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Susceptibility status of *Aedes aegypti* (L.) (Diptera: Culicidae) to temephos from three districts of Tamil Nadu, India

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ABSTRACT

Background & objectives: Dengue is the most rapidly expanding arboviral disease in India. *Aedes aegypti* is the primary vector of dengue fever. Chemical insecticides have long been used in the vector control programmes along with other control measures. However, continuous use of insecticides targeting *Ae. aegypti* may lead to development of insecticide resistance. Though resistance in *Ae. aegypti* has been reported, the mutation in *ace-1* gene associated with temephos resistance is not reported in natural populations. The present study aims to evaluate the susceptibility/resistance status of *Ae. aegypti* to temephos from three districts of Tamil Nadu.

Methods: Ae. aegypti larvae were sampled from different locations in three districts, viz., Dharmapuri, Salem and Namakkal. The standard WHO larval bioassay, biochemical assays and spotting of specific mutation (G119S) in the acetylcholinesterase gene, which is associated with organophosphate resistance, were carried out by PCR and sequencing.

Results: The results showed that larvae from Namakkal (NKL) population had an alteration in their susceptibility status (RR = 6.9 fold), while the other populations were moderately susceptible to insecticides. Biochemical assay showed increased activity for α- and β-esterase in NKL, as well as evidence of acetylcholinesterase insensitivity. G119S mutation was detected in this population with high frequency of 0.24.

Interpretation & conclusion: The high activity of esterase, mixed-function oxidase (MFO) and *ace-1* mutation frequency were closely associated with temephos resistance. Early detection of resistance alleles in natural vector population could be useful for the successful implementation of insecticide resistance management strategies. The results of this study provide baseline data on temephos resistance in *Ae. aegypti* populations.

Key words Dengue vector; G119S mutation; mutation frequency; temephos resistance

INTRODUCTION

Aedes aegypti (L.) (Diptera: Culicidae) is a primary vector of dengue and the most important vector-borne arbovirus in the world. It is the principal vector of yellow fever, chikungunya and dengue which are endemic in India and other countries. Due to the lack of vaccines for most vector-borne diseases[1], including dengue, vector control remains the best available strategy to control and prevent dengue transmission. The most widely used method for controlling immature *Ae. aegypti* is the periodic treatment of actual and potential breeding sites with chemical larvicide. Based on the data of NVBDCP, about 18,639 cases of chikungunya and 74, 454 cases of dengue with 167 deaths were reported in 2013[2]. Temephos is an organophosphate (OP) insecticide, which has been recommended to control immature dengue vectors due to its cost-effectiveness and community acceptance[3]. Earlier study from India shows that temephos is still effective as a larvicide for mosquito control[4]. Many other countries use temephos for controlling *Aedes* mosquito larvae. However, widespread use of temephos has led to the development of resistance in different countries, including Thailand, Brazil, Peru and Colombia[5-8].

Several studies have been conducted throughout the world to understand the metabolic and molecular mechanisms of resistance in mosquitoes. Biochemical assays have been used to detect the changes in metabolic enzyme activities of esterase, P450 mediated monooxygenase and acetylcholinesterase, the three major enzyme families principally involved in xenobiotic detoxification in mosquitoes[9].

Apart from metabolic resistance, target site insensitivity is also an important resistance mechanism in mosquitoes[10]. Target site insensitivity is caused by mutations that change the amino acid sequence of a target protein.

Insensitivity of acetylcholinesterase is one of the examples of target site resistance mechanisms, which is the target of OP and carbamate insecticides[11].

Ae. aegypti has been reported to show resistance to all four major classes of insecticides such as carbamates, pyrethroids, organochlorines and OPs[12]. So far, three loci have been described that present the major resistance alleles in different mosquito species. Two of these loci, *Est-2* and *Est-3*, have genes that confer resistance to OPs by overproducing esterase owing to the amplification or the up regulation of the target gene[13]. The third locus, *ace-1*, encodes an acetylcholinesterase and three different amino acid substitutions have been identified so far, resulting in reduced sensitivity to insecticides leading to single mutation in the gene: G119S in *Culex vishnui* (T), *Cx. pipiens* (L) and *Anopheles gambiae,* F290V in *Cx. pipiens* and F331W in *Cx. tritaeniorhynchus* (G)[8, 14–15].

