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Research Article

Insecticide Resistance and Occurrence of L1014F *kdr* Mutation in Wild *Culex quinquefasciatus* Populations from sub-Himalayan Region of West Bengal, India

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A B S T R A C T

Introduction: Culex quinquefasciatus has been known to be the prime vector of lymphatic filariasis in the sub-Himalayan regions of West Bengal. For the control of this vector species, synthetic pyrethroids are frequently used. These vector populations eventually develop insecticide resistance due to recurrent and rampant application of these synthetic pyrethroids.

Method: In this study, wild *Cx. quinquefasciatus* larvae are collected from various districts of the sub-Himalayan region of West Bengal and insecticide susceptibility status against 0.025% deltamethrin, 0.025% lambda-cyhalothrin, 0.25% permethrin, and 4% DDT was evaluated. The allele-specific PCR assay was performed for the detection of *kdr* mutation. The presence of the L1014F *kdr* mutation in Lambda-cypermethrin-resistant *Cx. quinquefasciatus* populations has been further confirmed by *vgsc* gene sequencing.

Result: The study revealed that *Cx. quinquefasciatus* populations from this region are highly resistant against synthetic pyrethroids and possess L1014F *kdr* mutation. The presence of *kdr* mutation in *vgsc* gene was a primary mode of insecticide resistance mechanism in *Cx. quinquefasciatus* populations.

Conclusion: The present study shows occurrence L1014F *kdr* mutation in synthetic pyrethroid resistant *Cx. quinquefasciatus* populations of the sub-Himalayan region of West Bengal. The study offers current insecticide resistance profile of *Cx. quinquefasciatus* populations from this region. Finding the vector populations' resistance mechanisms is essential to making the required implementations easier and reducing mistakes in vector control techniques.

Keywords: Lymphatic Filariasis, Knock Down Resistance Mutation, L1014F, *Culex quinquefasciatus*

Introduction

Culex quinquefasciatus Say, 1832, is one of the most prevalent mosquito species in Southeast Asia and is known to be the primary vector for numerous diseases, including West Nile Fever, Saint Louis Encephalitis, and lymphatic filariasis.[1] Recent studies also point to its potential involvement in the spread of the avian malaria-causing *Plasmodium relictum* and the Zika virus.[2,3] The parasitic infection of lymphatic filariasis still poses a severe threat to over 882 million people in 44 countries, and to impede its spread, preventive treatment is necessary.[4] India has 41% of the world's lymphatic filariasis cases and among them, 99.4% of infections are caused by *Cx. quinquefasciatus*.[5] Filariasis is endemic in 339 districts throughout 20 states and Union territories in India, potentially impacting over 650 million people.[6] Currently, West Bengal has 12 of its 23 districts endemic for filariasis, which adds to the country's current burden of lymphatic filariasis.[7]

Although there are other complementary preventative strategies, such as mass drug administration (MDA) and therapies for lymphatic filariasis, in India, controlling mosquito vectors using synthetic insecticides is consistently the main strategy, particularly in places where the disease is endemic. Chemical insecticides, especially synthetic pyrethroids, are therefore widely employed to reduce vector populations because of their rapid knockdown effect, low mammalian toxicity and less persistence in the environment.[8,9] In different parts of India, like West Bengal, Assam, Uttar Pradesh and Rajasthan this *Culex* species became highly resistant to synthetic pyrethroids.[10–13] One important factor contributing to this resistance was target site insensitivity. Target site resistance is a primary mode of insecticide resistance mechanism in mosquito species in which the targeted site of the insecticides is genetically modified; thus, it is no longer capable of interacting with neurotoxins and consequently eliminating the insecticidal effects. The Voltage-Gated Sodium Channel (*vgsc*) gene is targeted by DDT and synthetic pyrethroids, which changes its gating characteristics and ultimately cause a knockdown effect in mosquito vectors. Present in neuronal axons, it is a transmembrane protein with four homologous domains (I–IV) and each domain consists of six transmembrane segments (S1–S6) arranged in a circular pattern around a central ion pore. Insecticide resistance by point mutations in the transcript of these domains is commonly known as *kdr* (knockdown resistance) mutation.[14] The most frequent *kdr* mutation in *Cx. quinquefasciatus* is found in the 1014 position of the IIS6 region of the *vgsc* gene, with a change from leucine to phenylalanine (L1014F). This mutation has been found to provide strong resistance against synthetic pyrethroids and DDT and is found in many countries like Mexico, Nigeria, USA, Colombia, Sri Lanka and India.[15–20] Therefore, given the current circumstances, it is crucial to do a thorough investigation of the wild *Cx. quinquefasciatus* populations susceptibility status against commonly used synthetic pyrethroids and to screen for *kdr* mutations.

