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Molecular epidemiological surveillance of dengue virus and diversity of *Aedes* species in Tirunelveli district

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Abstract
Dengue is a re-emerging infectious diseases caused by any one serotype of dengue virus (DENV I - IV) and belongs to the family *Flaviviridae*. In recent years, an increasing number of dengue cases in Tirunelveli district present with unusual clinical manifestations including signs and symptoms involving the central nervous system and the neurological features. Therefore, there is an urgent need to assess the different serotypes and genotype determines dengue virus outbreak severity in the district of Tirunelveli.
Methods: A total of 2865 female adult *Aedes* mosquitoes and 420 blood samples were collected from suspected patients to have dengue fever were subjected to antigen capture ELISA. All the dengue positive samples, RNA were isolated by using virus nucleic acid extraction kit and the dengue RNA used as a template for cDNA synthesis by RT-PCR and fragmented using restriction endonuclease on the BanII enzyme.
Results: A total of 152 mosquitoes were DEN positive by either dengue IgM capture assay (n = 135) or antigen capture ELISA (n = 49) or both (n = 32). High rainfall and humidity with the temperature might have favored to the breeding and transmission of *Aedes*, in consequence leading to an increase in the number of dengue mosquitoes.
Conclusion: The up-to-date information of circulating dengue virus strains combined to the available epidemiological information aids in better understanding of the nature of the epidemics in the endemic areas.

Keywords: dengue virus strains, serological detection, RT-PCR and antigen capture ELISA.

1. Introduction
Dengue virus is a mosquito-borne flavivirus and the most widely prevalent arbovirus in tropical and subtropical regions of developing countries [1]. There are four distinct serotypes, DEN-1, DEN-2, DEN-3, and DEN-4, which belong as a positive stranded encapsulated RNA virus to the family *Flaviviridae*, which causes a spectrum of illness ranging from in apparent infection to moderate febrile illness and severe and fatal hemorrhagic disease. In recent years, dengue hemorrhagic fever (DHF) by hemorrhagic signs in infected cells and is also caused dengue shock syndrome (DSS) and is characterized by features of DHF plus evidence of circulatory failure manifested by hypotension or hypertension, cold clammy skin and restlessness, which emerged as a major public health problems, with expanded geographic distributions and increased epidemic activities [2-4]. Each serotype is sufficiently different that infection with one does not provide complete cross protection for the other serotypes [5]. DENV can be further divided into intra-serotypic categories called interchangeably as subtypes or genotypes based on their nucleotide sequence data [6-8]. However, the molecular epidemiology of dengue viruses has been used to determine the origin of the viruses that have caused outbreaks and epidemics, especially in the attempt to establish a correlation between the virulence of samples and the impact of these viruses on the population. The vector surveillance allows timely implementation of emergency mosquito control measures to kill adults and destruction of breeding places to limit an impending outbreak. Such surveillance based on the isolation and identification of dengue viruses in *Aedes* infecting the human population provides an important means of early detection for dengue outbreaks [9]. Lack of longitudinal research on dengue epidemiological surveillance has hampered our understanding of dengue serotype in Tirunelveli district, Tamil Nadu. In this study, we primarily determined the dengue serotype 2 isolates that were identified in rural and urban areas of Tirunelveli district using molecular and immunological techniques and it was associated dengue vectors diversities.

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2. Material and Methods

2.1 Study Area

Blood samples were collected by a trained nurse from consenting patients presenting with fever of ≥ 38 °C for less than 72 hours in the Tirunelveli district during the first week June 2013 to May 2014. The surveyed area is geographically located in the Southeastern portion of Tamil Nadu is triangular in shape (6759 sq. km). It lies between 8° 05' 10" N – 09° 30' 20" N latitudes and 77° 05' 07" E – 78° 25' 13" E longitudes. The district has peculiar climate and receives rainfall in all the seasons and maximum precipitation is received by the North East Monsoon (555.08 mm) followed by the South west monsoon (189.6 mm) and the Summer (127.7 mm) and the Winter (74.5 mm). The mean annual temperature is 26 °C – 28.5 °C. The district population is 3,077,233 and literacy rate is 82%. A network of government-run Primary Health Centres (PHCs) in rural areas and General Hospitals in urban areas serves the health needs of the people. Apart from dengue fever caused by dengue virus and transmitted by dengue vectors prevalent in the region due to mismanagement.

