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Population Genetics of Vector Mosquitoes Determines Endemic Zones of Dengue Fever - A Case Study on *Aedes Aegypti* & *Aedes Albopictus* in Ecologically Distinct Eco Zones of Thiruvananthapuram District, Kerala.

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KEYWORDS

Aedes aegypti, Aedes albopictus, Dengue. Hyper endemic zones, microhabitat specificity, Container Index, House index, Breteau index

ABSTRACT:

Introduction: Three ecologically distinct eco zones in the Thiruvananthapuram district of Kerala were discovered to have considerably varied corresponding distributions of *Aedes aegypti a*nd *Aedes albopictus*.

Objectives: The intended objective of the current study was to determine the shifting patterns of spread of dengue in connection with a microhabitat analysis of many Thiruvananthapuram city locations based on the vector density of mosquito species that are connected.

Methods: Mosquito populations were assessed using several indices: the House Index (HI) was over 10%, indicating a high dengue transmission risk in all zones.

Results: The Container Index (CI) was also high across the zones, suggesting increased risk of mosquito-borne diseases. According to the Breteau Index (BI), urban and suburban areas were classified as low-risk, while the coastal zone was high-risk. *Aedes aegypti* was most abundant in the coastal zone, while *Aedes albopictus* dominated the hilly, arid suburban zone. In the city, both species were found in roughly equal numbers. According to a study on Molecular Analysis, Type 1 was the most prevalent of the four dengue serotypes. The high frequency of dengue fever in the urban zone may be due to the abundance of microhabitats in Thiruvananthapuram that sustain *Aedes aegypti*.

Conclusions: The study found distinct distributions of *Aedes aegypti* and *Aedes albopictus* in Thiruvananthapuram, with *Ae. aegypti* dominating the coastal zone and linked to higher dengue cases, confirming it as the main dengue vector. Mosquito population indices showed high transmission risk across all zones, influenced by seasonal breeding behavior and socioeconomic factors like more breeding sites in households, highlighting the need for targeted vector control strategies.

1. Introduction

One of the most contagious illnesses in the world, dengue fever (DF) is spread in 128 nations. Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), which affect over half of the world's population, can spread epidemically and endemically in any location where *Aedes* mosquitoes (*Aedes aegypti, Aedes albopictus*) reproduce .It is more prevalent in urban and semi-urban areas of tropical and subtropical nations [1]. The virus can be passed from infected female *Aedes aegypti* and *Aedes albopictus* mosquitoes to their progeny through a mechanism called transovarian transmission [2].Dengue fever, dengue hemorrhagic fever, and mild acute febrile illness can all be brought on

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by the dengue virus. Patients may have headaches, fever, joint pain, nausea, and myalgia during the initial phase of fever [3].

Among the most significant clinical signs is dengue shock syndrome, which involves heightened vascular fragility, coagulation issues, and plasma leakage. An estimated 50–100 million cases of dengue fever are reported to the World Health Organization (WHO) annual [4]. In contrast to 23.28 million cases in 1990, there were 104.77 million dengue cases globally in 2017, according to a recent research [5]. The majority of the global dengue illness burden is borne by Asian countries. The areas where dengue is most prevalent include South-East Asia and the Western Pacific, with poorer nations being particularly vulnerable [6].Over the past ten years, dengue fever has increased thirty times, with an estimated 390 million cases year. The European Centre for Disease Prevention and Control revealed a considerable rise in dengue incidence in the past year alone, with notably high rates in the Americas and the Caribbean [7-9].

Dengue is a virus that is spread by mosquitoes and can cause symptoms that range from severe flu-like symptoms to subclinical (many are not even aware they are afflicted). An infection is categorized by the WHO as either "dengue" or "severe dengue." The latter is less frequent and is frequently linked to significant bleeding, organ damage, and plasma leakage. A person can contract dengue fever (DENV) four times before developing full immunity since there are four different forms of the virus (DENV1, DENV2, DENV3, and DENV4) (WHO, 2019). DENV1-4 is four closely related viral strains that cause dengue fever. The flaviviruses transmitted by mosquitoes are single-stranded RNA viruses that are antigenically different yet have 60-80% similarity. Infections provide long-term protection against one serotype but no resistance to other serotypes [10].