Materials and Method

Ethics Statement

There were no human trials or higher vertebrates involved in this work, thus the Institutional Animal Ethics Committee (IAEC), Department of Zoology, University of North Bengal (Regn. no. 840/GO/Re/S/04/CPCSEA), waived the need for ethics approval. The use of rats for blood feeding was also authorised by the IAEC (approval number IAEC/NBU/2022/22). All procedures were completed in accordance with relevant regulations and directions from the IAEC.

Collection of Mosquito Samples

Five districts namely Alipurduar, Cooch Behar, Jalpaiguri, Darjeeling and North Dinajpur from the sub-Himalayan region of West Bengal were screened for the collection of *Cx. quinquefasciatus* larvae. *Culex* mosquitoes were sampled from October to January during the years 2021 to 2023. Samples were collected in a 500 mL beaker from two different sites in each district. In the laboratory, larvae and pupae were reared up to F1 generation in a controlled environment (temperature 27˚C ± 2; relative humidity 75% ± 10%). A standard identification key was used to identify the larvae up to the species level.[21] F1 generation populations were used for adult susceptibility bioassay and *kdr* genotyping screening.

Insecticide Susceptibility Bioassay

Insecticide-impregnated papers (4% DDT, 0.025% Deltamethrin, 0.025% Lambda-cyhalothrin, 0.25% Permethrin) were prepared in the laboratory. 2–3 days old non-blood feed female mosquito larvae were used for adult susceptibility bioassay following standard WHO protocol.[22]

Isolation of Genomic DNA and AS PCR Assay

The genomic DNA of individual mosquitoes was collected following the High Salt protocol.[23] Quantification and purity checks of the isolated DNA were checked by using the SPECTRO star Nano fast scanning UV-visible Microplate Reader (Make-BMG Labtech, Germany). DNA with an OD_{260}/OD_{280} value of 1.8 to 2 was further selected for *kdr* mutation analysis. Allele Specific PCR (AS PCR) assay was carried out for the detection of L1014F *kdr* mutation at the *vgsc* gene following the standard protocol.[12] Four primers namely Cgd1 (5-GTGGAACTTCACCGACTTC-3),Cgd2 (5-GCAAGGCTAAGAAAAGGTTAAG-3), Cgd3 (5-CCACCGTAGTGATAGGAAATTTA-3) and Cgd4 (5-CCACCGTAGTGATAG GAAATTTT-3) were used for

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the reaction. Cgd1 and Cgd2 primers were used in one reaction to amplify the IIS6 region of *vgsc* gene containing *kdr* mutation site. Cgd3 and Cgd4 primers were allele-specific primers and used in different reactions to amplify knockdown resistant (Cgd3) and knock down susceptible (Cgd4) alleles. PCR conditions included initial denaturation at 95 ˚C for 15 min, followed by 30 cycles at 94 ˚C for 45 seconds, 49 ˚C for 45 secs, 72 ˚C for 45 secs, and a final extension of 10 mins at 72 ˚C. The amplified fragments were visualised under UV Transluminator by using 3% agarose gels with Ethidium bromide staining. Cgd 1–2 primers provided a characteristic product size of 540 base pair and Cgd3 and Cgd 4 primers amplified bands at 380 base pair respectively.[24]

Sequencing of *vgsc* Gene

A total of eight pyrethroid-resistant mosquitoes including all study sites were subject to partial sequencing of the IIS6 region of vgsc gene and the sequence has been submitted in GenBank (Accession no: PP597294). Sequencing was performed at Barcode Biosciences Pvt. Ltd. Bengaluru, Karnataka-560077 and aligned with other *vgsc* gene sequences of the GenBank database (Accession no: KM377241, KM377242, FJ182226, FJ970025) viz CLUSTALW. Sequences were analysed and plotted via Bioedit (Version 7.2.5).

