International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2023; 10(1): 56-61
© 2023 IJMR
www.dipterajournal.com
Received: 07-11-2022
Accepted: 23-12-2022

Biplob Sarmah
[A] Division of Medical Entomology, Arbovirology and Rickettsial Diseases, ICMR-Regional Medical Research Centre, N.E. Region, Dibrugarh, Assam, India
[B] Srimanta Shankaradeva University of Health Sciences, Guwahati, Assam, India

Dr. Siraj Ahmed Khan
Division of Medical Entomology, Arbovirology and Rickettsial Diseases, ICMR-Regional Medical Research Centre, N.E. Region, Dibrugarh, Assam, India

Dr. Prafulla Dutta
Division of Medical Entomology, Arbovirology and Rickettsial Diseases, ICMR-Regional Medical Research Centre, N.E. Region, Dibrugarh, Assam, India

Kamakhya Borah
Division of Medical Entomology, Arbovirology and Rickettsial Diseases, ICMR-Regional Medical Research Centre, N.E. Region, Dibrugarh, Assam, India

Corresponding Author:
Dr. Siraj Ahmed Khan
Division of Medical Entomology, Arbovirology and Rickettsial Diseases, ICMR-Regional Medical Research Centre, N.E. Region, Dibrugarh, Assam, India

Susceptibility status of *Cx. vishnui* subgroup of mosquitoes against different Insecticides and detection of novel mutations in esterase beta gene in *Cx. tritaeniorhynchus*

Biplob Sarmah, Dr. Siraj Ahmed Khan, Dr. Prafulla Dutta and Kamakhya Borah

DOI: https://doi.org/10.22271/23487941.2023.v10.i1a.662

Abstract
Japanese encephalitis (JE) is a mosquito-borne viral disease and several species of the *Culex* genera can transmit JE. The present study was conducted to observe resistance in *Cx. vishnui subgroup* against organophosphates, widely used in fogging and controlling JE. The study was conducted in 12 localities of Dibrugarh and Tinsukia districts of Assam, endemic for JE.
Susceptibility tests performed revealed both *Cx. pseudovishnui* and *Cx. tritaeniorhynchus* were found resistant against Organophosphates Malathion (5%) and Fenitrothion (1%). Molecular characterization of genes responsible for resistance depicted two non-synonymous polymorphisms for the primary JE vector *Cx. tritaeniorhynchus*.
To the best of our knowledge, this is the first report on detection of SNP's in esterase beta (*estβ*) gene in India for the species *Cx. tritaeniorhynchus*. As data regarding characterization of genes responsible for resistance in JE vectors is lacking from this country, this study will aid in understanding the characteristics of genes involved in resistance manifestation.

Keywords: Japanese encephalitis, *Cx. vishnui* subgroup, resistance, esterase ß gene

Introduction
Japanese encephalitis (JE) is one of the leading forms of acute encephalitis in the world prevalent mostly in Eastern and Southern Asia with a population exceeding 3 billion [1]. The disease is caused by a flavivirus with a single stranded RNA genome of approximately 11kb in length [2]. Around 35,000 to 50,000 cases of JE are reported annually with 10,000 deaths every year [3]. The disease was previously seen to be manifested in children but a surge in cases among the adult population has been reported in recent publications [4, 5]. The disease is endemic in most parts of South-East Asia including India [6]. The first case of JE infection in Assam was detected in 1974 [7]. Since then sporadic cases of JEV infection has been reported from most parts of North East India including Assam [8]. Infection in the *Culicine* mosquito is responsible for the transmission of the JE virus between ardeid birds and pigs in nature where humans are dead end host [9]. A myriad of species belonging to the Culicidae family has been reported to harbor the virus [10]. In India a total of 16 mosquito species has been incriminated for the JE virus of which 11 species are known to occur in this region [11, 12]. The *Cx. vishnui* subgroup comprising of *Cx. vishnui*, *Cx. pseudovishnui* and *Cx. tritaeniorhynchus* are considered the most important vectors and contribute significantly towards JE outbreaks in India [13]. People from North-East region of India especially Assam practices widespread paddy cultivation. Distribution of these paddy fields along with a tropical monsoon climate with high incidence of rainfall is closely associated with the relative abundance of *Cx. vishnui* subgroup in this region [14]. Among the *Cx. vishnui* subgroup, *Cx. tritaeniorhynchus* is the primary vector of JE from which the highest number of isolations have been reported [15]. Vector control has been one of the key strategies implemented globally for containment of arthropod associated diseases.

