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Direct one step-one tube multiplex PCR technique detects type 1 dengue virus in *Aedes albopictus* in certain eco zones of Kerala and confirm Trans-ovarian transmission of the virus through *Aedes aegypti*

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Abstract
Vector surveillance in relation to dengue viremia showed *Ae. aegypti* as chief vector of this disease and *Ae. albopictus* as an alternate vector in hyper endemic zones of Kerala. Among the four different serotypes DEN1 was identified as the most abundant virus in Kerala during 2016-17. *Ae. aegypti* larvae from dengue hyper endemic areas showed DEN1 in their bodies, which indicated trans-ovarian transmission of this virus and hence adult mosquitoes emerged from these larvae and are not exposed to blood of dengue viremia patients can be vectors of this disease. Presence of two different serotypes of DENV in a single mosquito can make it as a potential vector whose bite itself can cause DSS. Adult *Ae.albopictus* mosquitoes collected from Thiruvananthapuram city, which was a dengue hyper endemic zone during 2016-17 showed Denv1 in their body and simultaneous role played by both species of mosquitoes can make a particular zone which is hyper endemic to dengue fever. Direct one step-one tube Multiplex PCR technique is suggested as an efficient and cost effective technique for detecting dengue virus in mosquitoes and in human blood.

Keywords: *Aedes aegypti*, *Aedes albopictus*, dengue hyper endemic zones, Dengue serotype prevalence, Trans-ovarian transmission

1. Introduction
Dengue fever (DF), Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) are major public health problems in tropical countries [1]. Globally 50 million people are infected by this virus and annually 2.5 million people who are living in endemic areas are at risk. More than 70% of these cases are reported in Asian and Western Pacific Region [2]. Classic Dengue fever and the more severe Dengue Hemorrhagic fever-Dengue Shock Syndrome (DHF-DSS) are the clinical manifestations of the viral infection. The infection is caused by four distinct circulating serotypes of the Dengue virus (DENV), a *flavivirus*, transmitted to humans by the mosquito vectors mainly *Ae. aegypti* and also by another suspected vector *Ae. albopictus* [3, 4]. This virus exhibited four genetically distinct forms, called serotypes and are designated as serotypes DEN-1, DEN-2, DEN-3, and DEN-4 and they share 70% genetic homology with one another and sequential infection with the serotypes puts the individual at a higher risk of developing dengue shock syndrome(DSS), a severe form of disease [5, 6]. The disease is perceived to be highly endemic in India, with all the four serotypes circulating either individually or in combination [7]. Outbreaks of the disease are usually observed in a post monsoon pattern [8]. Kerala, one of the worst affected states in India, portrayed existence of all the four serotypes. Projections of increased prevalence and morbidity in future outbreaks can be made considering the circulation of the four serotypes in the population of Kerala [9]. As we understand the root cause for dengue fever outbreak is due to the bite by the mosquito vectors *Ae. aegypti* and *Ae. albopictus.* Successful management of ecosystem which restricts mosquito breeding has prime importance in preventing this disease. The aim of this study is to understand the presence of DENV in these mosquitoes and how they are transmitting to human.

Patient management is the only realistic option with Dengue fever considering the lack of a vaccine or a proper treatment regimen. Rapid diagnosis of early infection becomes absolutely necessary to effectively manage a patient with Dengue fever [10]. This presents a challenge for devising an affordable strategy for rapid screening, detection and typing the Dengue virus for future outbreaks. Dengue infection is usually diagnosed by viral isolation from acute phase serum, serological diagnosis or molecular assays [11]. Viral isolation, although accepted as the 'gold standard', from cell cultures are time consuming and impractical for outbreak scenarios which requires large number of samples to be screened and typed. Rapid serological identification of antibodies from serum samples does not furnish information about the prevailing serotype. PCR based molecular techniques offer alternatives to the conventional isolation technique [12]. The Reverse Transcriptase (RT) -PCR targeting the conserved regions of C-prM gene are widely employed, but a nested PCR is required for serotyping. This method also proves to be expensive, time consuming, and may possess a risk of contamination [13, 14]. To overcome these problems, we have slightly modified the widely accepted single tube multiplex PCR post amplification by Dengue RT-PCR to a direct one-step-one-tube Multiplex PCR from viral nucleic acid to achieve higher throughput in rapid detection and distinction of the four dengue serotypes in acute phase serum samples during outbreak scenarios [15].

