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Research Article

Monitoring of Insecticide Resistance and Exploring the Presence of Virus in Field Populations of *Culex gelidus* at Thiruvarur District of Tamil Nadu, India

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A B S T R A C T

Introduction: The introduction of potent synthetic insecticides into public health programmes has since beginning posed the challenge of development of resistance among the insect vectors against the insecticides. *Culex (Cx.) gelidus* is one of the vectors of JE which is abundantly found in southern India. Its breeding habitats are similar to the *vishnui* subgroup of *Culex* mosquitoes, the major vectors for transmission of JE. The present study was aimed to assess the susceptibility status of adult *Culex (Cx.) gelidus,* to insecticides, namely DDT (Dichlorodiphenyltrichloroethane), Deltamethrin, and Malathion.

Method: The field-collected mosquito larvae from ten villages of Thiruvarur district from December 2018 to May 2019 were reared in the laboratory until F1 generation and the emerged adults identified as *Cx. gelidus* were exposed to insecticide-impregnated papers supplied through World Health Organization (WHO). The adult susceptibility tests were carried out as per the protocol of WHO. Further, an attempt was made to check the presence of JE virus in *Cx. gelidus* and the virus detection was done by RT-PCR.

Results: The results indicated that the adult *Cx. gelidus* populations were susceptible to DDT, whereas they were resistant to Malathion and Deltamethrin. The possible reason of DDT susceptibility may be that DDT has not been used in Tamil Nadu since the year 1980 (about 40 years).

Conclusion: JE virus was not detected in the tested mosquitoes. The study suggests that insecticide resistance monitoring from time to time is required to facilitate vector control programmes in focusing on appropriate vector control measures.

Keywords: *Cx. gelidus,* DDT, Deltamethrin, Malathion, Thiruvarur, WHO Susceptibility Test

Introduction

Japanese encephalitis (JE) is a vector-borne viral infection, transmitted by mosquitoes. Japanese encephalitis virus (JEV), a single-stranded RNA genome of about 11kb in length, is a member of the family (Flaviviridae), genus (Flavivirus).[1] JEV was first isolated from the brain of the foetal human encephalitis case in Tokyo in 1934.[2]

Disease Burden

It has been reported from Eastern Asia, Southeast Asia, Northern Australia and Southern Asia.The countries that have reported JE are Bangladesh, Nepal, Sri Lanka, Thailand and Timor-Leste in South East Asia (SEA) Region and Malaysia, Indonesia, Philippines Cambodia, Vietnam, Laos, Fiji, China and Japan in Western Pacific Region.[3-9] In India, JE was first recognised to occur in Tamil Nadu in 1955, though, serological evidence for its prevalence was recorded in 1952.[10] JE in India is reported under the umbrella of Acute Encephalitis Syndrome (AES) which has been reported regularly from Andhra Pradesh, Assam, Bihar, Delhi, Goa, Haryana, Jharkhand, Karnataka, Kerala, Maharashtra, Manipur, Nagaland, Tamil Nadu, Uttar Pradesh, and West Bengal.

*Cx. tritaeniorhynchus, Cx. vishnui, Cx. pseudovishnui,*and*Cx. bitaeniorhynchus* under the subgroup *of Cx. vishnui* are important vectors for disease transmission. Though the JE virus has been isolated from 30 species of mosquitoes belonging to 5 genera viz., *Culex, Anopheles, Aedes, Mansonia* and *Armegeres* in India, viruses have been isolated from 17 species.[11-17] *Cx. gelidus* is reported to play a significant role in outbreaks, in addition to *Cx. tritaeniorhynchus, Cx. vishnui,*and *Cx.pseudovishnui*.[18-20]

The study on mosquito abundance, pig sero-positivity and human JEV cases indicated a strong correlation with the presence of *Cx.tritaeniorhynchus* and *Cx. gelidus* in Sibsagar and Kamrup districts of Assam, respectively.[21] Natural vertical transmission of *Cx. gelidus* was also documented from Madurai, Coimbatore, and Cuddalore districts of Tamil Nadu.[22-24] An increase in the population of *Cx. gelidus* was recorded in Thanjavur district which used to be less in earlier years. This study points out the exploration of *Cx. gelidus* both for its susceptibility to insecticides and presence of virus in Thiruvaur district of Tamil Nadu, where rice cultivation is carried out. *Cx. gelidus* breeds in rice fields in addition to polluted and dirty water,[25] since Thiruvarur harbours a variety of such breeding grounds, the possibility of an abundance of*Cx. gelidus* in the district is high.

