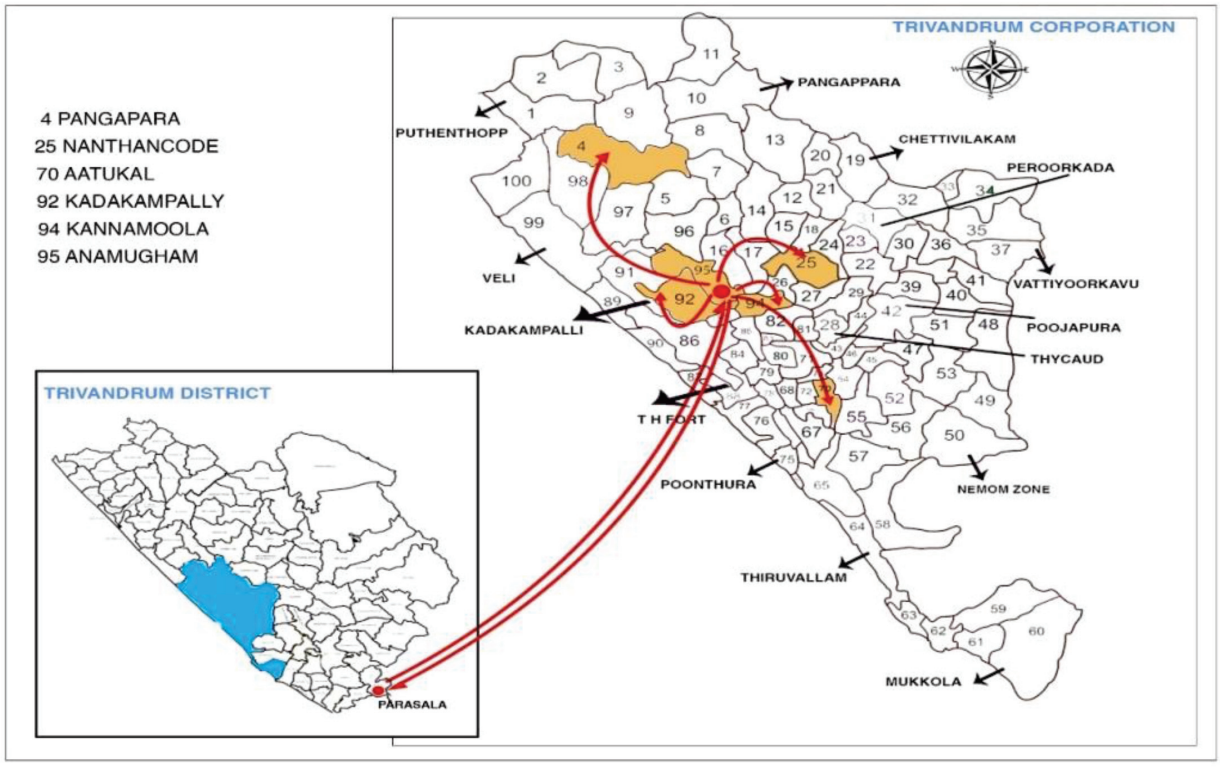
The first confirmed ZIKV case of Kerala was reported from Thiruvananthapuram on 8th July 2021. Since then, 83 ZIKV positive cases have been reported from the state. Study on vector prevalence in the outbreak area and their possible role in transmitting the virus forms an integral part in the assessment of the magnitude and severity of disease transmission in a locality. Hence, extended vector surveillance was carried out in the disease affected areas of Thiruvananthapuram district to ascertain the potential role of vector-pathogen relationship and dynamism of disease transmission.

MATERIALS AND METHODS

Study area: The first confirmed case of ZIKV was reported in a pregnant woman admitted in a hospital located in Anamugham (Ward No.95) of Thiruvananthapuram Municipal Corporation (TMC), Kerala. In the initial months, she stayed in Parassala (her native place), and during the final months of her gestation, she stayed in Nandancode (Ward No. 25), TMC (Map 1). Subsequent ZIKV cases had apparently contracted the infection from the limits of TMC. Hence, a study involving vector surveillance and vector incrimination was planned and carried focusing TMC area.