Data Analysis

Mosquito populations were classified as resistant (less than 90% death), probable resistant (90–98% mortality), and susceptible (98–100% mortality) based on the percentage of mortality against each insecticide.[25] Abbott's formula was used to adjust the data when the control setup showed a mortality rate of greater than 10%. KDT_{50} and KDT_{90} values were computed at a 95% confidence level using SPSS software version 21.0 by using probit analysis of knocked-down values.

Results

Study Area

The sub-Himalayan region of West Bengal has a huge epidemiologic importance as this area offers a favourable environmental condition for the spread of many mosquito-borne diseases. The current study areas (Figure 1) provide plenty of mosquito breeding sites, which are supported by average summer and winter temperatures that vary from 30 to 8 ^{O}C and 200 to 400 cm of yearly rainfall. The four distinct seasons that these regions encounter are the dry season (March to April), the rainy season (May to September), the autumn season (October), and the winter season (November–February). In addition, the geographic relevance of this study area is hugely important because it shares international borders with Nepal, Bhutan, and Bangladesh. Immature stages of Chironomids and drain flies were also found to be coexisting with *Cx. quinquefasciatus* larvae in most of the breeding sites. The drainage system was found to be the prime source of larvae within the study area. Table 1 provides detailed information on sample collection, type of sampling site, and larvae density of the

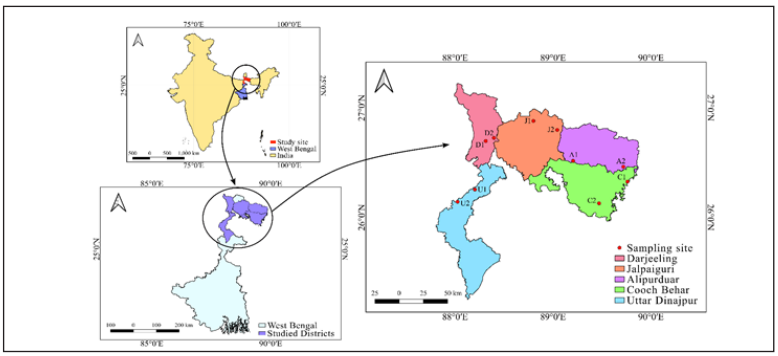

Figure 1.Geographical Distribution of Sampling Sites in the Sub-Himalayan Region of West Bengal (Sampling Sites - D1: Bagdogra, D2: Siliguri; J1: Chalsa, J2: Banarhat; A1: Falakata, A2: Kamakaguri; C1: Baxirhat, C2: Dinhata; U1: Ishlampur, and U2: Panjipara)

Table 1.Sampling Details of *Cx. quinquefasciatus* from the Sub-Himalayan Region of West Bengal, India

District	Sampling Site	Population Name	Average Larval Density/500 mL	Area Types
Cooch Behar	Boxirhat	BOX^{CX}	694.6	Rural
	Dinhata	DIN^{CX}	874.2	Semi-urban
Alipurduar	Kamakhaguri	KAM^{CX}	684.2	Rural
	Falakata	FAL^{CX}	732.5	Semi-urban

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Jalpaiguri	Banarhat	BAN^{CX}	680.3	Rural
	Chalsa	CHA^{CX}	588.2	Semi-urban
Darjeeling	Bagdogra	BAG^{CX}	774.1	Semi-urban
	Siliguri	SIL^{CX}	680.4	Urban
Uttar Dinajpur	Ishlampur	ISH^{CX}	568.2	Semi-urban
	Panjipara	PAN^{CX}	780.4	Rural

sampling site.