More than one million dengue cases were reported in India in 2016, and as of November 2017, 1,29,329 cases of infection and 200 fatalities have already surpassed the previous year's figures .This disease has become one of the most rapidly expanding vector-borne illnesses in India during the past few decades. In India, the danger of mosquito-borne illnesses like DENV, chikungunya virus, etc. is increased by the country's dense population, abundant greenery, and favourable abiotic conditions for mosquito reproduction [11]. Despite both *Aedes* species have overlapping geographic distributions, *Aedes aegypti* is thought to favour urban areas whereas *Aedes albopictus* favours rural regions, or places with greater vegetation [12]. One of the seven nations in the region that has been identified as consistently reporting DF/DHF outbreaks is India, which appears on track to become a major hyper endemic niche for dengue infection in the near future as the epidemic dengue spreads to more and more new places. Since the 1940s, when dengue infection was first established in India, various states have reported cases of the disease, which often occurs in epidemic proportions and frequently causes significant morbidity and death in both urban and rural settings.

Kerala, with an average population density of 819 people per square kilometre, provides adequate opportunities for vector mosquitoes to survive and it is one of the most often reported dengue fatality states in India, hyper endemic [13].There were 21,993 confirmed cases of dengue during the 2017 epidemic in Kerala, and 165 fatalities were reported. Thiruvananthapuram district accounts for about half of the cases in Kerala, despite the fact that all 14 districts have reported instances [14]. The drought may have contributed to the mosquito breeding problem by forcing individuals to stockpile drinking water in huge containers in order to deal with the shortage [15]. Thiruvananthapuram district was the most severely affected, with 3546 confirmed cases and 68 fatalities during Kerala's first pandemic in 2003. In 2006, a sparse distribution of Dengue Fever (DF) cases was recorded from various parts of Kerala, with Thiruvananthapuram district accounting for 65% of all human viremia cases. [16-17]. DF infection with several DSS and deaths was recorded from various regions of Kerala in 2017, with the Thiruvananthapuram district being the worst impacted in the state [18].

2. Objectives

The intended objective of the current study was to:

1. To analyze the mosquito index of different eco zones of Thiruvananthapuram District.
2. To study the role of environmental factors in the distribution and abundance patterns of *Ae. aegypti* & *Ae. albopictus* along an altitudinal gradient.

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3. Direct Multiplex RT-PCR for the detection of viral antigen.

Methods

Study site: The Indian state Kerala has a total area of 38863 km 2 and a population of 36.6 million, with 31.16% lives in urban areas. Thiruvananthapuram district of Kerala is fairly humid and warm throughout the year with relative humidity ranging from 70-90% and temperature ranging between 22-35.50 ºC respectively. The annual precipitation is high, reaching up to 300cm/ year (Meteorological Department, Meteorological Centre, and Thiruvananthapuram). The larval surveys were undertaken in 3 months. March, April and May (summer). Since Thiruvananthapuram forms the epicenter of this disease, sites such as Kannammoola , Pattom (Urban site), Sreekaryam, Pangappara (Sub-urban) and Poonthura,Vizhinjam (Coastal site) were selected for entomological and clinical study. 50 houses were selected from each site for the study. Urban sites are within the heart of the city and population density is highest among the three study sites. The study site in Sub-urban is moderately an elevated and arid zone (Table 1).

Entomological survey: In each of these representative sites, 50 houses were thoroughly checked for the breeding of *Aedes* mosquitoes. The survey was carried out on outdoors, indoors and also at premises of houses. The breeding sites such as cisterns, cement tanks, metal containers, plastic drums, grinding stones, mud pots, plastic bottles, flowerpots, flower vases, polythene sheets and natural breeding sites such as coconut shells, tree holes, fallen spates or bracts were observed from these localities. Among the above breeding sites mud pots were found to be possessing highest number of larval and pupal density.

For the collection mosquito juvenile stages, small containers (less than 20 litre) were emptied via a sieve into a white larval sampler (25 x 20 x 4 cm). Using a 250 ml larval dipper, samples were taken from large breeding locations such as cement tanks at ground level, fountains, etc. Every breeding location's surface water was sampled five times. The trial lasted for three months.

Table.1.Collection of *Aedes* egg and larvae from three different eco zones-urban, sub urban and coastal areas of Trivandrum district.

S I. N o.	Divisi ons	Study Site	No. of houses studied
1	Urban	Kannammoola, Pattom	50
2	Sub-urban	Sreekaryam, Pangappara	50
3	Coast al	Poonthura,Vizhi njam	50

Identification and maintenance of Experimental organism: The eggs were collected using ovitraps and the larvae were reared in bug dorm and fed with yeast or cat food. Standard taxonomic keys were used for larval identification [19-20].