Use of Insecticides for such containment strategies has been the key for decades. However extensive use of Insecticides has led to the development of resistance among mosquitoes and is a major hindrance in eradication of these diseases. The government in adherence with guidelines promulgated by NVBDCP initiated outdoor spraying of Malathion (5%) as a preventive step to curb the spread of JE during epidemics. The present study was undertaken to determine the susceptibility status of the *Cx. vishnui* subgroup against commonly used insecticides and detection of single nucleotide polymorphisms in estβ gene responsible for insecticidal resistance against Organophosphates in primary JE vector *Cx. tritaeniorhynchus.*

2. Materials and Methods

2.1 Selection of study sites

The study was conducted in the selected areas as per endemicity of JE in Assam, India. Two districts namely Dibrugarh (27.4728°N, 94.9120°E) and Tinsukia (27.4886°N, 95.3558°E) were selected for mosquito collection. Six localities per district were selected, from where; at least one JE case had been reported earlier according to data collected from line list obtained from PHC's. Simple random sampling method using Fisher and Yate's table of random numbers was adopted for the selection of these study sites.

2.2 Collection of mosquitoes

Location with human dwellings, mixed dwellings, cattle sheds and existing pig shelters were selected for collection of mosquitoes. For the study, outdoor resting mosquitoes from grasses, shrubs and bushes were collected with the help of a drop net of 2m×2m×2m size. Collection of resting adult full-fed female mosquitoes was also made in dusk hours, commencing from sunset and continuing for one hour.

2.3 Mosquito Rearing

In the interests of obtaining age-standardized results, susceptibility tests were performed on the F1 progeny of wild-caught female mosquitoes. Mosquitoes belonging to the *Cx. vishnui* subgroup were reared in the Insectary after morphological identification referring standard taxonomic keys using a stereoscopic microscope [16, 17]. Female mosquitoes of the potential JE vector species (at least 10 mosquitoes) were suspended in a 500 ml beaker partially filled with distilled water and covered with a net. All procedures were maintained at 27°C ± 1°C temperature and 70 ± 5% relative humidity. Beakers were exposed to ambient light and darkness. Egg-Rafts laid by the *Culex* mosquitoes were transferred to a flat plastic tray containing distilled water (about 1 liter) and a pinch of fine-ground larval food (dog biscuit and yeast powder mixed in 1:1 proportion). Larvae were monitored regularly by feeding them on larval food mixture and maintenance of ambient temperature and humidity conditions. Subsequently, the pupae were transferred to a petri dish containing distilled water that was placed inside a Barraud cage for emergence of adults. Adults were fed on cotton wad soaked in 10% sucrose solution. Adult female mosquitoes were subsequently used for insecticidal bioassay tests.

2.4 Insecticide Bioassay

Susceptibility tests against adult vector mosquitoes were conducted according to WHO standard guidelines [18]. Collected mosquitoes from index localities were exposed to WHO impregnated papers containing diagnostic concentrations of insecticides to be tested (DDT, Dieldrin, Malathion, Propoxur, Permethrin) using WHO insecticide susceptibility test kit. Full-fed mosquitoes collected in the field were brought to the laboratory and subjected to morphological identification without causing any harm to the mosquito. As per guideline, twenty-twenty five mosquitoes were used in tubes comprising of 4 replicates for test and 2 replicates for control group. Percentage mortality was determined depending upon the total number of live and dead mosquitoes in the replicates after 24 hours of holding period. If the control mortality exceeded 20% the tests were discarded. WHO Insecticide susceptibility kits and insecticide impregnated papers were obtained from WHO reference Center (Vector Control Research Unit, University Sains Malaysia, Penang, Malaysia) (Report of the WHO informal consultation, Geneva, 1998).

2.5 Homogenization and DNA extraction

Each mosquito pool was triturated mechanically in sterile mortar and pestles (chilled) in1ml of 2% Fetal calf serum (FCS) in minimum essential medium (MEM). The homogenate was centrifuged at 10,000 rpm for 30 min at 4ºC. The supernatant was filtered through a 0.2mm porosity syringe filter, aliquoted into vials and stored at -80ºC for further downstream molecular analysis. Mosquito DNA was extracted from the homogenized mosquito pools using QIAamp DNA mini-kit (Qiagen, Germany) as per manufacturer's instructions.

2.6 Polymerase Chain reaction

The 25µL amplification mixture for species diagnostic PCR assay included 2X Master Mix (Promega, USA), Nuclease Free Water (Promega, USA) as well as Forward primer and Reverse primer as previously described [19]. The amplification profile consisted of one cycle at 96°C for 12 min, 40 cycles at 96° C for 30s, 52°C for 30s, 72°C for 90 s and one cycle at 72°C for 4 min. Amplified products were identified on 1.2% agarose gel containing Ethidium Bromide. For negative control reaction mixture containing no template DNA was used.