Although, various mechanisms of insecticide resistance such as metabolic resistance [increase in metabolic capacity of detoxification enzymes, *i.e.* esterases, monooxygenases or glutathione-S-transferases (GSTs), resistance due to reduced penetration or behavioural resistance are reported in several vectors, generally it is governed by either involvement of metabolic mechanisms or alterations at target sites. Revealing the mechanism of resistance is equally important to that of monitoring resistance in mosquito vectors. Therefore, the aim of the present work was to evaluate the susceptibility status to temephos in *Ae. aegypti* populations from three districts of Tamil Nadu, India.

MATERIAL & METHODS

Study site

In the present study, *Ae. aegypti* larvae were collected from three locations, namely Dharmapuri (DPI), Salem (SLM) and Namakkal (NKL) in Tamil Nadu state during April 2011 and May 2012 (Fig. 1). These larvae were collected from indoor water containers and cement tanks from 10 houses in each location.

Colonization

The larvae were kept in dechlorinated water in a tray (30 × 20 cm) having a temperature of 27±1°C and relative humidity of 85±5%. Adult mosquitoes were maintained in a cage of 30×30×30 cm and their eggs were used for maintenance of the next generation. Larvae of the F1 and F2 generations were used for susceptibility testing. The susceptible (Sus) mosquito colony obtained from National Centre for Disease Control (NCDC),

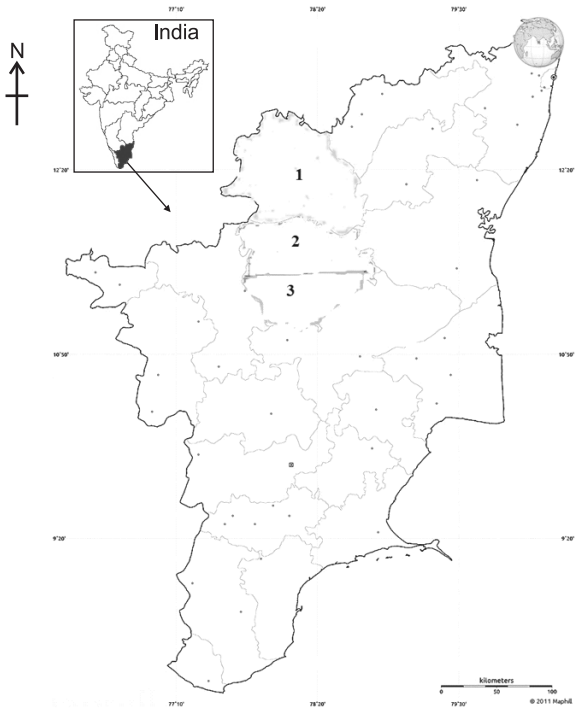

Fig. 1: Map of Tamil Nadu state showing sampling sites: 1—Dharmapuri; 2—Salem; 3—Namakkal.

Mettupalayam, Coonoor, India was used as the standard susceptible colony in all tests.

Insecticide

Commercial grade temephos insecticide as 50% emulsifiable concentrate (EC) was purchased from United Phosphorus Limited, Gujarat, India.

Larval susceptibility testing

Larval susceptibility testing was performed as described by the WHO[16] in order to estimate the lethal concentrations (LC_{50} and LC_{90}). For the larval bioassay several concentrations of the insecticide (0.001, 0.01, 0.1, 1, 2, 3, 4 and 5 ppm) were tested in triplicates. In each replicate, 25 early IV instar larvae were released. After a period of 24 h, larval mortality was recorded. Mortality was corrected by applying Abbotts formula[17]. The LC_{50} and LC_{90} were calculated through log-probit linear regression[18], using SPSS 8.0 for Windows. The larval resistance ratio (RR) was calculated by dividing the LC_{50} and LC_{90} of field population by the LC_{50} and LC_{90} obtained for susceptible colony respectively.

Biochemical assays

Biochemical tests were performed on III instar

larvae and with 1-day-old adult females from each site. The activity of esterases (α and β), acetylcholinesterase (AChE), GST and mixed-function oxidase (MFO) were evaluated according to the Brogdon method[19]. The substrate utilized in each assay included α- and β-napthyl for α- and β-Est respectively, reduced glutathione for GST, TMBZ (3,3',5,5'-tetramethylbenzidine dihydrochloride) for MFO, acetylthiocholine iodide for AChE activity and propoxur to inhibit this activity and measure insensitive AChE. Alteration in the activity of detoxification enzymes among populations were compared with susceptible colony and data were analyzed using Prism Graph Pad software (Version 6.0).

