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Research Article

Detection of Insecticide Resistance in *Aedes aegypti* from Dengue Endemic Areas of Northern India

Taruna Kaura[1], Seema Devi[2], Abhishek Mewara[3], Jaspreet Kaur[4], Lovleen[5], NazatInder Singh[6], Surya Kant Sharma[7], Jaspreet Takkar[8], Rakesh Sehgal[9], R K Ratho[10], Gagandeep Singh Grover[11]

[1,3,9]Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.
[2,5]Department of Zoology, Lovely Professional University, Jalandhar, Punjab, India.
[4,7]NIMR, Community Health Centre, Dhakoli, Punjab, India.
[6,11]Department of Health and Family Welfare, Chandigarh, India.
[8]Department of Physiology, Gian Sagar Medical College, Punjab, India.
[10]Department of Virology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.
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I N F O

Corresponding Author:
Gagandeep Singh Grover, Department of Health and Family Welfare, Chandigarh, India.
E-mail Id:
dr.gagangrover@gmail.com
Orcid Id:
https://orcid.org/0000-0002-2585-7030
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A B S T R A C T

Introduction: Aedes aegypti, a primary vector of arboviral diseases like dengue and chikungunya is distributed widely in the state of Punjab, India. The use of synthetic insecticides and source reduction are the most common methods used to control *Aedes* populations, although the development of insecticide resistance in *Aedes* worldwide has become a major challenge. The aim of this study was to investigate the status of resistance of *Ae. aegypti* to temephos and G119S mutation in the *Ace-1* gene which confers resistance toward it.

Method: For this, larval susceptibility to temephos was tested in five districts of Punjab at the WHO recommended concentration of 0.025 mg/L, followed by calculation of LC_{50} and LC_{90} at 24 hrs for each district using log-probit method. Based on larval resistance ratios (RR), the districts were categorised as having mosquitoes that were highly resistant, moderate or susceptible to temephos. The *Ace-1* gene was amplified and sequenced in resistant populations.

Results: We found that *Ae. aegypti* larvae were resistant to temephos in four out of five districts. Based on LC_{50} and RR ratios, *Ae. aegypti* larvae showed moderate resistance in three districts and were highly resistant in one, and susceptible to temephos in another district. However, the commonly described G119S mutation in the *Ace-1* gene was not found in any of the resistant populations.

Conclusion: In conclusion, temephos resistance is developing in *Ae. aegypti* in the state of Punjab, however, the genetic basis of the same needs further exploration in future studies. There is a need to develop an extensive database of the resistance profile of *Ae. aegypti* in order to guide the strategic plan of action for the control of *Ae. aegypti* populations.

Keywords: *Ae. aegypti*, Temephos, Susceptibility, Resistance, Mutation

Introduction

Tropical arboviruses like dengue, chikungunya and Zika virus transmitted by the vector species *Aedes* have raised public health concerns worldwide.[1,2] Due to the lack of any specific drug or vaccine for control of the dengue vectors, the vector control programs largely depend upon the use of larvicides to target the immature stages of the vector and the elimination of breeding sources. The larvicide temephos is one of the few organophosphates (OP) compounds which have been used under public health programmes since the 1980s.[3] Many studies have reported resistance of the vector populations to temephos in different countries including India for the last few years.[3–6] Periodic susceptibility testing in the vector populations can help in early detection of resistant individuals in an area which is essential for vector control programs in order to timely deploy alternative vector control strategies.

The primary target of organophosphate is acetylcholinesterase (AchE) and previous studies have demonstrated that a point mutation in the *Ace-1* gene at position 119, leading to amino acid substitution of glycine to serine (G119S, *Torpedo californica* numbering), is associated with insecticide resistance against this class of insecticide. This point mutation has been associated with resistance/ decreased sensitivity to this class of insecticide in several mosquito species.[7–9]

The state of Punjab in India has been highly endemic for dengue since 2013 with few cases of chikungunya reported every year (NCVBDC, Punjab). In recent entomological surveillance carried out in different districts in the state, a high prevalence of Ae. aegypti has been reported.[10] Under the Urban Malaria scheme, the larvicide temephos is being used to control the immatures of *Aedes*. However, there is no data available with regard to the status of insecticide resistance in *Ae. aegypti* for temephos from this region of Northern India. Thus, the aim of this study was to determine the susceptibility status of *Aedes* mosquitoes against temephos in five dengue-endemic districts of Punjab.