2.7 Esterase activity assay

Napthyl acetate assay: Adult individual female mosquitoes of each species were homogenized to determine the enzyme activity. Individual mosquitoes were homogenized in 50µl of distilled water in ice-cold conditions in 1.5ml centrifuge tubes and made upto a final volume of 200µl. Centrifugation at 14000 rpm was performed for the homogenates and the supernatant was used for performing the enzyme assays. Esterase activity assays were performed using the method described previously [20].

For control reaction, 10µl of distilled water was used in place of homogenate. End point enzyme activity was measured at 570nm. All activities were expressed in µg of product formed/ min/ mg protein based on standard curves of α and β napthols. Total protein content was estimated using Lowry's method of protein estimation.

2.8 Data Analysis

Regression analysis was done to determine the Lethal Time (LT_{50} and LT_{99}) by log-probit method using Log dose probit (Ldp) Line software (Ehabsoft, Egypt). The value for

exposure mortality E was corrected by using the Abbott's formula, and the exposure mortality is referred to as corrected percent mortality. DNA sequences obtained after sequencing were analyzed using BIOEDIT Software (Version 6.0) and inferred DNA sequences were subjected to pair wise as well as multiple alignments using the programme Clustal W.

3. Results and Discussion

A total of 3209 female mosquitoes comprising of 8 species and 2 genera were collected during the period of report as given in Table 1. *Cx. vishnui* was the most predominant species comprising 38.8% of the total mosquitoes collected. Other species collected were *Cx. tritaeniorhynchus* (24.49%), Cx. *pseudovishnui* (15.49%), *Ma. uniformis* (6.98%), *Cx. fuscocephala* (5.55%), *Cx. whitmorei* (3.55%), *Ma. annulifera* (3.18%) and *Cx. quinquefasciatus* (1.96%).

Table 1: Mosquitoes collected in two districts of Assam

Mosquitoes collected, n			
Species	**Dibrugarh District**	**Tinsukia District**	**Total (%)**
Ma. uniformis	114	110	224 (6.98)
Ma. annulifera	67	35	102 (3.18)
Cx. vishnui	578	667	1245 (38.80)
Cx. pseudovishnui	210	287	497 (15.49)
Cx. tritaeniorhynchus	433	353	786 (24.49)
Cx. whitmorei	42	72	114 (3.55)
Cx. fuscocephala	97	81	178 (5.55)
Cx. quinquefasciatus	25	38	63 (1.96)

3.1 Bioassay Test

3.1.1 *Cx. Vishnui*: The susceptibility status as well as base line susceptibility data of *Cx. vishnui* subgroup against different insecticides for the study sites is detailed in Table 2. *Cx. vishnui* was found susceptible to Organochlorides DDT (4%) and Dieldrin (4%) in both the districts (Mortality rate ≥ 98%). The Mortality rate calculated for organophosphate Malathion (5%) was 90.6% in Dibrugarh district and 98.33% in Tinsukia district. The species was found 100% susceptible for Fenitrothion (1%) in both the districts. For Permethrin the exposure mortality rate was calculated to be 91.6% and 96.6% for Dibrugarh and Tinsukia district respectively.

Table 2: Susceptibility status of *Cx. vishnui* in two districts of Assam

Insecticide	District	Conc. (%)	Exposure mortality (%)	LC10/LT10 [95% CI]	LC50/LT50 [95% CI]	LC99/LT99 [95% CI]	χ2	Slope	Status
DDT	Dibrugarh	4%	100	0.90[0.7-1.1]	2.82 [2.4-3.5]	22.40[13.3-52.5]	1.59	2.59 +/- 0.33	S
	Tinsukia	4%	100	0.95[0.7-1.2]	2.85[2.4-3.5]	20.85[12.7-46.9]	2.75	2.69+/- 0.34	S
Malathion	Dibrugarh	5%	90.6	19.58[15.5-22.8]	37.3[33.7-41.2]	119.9[93.8-177.8]	4.16	4.58+/-0.57	RS
	Tinsukia	5%	98.33	14.15[11.0-16.8]	27.64[24.6-30.6]	93.16[75.5-127.5]	4.19	4.41+/-0.48	S
Permethrin	Dibrugarh	0.75%	91.66%	23.7[18.9-27.7]	48.7[44.4-53.7]	179.6[138.5-269.1]	1.5	4.11+/-0.48	RS
	Tinsukia	0.75%	96.66%	23.51[18.8-27.3]	47.14[43.0-51.7]	166.67[130.7-242.2]	1.89	4.24+/-0.48	RS
Propoxur	Dibrugarh	0.1%	100%	17.43[7.2-18.8]	41.98[30.1-57.4]	206.93[209.1-843.6]	12.67	3.36+/-0.39	S
	Tinsukia	0.1%	100%	16.20[7.0-17.9]	39.45[28.3-52.5]	198.47[189.0-701.3]	11.74	3.32+/-0.38	S
Fenitothrion	Dibrugarh	1	100	10.37 [3.24-15.98]	29.64 [21.4-38.1]	199.26 [107.2-1136.0]	1.43	2.81	S
	Tinsukia	1	100	23.7[18.9-27.7]	48.7[44.4-53.7]	179.6[138.5-269.1]	1.5	4.11+/-0.48	S
Dieldrin	Dibrugarh	4	100	0.16[0.06-0.2]	0.78[0.5-1.7]	13.59[11.5-122.6]	17.65	1.87	S
	Tinsukia	4	100%	20.12[16.1-23.6]	48.15[44.0-53.0]	234.65[176.3-357.8]	7.33	3.38+/-0.41	S

