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Research Article

Detection of Insecticide Susceptibility Status and KDR Mutation in Field-Collected *Aedes Aegypti* from Different Districts of Punjab, India

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A B S T R A C T

Background: As the state of Punjab has become endemic for dengue, this study was planned to determine the susceptibility status for two different classes of adulticides and the VGSC gene polymorphism in domain II of *Aedes aegypti*.

Methodology: Adult bioassays were performed with pyrethrum, deltamethrin and malathion as per the WHO protocol. AS-PCR and sequencing of VGSC gene were carried out to detect V1016G and Kdr (Knockdown resistance) mutations.

Results: Ae. aegypti from the districts of Ludhiana and Patiala were found to be resistant to pyrethrum. The V/ V genotype frequency was found to be higher in all districts. Three known polymorphisms in VGSC gene were not recorded but one synonymous and non-synonymous mutation was found. Group B intron was found in domain II of the VGSC gene. For deltamethrin and malathion, 100% susceptibility was recorded for all four districts.

Conclusion: Thus, the present findings indicate 100% susceptibility towards deltamethrin and malathion whereas, moderate resistance towards pyrethrum was recorded. Therefore, more studies should be planned to study polymorphisms in the various domains of the VGSC gene.

Keywords: Susceptible, Resistance, VGSC, Mutation, Adulticides, Polymorphism

Introduction

Diseases transmitted by mosquitoes as vectors are of major public health importance worldwide and cause significant morbidity and mortality globally.[1] Malaria and dengue, mosquito-borne diseases, are accountable for more than 16% of the worldwide burden of all infectious diseases.[2] Recent studies have reported that the incidence of dengue cases has gone up dramatically around the world and approximately more than 390 million people get infected globally whereas, India alone contributes 34% of cases to it.[2,3] It is one of the most significant vector-borne viral infections in various parts of the world and has been reported from more than 100 countries including India where it is known to be endemic for over 2 centuries.[4,6]

The state of Punjab in India has been highly endemic for dengue fever for the last few years and large numbers of confirmed dengue cases have been recorded. Due to a lack of specific drugs and effective vaccines against dengue, vector control remains to be the mainstay of control measures towards the prevention of disease.[7,8]

Pyrethroids and organophosphates are the major classes of insecticides recommended for vector control under the National Centre for Vector Borne Diseases Control (NCVBDC) of the Government of India. Thus, the same are being used in the state of Punjab for the control of the vector of Dengue. In spite of the proper usage of insecticides, the development of resistance against pyrethroids, deltamethrin and malathion has been reported.[9,11] This is possibly by increasing the activity of insecticide detoxifying enzymes and target site insensitivity due to single or multiple-site mutations.[12] The most common insecticide gene mutation detected is the knockdown mutation (kdr) on the para Voltage-Gated Sodium Channel gene (VGSC). Several point mutation sites on the VGSC gene have been documented which confer resistance to pyrethroids and DDT in *Aedes aegypti*, the vector species of dengue.[13,15]

In a preliminary study carried out in different districts of Punjab to record the prevalence of dengue vectors, *Ae. aegypti* was reported to be the most prevalent species.[16] However, to date no study has been carried out on the effectiveness of insecticides which are being used for dengue vector control in Punjab. Thus, the present study is planned to determine the susceptibility status of *Ae. aegypti* against deltamethrin, malathion and pyrethroid as well as polymorphism in the VGSC gene in four dengue endemic districts of Punjab, India.

Materials and Method

Study Area

The study was carried out in different blocks of four dengue endemic districts of Punjab viz. Mohali, Fatehgarh Sahib, Patiala and Ludhiana from June 2019 to September 2019 (Figure 1). These districts were selected on the basis of the dengue cases reported in the last five years by the NCVBDC, Punjab, India.