2. Materials & Methods

2.1 Sample collection

Adult *Ae. aegypti* and *Ae. albopictus* and larvae were collected from three different zones of Kerala such as Thiruvananthapuram, Kollam(southern zone), Alappuzha, Palakkad, and Malappuram(central zone) Kozhikode and Kasargode (northern zone). The larvae were collected from accessible water holding containers in and around the houses. Small containers (< 20 litter) were drained through Strainer in to white larval sampling (25x20x4 cm) to collect the immature stages of mosquitoes. Large breeding places like ground level cement tanks; fountains etc. were sampled using a 250ml larval dipper. Five dips were taken from the surface water of each breeding place. The collected larvae were separately brought to the laboratory and identified to species level using standard larval identification key. The duration of the study period was three years from January 2014 to January 2017.

Adult mosquitoes were collected while resting or landing using the mouth aspirator and flash light. *Aedes* mosquitoes landing on human volunteers (with informed consent) were collected in the morning and evening. The mosquitoes were morphologically identified and separated by sex, pooled and stored in liquid nitrogen for further study/ analysis.

2.2 Viral Nucleic Acid (RNA) Isolation

The samples were subjected for cryopulverization using liquid nitrogen. The technique was done using a mortar and pestle, the samples were frozen in liquid nitrogen and grinded into a fine powder. The powder was then dissolved in cell lysis buffer and centrifuged at 12000 rpm for 10 minutes; the supernatant was collected and preceded to the next step. Viral nucleic acid isolation from the supernatant was carried out using QIAGEN's QIAamp Viral RNA Extraction Kit® (QIAGEN, Germany), following the manufacturers protocol.

2.3 One-step one-tube Multiplex PCR amplification with serotype specific primers

A direct one-step, one tube multiplex PCR amplification reaction was performed for differentiation of dengue virus serotypes. This method involves the usage of the isolated viral RNA directly with D1 consensus primer and serotype specific primers in a single tube reaction. The isolated viral RNA is directly used as the template. The PCR products were then electrophoresed on 1.5% Agarose gel in 1X TAE buffer. 0.5 µL each of the serotype specific primers TS1, TS2, TS3, TS4 as reverse primers, were added along with 0.5 µL D1 forward primer in one single tube along with TaKaRa's Prime Script One Step RT-PCR Master Mix (TaKaRa, Japan) consisting of 12.5 µL of 2X One Step RT buffer, 0.5 µL of Taq Polymerase (5 units/µL), 0.5 µL of 5X Reverse Transcriptase Enzyme and 3.0 µL of RNase Free dH_2O. 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 sec, denaturation at 94^0 C for 30 sec, 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.

Table 1: 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

3. Results

The 511 bp fragment obtained from the amplification of the Dengue viral nucleic acid was used as a cDNA template for a second round of amplification using a single tube multiplex PCR reaction. The same serotype specific primers (TS1, TS2, TS3, and TS 4) were used as reverse primers along with D1 consensus primer as the forward primer in a single tube. The amplification gave serotype specific fragments, which were observed on the gel. The fragment size of each serotype corresponded to the expected size.

The mosquito, *Ae. aegypti* collected from different parts of Kerala was subjected for the experiment. It was observed that the adult *Ae. aegypti* mosquito samples that were obtained from all the studied districts except Kasargode, showed the presence of dengue virus serotype 1 (DEN1) and the results are shown in Fig. 1. The band obtained from the samples collected from Kasargode was unable to sequence since the band was very indistinct.

Interestingly *Ae. aegypti* mosquitoes of Kozhikode district showed one additional band which indicated the presence of two different types of DENV in their body (Fig.1) *Ae. aegypti* larvae specifically from Alappuzha and Thiruvananthapuram

District which were imperiled for the experiment also showed the presence of Dengue virus serotype 1 (DEN1), confirming trans ovarian transmission of this virus (Fig.2).

Ae. albopictus adult mosquitoes collected only from Thiruvananthapuram district showed viraemia specifically of serotype-1(Fig.3), and *Ae. albopictus* from other six districts of Kerala didn't show vireamia which pointed a strong possibility that high prevalence of Dengue fever in Trivandrum district may be through the bites by both the species of mosquitoes on human victims. The serotype was confirmed by sequence analysis using Sanger sequencing.