PCR and sequencing of the ace-1 gene

Genomic DNA was extracted from 30 (5-day-old) female mosquitoes by using HiPurA™ insect DNA purification kit (Himedia, Mumbai), as recommended by the manufacturer. PCR was conducted to amplify a region of the acetylcholinesterase (AChE) gene, since the enzyme encoded by this gene is the target of OPs. The amplified region encompasses the two residues that have been found mutated in strains of *Cx. pipiens, Cx. tritaeniorhynchus, Cx. vishnui*, *An. gambiae* and *An. albimanus* resistant to OPs[20]. The mutant loci are located within the gene's 5th exon. For this assay Primers (F: 5'-CGATAACGAATGGGGAACG-3 and R: 5'-TCAGAGGCTCACCGAACACA-3') were designed based on the full length AChE cDNA (GenBank accession Number: EF209048). These primers amplify a region of around 500 bp of the gene. PCR conditions were: 3 min at 94°C, followed by 35 cycles of 2 min at 94°C, 1 min at 58°C and 2 min at 72°C, with a final extension for 7 min at 72°C. PCR amplicons were purified and sequenced using an ABI 3730 Biosystem. Sequences were edited and aligned through the Bio Edit (version 7.0.4.1) software. DnaSP (version 5.10.1) software was used to estimate the nucleotide diversity.

RESULTS

Larval susceptibility

The results obtained from the bioassays with the susceptible strain and *Ae. aegypti* larvae collected from revealed that individuals from SLM populations presented an LC_{50} value of 0.429 ppm, NKL presented LC_{50} of 0.925 and DPI presented LC_{50} of 0.305 respectively. With respect to the susceptible (Sus) colony the LC_{50} (0.062 ppm) was higher in all three field populations with RR of 6.9, 14.9 and 4.9 respectively (Table 1).

Biochemical assays

The results of biochemical assay with populations from SLM, NKL, DPI and the susceptible (Sus) colony are presented in Fig. 2. Individuals from SLM and DPI showed low to moderate alteration of enzyme activity compared to the Sus colony. NKL showed higher esterase and MFO activity than Sus, respectively ($p<0.001$). Moreover, 20% of individuals from NKL displayed insensitive AChE, whereas the activity of GST was not significantly different from Sus ($p<0.05$).

Ace-1 mutation

The 480 bp fragment of the *ace-1* gene was amplified (corresponding to nucleotides 1288–1708 of AChE ORF) in 75 individuals from the three populations studied. After measuring the quality of sequences and excluding those of poor quality, 432 bp long sequences were obtained. No indels (insertions or deletions) were detected in these sequences and only a single synonymous mutation was detected (position 1344, GGC to AGC, GenBank accession Number: KJ504172) when compared to the reference sequence of *Aedes aegypti* Rockefeller strain partial *ace-1* gene for acetylcholinesterase (Sequence ID: *emb|AJ621915.1|*) as shown in Fig. 3. Additionally, no amino acid polymorphic site was found within these sequences. The nucleotide diversity (π) was 0.00376.

Table 1. Toxicity of temephos against *Aedes aegypti* late III instar larvae from susceptible (Sus) colony and field populations—Salem (SLM), Namakkal (NKL) and Dharmapuri (DPI)

Population	n^a	LC_{50} ppm (FL 95%)[b]	RR LC_{50}[c]	LC_{90} ppm (FL 95%)[b]	RR LC_{90}[c]
Sus	840	0.062 (0.054–0.072)	–	0.091 (0.082–0.121)	–
SLM	840	0.429 (0.320–0.572)	6.9	0.643 (0.585–0.711)	7
NKL	800	0.925 (0.803–1.227)	14.9	1.520 (1.361–1.963)	16.7
DPI	820	0.305 (0.203–0.520)	4.9	0.830 (0.741–1.061)	9.1

n^a = No of larvae; [b]LC_{50} and LC_{90} = Lethal concentrations (in ppm) for 50 and 90% of larvae after 24 h exposure; FL = Fiducial limits 95% estimated using SPSS 8.0 software; [c]RR = Resistance ratio, calculated by dividing LC value of field populations divided by LC value of susceptible colony.