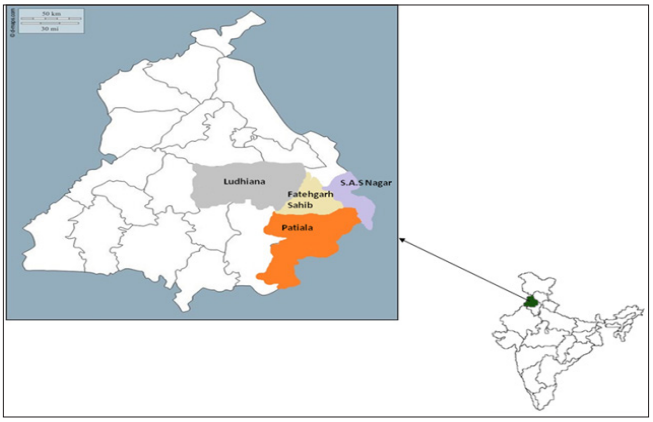

Figure 1.Study Areas in Punjab, India

Larval Collection, Rearing and Identification

From the selected study areas, the immature stages of Aedes were collected and reared up to the adult stage at standard conditions (Temperature - 25 ± 2 °C; Relative humidity-70-80%) in the Insectary of the Department of Medical Parasitology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh. The adults were identified by following the standard morphological keys.[17,19] The identified *Ae. aegypti* adults were subsequently used for insecticide susceptibility bioassays.

Adult Susceptibility Bioassays

Two-to-three-day-old F0 (adults developed after rearing collected larvae) female *Ae. aegypti* (unfed) were used for the insecticide susceptibility bioassay as per the standard WHO protocol.[20] The insecticides tested were pyrethrum 2% extract, deltamethrin 2.5%, and malathion 25% WDP (Hafed India).

Fogging/ Hand Spray Bioassay for Pyrethrum

For the bioassay, the WHO recommended concentration of pyrethrum was prepared by mixing 50 ml technical-grade insecticide solution with 950 ml kerosene oil. For the test, a hand-held spray pump (Ganesh pump) and a hand-fogging machine were used for indoor spray and for fogging the insecticide mixture of 1.0 L.[21,22] Cages (1 × 1 feet) covered with a mosquito net (1 mm mesh) were used to house groups of 25 adult female mosquitoes. Three cages each containing 25 mosquitoes were deployed at each test room separately. The formulation was diluted as 1:19 and applied using a hand fogger at room temperature of 27 ± 1 ºC and 70% humidity. In each trial, the knockdown effect was measured by counting the number of knocked-down and dead females 1 hr post-treatment (1 hKD) and after 24 hours.[23] 11 mosquitoes from a particular cage

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were sucked up into a sucking tube and stored for further molecular analysis. The susceptibility and resistance status of the mosquitoes was categorised according to the WHO guideline-mortality ≥ 97% indicated susceptiblility, ≤ 80% indicated confirmed resistance, and between 80-97% indicated possible resistance.[20]

CDC Bottle Bioassay for Deltamethrin and Malathion

The CDC bottle bioassay was performed following the standard protocol.[18,24] The stock and working solutions of deltamethrin (2.5%) and malathion (25%) were prepared as per the CDC guidelines. All 250 ml glass bottles were coated with respective insecticide-prepared solutions. For the bioassay, two bottles were treated for each insecticide and one as control (acetone only). In each coated bottle, 15-20 adult female mosquitoes were kept for 1 hr. After the exposure, the mosquitoes were transferred to paper-holding cups covered with a net for up to 24 hrs. The mortality was recorded after every 15 minutes up to a maximum of 2 hours and 24 hours. Mortality was considered to have occurred if the mosquito could not stand or fly.

The mosquitoes after the adult bioassays (both live and dead) were stored at-20 °C till further molecular analysis.

V1016G Allele-Specific PCR

All the mosquitoes viz. alive and dead, from each experiment, were processed for genotyping. Genomic DNA was extracted from individual mosquitoes by using Qiagen blood & tissue kit modified protocol with minor modifications i.e. time period of incubating the samples after adding proteinase K and lysis buffer was increased up to 4 hours, and in the last step, DNA was eluted in 25 µl AE. The PCR was performed by using primers viz. FP-5'-AC CGACAAATTGTTTCCC-3';RPG5'-GCGGGCAGGGCGGCGGG GGCGGGGCCAGCAAGGCTAAGAAAAGGTTAACTC-3';RPV 5'-GCGGGCAGCAAGGCTAAGAAAAGGTTAATTA-3'.[25] The cycling conditions were the initial step of denaturation at 94 ºC for 5 mins, followed by 35 cycles of amplification at 94 ºC for 1 min, 58 ºC for 1 min, and 72 ºC for 1 min, and final extension at 72 ºC for 10 mins.

PCR for the Sequencing of Domain II of VGSC Gene

The amplification was performed by using specific primers viz. FP: - ATTGTATGCTTGTGGGTGAC and RP: - AACTGAGATGATTGTGCTGC which were designed to amplify a fragment of domain II in the sodium channel gene (Gen Bank Accession No. NC_035109) approximately 536 bp that encompasses the known mutations A1007G, I1011M, and V1016G. For this, PCR was performed with an initial step of denaturation at 94 ºC for 5 mins, followed by 35 cycles of amplification at 94 ºC for 1 min, 60 ºC for 1 min, and 72 ºC for 1 min, and final extension at 72 ºC for 10 mins.