2.2 Sampling design

Based on the occurrence of mosquitoes breeding sites, the areas are identified as dengue infection risk and non risk areas. The availability of breeding sites is to favor the increase the population of dengue vectors. Therefore, the selected site was divided into 15 × 15 km grids, so that each grid would contain at least one village (rural) or ward (urban). Since a total 25 and 10 sites (intersection points – villages/wards) were selected on a systematic random basis, to minimize the cost of survey but at the same time ensured a fair spatial representation of the area for risk and non-risk zones respectively. In the non-risk zone, all the villages/wards falling on either intersection points or nearer to them were selected for the survey, since the negative estimation in the site was more critical from the application point of view. A total of 35 sites were surveyed from the study area on a systematic random basis.

2.3 Mosquito collection

Adult female mosquitoes were collected from inside houses including houses with the help of mouth aspirators, aided by torch lights (June 2013 to May 2014). Most of thehuman dwellings were permanent houses. Outdoor resting habitats consisted of grasses, baddy, sugarcane, banana and cotton fields. All adult specimens were anesthetized, labeled and identified to species by using microscopic examination and relevant taxonomic references [10].

2.4 Samples screening

The collected female *Aedes* were dissected individually to separate head, thorax and abdomen. The thorax was triturated with 1 ml mosquito tissue grinders in a solution of 20% acetone extracted normal human serum in PBS, pH 7.4. The suspension was directly screened against dengue virus, using the ELISA technique. The procedure for antigen capture ELISA was carried out as previously described, with modification [11]. In brief, micro well plates were coated with 100 μl/well of polyclonal antibodies overnight at 4 °C, and then the wells were incubated with a 1% Bovine serum albumin (BSA). After removal of the BSA solution, a series of diluted 100 μl/well samples was added and incubated for 1 h at 37 °C. After the plates were washed, 100 μl/well of diluted HRP-conjugated MAb was added (gifted by Dept. of Immunology, MKU) and incubated for 30 min at 37 °C. After further washing, 100 μl/well of TMB solution was added, and the reaction was stopped after incubation for 10 min with 100 μl/well of 1 N sulfuric acid and absorbance was read at 450 nm in a microplate reader. For serosurveillance in the district, suspected patient blood samples were directly screened against dengue virus, using the antigen capture ELISA technique. A total of 420 suspected patient blood samples were collected in selected 35 sites during the epidemic of dengue. Dengue positive samples were stored at -20 °C for further use.

2.5 Viral RNA extraction

Total viral RNA was extracted from acute phase plasma collected from dengue positive patients in the epidemiologic surveillance program using the viral nucleic acid extraction kit (GIBCO BRL, USA) according to the manufacturer's protocol. The dengue RNA was suspended in 50μl double distilled water treated with diethylpyrocarbonated (Sigma-Aldrich) used as a template for cDNA synthesis. The cDNA was synthesized by reverse transcription from 10 μL of RNA at 42 °C for 50 min in a 50 μL solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 100ng of the random hexamer primers, 200 U of Superscript II reverse transcriptase (Invitrogen), 25 U of RNAs in (Invitrogen) and 0.5 mM dNTPs. The PCR was performed in a 25 μl mixture containing 2 μl of cDNA, 10 mM Tris/HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl2, 100 μM each dNTP, 1 U Taq DNA Polymerase (Bangalore genei, India), 0.25 μM each primer (anti-sense primer 5′- GTTTCACCTAGAATGGCCATT-3′; sense primer 5′-AAGTGCAAAGTCCG TATGGAG-3′). The amplification reactions consisted of an initial denature step of 3 min at 94 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C. All positive sera (dengue virus - cDNA) were initially fragmented using restriction endonuclease on the BanII enzyme. This investigation was directly applicable to the determination of circulating viruses, identifying the different genotypes for the DEN- 1 and DEN-2 viruses.

3. Result and Discussion

The adult *Aedes* mosquitoes were mainly identified by the exposed patterns of the thorax formed by black, white or silvery scales and the legs were often black with white rings [10]. In this study, the adult female mosquitoes were subjected for dengue virus detection by ELISA. The *Aedes* thorax suspension was directly screened and considered positive if their optimum density values were greater than negative control values. A total of 2865 mosquitoes were screened for dengue virus infection by antigen capture ELISA from the selected 35 sites. All the samples were tested as described above and the results are given in Table 1. A total of 862 samples were tested from "non-risk" area and 5 samples were positive for DENV and 2003 samples were tested from risk area. A total of 152 mosquito samples were positive for DENV recorded from the "risk" and non-risk areas in Table 1. The present results were also confirmed that showed positive to the dengue virus by immunological assay [12]. The study shows that the three species of *Aedes* mosquitoes (*Ae. aegypti* 389 (56.05%), *Ae. vittatus* 268 (38.62%) and *Ae. albopictus* 37 (5.33%)) were abundantly distributed and had expanded their range in all the selected areas of Tirunelveli district. Nearly, five hundred twenty patients were seen between the study periods (June 2013 to May 2015). Two hundred and eighty suspected patients were identified to be suffering from acute fever, headache, and/or arthralgia, myalgia and were clinically abnormal by possible investigations. The remaining 140

