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Original Article

Insecticide Resistance Dynamics in *Aedes aegypti* (Diptera: Culicidae) from Different Habitats in the Punjab, Pakistan

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Abstract

Background: The mosquito *Aedes aegypti* is the most important vector of many viral diseases, such as dengue virus. Thus, various chemical insecticides are being used to control this vector. On the other hand, due to the intensive blind use of chemical insecticides, mosquito vectors are rapidly developing resistance against most of them.

Methods: The present study was conducted during 2024–25 to explore the development of resistance in three *Ae. aegypti* strains with a previous history of insecticidal records compared with Laboratory (LAB) and Tidiwala (TDW) strains collected from localities (Tidiwala and rural barren land) with no or minimal use of insecticides. Resistance development was recorded by utilizing five commonly used insecticides against mosquitoes in Punjab, Pakistan.

Results: Results showed that deltamethrin was the best during 2024 when compared with alpha-cypermethrin, temephos, lambda-cyhalothrin and *Bacillus thuringiensis israelensis*. All field-collected strains revealed significant resistance to the tested insecticides compared with LAB and TDW strains. The same findings were reflected in the enzyme spectrum (glutathione-S-transferase, mixed-function oxidases, esterase, and acetylcholinesterase).

Conclusion: These results may help to devise a comprehensive future strategy for mosquito control through selective insecticides by limiting the spread of resistance.

Keywords: Dengue; Enzymatic analysis; Temephos toxicity; *Bacillus thuringiensis*; Pyrethroids

Introduction

Mosquitoes are recognized as one of the most life-threatening vectors on Earth, responsible for spreading fatal diseases such as malaria, dengue, Zika Virus, yellow fever, Japanese encephalitis, filariasis and Saint Louis encephalitis (1). These are found worldwide (2) and transmit illnesses to humans and animals through their bites. *Aedes aegypti* (Linnaeus) is becoming very notorious in this regard as it possesses great flexibility to adopt a new locality in tropical, subtropical and temperate regions (3). Each year, WHO detects 390 million individuals with dengue fever, including 96 million with clinical symptoms (4, 5). Much of the tropical globe has become endemic for one or more of the five dengue virus (DENV) serotypes. However, in the Americas between 2013–2015, massive Chikungunya virus (CHIKV) and Zika virus (ZIKV) outbreaks spread quickly among a huge, vulnerable population (6, 7). Climate change has an impact on mosquito survival, dispersal and transmission rates of dengue fever (8). In Pakistan, a total of 25,932 confirmed dengue cases with 62 deaths were reported after heavy flooding during 2022 (9).

Eliminating mosquitoes in their larval instars is the most effective way to lower adult mosquito population densities (10), which has an impact on disease transmission. The most extensively used and trusted form of mosquito control is synthetic insecticides. Insecticides are vital not only for controlling pests in agriculture, but also for reducing the number of vectors, such as mosquitoes, which have a direct influence on public safety (11). *Aedes aegypti*

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management tactics rely on the use of pyrethroids, organochlorines, organophosphates and carbamates. Among these, a few chemical insecticides are licensed for public health usage like deltamethrin, cypermethrin, lambda-cyhalothrin, permethrin and alpha-cypermethrin. Pyrethroid insecticides like deltamethrin, cypermethrin and permethrin are widely employed due to their low toxicity to animals and excellent efficiency against vectors (12).

Bio-pesticides like *Bacillus thuringiensis israelensis* (Bti) are also used against mosquito larvae, and they are effective because it disrupts the developmental stages and growth. Synthetic pesticides, on the other hand, have several harmful effects on human health and the environment, and numerous mosquito species have acquired resistance to them. Previous research also confirmed the role of metabolic enzymes in insecticidal resistance; the higher activity of α-esterase, β-esterase, and mixed-function oxidases may contribute to resistance to organophosphates and pyrethroids, respectively (13). Because of the indiscriminate use of pesticides, vector mosquitoes (*Aedes*) have acquired resistance to many synthetic pesticides, including pyrethroids in various districts of the province Punjab (14) and Khyber Pakhtunkhwa (15), Pakistan. Similarly, resistant *Aedes* mosquitoes against pyrethroids were also prevalent in the neighboring province of Punjab, New Delhi (Indian capital) (16). Insecticidal resistance has also been reported in *Ae. aegypti* for temephos from different districts of the Punjab (13).

Insecticide resistance is the main problem in vector control. Variation in patterns of resistance development is a big challenge for controlling vector; exceptionally when a large geographical area is targeted for vector control. Insecticide susceptibility surveillance in field populations of *Aedes* is essential to identify the levels, processes and geographical distribution of resistance. So, effective pesticides will be selected and utilized based on this evidence. The prevention of dengue is highly dependent on the successful regulation of vectors along with vaccination and community engagement. So, the data on prevailing resistance status and stated mechanisms of resistance in field inhabitants' mosquitoes of dengue can assist in developing effective strategies for mosquito control (17). To understand the level of resistance development in the local mosquito population (Faisalabad), their larvae were investigated by using different insecticides recommended by WHO.