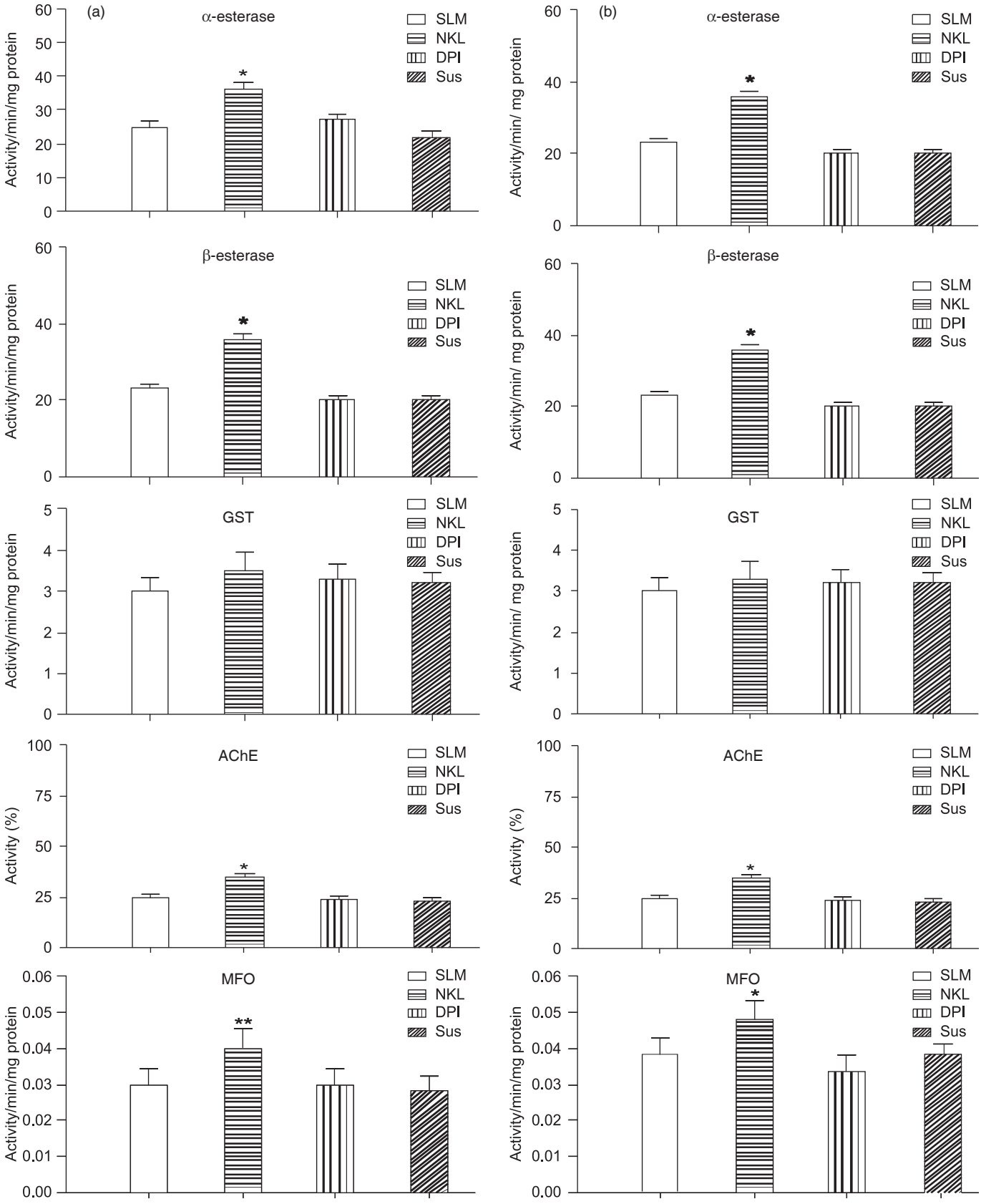

Fig. 2: Activity of α- and β-esterase, GST, AChE and MFO in larvae (a) and adult (b) *Aedes aegypti* individuals from the field Salem, Namakkal and Dharmapuri (SLM, NKL and DPI) and susceptible (Sus) colony; *indicates significant differences in enzyme activity among the groups *p<0.05; **p<0.001.