Insecticide Resistance Profile of Cx. quinquefasciatus

Cx. quinquefasciatus population from all ten sites of the study area were completely resistant to the tested insecticides. Corrected mortality percentages against all the tested insecticides were plotted in Figure 2. Mortality percentage ranged from 9.61 to 54.13 for permethrin, 12.08 to 48.94 for lambda-cyhalothrin, 20.71 to 55.96 for deltamethrin, 2.10 to 38.99 for DDT (Appendix: Table 1). DDT was found to be the least effective insecticide with the highest mortality against the BOX^{CX} population. Against permethrin highest mortality was reported in the CHA^{CX} population and the lowest mortality was reported against the BAN^{CX} population. The highest mortality against lambda-cyhalothrin was found in the KAM^{CX} population, whereas the lowest mortality rates were found in the CHA^{CX} population. In comparison to deltamethrin, BAN^{CX} population had the

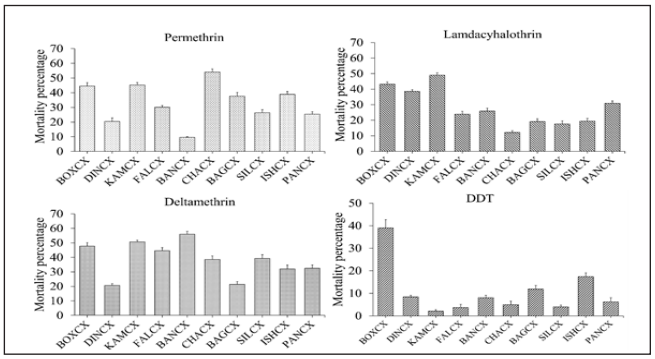

Figure 2.Mortality Percentage of *Cx. quinquefasciatus* (n ≥ 100) against Permethrin, Lambda-Cyhalothrin, Deltamethrin and DDT from Sub-Himalayan Region of West Bengal, India

highest mortality rate and DIN^{CX} population had the lowest mortality rate.

Knock Down Rates

All studied populations showed an elevated KDT value against the tested insecticides. Figure 3 shows different knockdown times (KDT_{50} & KDT_{90}) against the tested insecticides in the studied populations. A higher KDT value indicates the emergence of insecticide resistance probably due to the presence of *kdr* mutation. The ISH^{CX} population has the highest KDT (KDT_{50} & KDT_{90}) values against DDT. In comparison to permethrin, the KDT_{90} value for the BAG^{CX} population was the highest, while the KDT_{50} value belonged to the SIL^{CX} population. The highest KDT_{90} and KDT_{50} values were seen in the ISH^{CX} and FAL^{CX} populations when

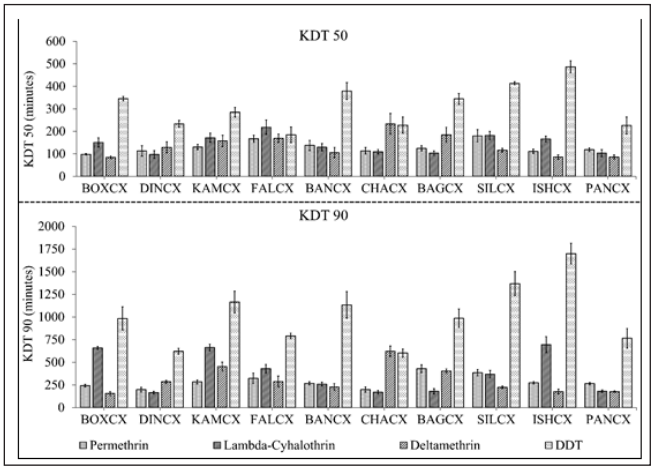

Figure 3.Knockdown Time (in minutes) of *Cx quinquefasciatus* (n ≥ 100) against Permethrin, Lambda-Cyhalothrin, Deltamethrin and DDT from Sub-Himalayan Region of West Bengal, India

compared to lambda-cyhalothrin. In CHA^{CX} populations, the KDT_{50} and KDT_{90} values against deltamethrin were highest (Appendix: Table 2 and Figure 1).