Materials and Method

Study Area

Surveys were carried out from June 2019 to September 2019 for the collection of larvae of *Aedes* from selected areas of five dengue-endemic districts of Punjab based on the retrospective data (NCVBDC, Punjab). Out of the five districts, three are known highly endemic districts for dengue - SAS Nagar, Ludhiana and Patiala, whereas two are moderately endemic for dengue - Fatehgarh Sahib and Hoshiarpur (Figure 1). The larvae were brought to the Insectary Unit, Department of Medical Parasitology, PGIMER, Chandigarh and identified up to species by using morphological keys.[11] Larvae of *Ae. aegypti* were then reared up to the third and fourth instar stages at room temperature of 27 ± 1 °C and 70% humidity for performing susceptibility bioassays.[12]

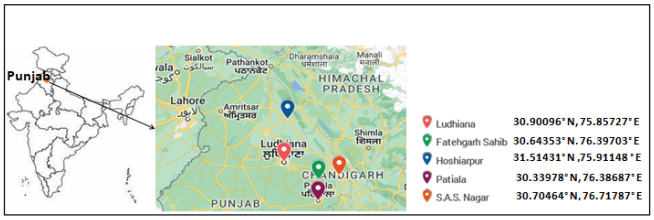

Figure 1.A Map of Punjab Showing the Districts Selected for Sampling

Temephos Bioassay

The bioassays for susceptibility to temephos were done with late III/ early IV instar larvae of *Ae. aegypti*.[11] Each bioassay was performed with standard concentrations in the WHO kits, i.e., 31.25 mg/L, 6.25 mg/L, and 1.25 mg/L, which correspond to final concentrations of 0.125 mg/L, 0.025 mg/L and 0.005 mg/L, respectively when one mL of each technical grade concentration was added to 249 mL of water.[13] For each concentration, four replicates of 25 larvae were used, while 2 replicates were used as control. The larval mortality for each concentration and control was recorded after 24 hours of exposure and larvae without movement on the water surface were considered dead. If the larval mortality was between 98 and 100%, then susceptibility is indicated, for mortality less than 98%, probable resistance is suggested, while for mortality between 90 and 97%, resistance is indicated but needs confirmation, while less than 90% mortality indicates confirmed resistance.[13] The corrected mortality was determined using Abbott's formula and results were analysed by probit (Finney 1971) to obtain LC50 and LC90.3,14,15 The larval resistance ratios (RR) were calculated by dividing the LC50 and LC90 of the field population by the LC50 and LC90 obtained for the susceptible colony (Rockefeller strain), respectively. If RR < 5, the field population is considered susceptible, for values between 5 and 10 mosquitoes, it is considered to have moderate resistance, while for RR 10, the mosquitoes are considered to be highly resistant.[13]

PCR and Sequencing of *Ace-1* Gene

Genomic DNA was extracted from exposed 4th instar larvae in a pool of n = 4 by a modified protocol of Qiagen DNA extraction kit (blood and tissue). PCR was conducted to amplify a region of the *AChE* gene since the enzyme encoded by this gene is the target of OPs. 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 used by following the protocol of Muthusamy and Shiv Kumar.[4] These primers amplified a region of around 500 bp of the gene.

Sequencing

The PCR-amplified products were purified and sequenced (Agrigenome Pvt. Limited, Bangalore, India). The sequences

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obtained were submitted to GenBank and accession numbers were obtained. The sequences were aligned with the reference sequence for *Ae. aegypti* (GenBank accession no. AJ621915.1) using the ClustalW multiple sequence alignment tool to study the known mutations.

Ethics Approval and Consent to Participate

The project was ethically approved by the IEC committee of PGIMER, Chandigarh vide letter no. PGI/IEC/2019/001118. The present study doesn't include any patient samples, thus no consent to participate was required.

Data Analysis

The populations were considered resistant, if the RR50 was over 10, moderately resistant with RR50 between 2 and 10, and susceptible if the RR50 was under 2 by following the standard WHO criteria.[13]

Results

Larval Bioassay

The larval bioassays were carried out at three concentrations, viz. 1.25 mg/L, 6.25 mg/L and 31.25 mg/L. The *Ae. aegypti* larvae from all the districts were found to be resistant at a dose of 1.25 mg/L, whereas they were susceptible at a dose of 31.25 mg/L, respectively (Table 1).