Viral Nucleic Acid (RNA) Isolation: Liquid nitrogen was utilized to cryopulverize the samples. Using a mortar and pestle, the samples underwent freezing in liquid nitrogen before being ground into a fine powder. After dissolving the powder in cell lyses buffer, the mixture was centrifuged for 10 minutes at 12,000 rpm, collecting the supernatant before moving on to the following stage. Using QIAGEN's QIAamp Viral RNA Extraction Kit® (QIAGEN, Germany), viral nucleic acid was isolated from the supernatant in accordance with the manufacturer's instructions.

Multiplex PCR amplification in one step and one tube using primers unique to serotypes: To differentiate between dengue virus serotypes, a straightforward one-step, one tube multiplex PCR amplification procedure was carried out. In a single tube reaction, this approach uses the extracted viral RNA in conjunction with serotype-specific primers and the D1 consensus primer (Table 2). The template is directly made from the extracted virus RNA. After that, the PCR products were electrophoresed in 1X TAE buffer on a 1.5% Agarose gel. In one single tube, 0.5 µL of each of the serotype-specific primers (TS1, TS2, TS3, TS4), 0.5 µL of D1 forward primer, and 12.5 µL of 2X One Step RT buffer were added. 0.5 µL of Taq polymerase (5 units/µL), 0.5 µL of Taq polymerase (5 units/µL), 0.5 µL of 5X reverse

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transcriptase enzyme, and 3.0 µL of RNase Free dH2O were included in TaKaRa's Prime Script One Step RT-PCR Master Mix (TaKaRa, Japan). A 25µL reaction volume was used for 40 cycles of PCR, which included 420 C for 5 min of initial cDNA synthesis, 940 C for 10 sec of initial denaturation, 940 C for 30 sec of denaturation, 550 C for annealing, 720 C for 60 sec of extension, and 720 C for 60 sec of final extension. The products were examined as bands in 1X TAE buffer on a 1.5% Agarose gel.(Table 2).

Table 2: Oligonucleotide primers and their sequences used in RT-PCR

Name	Nucleotide sequence (5' to 3')	Product size (bp)
D1	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	511
TS1	CGT CTC AGT GAT CCG GGG G	482
TS2	CGC CAC AAG GGC CAT GAA CAG	119
TS3	TAA CAT CAT CAT GAG ACA GAG C	290
TS 4	TGT TGT CTT AAA CAA GAG AGG TC	392

Data analysis: From the entomological data the following indices were calculated as described in standard methods, House Index (HI), Container Index (CI) and Breteau Index (BI) (WHO, 2009).

House Index (HI) =number of houses positive for *Aedes* breeding/ houses checked X 100

Container Index (CI) = number of positive containers/ total containers checked X 100

Breteau Index (BI) = number of positive containers/total number of houses searched X 100