L1014F kdr Genotyping in Cx. quinquefasciatus

The AS-PCR assay was performed on 250 individual samples and all three different genotypes were found. Among all the studied populations highest percentage (40%) of susceptible genotype (L/L) was found in BAN^{CX} population with an L allele frequency of 0.52. ISH^{CX} population has the highest (72%) homozygote resistance (F/F) genotype with a resistant F allele frequency of 0.76, followed by the population of SIL^{CX} population. BAG^{CX} population reported the highest (46.42%) percentage of heterozygote resistant (L/F) genotype among all studied populations. Mutated F allele frequency ranges from 0.48 to 0.76, with the lowest frequency in BAN^{CX} population (Table 2). Analysis of sequences obtained from the current study confirms the

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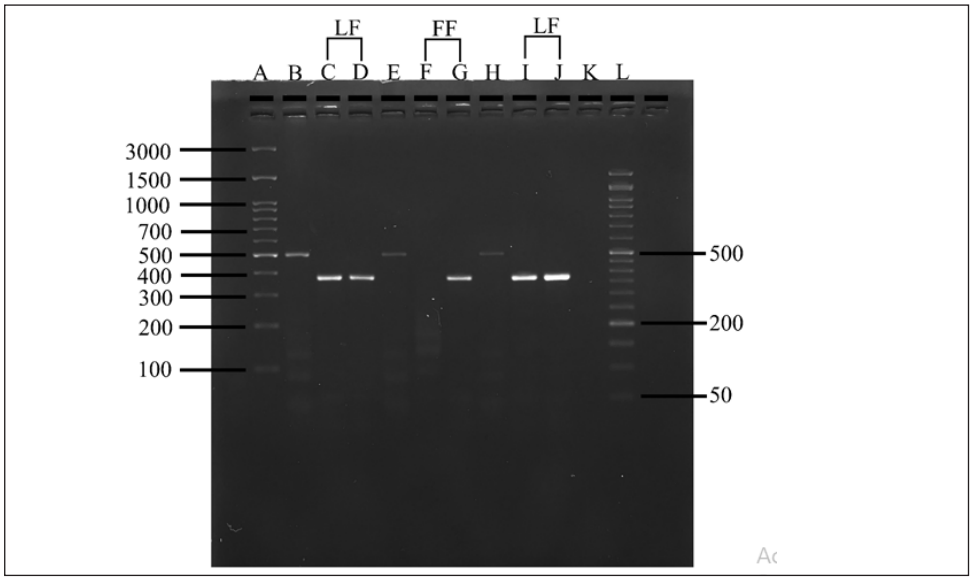

Figure 4.Gel Electrophoresis Image Illustrating Allelic-Specific PCR (AS-PCR) Results for the L1014F *kdr* Mutation in the vgsc Gene of *Cx. quinquefasciatus* from the Sub–Himalayan Region of West Bengal, India. Lane A and L: 100 bp and 50 bp DNA ladders respectively. Lanes B, E, and H exhibit PCR products at 540 bp indicative of the *vgsc* mutation region. Lanes C, and I display PCR products at 380 bp representing the L allele, while lanes D, G, and J show PCR products at 380 bp representing the F allele. Lane K serves as the negative control

Table 2.Genotypic and Allelic Frequencies of L1014F kdr Mutations in Cx. quinquefasciatus from the Sub-Himalayan Region of West Bengal, India

Mosquito Population	Total PCR Sample	Genotype			F-Allele Frequency	Inbreeding Coefficient
		LL	LF	FF		
BOX^{CX}	25	6	3	16	0.700	0.714
DIN^{CX}	25	4	8	13	0.680	0.265
KAM^{CX}	26	8	10	8	0.500	0.231
FAL^{CX}	22	9	4	9	0.500	0.596
BAN^{CX}	25	10	6	9	0.480	0.519
CHA^{CX}	25	6	4	15	0.680	0.632
BAG^{CX}	28	4	13	11	0.625	0.010
SIL^{CX}	26	5	3	18	0.750	0.692
ISH^{CX}	25	5	2	18	0.760	0.781
PAN^{CX}	23	9	5	9	0.500	0.565

Cx = Culex

occurrence of L1014F *kdr* mutation in *Cx. quinquefasciatus* populations with the presence of TTT codon (phenylalanine) instead of TTA (leucine) in the IIS6 region of the *vgsc* gene (Figure 4).