*S (Susceptible), R (Resistant), RS (Resistance suspected)

3.1.2 *Cx. pseudovishnui*

The susceptibility status of *Cx. pseudovishnui* against recorded doses of Insecticides is shown in Table 3. *Cx. pseudovishnui* was found to be susceptible to DDT and Dieldrin with mortality ≥ 98% in both the districts. The species was found to be resistant against Malathion with mortality rate calculated to be 63.33% in Dibrugarh district and 70% in Tinsukia district. For the Organophosphate Fenitrothion 68.3% and 81.6% in exposure mortality was calculated for the respective districts. The species was found to be susceptible in Dibrugarh district against Permethrin (0.75%) but suspected to be resistant in Tinsukia district (Mortality rate = 96.6%). For Propoxur the mortality rate was calculated to be 86.6% in Tinsukia district but susceptible in Dibrugarh district with mortality recorded to be 100%.

Table 3: Susceptibility status of *Cx. pseudovishnui* in two districts of Assam

Insecticide	District	Conc. (%)	Exposure mortality (%)	LC10/LT10 [95% CI]	LC50/LT50 [95% CI]	LC99/LT99 [95% CI]	χ2	Slope	Status
DDT	Dibrugarh	4%	100	0.67[0.4-0.9]	2.50 [2.1-3.2]	27.03[14.9-74.6]	1.29	2.25+/- 0.31	S
	Tinsukia	4%	100	0.68[0.5-0.9]	2.30[1.9-2.8]	20.80[12.3-48.9]	1.96	2.43+/- 0.31	S
Malathion	Dibrugarh	5%	63.33	8.00[3.2-12.3]	35.36[28.9-43.8]	525.21[243.2-2849.1]	1.21	1.99+/-0.38	R
	Tinsukia	5%	70.00	8.93[4.3-13.0]	33.56[27.9-40.2]	371.36[198.2-1315.4]	0.18	2.23+/-0.39	R
Permethrin	Dibrugarh	0.75%	100	4.79[2.2-7.2]	17.39[13.9-20.6]	180.69[104.4-527.5]	0.14	2.29+/-0.39	S

	Tinsukia	0.75%	96.66	5.49[2.8-7.9]	18.45[15.2-21.6]	166.68[100.5-432.4]	0.13	2.43+/-0.39	RS
Propoxur	Dibrugarh	0.1%	100%	20.12[16.1-23.6]	48.15[44.0-53.0]	234.65[176.3-357.8]	7.33	3.38+/-0.41	S
	Tinsukia	0.1%	86.66%	19.69[8.0-20.9]	46.91[34.5-66.7]	226.78[242.1-1113.8]	12.84	3.40+/-0.41	R
Fenitothrion	Dibrugarh	1	68.33	28.74[21.1-34.5]	66.83 [60.5-75.4]	309.19 [212.7-597.6]	2.65	3.50+/-0.50	R
	Tinsukia	1	81.66	30.06[22.7-35.6]	66.91[60.8-75.0]	285.93[202.3-519.3]	3.66	3.69+/-0.51	R
Dieldrin	Dibrugarh	4	98.33	0.08[0.0-0.1]	0.55[0.3-1.2]	19.93[15.2-248.8]	15.13	1.49+/-0.13	S
	Tinsukia	4	100	0.08[0.0-0.1]	0.52[0.3-1.0]	17.48[11.8-152.4]	12.99	1.53+/-0.13	S

3.1.3 *Cx. tritaeniorhynchus:*

The susceptibility status as well as base line susceptibility data of *Cx. vishnui* subgroup against different insecticides for the study sites is detailed in Table 4.