Vector control and reduction in human-vector contact by reducing the vector population have been the strategic component in the prevention and control of JE. Mosquitoes are also exposed to the insecticide used for elimination or control of other vector-borne diseases under the programme in addition to the insecticides used for agricultural purposes. The insecticide resistance monitoring (IRM) among vector mosquitoes has been emphasised in the Global vector control response (GVCR).[26] Since *Cx. gelidus* is a known secondary vector in various districts of Tamil Nadu,[22-24] the virus isolation was also explored among the field caught mosquitoes along with resistance monitoring, so that, the current insecticide resistance and presence of circulating virus among this vector species if any, may be evidenced to facilitate the public health programme for the selection of appropriate and effective insecticide to be used under the vector control programme. The prevalence of *Cx. gelidus* though reported to be high in southern India, no study has been reported on the insecticide susceptibility status of *Cx. gelidus* in Thiruvarur district of Tamil Nadu. Considering the significance of IRM for JE vectors, the present study was undertaken to monitor the insecticide susceptibility status of *Cx. gelidus* populations in Thiruvarur district.

Material and Method

Since this study involves mosquitoes, it does not require any ethical committee approval.

Study Area and Period of Study

The study was performed in Thiruvarur district. It covers an area of 2374 sq. km. It lying between 10°20' and 11°07' North latitude and 79°15' and 79°45' East longitude.[27] Agriculture is the principal occupation of the district. The average annual rainfall in Thiruvarur District is 1173 mm. It has a tropical climate, and the average temperature is 28.6°C. The temperature is high in May (around 35.9°C) and is low in January (around 21.3°C).[28] The study was undertaken from December 2018 to May 2019.

Sample Collection

Mosquito larvae were collected by dipping and pipetting methods from ten different pools of rural areas of the district Thiruvarur. The location of the sites using software ArcGIS 10.4 is shown in Figure 1. The mosquito larvae and pupae were collected from different sites of Thiruvarur district by dipping and pipetting methods (Figure 2a).[29] The collected larvae and pupae were transferred into labelled plastic containers and transported to the Vector Biology Research Laboratory of the Department of Life Sciences, Central University of Tamil Nadu, Thiruvarur.

Rearing and Identification

The temperature between 25°C-28°C and relative humidity between 80%-90% were maintained in the laboratory. After emergence, the adult mosquitoes were identified morphologically according to the key of "A catalogue of Indian mosquitoes" from International Journal of Mosquito (2015).[30]

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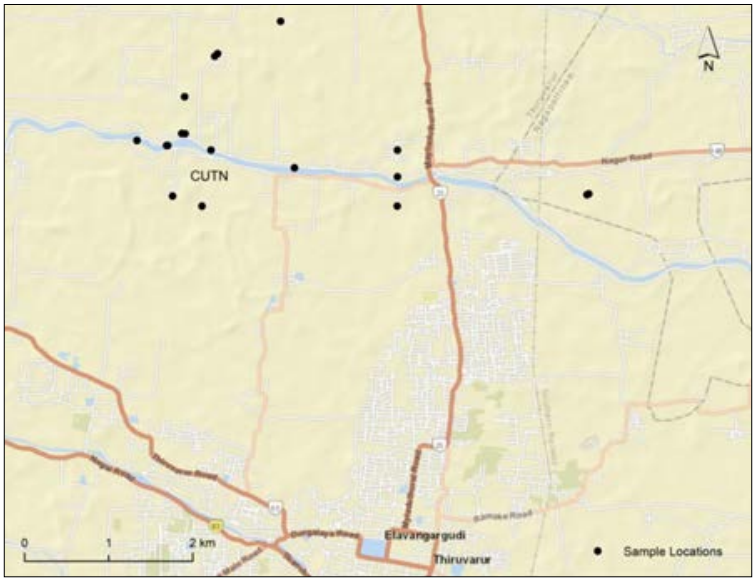