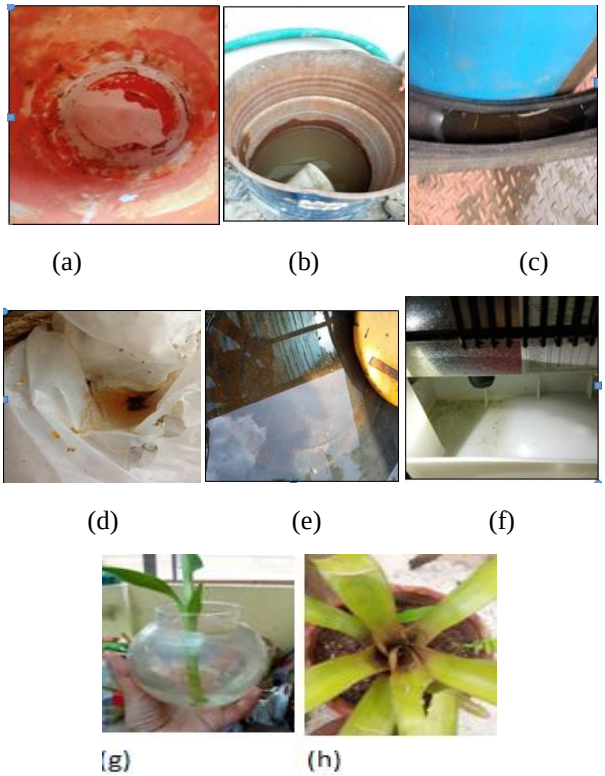
3. Results

The study is on the distribution of different species of mosquitoes in the selected sites such as urban, semi urban and coastal zones of Thiruvananthapuram City proved that *A.aegypti* and *A.albopictus* are the dominant mosquito species. Sporadic occurrence of *Culex quinquefasciatus, Anopheles stephensi* and *Armigerus subalbatus* were also located in the study sites. Occurrence of *Culex quinquefascitus* was observed in foul smelling water collection such as leakages of drainage vessels and septic tanks possessing rich sources of putrefied animal wastes. Larvae of *Ae. aegypti* and *Ae. albopictus* were observed in comparatively less polluted water with no foul smell. Even indoor collection of water for drinking purposes, stored in closed containers possessing very little space between the lid and rim of containers were the breeding sites of both species of *Aedes*. More than 8 types of habitat diversity is noted from the study including barrels/drums, plastic tanks, cement tanks, coconut shells, discarded tyres, flower pots, minor plastic containers, flower vases/pots for keeping money plants or lucky bamboos, drip trays of refrigerators or coolers, leaf axils etc (Fig 1 and Table 3).

Table 3. Habitat Diversity in door and out door

BREEDING HABITATS	
OUTDOOR	**INDOOR**
Barrels/drums	Flower vases
Plastic tanks/Containers	Pots
Cement tanks	Drip trays -refrigerators
Coconut shells	Drip trays in coolers
Discarded Tyres	Leaf axils
Flower pots	

Fig 1. Habitat diversity outdoor and indoor: a- plastic container, b-drum, c-tyre, d- plastic cover, e - cement tank, f-drip tray in refrigerator, g- flower pot, h-leaf axil.



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The three eco zones of Thiruvananthapuram city showed marked variations on the occurrence of *Ae. aegypti* and *Ae. albopictus.* The semi urban zone of Thiruvananthapuram city is a dry and elevated site from sea level, where almost 90% of the mosquito larvae observed was *Ae. albopictus* and the remaining was shared by *Ae. aegypti* and no other species of mosquito was observed. In coastal zone, almost 90% of the mosquito larvae were that of *Ae. aegypti* and remaining of the larvae were *Ae. albopictus*. Coastal zone possessed a scanty distribution of *Anopheles stephensi* larvae, which were observed in water collection with less pollution or less stench. In the city core area both *Ae. aegypti* and *Ae. albopictus* were observed almost equal proportion. The vector density of the three eco zones was different. The larval indices of each study site have a stable density of mosquitoes. The median (HI) of coastal zone and sub urban area is almost same 30 and 31 and in case of BI its same, that is 32.The urban zone the all the vector indices are lower than the sub urban and coastal (Table 4).The CI of coastal zone is higher from all the other areas.

The dominant vector mosquitoes observed in three study sites exhibited sharp variation in terms of species and number. The sub urban zone is dry and elevated site from the sea level almost 90 % of the mosquito larvae were *Ae. albopictus* and the remaining were shared by *Ae. aegypti.* In coastal zone, almost 90 % of the larvae were that of *Ae. aegypti* and 10% of the larvae were *Ae. albopictus*.(Table 2).Coastal zone possess a scanty distribution of *Anopheles stephensi* larvae. In the urban area 80% of larvae were *Ae. albopictus* and the remaining is *Ae. aegypti*. Presence of Culex, Anopheles and *Aedes vittatus* were observed as part of study. The vector density of the 3 eco zones was different. The larval indices such as HI, CI and BI were high in sub urban and coastal zones.

Table 4. Larval indices of three eco-zones of Thiruvananthapuram city and the distribution of *Ae. aegypti* and *Ae. albopictus*

Study Site	**URBAN**			**SUB-URBAN**			**COASTAL**		
Index	**HI**	**CI**	**BI**	**HI**	**CI**	**BI**	**HI**	**CI**	**BI**
Months									
MARCH	**10**	**25**	**10.5**	**30**	**49**	**29**	**35**	**50**	**32**
APRIL	**15**	**20**	**19**	**40**	**35**	**32**	**31**	**47**	**43**
MAY	**7**	**11**	**13**	**28**	**40**	**33.6**	**36**	**54**	**30**
MEDIAN	**10**	**20**	**10.5**	**30**	**35**	**32**	**31**	**47**	**32**
Proportion *of Ae. aegypti* & *Ae. albopictus*	**1:2**			**1:9**			**9:1**		

Analysis of the epidemiology of dengue viremia in Kerala during the periods of 3 of months, provided that the April month has high dengue viremia comparing to other months (Table 5 and Fig 2).So in Kerala during the month of march 136 DF cases were reported and out of which 27 were reported from Thiruvananthapuram and 1 death case also reported & during month of April out of 164 cases, 37 cases were reported from Thiruvananthapuram. In May 113 cases were reported from the whole Kerala state and out of which 25 were reported from the state capital and also 1 death case were reported.