The results obtained at 0.025 mg/L which is the diagnostic dose recommended by WHO revealed that larvae of *Ae. aegypti* collected from four districts, viz. SAS Nagar, Ludhiana, Hoshiarpur and Fatehgarh Sahib showed confirmed resistance as mortality was less than 90%, whereas in the district of Patiala, less than 98% mortality suggested probable resistance (Table 2).

The susceptibility tests show that the LC50 value of Ae. aegypti larvae collected was 0.068 from district SAS Nagar, 0.039 from district Ludhiana, 0.017 from district Patiala, 0.032 from district Hoshiarpur, and 0.024 from district Fatehgarh Sahib (Table 2). The LC50 was found to be high in all the districts studied as compared to the susceptible colony (Rockefeller strain) which had an LC50 value of 0.0042 ppm. The RR values indicated high resistance to temephos in district SAS Nagar and moderate resistance in three districts of Hoshiarpur, Ludhiana, and Fatehgarh Sahib, whereas the population from district Patiala was found to be susceptible (Table 2).

Table 1.Insecticide Susceptibility Status of the Larvae of *Ae. aegypti* against Temephos in Different Districts of Punjab

District	No. of *Aedes* Larvae Exposed	% Mortality for Control	% Mortality for Control	% Mortality per Test for Exposed Larvae	Susceptibility Status
	Test	Control			
SAS Nagar	100	50	0	24	CR
Ludhiana	100	50	0	44	CR
Patiala	100	50	0	90	RS
Hoshiarpur	100	50	0	55	CR
Fatehgarh Sahib	100	50	0	75	CR

CR: Confirm resistance, RS: Resistance suggested

Table 2.Lethal Concentrations of Temephos for 50% (LC50) and 90% (LC90) Mortality of *Ae. aegypti* in Different Districts of Punjab

District	LC50 (Confidence Limits)	RR LC50	LC90 (Confidence Limits)	RR LC90
SAS Nagar	0.068 (0.037–0.050)	16.1	0.132 (0.125–0.1875)	4.64
Ludhiana	0.027 (0.025–0.037)	6.4	0.039 (0.087–0.125)	1.37
Patiala	0.017 (0.0125–0.018)	4.0	0.026 (0.025–0.0375)	0.91
Hoshiarpur	0.032 (0.02–0.0225)	7.6	0.094 (0.087–0.125)	3.30
Fatehgarh Sahib	0.024 (0.0125–0.018)	5.7	0.034 (0.0437–0.0625)	1.19

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Ace-1 Mutation Assay

The 480 bp fragment of the *Ace-1* gene was amplified (corresponding to nucleotides 1288–1708 of AChE ORF) from the resistant larvae of Ae. aegypti (Figure 2).

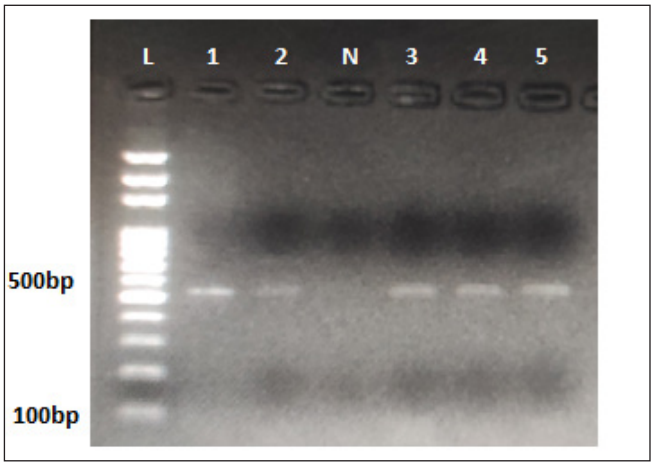

Figure 2.Gel Run Showing Amplification of *Ace-1* Gene from Susceptible and Resistant Larvae of *Ae. aegypti*

The sequences obtained from mosquitoes of all the districts were studied for the most common mutation due to organophosphate at position 1344, i.e., GGC to AGC leading to G119S substitution. However, no such substitution was observed in any of the sequences (Figure 3). Moreover, no indels (insertions or deletions) were detected in the sequences from all five districts when compared to the reference sequence of Ae. aegypti Rockefeller strain partial *Ace-1* gene for acetylcholinesterase (AJ621915.1) and KJ504172 (Figure 3). A reference sequence was submitted from each district and accession numbers obtained (SAS Nagar - MT993470, Ludhiana - MW690112, Patiala - MW380118, Hoshiarpur - MW380119, and Fatehgarh Sahib - MW380117).