Figure 1.GPS Co-ordinates of Various Locations at Thriuvarur district, Tamil Nadu, India

Figure 2.Surveillance and Sample Processing Images

Susceptibility Test of *Cx. gelidus*

Two to three days old laboratory hatched female mosquitoes of F1 generation were used for the insecticide susceptibility bioassay (Figure 2b). The susceptibility tests were performed using WHO supplied kits. The non-blood fed mosquitoes were exposed to DDT (4%), Deltamethrin (0.05%), and Malathion (5%). A total of 80 mosquitoes were exposed to insecticide-impregnated papers (4 replicates of 20 mosquitoes each). The control tests were also carried out using 40 mosquitoes exposed to control papers (2 replicates of 20 mosquitoes each). After one hour, the mosquitoes were transferred from exposure tubes to holding tubes

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and mortality was observed after one hour and 24 hours. The temperature and relative humidity were maintained throughout the test and exposure period.

The knockdown rate (KDR) was calculated by observing the number of knocked-down mosquitoes after 10, 20, 30, 40, 50, and 60 min during the hour-long exposure period.

RNA Extraction and Reverse Transcription

A pool of mosquito samples was homogenised to extract RNA using Trizol method, out of which 25mg of tissue was used for desirable yield before performing the experiments.[31] The gel tank, the beakers, and micro tips were soaked in 0.1% DEPC water overnight and then autoclaved. Then the 25mg of tissue was homogenised thoroughly with 500µl of Trizol and kept for 2 days of incubation at -80°C. The samples were then processed by adding 100µl of chloroform and centrifuged at 12,000 rpm for 15min at 4°C. The supernatant containing RNA was aspirated from the tube and mixed with 250µl of isopropanol with a slight shack and kept for 10min at room temperature. Then the samples were centrifuged at 12,000 rpm for 15min at 4°C. The pellet of RNA was washed with 75% of ice-cold ethanol and again centrifuged at 12,000 rpm for 1min at 4°C. Finally, the samples were allowed to air dry for 20 min and re-suspended with 30µl of RNase free water as shown in Figures 2(c) and 2(d). Bio-spectrometer (Eppendorf, Germany) was used to measure the ratio of absorbance at 260/280nm to assess the purity of RNA sample (ratio of ~2is pure for RNA) and sample concentration was noted. Agarose gel electrophoresis was used to visualise the RNA band. As a next step, the reverse transcription was carried out from the RNA sample, according to the manual of cDNA synthesis kit (Prime Script ™RT Reagent kit, Takara). cDNA was synthesised in a total volume of 10µl reaction mixture containing 5X Prime Script (2µl), Prime Script RT Enzyme Mix (0.5µl), OligodT Primer (0.5µl), Random Hexamer (0.5µl), Total RNA(2µl) and RNase free water (4.5µl) as shown in Table 1. The reaction mixture was incubated under the following conditions at 37°C for 15 min, 85°C for 5 sec and 4°C for infinite (∞) time.

Polymerase Chain Reaction for cDNA Amplification and Viral Detection

The polymerase chain reaction (PCR) was performed using the kit method (Emerald Amp® GT PCR Master Mix, Takara). The following mixture was added in a PCR tube with a total volume of 50µl: Emerald Amp GT PCR Master mix (25µl), JEV – prM Forward primer 0.2µm (2µl), JEV - prM Reverse primer 0.2µm (2µl), cDNA (5µl) and distilled water (16µl) were added as shown inFigure 2(d). Afterwards, 30 thermal cycles were performed, as from an initial denaturation at 94for 10min, denaturation at 94°C for 60 sec, annealing at 60°C for 60 sec, extension at 72°C for 60 sec, final extension at 72°C for 10 min and cooling at 4°C for infinite (∞) time. The presence of cDNA was also checked by using Cytochorme C Oxidase subunit 1(COX) primer as control as shown in Table 1. A total of 35thermal cycles were performed, beginning with an initial denaturation at 95°C for 5min, denaturation at 94°C for 40 sec, annealing at 45°C for 1 min, extension at 72°Cfor 1min, final extension was carried out at 72°C for 5min and samples were kept for cooling at 4°C for infinite (∞) time. The obtained PCR products were visualised by using 2% agarose gel and observed at a UV transilluminator (Bio pirnt – Gel Doc system ST4, Valleecder, France).