The species was found resistant against Organophosphates Malathion (5%) and Fenitrothion (1%) with mortality rate 61.6% and 71.6% in Dibrugarh district and 65% and 70% in Tinsukia district respectively. The mortality rate for Permethrin was calculated to be 93.3% in Dibrugarh district but found to be susceptible in Tinsukia district with mortality rate 100%.

Table 4: Susceptibility status of *Cx. tritaeniorhynchus* in two districts of Assam

Insecticide	District	Conc. (%)	Exposure mortality (%)	LC10/LT10 [95% CI]	LC50/LT50 [95% CI]	LC99/LT99 [95% CI]	χ2	Slope	Status
DDT	Dibrugarh	4%	100	0.67[0.4-0.9]	2.50 [2.1-3.2]	27.03[14.9-74.6]	1.29	2.25+/- 0.31	S
	Tinsukia	4%	100	0.68[0.5-0.9]	2.30[1.9-2.8]	20.80[12.3-48.9]	1.96	2.43+/- 0.31	S
Malathion	Dibrugarh	5%	61.66	17.92[11.9-22.5]	52.02[44.6-65.3]	360.0[204.2-1087.0]	1.50	2.77+/-0.46	R
	Tinsukia	5%	71.66	18.80[13.6-22.9]	46.05[40.6-53.9]	234.25[154.3-491.6]	3.79	3.29+/-0.48	R
Permethrin	Dibrugarh	0.75%	93.33	5.61[2.6-8.5]	17.67[13.1-21.4]	142.04[94.9-300.3]	2.18	2.29+/-0.41	RS
	Tinsukia	0.75%	100	5.20[2.3-8.1]	16.44[11.8-20.1]	132.94[89.3-279.8]	4.18	2.56+/-0.42	S
Propoxur	Dibrugarh	0.1%	100%	21.32[16.8-25.1]	30.38[26.0-34.1]	174.79[135.1-259.2]	5.93	3.95+/-0.44	S
	Tinsukia	0.1%	100%	20.64[7.9-22.7]	42.09[29.4-56.8]	153.42[147.3-646.0]	9.27	4.14+/-0.44	S
Fenitothrion	Dibrugarh	1	65	11.17[5.3-16.0]	46.31 [38.4-61.1]	611.9 [275.2-3554.8]	0.36	2.08+/-0.40	R
	Tinsukia	1	70	11.49[5.8-16.1]	44.70[37.4-57.3]	526.03[252.1-2505.6]	0.07	2.17+/-0.40	R
Dieldrin	Dibrugarh	4	100	0.08[0.0-0.1]	0.55[0.3-1.2]	19.93[15.2-248.8]	15.13	1.49+/-0.13	S
	Tinsukia	4	100	0.08[0.0-0.1]	0.52[0.3-1.0]	17.48[11.8-152.4]	12.99	1.53+/-0.13	S

3.2 Molecular detection of SNP'S in esterase beta gene in *Cx. tritaeniorhynchus*

PCR based assays revealed two non-synonymous polymorphism in the esterase beta gene of *Cx. tritaeniorhynchus.* For comparative analysis a susceptible strain of *Cx. tritaeniorhynchus* was obtained from GenBank with accession number AF177382.1. Multiple alignments with the reference gene depicted a L354M mutation and A389F mutation in the esterase beta gene of *Cx. tritaeniorhynchus*. Mutations at other positions do not lead to any change in amino acid sequence and hence are synonymous in origin.

3.3 Esterase activity assay

The mean value of α and β esterase activity for *Cx. vishnui* subgroup of mosquitoes are given in Table 5. The lowest mean value for α and β esterase activity (0.098 and 0.104) was calculated for *Cx. vishnui* in Tinsukia District and highest (0.237 and 0.244) was calculated for *Cx. triteaniorhynchus* in Dibrugarh district. There was a 0.55 and 1.30 times increase in enzyme activity of *Cx. pseudovishnui* and *Cx. tritaeniorhynchus* respectively as comparison to *Cx. vishnui* in Tinsukia district. Similarly 1.10 and 0.90 times increase in enzyme activity was calculated for *Cx. pseudovishnui* amd *Cx. tritaeniorhunchus* as compared to *Cx. vishnui* in Dibrugarh district respectively. The proportion of population showing activity for *Cx. vishnui* subgroup is shown in Figure 1.