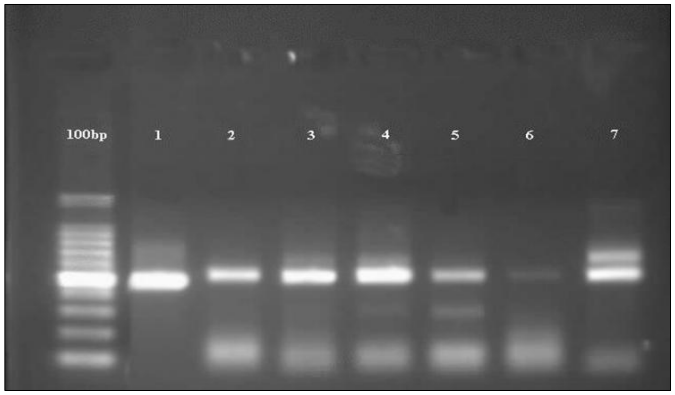

Fig 1: Agarose gel analysis showing dengue viremia in *Ae. aegypti* from different districts of Kerala. 1. Thiruvananthapuram, 2. Kollam, 3. Alappuzha, 4. Palakad, 5.Malappuram, 6.Kasargode, 7.Kozhikode

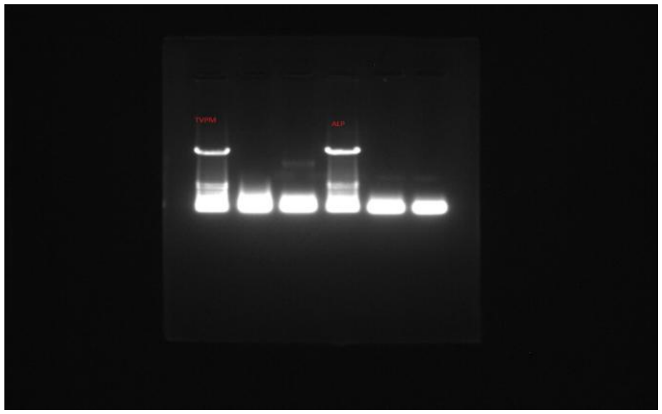

Fig 2: Agarose gel analysis of dengue serotyping showing dengue viremia (Serotype1) in *Ae. aegypti* larvae from two dengue hyper endemic districts.

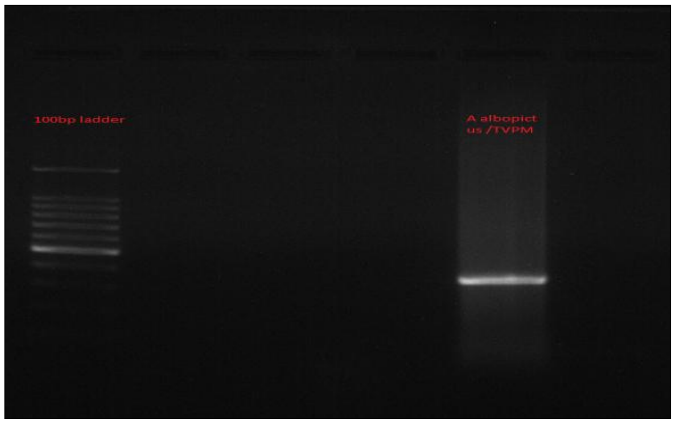

Fig 3: Agarose gel analysis of dengue serotyping showing dengue viraemia (Serotype1) in *Ae. albopictus* from Thiruvananthapuram district of Kerala.

4. Discussion

Kerala state is fast emerging as a region of high to very high endemicity for dengue infection. Reports of Dengue Shock Syndrome (DSS) and Dengue Hemorrhagic Fever (DHF) are increasing every year. Cyclic outbreaks, yearly and post monsoon is transferring the southern state of India into a hyper endemic niche [16]. *Ae. aegypti* mosquitoes collected from all the three different regions of Kerala such as southern, central and northern regions, represented by seven districts among the total of 14 districts of Kerala. Direct one step one tube multiplex PCR techniques is used in the present study for the detection of viral antigen from the body of mosquitoes. This is the state of the art technology that can be used for the rapid detection of dengue viral serotypes more accurately. The direct one step one tube multiplex PCR which consist of a common forward primer and four serotype specific primers which will amplify only the specific serotype. The advantage of this PCR is that we can analyze the samples in less than 3 hours. This study showed the presence of Denv1in all the *Ae. aegypti* mosquitoes that were collected from all the three different regions of Kerala (Fig.1). Adult *Ae. aegypti* from Kasargode district (northern region) showed only indistinct band and Kozhikode district showed two bands, indicating the presence of two different types of virus (Fig1), which pointed the possibility of Kozhikode district to become a hyper endemic region of DENV in future. Kasargode district of Kerala is characterized by dry laterite soil and dry weather and human dengue viraemia is not common in this district.