Table 1.Primer Sequence Details

Primer	Sequence 5′ to 3′
JEV- prM (Forward)	CGTTCTTCAAGTTTACAGCATTAGC
JEV – prM (Reverse)	CCYRTGTTYCTGCCAAGCATCCAMCC
COX 1 (Forward)	GGATTTGGAAATTGATTAGTTCCTT
COX 1 (Reverse)	AAAAATTTTAATTCCAGTTGGAACAG

JEV-Japanese encephalitis virus; *COX 1-Cytochrome c oxidase gene 1.*

Table 2.Susceptibility Status of *Cx. gelidus* Adult Mosquitoes to Various Insecticides in Thiruvarur District, Tamil Nadu

Name of the Place	Insecticide (% Con.)	No of Mosquitoes Exposed		No of Mosquitoes Dead (After 24 Hrs)		Observed Mortality (OM) %	Status (S/PR/R)
		T	C	T	C		
Thiruvarur	DDT (4)	80	40	80	2	100	Susceptible
	Malathion (5)	80	40	75	3	93	Possible resistance
	Deltamethrin (0.05)	80	40	76	4	95	Possible resistance

T- Test; C- control

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Results

Adult Susceptibility Status

The adult susceptibility bioassay results of *Cx. gelidus* against three different insecticides are indicated in Table 2. There was no mortality observed in control tests, so Abbott's formula was not required to be applied.[32] After 24 hours of exposure, the mortality of 100% was observed against 4% DDT indicating that *Cx. gelidus* population in the study areas are highly susceptible to DDT. The mortality against 5% Malathion and 0.05% Deltamethrinwasrecorded as 93% and 95% respectively, which indicates possible resistance among *Cx. gelidus* populations against Malathion and Deltamethrin. This is according to WHO criteria, the mortality range of 98-100% indicates susceptibility and less than 98% indicates the possible existence of resistance and requires further investigations.

The knockdown was observed at the KDT50 value of 36.6 mins for DDT, 23.0 mins for Deltamethrin and 43.73mins for Malathion. Knockdown Rate (KDR) of *Cx. gelidus* against DDT, Deltamethrin, and Malathion for an exposure time of 1 hour at different time intervals are shown in Figures 3 and 4 and Table 3. Among all the three insecticides, it has been observed from the results that the time taken for 50% knockdown in *Cx. gelidus* was found to be less in 0.05% Deltamethrin as compared with DDT and Malathion. Subsequently, the lowest KDT95 is observed in Deltamethrin, wher eas the highest value is observed in DDT (143.7 min) in Thiruvarur District, Tamil Nadu.

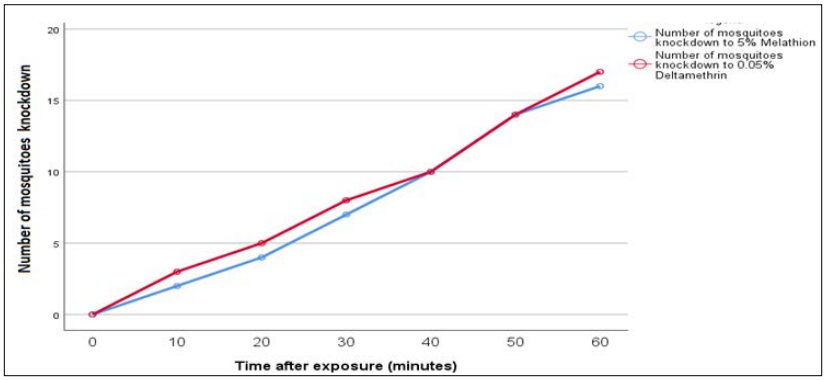

Figure 3.Graphical Representation depicts the Knockdown Rate of Malathion (5%) and Deltamethrin (0.05%)
From the graph, it is clearly observed that the *Cx. gelidus* population from the study area has a more knockdown sensitivity to Deltamethrin (0.05%) as compared toMalathion (5%)