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SLM   ACACCCCAACGTTCGCTAGCCAGTGGTAGGTTTAAGAAGACGGACATCCTAACCAGGAGT 60
       T  P  Q  R  S  L  A  S  G  R  F  K  K  T  D  I  L  T  S S
NKL   ACACCCCAACGTTCGCTAGCCAGTGGTAGGTTTAAGAAGACGGACATCCTAACCAGCAGT
DPI   ACACCCCAACGTTCGCTAGCCAGTGGTAGGTTTAAGAAGACGGACATCCTAACCAGGAGT
       T  P  Q  R  S  L  A  S  G  R  F  K  K  T  D  I  L  T  G  S
Sus   ACACCCCAACGTTCGCTAGCCAGTGGTAGGTTTAAGAAGACGGACATCCTAACCGGCAGT 1347

       N  T  E  E  G  Y  Y  F  I  I  Y  Y  L  T  E  L  L  R  K  E
SLM   AATACGGAGGAAGGTTATTACTTCATAATATACTACTTGACTGAACTATTGCGGAAAGAG 120
NKL   AATACGGAGGAAGGTTATTACTTCATAATATACTACTTGACTGAACTATTGCGGAAAGAG
DPI   AATACGGAGGAAGGTTATTACTTCATAATATACTACTTGACTGAACTATTGCGGAAAGAG
       N  T  E  E  G  Y  Y  F  I  I  Y  Y  L  T  E  L  L  R  K  E
Sus   AATACGGAGGAAGGTTATTACTTCATAATATACTACTTGACTGAACTATTGCGGAAAGAG 1407

       E  G  V  T  V  S  R  E  E  F  L  Q  A  V  R  E  L  N  P  Y
SLM   GAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCAGGCCGTTAGAGAACTGAATCCTTAC 180
NKL   GAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCAGGCCGTTAGAGAACTGAATCCTTAC
DPI   GAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCAGGCCGTTAGAGAACTGAATCCTTAC
       E  G  V  T  V  S  R  E  E  F  L  Q  A  V  R  E  L  N  P  Y
Sus   GAGGGTGTCACAGTTTCACGGGAGGAGTTCTTGCAGGCCGTTAGAGAACTGAATCCTTAC 1467

       V  N  G  A  A  R  Q  A  I  V  F  E  Y  T  D  W  T  E  P  E
SLM   GTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGAGTACACCGACTGGACTGAACCGGAA 240
NKL   GTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGAGTACACCGACTGGACTGAACCGGAA
DPI   GTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGAGTACACCGACTGGACTGAACCGGAA
       V  N  G  A  A  R  Q  A  I  V  F  E  Y  T  D  W  T  E  P  E
Sus   GTGAACGGAGCCGCGAGGCAGGCTATCGTGTTCGAGTACACCGACTGGACTGAACCGGAA 1527

       N  P  N  S  N  R  D  A  L  D  K  M  V  G  D  Y  H  F  T  C
SLM   AATCCCAACAGCAATCGGGATGCATTGGACAAAATGGTCGGAGATTATCACTTCACGTGT 300
NKL   AATCCCAACAGCAATCGGGATGCATTGGACAAAATGGTCGGAGATTATCACTTCACGTGT
DPI   AATCCCAACAGCAATCGGGATGCATTGGACAAAATGGTCGGAGATTATCACTTCACGTGT
       N  P  N  S  N  R  D  A  L  D  K  M  V  G  D  Y  H  F  T  C
Sus   AATCCCAACAGCAATCGGGATGCATTGGACAAAATGGTCGGAGATTATCACTTCACGTGT 1587

       N  V  N  E  F  A  Q  R  Y  A  E  E  G  N  N  V  Y  M  Y  L
SLM   AATGTGAATGAGTTTGCCCAGCGATATGCAGAAGAAGGCAACAATGTGTACATGTATCTG 360
NKL   AATGTGAATGAGTTTGCCCAGCGATATGCAGAAGAAGGCAACAATGTGTACATGTATCTG
DPI   AATGTGAATGAGTTTGCCCAGCGATATGCAGAAGAAGGCAACAATGTGTACATGTATCTG
       N  V  N  E  F  A  Q  R  Y  A  E  E  G  N  N  V  Y  M  Y  L
Sus   AATGTGAATGAGTTTGCCCAGCGATATGCAGAAGAAGGCAACAATGTGTACATGTATCTG 1647

       Y  T  H  R  S  K  G  N  P  W  P  R  W  T  G  V  M  H  G  D
SLM   TACACTCATAGAAGCAAAGGTAACCCCTGGCCACGGTGGACCGGTGTGATGCATGGTGAC 420
NKL   TACACTCATAGAAGCAAAGGTAACCCCTGGCCACGGTGGACCGGTGTGATGCATGGTGAC
DPI   TACACTCATAGAAGCAAAGGTAACCCCTGGCCACGGTGGACCGGTGTGATGCATGGTGAC
       Y  T  H  R  S  K  G  N  P  W  P  R  W  T  G  V  M  H  G  D
Sus   TACACTCATAGAAGCAAAGGTAACCCCTGGCCACGGTGGACCGGTGTGATGCATGGTGAC 1707