The PCR-amplified products were purified and sequenced (Agrigenome Pvt. Limited, Bangalore, India). The sequences obtained were submitted to GenBank and the accession numbers were obtained.

Analysis of Gene Sequences

For adult bioassays, the observed mortality was calculated by the formula: observed mortality (%) = (Total no. of dead mosquitoes/Total mosquitoes exposed) x 100. For adult bioassays, resistant/ susceptibility status was defined according to the WHO recommendations.[20] The analysis of genotype and allele frequencies among the districts was performed using the Pearson chi-square test with a $p < 0.05$ significance level and the Hardy-Weinberg equilibrium (HWE).[26] The sequences were aligned with the reference sequence for *Ae. aegypti* (GenBank accession no. NC_035109) using an online multiple sequence alignment tool (Clustal W).

Institutional Review Board Statement

This study was carried out according to Institutional Ethical guidelines and was approved by the Institutional Ethics Committee of PGIMER, Chandigarh vide no. PGI/IEC/2019/001118.

Result

Adult Bioassay

The adult susceptibility bioassay test results with pyrethrum fogging for *Ae. aegypti* revealed that specimens from only one district Fatehgarh Sahib exhibited 88% mortality while those from district Ludhiana showed probable resistance with 92% mortality. The specimens from the other two districts were found to be 100% susceptible. With pyrethrum mist, specimens from the district of Ludhiana and Patiala were found to be resistant with 84% and 88% mortality respectively and there was probable resistance in those from the district of Fatehgarh Sahib with 92% mortality (Table 1).

Allele-Specific PCR for V1016G Mutation

A total of 558 susceptible and 42 resistant adult female mosquitoes from all the study areas were genotyped for V1016G mutation. The allele frequencies for Val/ Val, Val/ Gly and Gly/ Gly were also calculated and Val/ Val was found to be high in all districts (Table 2). The frequency of heterozygotes among resistant specimens was observed to be more in the district of Ludhiana followed by Fatehgarh Sahib. All the districts were found to be in concordance with HWE and $p \leq 0.05$ was considered to be significant.

PCR for Sequencing of VGSC Gene

To study the kdr mutations, an approximate 500 bp fragment of domain II of the VGSC gene was amplified from 58 adult mosquitoes, of which 42 were resistant and

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16 were susceptible. The sequences obtained were studied for the already known common mutations associated with pyrethroid resistance viz. A1007G, I1011M, and V1016G but no such substitution was observed in any of the sequences from resistant mosquitoes. Moreover, no indels (insertions or deletions) were detected in these sequences when compared to the reference sequence (LC485541) of *Ae. aegypti* (Figure 2). In addition, only one synonymous mutation A1007A (GCC-GCT) and one non-synonymous mutation L1006S (TTG-TCG) in domain II were recorded from the sequences of resistant *Ae. aegypti* mosquito from all districts (Figure 2). In all the sequences, the group B intron was found in domain II of VGSC gene.

The presence of non-synonymous mutations in the concerned gene position indicated that regularly more such studies should be planned by screening large populations with various domains of VGSC to find out the role of this mutation in pyrethroid resistance.

CDC Bottle Bioassay

When lab-reared F1 generation adults of *Ae. aegypti* were exposed to discriminating dosages of malathion and deltamethrin, 100% mortality was recorded from all four districts (Table 3) indicating that this species was highly susceptible to these two insecticides.

Table 1.Results of Adult Susceptibility Bioassay in *Aedes aegypti* against 2% Pyrethrum in Four Districts of Punjab

Name of District	Type of Test	Number of Mosquitoes Exposed	Number of Mosquitoes Knockdown/ Dead after 30 Min	Number of Mosquitoes Knockdown/ Dead after 60 Min	Number of Mosquitoes Dead after 24 Hrs.	% Mortality	Susceptibility Status
SAS Nagar	Fogging	25	7	25	25	100	Susceptible
	Mist	25	6	13	25	100	Susceptible
	Control	25	0	0	0	0	-
Fatehgarh Sahib	Fogging	25	22	22	22	88	Resistance
	Mist	25	23	23	23	92	Probable resistance
	Control	25	0	0	0	0	-
Ludhiana	Fogging	25	23	23	23	92	Probable resistance
	Mist	25	12	21	21	84	Resistance
	Control	25	0	0	0	0	-
Patiala	Fogging	25	14	25	25	100	Susceptible
	Mist	25	0	22	22	88	Resistance
	Control	25	0	0	0	0	-