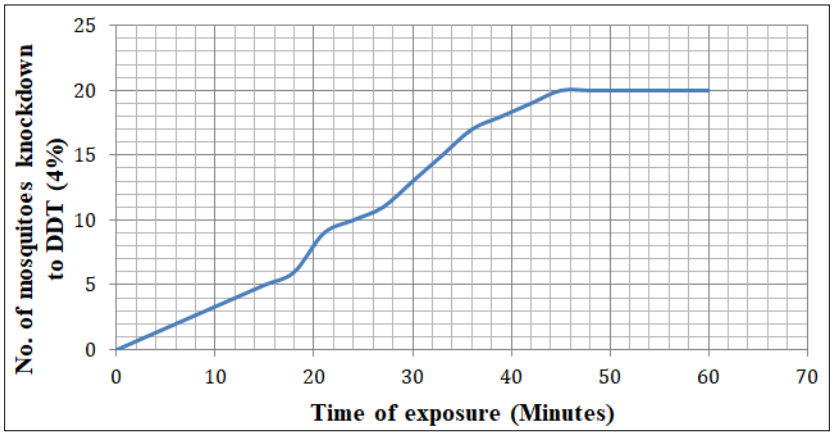

Figure 4.Knockdown Rate of DDT (4%)
Cx. gelidus population from the study area showed a gradual increase in knockdown. Further, the population was found to be susceptible which showed a complete knockdown at 60 min

Table 3.Knockdown Rate of Various Insecticides

S. No.	District Name	Insecticide	(KDT_{50}) Min	(KDT_{95}) Min
1.	Thiruvarur	DDT (4%)	36.6(26.8-45.8)	143.7(87.2-496.9)
2.		Malathion (5%)	43.73(35.4-49.0)	90.6(72.6-171.8)
3.		Deltamethrin (0.05%)	23.0(19.7-25.3)	42.2(38.7-47.9)

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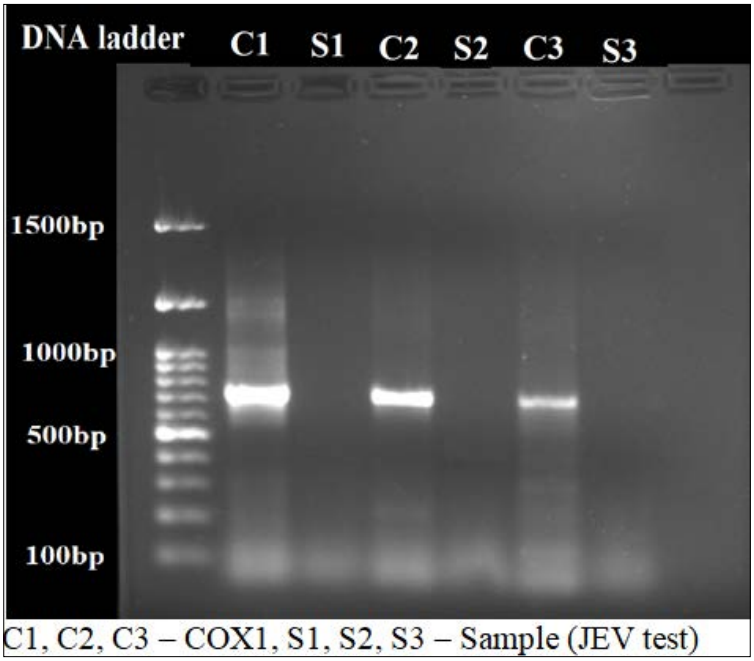

Figure 5.1.5% Agarose Gel represents the RT-PCR Analysis of JEV and COX Gene Expressions in the Field Collected F1 Generation of *Cx. gelidus*