patient sera were collected from government hospital to be taking treatment for various illnesses. Four patients had Dengue Hemorrhagic Fever (DHF) out of 420 suspected cases. For surveillance of dengue in risk and non risk, altogether 420 blood samples were collected from suspected patients in selected 35 sites of Tirunelveli district during the study period and suspected samples were examined in the dengue virus by antigen capture ELISA A total of 40 dengue (both primary and secondary) cases were obtained, constituting 9.52% of the people examined (Table 2).

Table 1: The number of field caught female *Aedes* positive for dengue virus from selected sites by antigen capture ELISA technique

Risk status	No. of sites selected	No. of sites Positive for DV	No. of individuals tested		
			Positive	Negative	Total
"Non-risk"	20	02	005	857	862
"Risk"	15	12	147	1856	2003
Total	35	14	152	2713	2865

Table 2: The number of dengue positive sera from selected sites by antigen capture ELISA technique.

Risk Status	No. of sites selected	No. of sites positive for DV	No. of individuals tested		
			Positive	Negative	Total
Non-risk	20	04	08	180	188
Risk	15	12	32	200	232
Total	35	16	40	380	420

Fig 1: Restriction analysis of dengue virus serotype-specific RT-PCR assay products on a 1% Agarose gel electrophoresis. Lane M, 100-bp DNA ladder (Genei Bangalore); lane 1, RT-PCR assay amplification with RE (BanII) digestion of DEN-1 product, 110 bp; lane 2, RT-PCR assay amplification with RE (BanII) digestion of DEN-2 product, 130 bp.

Dengue virus was isolated from acute phase serum samples in Tenkasi Government Hospital and the antibody responses of the patient were found to be cross-reactive and non typable. These results provided evidence that the infection was caused by dengue virus as the titers were higher to dengue than to JEV. The IgM titers were found to be higher against DENV- I and DENV- II than against the rest of the dengue virus types suggesting that these DENV types have been involved. The RT- PCR product was amplified in dengue virus typing RT-PCR from the C-preM region (454 bp) was sequenced and used for the phylogenetic analysis. The results showed the strains to represent first two serotypes in Figure 1. It confirmed the source of the DEN-1 and DEN-2 viruses circulating in Tamilnadu and identified the presence of this genotype only. There was demonstrating the spread of this virus from northern region to other district of Tamil Nadu and was also great uniformity among our samples, which underwent few modifications over the course of the years in which they circulated in the state. The samples collected from high density dengue mosquito population in Tenkasi taluk in 2014 were the only ones to present a larger number of alterations in the nucleic acid bases, reflecting the evolution of the DEN-2 viruses since their introduction into the identification of virus-specific serotype. All samples were identified as DENV-2 by using an antigen-capture ELISA as described earlier. The DENV-2 identification of all samples was also confirmed by using RT-PCR [13].

The four dengue virus serotypes can co-circulate in the endemic areas because the immunity to one serotype does not protect from the infection by a heterologous serotype. This is likely a result of selection that was driven by the respective effects of the cross-protective antibodies raised against heterologous serotype. The dengue virus strains, which were able to escape this neutralization, had a significant competing advantage and became the dominant lineages. This evolutionary adaptation not only enabled the co-circulation of the four serotypes but also had a great influence to their pathogenicity for humans [14]. Transmission of Dengue virus is mainly influenced by demographic factors viz., population density, occupation, literacy, migration, economic status and health seeking behavior on the occurrence of mosquito-borne diseases at micro level has been described [15-17]. Similarly, the vector abundance may be depending on the geo-physical and human associated factors, but the vector survival and the virus transmission are greatly determined by the environmental factors. However, through this attempt, it can be possible to identify the positive cases from the potential "risk" area with any of the existing tools. Therefore, the immunological assay is an alternative tool for epidemiological surveillance for dengue virus in mosquitoes. The active surveillance is not currently performed in many countries mainly because of the lack of human resources. Infected people are played a major role in introducing dengue virus to new areas, but also provide a way to study these viruses from endemic areas.

5. References

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