A sample survey on the serological study of 50 dengue patients of Thiruvananthapuram city proved that all the four serotypes are seen in the city and among them DEN1 is the most abundant virus which is also agreeing with the present entomological study [17]. Reports by the previous investigators showed that prevalence of any one of the four dengue serotypes is not static but changes occur in relation to time and space. In Kerala and Tamil Nadu, dengue outbreak of 2012 showed the complete dominance of Denv4 [18]. In the outbreak in Delhi during 1996, DEN2 was the abundant virus [19], but in 2003, DEN3 was the prevalent virus [7]. Major cities of India such as Delhi and Mumbai, reported DSS with all the four circulating serotypes in 2005 and increased vectors density supported by anthropogenic factors was major reason [20].

Ae. aegypti larvae collected from Alappuzha and Thiruvananthapuram districts of Kerala with vast areas of coastal belt, representing the central and southern region of Kerala showed the presence of DEN1 which indicated trans-ovarian transmission of dengue virus (Fig 2). The above cited districts are considered as region which is hyper endemic to dengue virus [21]. Vector mosquitoes fed with the blood of dengue fever patient, which are transmitting the virus into their eggs and multiplication of virus in the larval body indicated that mosquitoes which were not exposed to blood possessing viraemia can act as vectors of dengue fever. Mosquitoes living in dengue hyper endemic region getting multiple chances to feed blood possessing dengue virus may be the reason for trans-ovarian transmission of dengue virus. Trans-ovarian transmission of all the four types of virus was experimentally demonstrated in *Ae. aegypti* and *Ae. albopictus* under laboratory conditions during 1980s. In these studies DEN1 was found to be efficiently passing from mother to eggs when compared to other strains, [22] the same virus was located in *Ae. aegypti* under field condition in

Amazon Basin [23] and in Malaysia [24]. In Kerala, Thiruvananthapuram and Alappuzha districts are reported as hyper endemic sites of Dengue virus. Prevalence of Denv1 in all zones of Kerala and the localization of this particular serotype, in both species of *Aedes* mosquitoes indicated that DEN1 has more adaptive efficiency than other serotypes in acting as a pathogen and also to thrive more efficiently in the body of vector mosquitoes. Laboratory demonstrations in South American states also agree with the suggestion [22]. A favorable environment offered by selection pressure coupled with good incubation and thriving conditions may be responsible for directing the selection of a particular strain from the circulating serotypes.

Adult *Ae. albopictus* mosquitoes collected from different regions of Kerala did not show any viraemia when tested by PCR technique, but specifically from Thiruvananthapuram district showed the presence of DEN1. These results suggested the alternate role played by *Ae. albopictus* mosquitoes in endemic areas. In almost all instances of dengue out breaks in India, *Ae. aegypti* was the major vector [19, 25, 26]. There are some reports, especially from India that *Ae. albopictus* has some role in transmission of dengue virus. Reports from West Bengal [27] and Kerala [28] showed the presence of dengue viraemia in *Ae. albopictus* mosquitoes indicating this mosquito as an alternate vector of DENV.

Ae. albopictus is reported to be more aggressive than *Ae.ae gypti* and invading in many peridomestic zones exclusively used by *Ae. aegypti* as their breeding sites [29]. Studies by the investigators showed that *Ae. aegypti* and *Ae.albopictus* showed equal distribution in costal zones of Thiruvananthapuram City but in arid hilly suburban zone *Ae.albopictus* was the abundant vector [17]. Previous studies by the investigators also proved that costal zones in Thiruvananthapuram City exhibited highest number of human DENV and in *Ae.albopictus* abundant arid hilly zone of Thiruvananthapuram City, human dengue viraemia was very low[17]. Habitat specificity of the vector mosquitoes is different in different Eco zones of Kerala has very important role in occurrence of vector-borne diseases especially Dengue fever. In Trissur district of Kerala, it was reported that the coconut shells used in the rubber plantations for collecting rubber latex form an efficient breeding site for mosquitoes during rainy season but only *Ae.albopictus* were reported from such man made containers [30].

5. Conclusion

Ae. aegypti mosquitoes are the most efficient vector of Dengue fever and in hyper endemic region of Kerala, continuous and repeated blood meal possessing the viral pathogen by *Ae.albopictus* makes them alternate vectors of this disease. *Ae. aegypti* larvae from hyper endemic zones of Kerala showed viraemia in their body and that also may be due to continuous blood meal possessing virus particles by the mother mosquitoes. Presence of more than one type of virus in *Ae. aegypti* mosquitoes of non-hyper endemic zone points the possibility of Kozhikode district of Kerala to become a hyper endemic zone of DENV in future. Ability of a mosquito to be a carrier of a viral disease is only because of its susceptibility of its immune system and genome to that particular type of virus. In this context all the mosquitoes of genus *Aedes* may become vectors of this virus if they are getting a chance to continuously feed the blood of dengue patients and is augmented by adaptive efficiency and selection pressure which act among the different competing strains of Dengue virus.

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