Entomological surveillance: During the recent ZIKV outbreak, detailed vector surveillance was carried out (from 8th to 27th July 2021) in 10 wards in TMC (Anamugham (Ward No. 95), Nandancode (Ward No. 25), Parassala, Palkulangara (Ward No.85), Kadakampally (Ward No.92), Kunnukuzhi (Ward No.26), Kannanthura (Ward No.84), Thycaud (Ward No.28), Pattom (Ward No.17), Balaramapuram (Ward No.19) and Anayara (an area within Ward No.86), and 80% of them were micro containment wards for infection. All accessible water holding containers /habitats in and around houses/other building premises were checked for the presence of mosquito larvae/pupae (Rajendran *et al*., 2021) in the Zika outbreak areas. Larvae/pupae collected from each of the containers/sources were kept in separate vials labeled with date of collection, locality, house

MAP 1. ROUTE MAP OF FIRST ZIKA CONFIRMED CASE IN THIRUVANANTHAPURAM, KERALA



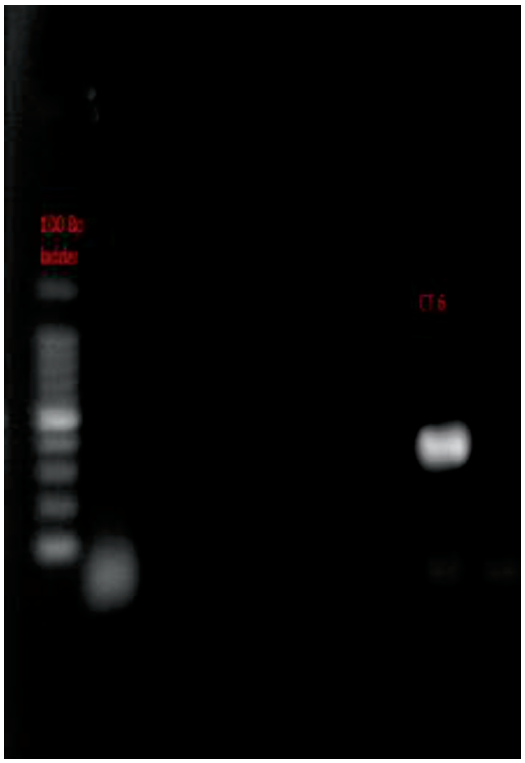


Fig.1 ZIKA virus positive gel image

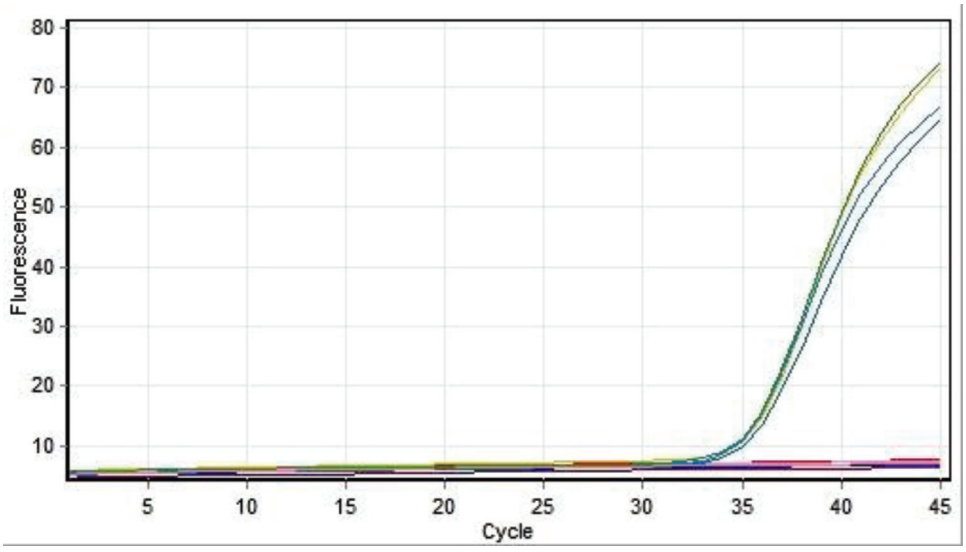


Fig. 2 Raw Data for Cycling A. Green

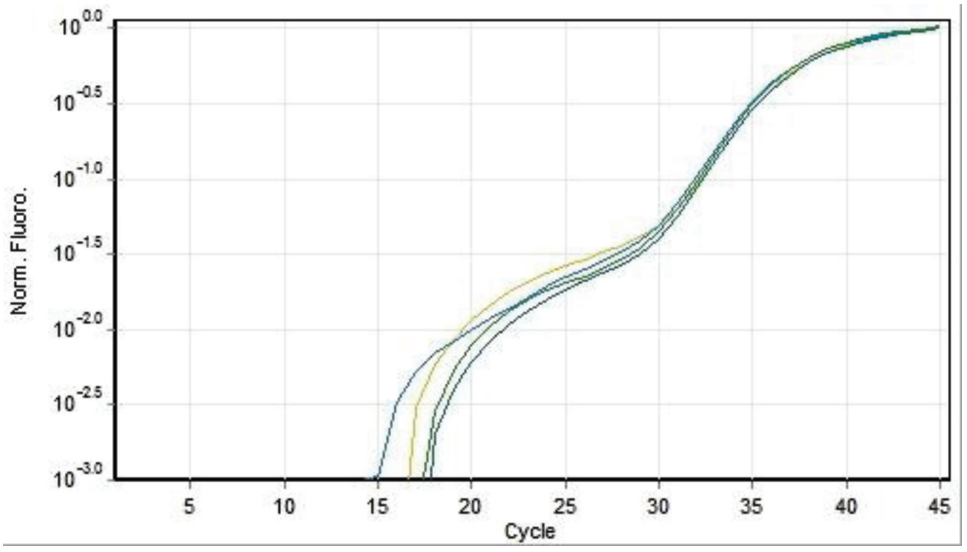


Fig. 3 Quantitation data for Cycling A.Green

number and breeding source (container type/ habitat), and reared in the State Entomology Unit laboratory attached to the Directorate of Health Services (DHS), Thiruvananthapuram, Kerala. Collected larvae/pupae were reared in jars filled with 150 ml fresh water, covering with fine mosquito net. The field collected adult mosquitoes as well as the adults emerged from the larvae/pupae were identified using standard key (Rueda, 2004; WHO, 2020). The identified male and female mosquitoes were kept in separate pools for virus detection as per standard procedure.