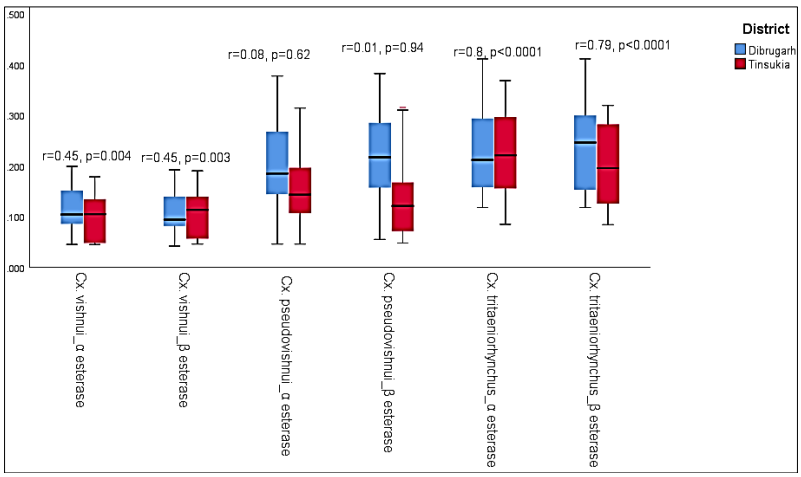

Fig 1(a): α-esterase activity and (b) β-esterase activity of *Cx. vishnui*, *Cx. pseudovishnui* and *Cx. tritaeniorhynchus* in Dibrugarh and Tinsukia district of Assam

Table 5: Mean α- and β-esterases activity (µmol/min/mg) in *Cx.* *vishnui* subgroup from the study districts

Mosquito strain (n)	Activity (µmol/min/mg) ± SD	
	α-esterase	β-esterase
Cx. vishnui DIB	0.111±0.05	0.106±0.04
Cx. vishnui TSK	0.098±0.045	0.104±0.044
Cx. pseudovishnui DIB	0.202±0.09	0.223±0.08
Cx. pseudovishnui TSK	0.152±0.08	0.132±0.07
Cx. tritaeniorhynchus DIB	0.237±0.08	0.244±0.09
Cx. tritaeniorhynchus TSK	0.226±0.08	0.201±0.08

3.4 Discussion

Cx. vishnui subgroup comprising of *Cx. vishnui*, *Cx. pseudovishnui* and *Cx. tritaeniorhynchus* are the major vectors responsible for JE throughout the South-East Asian belt [21]. The prevalence of *Cx. vishnui* subgroup is a significant contributing factor towards the rise of JE cases in India. Along with vaccination, reduction in mosquito density using insecticides is a formidable and effective control measure for JE outbreak in India [22, 23]. But development of resistance to Insecticides in JE vectors is reported from different parts of the country periodically [24, 25, 26, 27, 28]. This creates major hindrance in implementation of vector control programmes, as use of right insecticide is paramount for effectiveness of these control programmes promulgated by the health departments concerned. Although, phenotypic studies to determine susceptibility status of major JE vectors have been carried out earlier but no molecular studies as such have been carried out in regards to *Cx. vishnui* subgroup in Assam [25, 27]. The present study was conducted in two JE endemic districts of Assam *viz*., Dibrugarh and Tinsukia. The susceptibility status of the *Cx. vishnui* subgroup against commonly used insecticides was investigated and it was found that *Cx. tritaeniorhynchus* and *Cx. pseudovishnui* exhibited significant resistance against organophosphates Malathion and Fenithothrion. Resistance developed against Organophosphates is a matter of serious concern because outdoor spraying of Malathion is a formidable combat option against the rising cases of JE in endemic areas. Resistance against organophosphates was previously reported in South Odisha in both *Cx. vishnui* and *Cx. tritaeniorhynchus* [24]. In another study conducted in three districts of West Bengal, *Cx. tritaeniorhynchus* was reported to be resistant against Malathion, but both *Cx. vishnui* and *Cx. pseudovishnui* was found susceptible [26]. In JE endemic Sibasagar district of Assam all species belonging to the *Cx. vishnui* subgroup was found susceptible against Organochloride DDT and pyrethroid Deltamethrin [25]. This is in accordance to the study conducted by us, as we found the three species to be susceptible to DDT 4%.

Elevated alpha and beta esterase activity had previously been reported in many Insecticide resistant vectors of JE with particular reference to organophosphates [29]. The present study recorded elevated levels of alpha and beta esterase activity in both *Cx. tritaeniorhynchus* and *Cx. pseudovishnui* in both the study sites in Assam. However, esterase activities exhibited by *Cx. vishnui* were low in comparison to the other two species. This is in conformity with the phenotypic resistance patterns studied for these vector species. Both *Cx. tritaeniorhynchus* and *Cx. pseudovishnui* were found to be resistant to organophsphates as compared to *Cx. vishnui* which was found completely susceptible to Fenithothrion and Malathion except in Dibrugarh district where mortality rate was recorded to be >90% for Malathion. Although no evidence of elevation in esterase activity was recorded for *Cx. vishnui* against Malathion in Dibrugarh district, the observed resistance phenomenon might be attributed to some other mechanism. Resistance observed in the two species of *Cx. vishnui* subgroup against Organophosphates along with elevated esterase activities is a matter of serious concern and periodic monitoring of these vector species is a necessity.