Lanes C1, C2, and C3 show the mRNA expression of mitochondrial Cytochrome c oxidase subunit 1 and this housekeeping gene was analysed to validate the cDNA. Here, the amplified COX1 gene product size is 758bp and it confirms the presence of cDNA. In parallel, the lanes of S1, S2, and S3 wereloaded with JEV tested study samples and it should be noted that there was no amplification observed. It indicates that the samples were not infected with JEV

In addition to the susceptibility status, the JE viral detection was explored using RT-PCR technique in *Cx. gelidus* to endorse the presence of JE virus in field-collected samples and depicts the representative agarose gel showing the level of expression of JEV in samples 1, 2, and 3. This analysis was performed to provide an extra arm to this study. In parallel, COX the housekeeping gene was also analysed (Figure 5, lane C1, C2 and C3) to validate the presence of cDNA and the gene expression. The obtained result reveals that the analysed mosquitoes are non-infected with JEV because there was an absence of expression in JEV analysed samples. More samples are needed to be analysed for further confirmation.

Discussion

Cx. gelidus is reported to be the predominant secondary vector of JE in peri-urban and rural areas of India.[33] The present study was undertaken to assess the insecticide susceptibility status of *Cx. gelidus* in Thiruvarur district of Tamil Nadu as no such information was available from this area. The mortality of 100% was observed against 4% DDT which indicates the susceptibility of *Cx. gelidus* population to DDT, while the mortality of 93% against 5% Malathion and 95% against 0.05% Deltamethrin indicates the possible resistance among *Cx. gelidus* populations against Malathion and Deltamethrin. This is according to WHO criteria, mortality range of 98-100% indicates the susceptibility and less than 98% indicates the possible existence of resistance.

Further, in the present study, it was found that KDT50 and KDT95 values for DDT are 36.6 and 143.7 min respectively, and for Deltamethrin, the lowest rates were observed (23 and 42 min respectively). In addition, the knockdown values for Malathion were also observed within the denoted time (43.73 and 90.6 min, respectively). Among all the three insecticides, it has been observed that the *Cx. gelidus* was sensitive to Deltamethrin. Although Deltamethrin showed the lowest knockdown values, the mortality rate is higher in DDT which indicates that the *Cx. gelidus* is more susceptible to DDT as compared to Malathion and Deltamethrin. A similar observation was recorded in a study conducted at Sibasagar, Assam by Dhiman et al. In the study, it was found that the KDT50 and KDT95 values (47.81 and 304.97 min, respectively) were highest for DDT, where as the lowest rates of knock down values were reported for Deltamethrin in *Cx. gelidus* (27·26 min).[34]

On the other hand, *Cx. quinquefasciatus* is resistant to DDT and Malathion and 100% susceptible to Deltamethrin as shown in a study conducted at major filarial endemic districts (Chandauli and Varanasi) of Uttar Pradesh, North and army cantonments and neighbouring villages of Northeastern India.[35,36] This indicates that at several places in India, the resistance status for various insecticides to different vector species or same species has been reported as varying.

In the present study, the possible resistance observed to Malathion and Deltamethrin can be correlated with the

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use of these insecticides in agricultural practices in the district. There are similar scientific reports of resistance in *An. Culicifacies* the malaria vector against Malathion and Deltamethrin in the Odisha state of India.[37] *Cx. gelidus* has shown resistance to Deltamethrin and susceptibility to Malathion in a study undertaken in West Bengal, India.[33] The reason for variation in the susceptibility status of same insecticides to same vectors at different places may be due to insecticide pressure but it needs further exploration of resistance mechanisms. Further, in our study, the virus could not be detected in mosquitoes for which more samples needed to be analysed but could not be done due to the limitation of field visits during COVID-19.

Conclusion

The present study concludes that *Cx. gelidus* one of the JE vectors, was found to be susceptible to DDT whereas, possible resistance was noted against Malathion and Deltamethrin in Thiruvarur district of Tamil Nadu. The findings of the present study could be highly useful in the management of vector control targeting vectors like *Cx. gelidus*. Further, viral detection in the *Cx. gelidus* was investigated which revealed that the analysed mosquitoes were non-infected with JEV. However, more sampling and its analysis may facilitate in confirming the evidence of virus isolation from *Cx. gelidus*.

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Conflict of Interest: None

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