Detection of Zika virus: Samples of mosquitoes from different pools labeled with identity details were analysed for virus detection using RT-PCR/ virus isolation protocols at Rajiv Gandhi Centre for Biotechnology (RGBC), Thiruvananthapuram, Kerala.

Viral Nucleic Acid (RNA) isolation: The specimens stored in 70% ethanol were put into a fresh sterile tubes and added 100µl 1x PBS to each tube. The specimen was washed with 1X PBS by tapping the tube gently and 1X PBS was decanted. This procedure was done to wash off the ethanol thoroughly, as traces of ethanol will inhibit PCR. Cryogenic grinding, otherwise known as freeze grinding, technique was used for grinding tissue samples to extract nucleic acid. The specimens were cooled below -80^{0}C by adding liquid nitrogen. The specimens were subjected to cryogenic grinding using liquid nitrogen in mortar and pestle under aseptic conditions. 200µl 1X PBS (lysis buffer) was added to the mortar with the powdered specimen. The powder was dissolved completely. The solution was then transferred to 1.5ml micro centrifuge tube and was centrifuged at 13,000 rpm for 5 minutes at room temperature. The supernatant was discarded. 200µl 1X PBS was again added to the pellet and was vortexes for 1 minute until the pellet was completely dissolved. Viral nucleic acid (RNA) isolation from the supernatant was carried out using QIAGEN s QIAamp Viral RNA Extraction Kit®(QIAGEN, Germany), following the manufacturer's protocol.