Although amplification of esterase gene has been studied in detail in the primary vector *Cx. tritaeniorhynchus*, no mutational study has been carried out as such. The L354M and A389F mutation observed in this study had previously been not reported anywhere. A similar study conducted in Gorakhpur in Uttar Pradesh reported the F331W mutation in *Cx. tritaeniorhynchus* and G119S mutation in *Cx. quinquefasciatus* in the *ace* gene. In another study conducted in army cantonments and surrounding villages in Assam, Leucine to phenylalanine change was observed in IIS6 domain of VGSC gene. To the best of our knowledge this is the first report of SNP in the esterase beta gene in *Cx. tritaeniorhynchus*. Due to lack of whole genome sequence of these primary vector species of JE, the study of genetic makeup of these vector species is very challenging. The L354M and A389F mutations reported in the esterase beta gene might be responsible for such resistance manifestation observed phenotypically in these vectors, apart from having a high copy number and elevated enzyme production. But the role of these mutations to confer resistance if any can only be confirmed upon further molecular investigations and with the availability of databases conferring the whole genome sequences of these vector species.

4. Conclusions

The study was conducted to determine the susceptibility status of *Cx. vishnui* subgroup against different classes of Insecticides and identify single nucleotide polymorphism in *estβ* gene responsible for conferring insecticidal resistance in the primary JE vector *Cx. tritaeniorhynchus*. To the best of our knowledge, this is the first report on detection of SNP's in esterase beta (estβ) gene in India for the species *Cx. tritaeniorhynchus*. As data regarding characterization of genes responsible for resistance in JE vectors is lacking from this country, this study will aid in understanding the characteristics of genes involved in resistance manifestation. Incorporating this knowledge will further assist in devising new strategies in JE vector control programmes.

Acknowledgments

Authors are thankful to (Department of Health Research), Ministry of Health and Family Welfare, Government of India for providing funds for the study and all the staff involved in entomological surveys. Sincere thanks is also due to MO, SMO and health workers of respective districts of the states who supported during field surveys and provided valuable information related to visited places.

References

1. Ghosh D, Basu A. Japanese encephalitis - A pathological and clinical perspective. PLoS Negl Trop Dis. 2009, 3(9).
2. Sumiyoshi H, Hoke CH, Trent DW. Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates. Journal of virology. 1992 Sep 1;66(9):5425-31.
3. Solomon T, Ni H, Beasley DW, Ekkelenkamp M, Cardosa MJ, Barrett AD. Origin and evolution of