       E  I  N  Y
SLM   GAGATCAATTAT 432
NKL   GAGATCAATTAT
DPI   GAGATCAATTAT
       E  I  N  Y
Sus   GAGATCAATTAT 1719
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Fig. 3: Alignment of *ace-1* proteins of *Aedes aegypti* (SLA, NKL and DPI) compared with Rockefeller strain sequence corresponding to position of 1211–1718. G119S mutation is underlined.

Four homozygous individuals for the G119S mutation were identified in NKL, with an *ace-1* frequency of 0.24.

DISCUSSION

Temephos resistance level

The results obtained in the present study showed that only NKL population exhibited high level of resistance to temephos as compared to the susceptible colony. SLM and DPI population showed moderate resistance level. Robertson *et al*[21] reported that natural variation in bioassays may exist due to environmental factors, as observed in SLM and DPI, which were moderately susceptible than NKL as compared to Sus. The high to moderate resistance status observed among populations was due to intense use of temephos against *Ae. aegypti*. Our present

study showed RR of 6.9, 14.9 and 4.9-fold resistances in NKL, SLM and DPI populations. Paeporn *et al*[22] reported 5-fold resistance in *Ae. aegypti* to temephos selection. Wirth and Georghiou[23] reports that the laboratory selected temephos resistant *Ae. aegypti* colony, presented a 4-fold increased RR. Tikar *et al*[24] observed an increase of 20.3-fold resistance *Ae. aegypti* strain (GA1), after 24 generations. Opposing our data Ponlawat *et al*[5] reported low level of resistance to temephos in *Ae. albopictus* from different areas of Thailand. The differences in the development of temephos resistance observed in all these population could be due to, among other factors, levels of pre-existent resistance to the chemicals, frequencies of genes involved in resistance, and to distinct resistance mechanisms with different mode of inheritance. All this emphasizes the importance of resistance in mosquito populations.

Detoxifying enzymes

Biochemical profile of detoxification enzyme showed increased activity for α-esterase, β-esterase and MFO, in 59 and 60% of the individuals for NKL compared to the susceptible colony's 99th percentile. *Ae. aegypti* from SLM and DPI were considered to be moderately resistant and showed no markable variation in enzyme activity compared to Sus colony. In the larval breeding site, the preexistence of temephos residues could enhance *Ae. aegypti* detoxification system, which may accelerate the selection process of resistance. The increased activity of esterase enzyme system in insects has been reported to be involved in insecticide resistance to OPs, carbamates[22, 25] and pyrethroids[26] in mosquitoes and other insects. Studies have reported that elevated MFO and esterase levels are indicators of temephos resistance in *Ae. aegypti*[27]. Similarly, this study also reports higher activity of esterase and MFO enzymes in field population than susceptible colony.

Ace-1 mutation analysis

Despite increasing reports of temephos resistance in *Ae. aegypti*, the molecular mechanisms underpinning it are not well-characterized. In several mosquito species of medical importance, such as *An. gambiae*, *Cx. pipiens* and *Cx. tritaeniorhynchus*, mutations on the acetylcholinesterase (*ace-1*) gene have been associated with OP resistance[8, 20, 28]. The widespread mutation in the acetylcholinesterase (*ace-1*) gene leading to G119S substitution is responsible for insensitivity to OP and carbamate insecticides in *An. gambiae*[29], *Culex* populations[14] and in many other species[30]. This mutation was found only in NKL, with an allele frequency of 0.24, which supports biochemical data showing changes in acetylcholinesterase sensitivity in 15% of the individuals sampled.

CONCLUSION

The data presented here suggest that temephos resistance in *Ae. aegypti* field populations may be existed through continuous exposure to temephos and other xenobiotics from the larval breeding sites. The presence of resistance alleles in *Aedes* natural populations could also poses a threat for future control strategies. In addition, the early detection of resistance alleles is essential for the successful implementation of insecticide resistance management strategies, such as rotation of insecticides with different modes of action. Moreover, the sustainability of the vector control programme should be supplemented by other environmental interventions such as cleaning of tanks, tyres and elimination of breeding sites where possible to improve the efficiency of vector control programmes.

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