Identification of Zika virus diversity in vector mosquito: A direct PCR amplification reaction was achieved for identification of zika virus using remoted viral RNA immediately with unique primers. The isolated viral RNA was directly used as the template. The PCR merchandise was then electrophoresed on 1.5 per cent agarose gel in 1x TAE buffer. 0.5 µl of forward and reverse primers along with TaKaRa's prime script one step Rt-PCR master blend (TaKaRa, Japan) including 12. 5 µl of 2x one step RT buffer, 0.5 µl of taq polymerase (5 units/µl), 0. 5 µl of 5x different transcriptase enzyme and 3.0 µl of RNAse unfastened dH2O. A total reaction volume of 25µL was subjected to PCR for 40 cycles, with an initial cDNA synthesis step at 42^0 C for 5 min., initial denaturation at 94^0 C for 10 secs, denaturation at 94^0 C for 30 secs, annealing at 55^0 C for 60 sec., and extension at 72^0 C for 60 sec., and a final extension at 72^0 C for 60 sec. The products were analyzed as bands on a 1.5% Agarose gel in 1X TAE buffer at 250 Bp. Positive bands obtained were extracted from the gel and purified. The purified product was then subjected for cycling sequencing followed by Sanger Sequencing. The sequence obtained was analysed using NCBI BLAST. RT-PCR was performed using RealStar® Zika Virus RT-PCR Kit 1.0, Altona Diagnostics, GmBH, Germany according to manufacturer's protocol.

RESULTS AND DISCUSSION

The first case of ZIKV in Kerala was reported from Nandancode (Ward No.25) of TMC area. On suspected symptoms, the serum sample of the patient was subjected to laboratory tests and confirmed for Zika positive by National Institute of Virology, Pune. The investigation team visited the residential area of the first Zika confirmed case on 8th July 2021 to trace the primary source of the infection. The duration of the stay of the first Zika confirmed case in the assigned area, incubation period of the disease and vector proliferation magnitude suggest that the first confirmed case might have interacted with many people in Nandancode, where the patient resided during the onset of illness. There had been a history of as many as 14 staff of the mentioned hospital presented with fever symptoms as early as in mid of May 2021, and 19 samples of staff subjected to test for measles, rubella, dengue and chikungunya

found negative at NIV, Pune, but test for Zika was not done at that time due to lack precedence. However, after the confirmation of first ZIKV case, the aforesaid 19 archived samples were retrieved and tested for Zika, of which 14 were found positive.

Extensive vector surveillance carried out in Zika affected areas revealed the presence of aedine mosquitoes *viz.*, *Ae. aegypti, Ae. albopictus* (Skuse, 1894) and *Ae. vittatus* (Bigot, 1861) and non-aedine mosquitoes *viz*., *Anopheles stephensi* Liston, 1901, *Mansonia uniformis* (Theobald, 1901), *Culex tritaeniorhynchus* Giles, 1901, *Cx. gelidus* Theobald, 1901. A total of 137 males and 108 females were collected from the wards. Further the State Entomology team surveyed in and around the house of the first Zika confirmed case in Parassala, but could not detect any mosquito breeding habitats. However, the team could collect five *Anopheles* fourth instar larvae and four pupae from a deserted fish (cement) tank in the terrace of a neighbouring building. On emergence (only three adults), the mosquitoes were identified as *An. stephensi,* a known vector of malaria in Kerala and elsewhere.

All the samples (adult mosquitoes that emerged from fourth instar larvae collected) were screened primarily using Real Time PCR for the detection of Zika Virus. Fig.1 shows ZIKA virus positive gel image. The four sigmoid graphs in the raw data (obtained in real time PCR) represents positive control (Fig. 2); while the four sigmoid graphs represents PCR positive samples isolated from the adults of *Ae. aegypti, Ae. vittatus* and *An. stephensi* (Fig. 3). Based on the diagnostics, ZIKV was detected in the *Ae. vittatus* mosquitoes (7 males) from Nandancode and in *Ae. aegypti* (3 males and 3 females) from Anamugham. Three female *An. stephensi* mosquitoes obtained from rearing were found positive for Zika virus (Figs. 1, 2, 3). Detection of ZIKA virus in these three species, (reared from fourth instar larvae from Zika affected localities), indicates the transovarial transmission of the virus.