Japanese encephalitis virus in Southeast Asia. Journal of virology. 2003 Mar 1;77(5):3091-8. (Vancouver)
4. Lee EJ, Cha GW, Ju YR, Han MG, Lee WJ, Jeong YE. Prevalence of neutralizing antibodies to Japanese encephalitis virus among high-risk age groups in South Korea, 2010. PLoS One. 2016 Jan 25;11(1):e0147841.
5. Xiong W, Lu L, Xiao Y, Li J, Zhou D. Mortality and Disability Due to Japanese Encephalitis in Elderly Adults: Evidence From an Adult Tertiary Care Center in West China. Frontiers in neurology. 2019 Aug 22;10:918.
6. Solomon T, Dung NM, Kneen R, Gainsborough M, Vaughn DW, Khanh VT. Japanese encephalitis. Journal of Neurology, Neurosurgery & Psychiatry. 2000 Apr 1;68(4):405-15.
7. Phukan AC, Borah PK, Mahanta J. Japanese encephalitis in Assam, northeast India. Southeast Asian journal of tropical medicine and public health. 2004 Sep 1;35:618-22.
8. McNaughton H, Singh A, Khan SA. An outbreak of Japanese encephalitis in a non-endemic region of north-east India. JR Coll Physicians Edinb. 2018 Mar 1;48(1):25-9.
9. Impoinvil DE, Baylis M, Solomon T. Japanese encephalitis: on the one health agenda. One health: the human-animal-environment interfaces in emerging infectious diseases, 2012, 205-47.
10. Pearce JC, Learoyd TP, Langendorf BJ, Logan JG. Japanese encephalitis: the vectors, ecology and potential for expansion. Journal of travel medicine. 2018 May;25(1):S16-26.
11. Samuel PP, Hiriyan J, Gajanana A. Japanese encephalitis virus infection in mosquitoes and its epidemiological implications. ICMR Bulletin. 2000;30(4):37-43.
12. Khan SA, Narain K, Handigue R, Dutta P, Mahanta J, Satyanarayana K, *et al*. Role of some environmental factors in modulating seasonal abundance of potential Japanese encephalitis vectors in Assam, India. The Southeast Asian journal of tropical medicine and public health. 1996 Jun 1;27(2):382-91.
13. Murty US, SatyaKumar DV, Sriram K, Rao KM, Singh TG, Arunachalam N, Samuel PP. Seasonal prevalence of Culex vishnui subgroup, the major vectors of Japanese encephalitis virus in an endemic district of Andhra Pradesh, India. Journal of the American Mosquito Control Association. 2002 Dec 1;18(4):290-3.
14. Barik M, Bhattacharjee I, Ghosh A, Chandra G. Larvivorous potentiality of Puntius tetrazona and Hyphessobrycon rosaceus against Culex vishnui subgroup in laboratory and field based bioassay. BMC research notes. 2018 Dec;11(1):1-5.
15. Pearce JC, Learoyd TP, Langendorf BJ, Logan JG. Japanese encephalitis: the vectors, ecology and potential for expansion. Journal of travel medicine. 2018 May;25(1):S16-26.
16. Barraud PJ. The Fauna of British India, including Ceylon and Burma. Diptera. Vol. 5. Family Culieldae. Tribes Megarhinini and Culicini. The Fauna of British India, including Ceylon and Burma. Diptera. Vol. 5. Family Culieldae. Tribes Megarhinini and Culicini; c1934.
17. Reinert JF, Harbach RE, Kitching IJ. Phylogeny and classification of tribe Aedini (Diptera: Culicidae). Zoological Journal of the Linnean Society. 2009 Dec 1;157(4):700-94.
18. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes.
19. Paton MG, Karunaratne SP, Giakoumaki E, Roberts N, Hemingway J. Quantitative analysis of gene amplification in insecticide-resistant Culex mosquitoes. Biochemical Journal. 2000 Feb 15;346(1):17-24.
20. Penilla PR, Rodriguez AD, Hemingway J, Torres JL, Arredondo-Jiménez JI, Rodriguez MH. Resistance management strategies in malaria vector mosquito control. Baseline data for a large-scale field trial against Anopheles albimanus in Mexico. Medical and veterinary entomology. 1998 Aug;12(3):217-33.
21. Toma T, Miyagi I, Crabtree MB, Miller BR. Identification of Culex vishnui subgroup (Diptera: Culicidae) mosquitoes from the Ryukyu Archipelago, Japan: development of a species-diagnostic polymerase chain reaction assay based on sequence variation in ribosomal DNA spacers. Journal of medical entomology. 2000 Jul 1;37(4):554-8.
22. What is the prevention and control of JE? (https://nvbdcp.gov.in/index4.php?lang=1&level=0&linkid=484&lid=3761) accessed on 26/0802021.
23. Kulkarni R, Sapkal GN, Kaushal H, Mourya DT. Suppl-2, M8: Japanese encephalitis: A brief review on Indian perspectives. The open virology journal. 2018;12:121.
24. Sahu SS, Sonia T, Dash S, Gunasekaran K, Jambulingam P. Insecticide resistance status of three vectors of Japanese encephalitis in east central India. Medical and veterinary entomology. 2019 Jun;33(2):213-9.
25. Dhiman S, Rabha B, Talukdar PK, Das NG, Yadav K, Baruah I, Singh L, Veer V. DDT & deltamethrin resistance status of known Japanese encephalitis vectors in Assam, India. The Indian journal of medical research. 2013 Dec;138(6):988.
26. Misra BR, Gore M. Malathion resistance status and mutations in acetylcholinesterase gene (Ace) in Japanese encephalitis and filariasis vectors from endemic area in India. Journal of medical entomology. 2015 May 1;52(3):442-6.
27. Bhattacharyya DR, Handique R, Prakash A, Dutta P, Mahanta J. Insecticide susceptibility status of potential vectors of Japanese encephalitis in Dibrugarh district, Assam. Journal of communicable diseases. 1996;28(1):67-9.
28. Sharma RS, Sharma SN, Kumar A. Susceptibility status of Japanese encephalitis vectors in Kurnool and Mehboobnagar districts of Andhra Pradesh, India. The Journal of communicable diseases. 2003 Jun 1;35(2):118-22.
29. Hemingway J, Ranson H. Insecticide resistance in insect vectors of human disease. Annual review of entomology. 2000 Jan;45(1):371-91.