Discussion

The current study found that wild populations of *Cx quinquefasciatus* from the sub-Himalayan region of West Bengal possess an elevated level of insecticide resistance along with the presence of *kdr* mutation. The studied area is crucial in the context of neglected tropical diseases. Besides providing an ample area for mosquito breeding sites, the environmental condition of this area enhances the rapid spreading of mosquito-borne diseases. The drainage system throughout the study area provides an excellent habitat for *Cx. quinquefasciatus* population especially during the

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March–April months of every year.

Wild populations of *Cx. quinquefasciatus* from the sub-Himalayan region of West Bengal showed severe resistance against a wide range of insecticides.[10,20] The Present study includes both type-I and type-II synthetic pyrethroids as well as DDT as these insecticides largely triggered *kdr* genotypes. There are no reports of the direct application of synthetic pyrethroid in West Bengal to manage the *Cx. quinquefasciatus* population. The most likely reason for this resistance development is extensive domestic uses of these insecticides for mitigating mosquito-borne nuisances and controlling household pests. Synthetic pyrethroid-containing products, like fumigants, mosquito coils, oils, and sprays play a key role in resistance development. Moreover, the application of pyrethroids in agricultural fields may also exert insecticidal selection pressure on the mosquito population.[26] The higher KDT_{50} and KDT_{90} values indicate that DDT and synthetic pyrethroids have a delayed effect on *Cx. quinquefasciatus* populations of the study area. Earlier studies in *Cx. quinquefasciatus* from this area suggests that an increased level of detoxifying enzymes i.e., Monooxygenase, GSTs and esterase may serve as the main machinery behind resistance development.[10,20] An increased expression of CYP450 metabolic enzymes has a strong attribution behind the resistance development against synthetic pyrethroids in many insects.[27] However, results from synergist assay with PBO along with prolonged knockdown times indicate the involvement of *kdr* mutation behind this resistance development.[10] As L1014F mutation is the most frequent *kdr* mutation in *Cx. quinquefasciatus* population worldwide thus, in the current study AS-PCR technique was utilised to screen the L1014F *kdr* mutation. A total of 250 individual mosquitoes were screened for this mutation and all studied populations were found to carry the mutated F allele. Partial sequencing of the IIS6 *vgsc* gene from homozygote-resistant individuals further confirms the presence of a polymorphic site (TTA to TTT) at codon 1014, which results in a shift from leucine (TTA) to phenylalanine (TTT) (Figure 5). Studies around the globe suggest that the L1014F *kdr* mutation had a substantial role in insecticide resistance development against DDT and synthetic pyrethroids in *Cx. quinquefasciatus* population.[15–20] The current study advocates that insecticide resistance was prevalent among the wild population of *Cx. quinquefasciatus* from the sub-Himalayan region of West Bengal and L1014F *kdr* mutation in the *vgsc* gene plays the potential role behind this resistance development. The existence of a *kdr* mutation in the wild population poses serious issues in the near future, particularly in areas

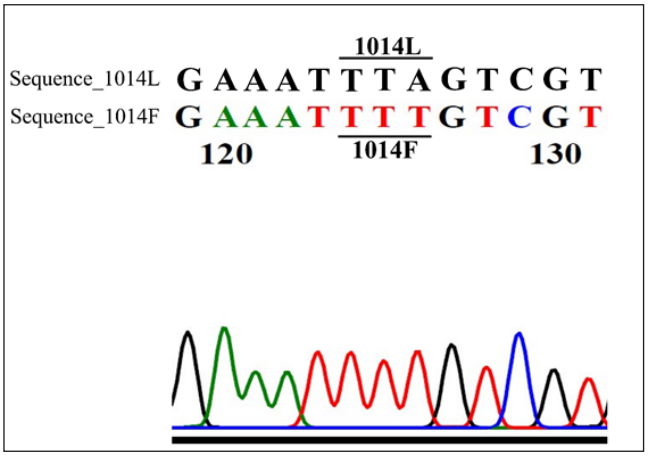

Figure 5.DNA Chromatogram Showing Occurrence of TTT Codon for Phenylalanine Allele in *Cx. quinquefasciatus* from Sub-Himalayan Region of West Bengal, India

where the disease is endemic, in addition to an enhanced expression of detoxifying enzymes. Thus, a more scientific vector control approach must be needed, as the genetic makeup of the mosquito is rapidly changing over time due to the immense selection pressure of the commonly used insecticides.