ZIKV detected in *Ae. aegypti* mosquitoes collected from Zika outbreak areas of Rajasthan in 2019 (Singh *et al*., 2019) was the first report of the detection of ZIKV in *Ae. aegypti* mosquitoes in India. Sasi *et al.* (2021) reported detection of ZIKV in the *Ae. aegypti*, *Ae. albopictus* and *Ae. vittatus* from Thiruvananthapuram, Kerala. *Anopheline* mosquitoes all over the world indicate that only *An. africanus*, *An. coustani* Laveran, 1900 and *An. gambiae* Giles, 1902 are incriminated vectors of ZIKV. As of the case, this is the first ever global report of *An. stephensi* for ZIKV detection and transovarial transmission. During the present investigation, ZIKV could not be detected in *Mansonia uniformis* (Theobald, 1901), *Culex tritaeniorhynchus* Giles, 1901, *Cx. gelidus* Theobald, 1901 mosquitoes collected from Zika affected areas. The detection of ZIKV in the reared *Ae. aegypti*, *Ae. albopictus* and *Ae. vittatus* confirms the transovarial transmission of the virus as reported by Lai *et al*. (2020).

Since first ZIKV was detected in *Ae. africanus* mosquitoes collected from Zika forest, Uganda in 1948 (Dick *et al*., 1952), its positivity has been found in 20 species of *Aedes* mosquitoes, one species each of *Mansonia* and *Culex* and three species of *Anopheles* mosquitoes so far from different Zika affected areas of the world. In India, ZIKV has been detected in *Ae. aegypti, Ae. albopictus and Ae. vittatus* mosquitoes. As per the current report, there are 31 wild-caught mosquito species infected with ZIKV worldwide. These mosquitoes belong to *Aedes* (22 species), *Culex* (4 species), *Anopheles* and *Eretmapodites* (2 species each) and *Mansonia* (1 species). Among the *Aedes* mosquitoes, ZIKV could be detected from nine species of mosquitoes belonging to the subgenus *Stegomyia*. This suggests that *Aedes* genus, especially *Stegomyia* subgenus is the most significant taxon involved in ZIKV transmission. So far, ZIKV could be detected in 21 species of mosquitoes collected from sylvatic settings. However only six species (*Ae. aegypti*, *Ae. albopictus*, *Ae. vexans* Meigen, 1830, *Cx. quinquefasciatus* (Say), *Cx. coronator* (Dyar and Knab) and *Cx. tarsalis* Coquillett, 1896 have been identified as ZIKV vectors in urban settings (Smartt *et al*., 2017; Elizondo-Quiroga *et al*., 2018).

The present study reveals that in addition to the conventional *Aedes* mosquitoes, Zika virus also has

An. stephensi as a potent vector. Significantly this is the first ever report of ZIKV detecting in *An. stephensi* in the world. *Aedes* adults (male and female) and *An. stephensi* emerged from fourth instar larvae and pupae were found to have ZIKV, indicating transovarial transmission of the virus. This obviously calls for a change in vector control strategy, especially in the areas where these mosquitoes are abundant. Most of the districts of Kerala, especially coastal areas, are conducive for profuse breeding of *Ae. aegypti*, *Ae. albopictus*, *Ae. vittatus* and *An. stephensi*, the vectors of ZIKV in the recent outbreak. There is a need for continuation of human, veterinary, entomological and environmental surveillance to ascertain the incidence and geographical distribution of ZIKV, host and vector diversity, virus strain and its transmission potential. Effective surveillance and appropriate vector control strategy are a must to avert future outbreaks.

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