Table 5. Incidence of dengue fever in Kerala and respect of Thiruvananthapuram district (Directorate of Health Services, Kerala)

MONTH	**Thiruvananthapuram District**		**Kerala State**	
	Dengue Fever	**Death**	**Dengue Fever**	**Death**
March	27	1	136	1
April	37	-	164	-
May	25	-	113	1

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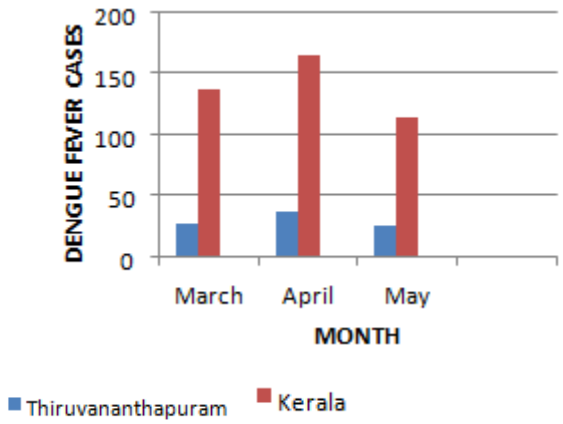
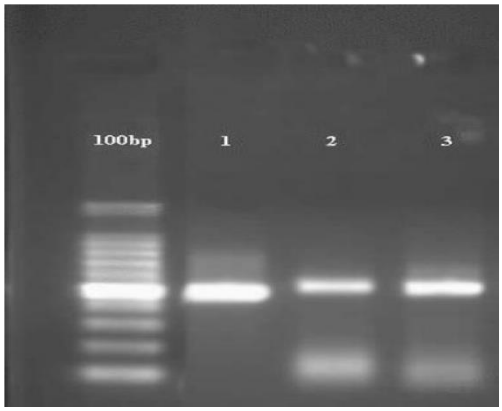
Fig 2.Dengue fever cases in Kerala and Thiruvananthapuram



Utilizing a single tube multiplex PCR reaction, a second round of amplification was conducted with the 511 basepair fragment that was acquired from the Dengue virus nucleic acid amplification. The same serotype specific primers (TS1, TS2, TS3, and TS 4) were used as reverse primers together with D1 consensus primer as the forward primer in a single tube. On the gel, pieces unique to each serotype were obtained by amplification. Every serotype's fragment size matched the size that was anticipated. From the genetic analysis of Fig. 3, it is evident that dengue cases were recorded from all three zones.

Fig: 3 Agarose gel electrophoresis showing dengue viremia from 3 eco zones – 1. urban 2. Sub urban 3.Coastal zone



4. Discussion

The present study clearly showed that the species diversity of mosquitoes in three eco-zones of Thiruvananthapuram city exhibited contrasting difference especially on the distribution of *Ae. aegypti* and *Ae. albopictus*. In the coastal zone *Ae. aegypti* was the dominant species and in the hilly suburban zone *Ae. albopictus* was the dominant species. In the city core area (urban) almost equal distribution of both species of *Aedes* mosquitoes were observed. Similar observations were reported from Rajasthan in which different eco zones such as desert area, forest and river area and semi arid area exhibited sharp difference on the distribution of *Ae. aegypti* and different strains of DENV. Both species of mosquitoes are highly precise on their preference in niche selection. Previous reports also supports the present investigation that, in Thrissur district of Kerala, the rubber plantations possessing coconut shells used for collecting rubber latex showed the larvae of *Ae. albopictus* only during rainy season and no *Ae. aegypti* larvae in the whole plantation area[21].

A very remarkable observation made in the present study is the relationship between dengue viremia in the study sites and the distribution of mosquito population. In sub urban site, *Ae. albopictus* was the dominant mosquito, in which Dengue infection were moderate but in the other study site, the coastal zone, there was high prevalence of Dengue infection, in which the dominant mosquito vector was *Ae. aegypti* [22]. This clearly indicates that *Ae. aegypti* is one of the true vectors of Dengue Fever and the role of *Ae. albopictus* role is significant.