Conclusion

The wild populations of *Cx. quinquefasciatus* from the sub-Himalayan region of West Bengal are highly resistant to DDT and synthetic pyrethroids. The occurrence of L1014F *kdr* mutation in this vector population may serve as the key machinery behind this resistance development. Data obtained from the current study provide valuable insights for creating and implementing resistance management strategies against this species, a potential arbovirus vector, and for establishing trustworthy diagnostic techniques. Finding a particular mutation causing pyrethroid resistance can be useful in monitoring and mapping the spread of resistance as well as gauging mosquito populations' reactions to upcoming insecticide-based treatments.

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Conflict of Interest: None

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Appendix

Table 1: Insecticide resistance profile of *Cx. quinquaefasciatus* (n ≥ 100) from sub-Himalayan region of West Bengal, India against DDT and synthetic pyrethroids. M%-Mortality percentage; S.E-Standard Error; n-total number of adult mosquitoes

Population Name	Permethrin M % ± S.E	Lambda-cyhalothrin M % ± S.E	Deltamethrin M % ± S.E	DDT M % ± S.E
BOX^{CX}	44.46 ± 2.35	43.22 ± 1.40	47.72 ± 2.42	38.99 ± 3.69
DIN^{CX}	20.46 ± 2.39	38.69 ± 0.95	20.71 ± 1.10	8.46 ± 0.72
KAM^{CX}	45.28 ± 1.68	48.94 ± 1.62	50.61 ± 1.34	2.10 ± 0.64
FAL^{CX}	30.07 ± 1.24	23.97 ± 1.74	44.68 ± 2.02	3.72 ± 1.40
BAN^{CX}	9.61 ± 0.35	25.85 ± 1.89	55.96 ± 2.00	8.07 ± 1.10
CHA^{CX}	54.13 ± 2.03	12.08 ± 1.26	38.52 ± 2.49	5.02 ± 1.56
BAG^{CX}	37.61 ± 2.52	19.06 ± 1.99	21.48 ± 1.77	11.87 ± 1.63
SIL^{CX}	26.35 ± 2.05	17.54 ± 2.01	39.33 ± 2.49	4.02 ± 0.75
ISH^{CX}	38.87 ± 1.97	19.30 ± 1.93	32.07 ± 2.74	17.38 ± 1.77
PAN^{CX}	25.40 ± 1.72	30.92 ± 1.49	32.41 ± 2.37	6.17 ± 1.18

Table 2: Knock down time (in minutes) of *Cx. quinquaefasciatus* (n ≥ 100) against DDT and synthetic pyrethroids from sub-Himalayan region of West Bengal, India

Population Name	Permethrin		Lambda-Cyhalothrin		Deltamethrin		DDT	
	KDT_{50}	KDT_{90}	KDT_{50}	KDT_{90}	KDT_{50}	KDT_{90}	KDT_{50}	KDT_{90}
BOX^{CX}	98.86v	243.25	150.16	658.48	84.31	154.14	344.42	984.87
DIN^{CX}	112.31	197.71	97.01	166.33	129.44	283.146	233.19	620.67
KAM^{CX}	129.44	283.14	171.28	662.79	157.42	452.89	285.11	1164.64
FAL^{CX}	167.54	321.67	218.39	428.46	168.74	287.35	184.60	789.56
BAN^{CX}	137.51	268.28	129.56	258.60	105.39	226.38	378.90	1132.28
CHA^{CX}	113.57	197.80	109.46	169.35	233.19	620.67	228.01	602.04
BAG^{CX}	124.07	428.56	103.53	179.79	184.46	403.18	344.42	984.87
SIL^{CX}	179.87	384.46	181.79	368.68	116.12	223.02	413.13	1367.56
ISH^{CX}	110.62	273.82	166.08	694.12	85.56	176.30	486.34	1398.47
PAN^{CX}	118.63	264.93	103.53	179.79	85.58	176.30	226.24	764.86