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CLUSTAL 2.1 Multiple Sequence Alignment


M_              TTTAAGAAGACGGACATCCTAACCAGCAGT
1_              TTTAAGAAGACGGACATCCTAACCGGCAGT
2_              TTTAAGAAGACGGACATCCTAACCGGCAGT
3_              TTTAAGAAGACGGACATCCTAACCGGCAGT
4_              TTTAAGAAGACGGACATCCTAACCGGCAGT
5_              TTTAAGAAGACGGACATCCTAACCGGCAGT
RF_             TTTAAGAAGACGGACATCCTAACCGGCAGT
                ************************* *****
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Figure 3.Multiple Sequence Alignment of 11 *Ace-1* Sequences Obtained from Five Districts with the Susceptible Rockefeller Strain (AJ621915.1) and Resistant KJ504172 by Using Clustal W

Discussion

A very important obstacle for vector control programmes is the development of resistance to larvicides or insecticides being used. Temephos is one of the most common organophosphates used against the larvae of *Ae. aegypti* worldwide.[16] It is also being used under the National Centre for Vector Borne Disease Control (NCVBDC) of India and is also being implemented in the state of Punjab. Studies on temephos resistance in *Aedes* have been undertaken from different parts of the country[7,16,17] but no prior studies have been carried out in the present study area. As reported in an earlier surveillance-based study, the dominant vector species of *Aedes* in different districts of the state of Punjab is *Ae. Aegypti*.[10] Thus, in this study, the larval susceptibility tests for temephos were performed on larvae of *Ae. aegypti* collected from field areas from specific sites selected in five dengue-endemic districts of Punjab. From the larval bioassays, four out of five districts recorded confirmed resistance to temephos. In a similar study carried out in different areas of Delhi on field-collected larvae of *Ae. aegypti*, development of resistance towards temephos has been reported,[3] although a few studies from India have also reported that *Aedes* are still susceptible to temephos.[18,19] In another study carried out in three districts of Tamil Nadu, not only resistance to temephos with high LC50 value but increased activity for α- and β-esterase and G119S mutation was also recorded with a frequency of 0.24.[4] The authors also suggested that the increased use of temephos may be responsible for the development of resistant populations.

In our study, based on RR ratios, moderate resistance was reported among mosquito populations from three districts, while the population of one district recorded high resistance. Our findings are in concurrence with earlier studies carried out in different districts of Tamil Nadu, urban areas of Delhi, Mumbai, Jodhpur, Chennai, and Coimbatore where high RR ratios were observed indicating resistance towards temephos.[4,20] Moreover, studies carried out in different parts of the world such as Japan, Saudi Arabia, Brazil, and Costa Rica have also reported moderate to high resistance of *Ae. aegypti* towards temephos.[21–27] Despite increasing reports of temephos resistance in *Ae. aegypti* in the world, only a few studies have been carried out in India to screen the populations for the most common G119S mutation.[4] In a study carried out in Colombia detected no amino acid change in the *Ace-1* gene from the resistant strain and it has also been observed that target site mutation has a minor role in conferring temephos resistance to *Aedes*.[7,28] Moreover, it has also been hypothesised that this G119S mutation is most unlikely to occur frequently in *Ae. aegypti*.[29] Therefore, other genetic mechanisms of the development of resistance to temephos must be explored in future studies.

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Conclusion

In conclusion, the present study highlights the importance of detecting insecticide resistance in vectors. This is the first such surveillance study to infer the insecticide susceptibility status of the primary vector of dengue in this part of India. The control of the *Aedes* vector presently relies only on the use of insecticides and habitat management by source reduction, however, due to the rapid spread of insecticide resistance, it is essential to generate area-specific data on the prevalence and development of resistance in the vector population. Thus, there is a need to increase efforts for rapid and timely screening of larval populations of *Aedes* for resistance to temephos which is important for guiding the development of policies for vector control programs.

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Conflict of Interest: None

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