Table 2.Detection of V1016G Mutation in VGSC Gene of *Aedes aegypti* from Four Districts of Punjab

Name of District	Type of Test	N	Val/ Val	Val/ Gly	Gly/ Gly	Chi-Square (χ^2)
SAS Nagar	Pyrethrum thermal fogging	75	1	-	-	-
	Pyrethrum mist	75	0.9867	0.026	0.0133	0.0137
Fatehgarh Sahib	Pyrethrum thermal fogging	75	0.98	0.0392	0.02	0.0312
	Pyrethrum mist	75	0.9867	0.026	0.0133	0.0137
Ludhiana	Pyrethrum thermal fogging	75	0.993	0.0133	0.0067	0.0034
	Pyrethrum thermal mist	75	0.9733	0.089	0.026	0.050
Patiala	Pyrethrum thermal fogging	75	1	-	-	-
	Pyrethrum thermal mist	75	0.9867	0.0133	-	0.0137

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Table 3.Insecticide Susceptibility Status of *Ae. aegypti* against 2% Deltamethrin and 25% Malathion inFour Districts of Punjab

District	Insecticide	No. of Mosquitoes Exposed Per Test		No. of Mosquitoes Dead Per Test		Corrected Mortality (%)
		Test	Control	Test	Control	
SAS Nagar	Deltamethrin	15	15	15	0	100
	Malathion	15	15	15	0	100
Fatehgarh Sahib	Deltamethrin	15	15	15	0	100
	Malathion	15	15	15	0	100
Ludhiana	Deltamethrin	15	15	15	0	100
	Malathion	15	15	15	0	100
Patiala	Deltamethrin	15	15	15	0	100
	Malathion	15	15	15	0	100