House index (HI) describes of vector mosquitoes in a region. According to WHO estimates, an area with high HI (higher than 10%) is considered as high risk zone for the dengue fever, and a zone with low HI (less than 1%) is safer and low risk zone. The present study demonstrated that all three areas exhibited HI> 10% which indicated that all 3 zones are high risks for dengue transmission. Container index (CI) illustrated the number of positive water reservoirs which contains larvae. The higher value of CI in a place indicates the high risk of occurrence and spread of mosquito-borne disease. The standard value set by WHO for CI are less than 5 %.The CI was high is all 3 sites indicates the higher risk of occurrence and spread of disease. The Breteau Index (BI) establishes a relationship between several positive containers and houses.BI indicates prevalence rather than abundance. Among the 3 indices, BI is considered as the best which is more qualitative, and it has more epidemiological significance. As per the WHO criteria, a region with BI lower than 5 is considered as safe zone

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and BI 5to 20 and 35 to 50 are considered as regions with low risk and higher risk, respectively. The fact is that if it refers to the WHO criteria, there will be no area that is safe from dengue infection. In our study site the urban and sub urban sites falls under the category of low risk area while coastal zone shows high risk status.

Clinical data from the Directorate of Health Services, Govt. of Kerala, clearly showed that, Thiruvananthapuram district of Kerala carry the major share of DF. This clearly indicated either ecological factors or genetic factors of vector mosquitoes is favouring Thiruvananthapuram district to be the most favourable zone in Kerala for maintaining dengue virus. Similar type of observations is reported from Australia in which socio demographic and ecological features play a significant role on the distribution of *Ae. aegypti.* People of high economic group possessing rain water harvest tank above the houses provide ample chances to this mosquito to breed in this tanks, but the people living in small houses, where the disease is uncommon because the abundant vector in such places is *Ae. albopictus* [23]. Compared to Thiruvananthapuram species level difference on the distribution of DENV, it is not a rare phenomenon preference in relation to egg laying behaviour of *Ae. aegypti* changed in accordance with season. During summer, the preferred egg laying sites for *Aedes* mosquitoes were indoor collections of water, but after summer rains and during rainy seasons the preferred sites were outdoor collection of water in discarded containers. Dengue infection in three study sites showed a co-relation with species level distribution of *Ae. aegypti* and *Ae. albopictus*. Dengue infection in areas with abundance of *Ae. aegypti* was higher than that of the areas with abundance of *Ae. albopictus.* The present study proved that *Ae. aegypti* and *Ae. albopictus* exhibits microhabitat specificity on their distribution and this has major influence on the transmission of DENV on each micro habitat. Agarose gel electrophoresis revealed the presence of dengue virus in three eco zones, supporting the studies finding.

The distribution and ecological parameters affecting the variety of mosquito species, particularly *Ae. aegypti* and *Ae. albopictus,* were investigated in this study in three eco-zones of Thiruvananthapuram city (coastal, hilly suburban, and urban). *Ae. aegypti* was the dominating species in the coastal zone, whereas *Ae. albopictus* was the dominant species in the hilly suburban zone. Both species were distributed almost equally across the urban core. These results are consistent with other studies that shown species variety across several eco-zones. Areas dominated by *Ae. aegypti* (coastal zone), a key vector for the dengue virus, had a greater prevalence of dengue. Conversely, dengue incidence was modest in the suburban zone, which has a greater proportion of *Ae. albopictus*. This confirms that *Ae. aegypti* is a more important dengue disease vector. The research evaluated mosquito populations using a number of indices such as House Index (HI), Container index (CI) and Breteau Index (BI).
There was a high risk of dengue transmission in all three zones, as shown by the House Index (HI), which was greater than 10%.Container Index (CI). CI was high across all zones, indicating a higher risk of illnesses spread by mosquitoes. The coastal zone was categorized as high-risk based on the Breteau Index (BI), whilst urban and suburban areas were categorized as low-risk. These indicators show the degree of dengue transmission risk in each area. By conducting multiplex RT-PCR, virus serotype 1 was found from all three eco zones, supporting the existence of dengue viraemia. Mosquito breeding behaviour was impacted by ecological variables, including water reservoirs and seasonal variations (such as the rainy season). *Ae. aegypti* switched to outdoor water collections in abandoned containers during the wet season, but preferred inside water collections during dry seasons. The distribution of mosquitoes was also influenced by socio demographic characteristics; more affluent families offered more breeding grounds for *Ae. aegypti* (such as rainwater collecting tanks).

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