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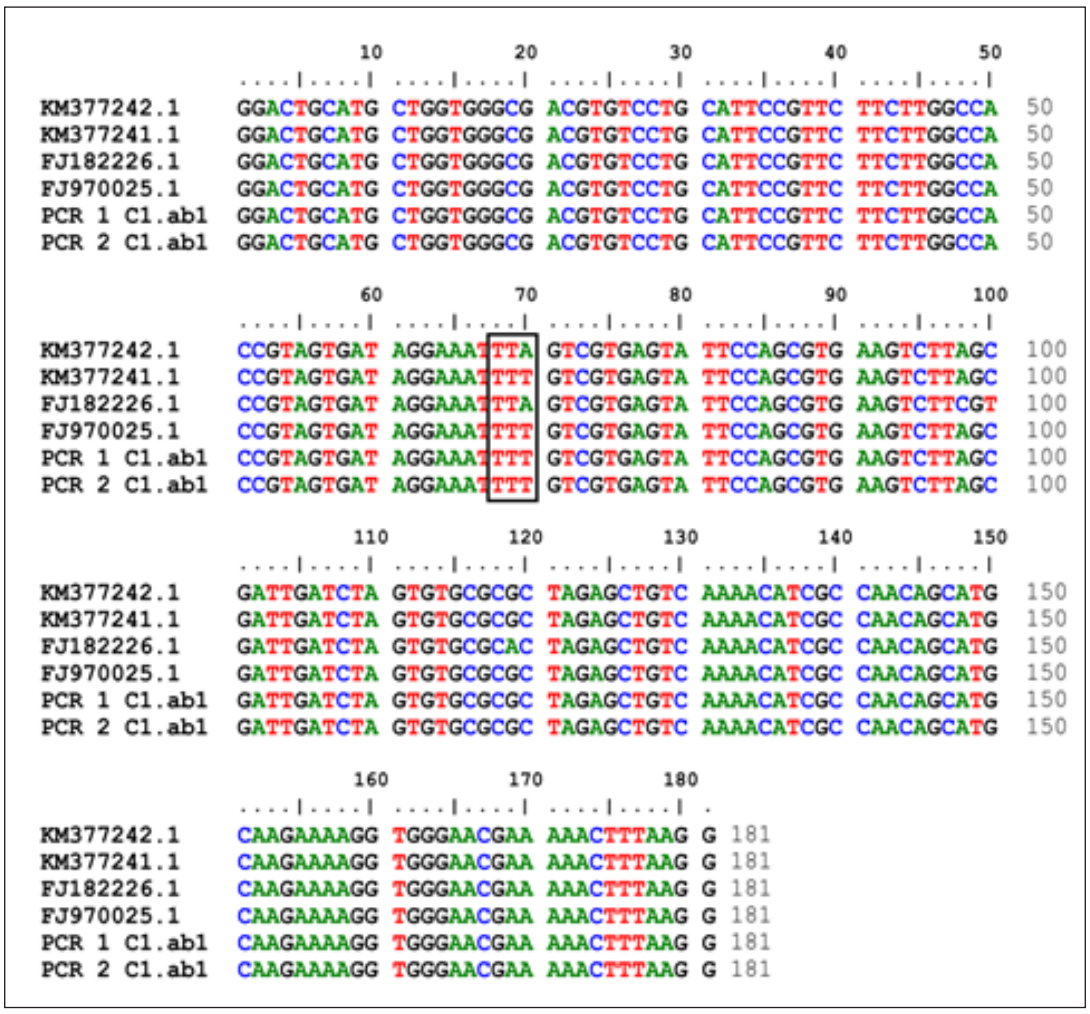

Figure 1.Nucleotide diversity in the IIS6 region of wild *Cx. quinquefasciatus* from sub-Himalayan region of West Bengal, India, showcasing the transition from TTA to TTT at position 68-70, highlighted within the black box

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