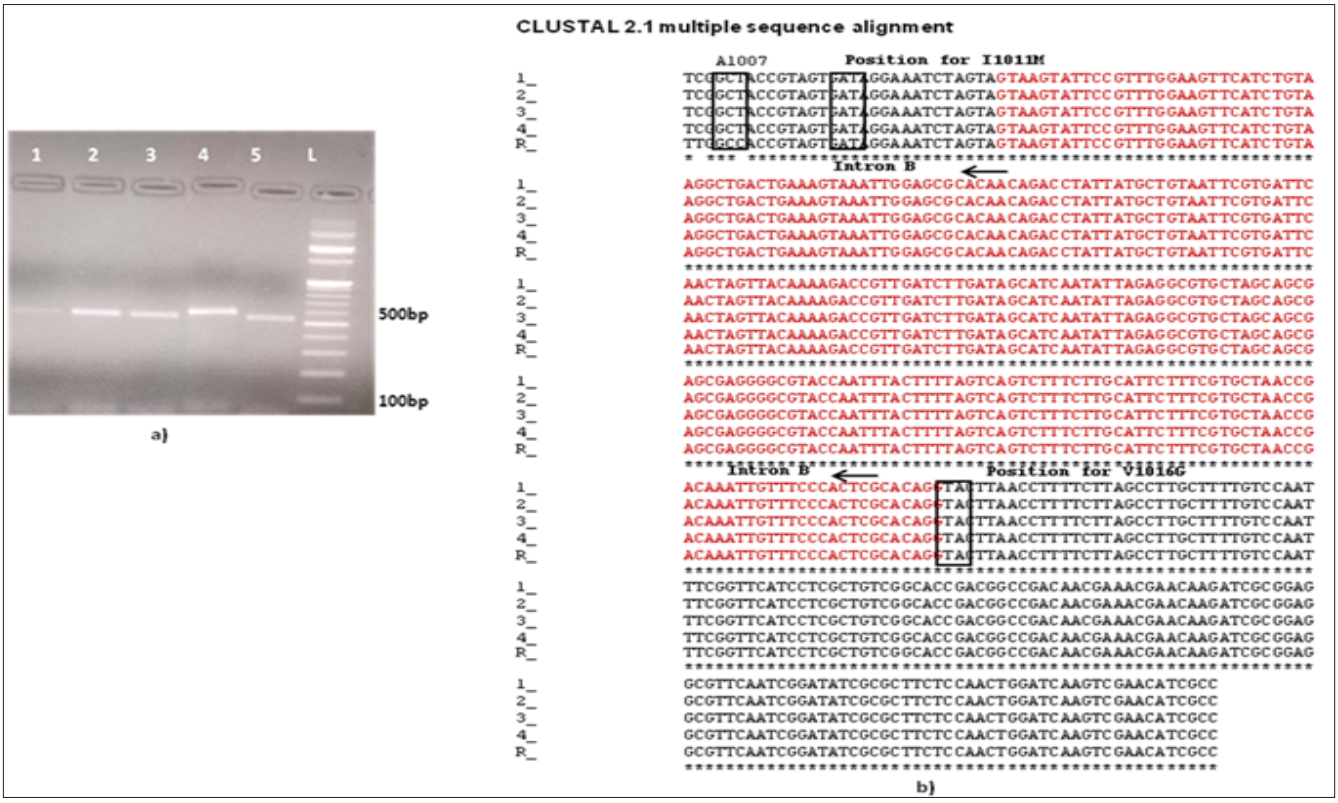

Figure 2.Gel Run showing (a) Amplification of VGSC Gene Domain II from Susceptible and Resistant Adults for Pyrethroid of Aedes aegypti and (b) Multiple Sequence Alignment of Domain II of the VGSC Gene Sequences of Resistant Mosquitoes obtained from Four Districts of Punjab with Reference Sequence (R)

Discussion

Dengue is a vector-borne disease which is highly endemic in the tropical world. One of the most effective approaches to control this disease is to control the population of its principal vector Aedes in order to prevent its transmission.[27] As per the guidelines of NCVBDC, India for integrated vector management to control dengue, the adulticides being recommended are pyrethroids and malathion. Several studies have illustrated that pyrethroids are effective for the control of insects and it has minimal health risks to people.[28,29] However, due to the widespread use of insecticides for vector control, the resistance developed for insecticides as well as the adverse effects of these on the environment and human health constituted the raised concerns.[14] Moreover, regular monitoring of vector susceptibility to the commonly used insecticides is a prerequisite for national programmes as this information is important to devise strategies for vector control.[27]

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In the present study, the susceptibility status of *Ae. aegypti* for the adulticides being used for the control of dengue in Punjab, India, was determined. In the insecticide susceptibility bioassays, 100% susceptibility was recorded for deltamethrin (2%) and malathion (25%) whereas, for 2% pyrethrum extract, resistance was found in only two districts. The frequency of homozygotes for Val/ Val, Gly/ Gly and heterozygotes Val/ Gly were calculated to detect V1016G mutation. It was found that the frequency of Val/ Val homozygotes was higher in all districts while the frequency of the heterozygote Val/ Gly was high in those two districts which showed resistance in insecticide susceptibility bioassays.

Mutation in the VGSC gene is the mechanism by which Aedes mosquitoes show resistance to pyrethroids. Therefore, studying polymorphisms in marker genes such as VGSC is an additional method to understand potential mechanisms by which the insects develop resistance.[30] We amplified and sequenced domain II of the VGSC gene to look for the most common mutations associated with pyrethroid resistance. Heterozygotes for V1016G mutation were found in two districts. Although no polymorphism was recorded for the same after sequencing, a non-synonymous mutation L1006S was detected in both susceptible and resistant mosquitoes. More such studies are required to explore the role of this mutation in pyrethroid resistance. These findings are found to be in concurrence with previous studies where resistance to pyrethrum has been reported but no VGSC gene mutation was detected in spite of a high number of heterozygote genotypes.[25,31,32]

In recent studies, it has been documented that the VGSC gene exons 20 and 21 are separated by an intron which is polymorphic based on their base pair length and is classified as intron A and B. It has been reported that polymorphic sites S989P, V1016G, and D1763Y are linked with intron A while the F1534C mutation is linked with the intron B.[14,15] We also identified a similar type of intron in our sequences and it was found to be intron B.

Conclusion

This is the first study to determine the efficacy of adulticides being used in dengue-endemic districts of the state of Punjab for the control of Aedes. Resistance was recorded in two districts for pyrethrum and the frequency of Val/ Gly heterozygotes was also high though no kdr mutation was found. Therefore, more studies are required to study polymorphisms in various domains of the VGSC gene to determine its efficacy for the control of *Ae. aegypti*.

Supplementary Information

Accession Numbers

GeneBank accession Number: Ludhiana-MW690112, Patiala-MW654198, FG Sahib-MW602461.

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Conflict of Interest Statement

The authors declare that they have no known competing interests.

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