Materials and Methods

Study area and field collection of mosquitoes

Field strains of mosquitoes were collected from standing water at larval and pupal stages with a plastic dipper (BioQuip, United States of America) from four different locations of district Faisalabad (31° 27' 1.3176" N, 73° 8' 5.8596" E, 185.856 m elevation) based on mosquito prevalence during 2024-25;

The first, rural locality (Theekriwala, TKW), was a village (30° 50' 30" N, 72° 45' 0" E, 182.67 m elevation) where most people used different kinds of pesticides to protect their crops and in houses for personal protection from household pests like mosquitoes, ticks, cockroaches, etc. The second, urban locality (Jinnah Colony, JC), was an urban place (31° 25' 12" N, 73° 3' 59" E, 185 m elevation) where the city government sprayed different pesticides to save people from different pests (mosquitoes, house flies, cockroaches). The third, University of Agriculture (UAF), was a blend of rural and urban life (31° 25' 46.8048" N, 73° 4' 14.3112″ E, 184.678 m elevation), where different kinds of vegetation, crops, animals, and birds are present. The university administration sprays different kinds of pesticides (deltamethrin) on its premises. These locations were selected based on the history of extensive mosquito control applications with an average of six to seven applications in each season (personal interaction with inhabitants). The fourth, the semi-urban locality (Tidiwala, TDW)

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(31° 24' 56" N, 73° 5' 23" E, 182.56 m elevation) strain was collected because the inhabitants don't spray or were reluctant to spray and protect themselves by utilizing screens on doors, windows, and vents and full-sleeve clothes.

The LAB strain reared at Government College, University, Faisalabad (31° 24' 32.21" N, 73° 04' 5.86" E, 184.505 m elevation) and maintained without insecticide exposure as detailed below.

Mosquito rearing

Field collected larvae were brought to laboratory of the Department of Zoology, Government College University, Faisalabad and identified up to species level by using identification keys of Qasim et al. (18). For bioassay stock cultures, rearing of these larvae was carried out separately for each locality under laboratory conditions (25±2 °C, 60±5% RH and 16:08 h (L:D)) for one generation before bioassay studies. This process was carried out in mosquito rearing cages having plastic trays (35x30x5 cm) with fish feed. After emergence of adults, rearing was again carried out separately for each locality in rearing cages (90× 90×90 cm) having plastic trays (35x30x5 cm). The adults (males) were fed with a 10% sucrose solution, and blood of a rat was used to feed female mosquitoes within the same cage. After hatching, 0.02% yeast suspension was given daily to 1st and 2nd instar larvae and powdered fish feed to 3rd and 4th instar larvae (15).

Insecticides

Commercially available insecticides used in this study were alpha cypermethrin (Fendona 10% SC, Swat Agro Chemicals, Pakistan), deltamethrin (Guardian 1.5% EC w/v, Ali Akbar Group, Pakistan), Bti (BTI Mosquito Dunks® 10.31%, Summit®, USA), lambda cyhalothrin (Lambda-cyhalothrin 2.5 EC @ 20ml/L, Engro Pesticides Ltd., Pakistan), temephos (Abate® 500E @ 500g/L, BASF S.A., Brazil) were obtained from pesticide market and utilized for estimation of *Ae aegypti* resistance in different field strains. Each insecticide comprised of following concentrations; alpha cypermethrin (11, 1.1, 0.11, 0.011, 0.0011 ppm) with 11% stock solution, temephos (7, 0.7, 0.07, 0.007, 0.0007 ppm) with 7% stock solution, lambda cyhalothrin (100, 10, 1, 0.1, 0.01 ppm) with 100% stock solution, deltamethrin (3, 0.3, 0.03, 0.003, 0.0003 ppm) with 3% stock solution and Bti (10, 1, 0.1, 0.01, 0.001 ppm) with 10% stock solution. Doses were prepared according to the recommended dose of the Health Department, district Faisalabad, following the formula $C_1V_1 = C_2V_2$ (19, 20). Dose-response bioassays were executed according to WHO (21) recommendations. The Standard mosquito resistance detection procedure was adopted according to Li et al. (17). Five concentrations of each treatment (insecticide) were applied to larvae (late 3rd or early 4th instar), and 20 larvae for each replication separately for each locality. The experiment was repeated four times, and the mortality data were recorded after 24 h, 48 h and 72 h, by probing ice-cream-stick to each larva. The % mortality was corrected with the use of Abbott's formula (22).

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment} - \text{Observed mortality in control}}{100 - \text{Control mortality}} \times 100$$

Metabolic enzyme activity assays

For enzyme analysis, 20 larvae from each treatment were stored in 75% alcohol, and the enzyme assay was performed according to Li et al. (23). At the time of analysis, larvae were washed thoroughly with dH_2O and dried using blotting paper. After homogenization of larvae with buffer, it was centrifuged at 8000 rpm and 4 ºC for 20 minutes to collect supernatant for further assays. To assess acetylcholinesterase enzyme, 25 µl of homogenate was added to 96-well plates with 145 µl of Triton/Na phosphate and 10 µl of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB/Na) phosphate. After the addition of 10 mM acetylcholine iodide in water, the acetylcholinesterase plates were incubated for 1 hr at 25 ºC and absorbance was noted at 414 nm. To assess Mixed

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Function Oxidase, 20 µl of homogenate was mixed with 60 µl of 90 mM K_2SO_4 buffer and 200 µl Na acetate/TMBZ (3,3',5,5'-tetramethylbenzidine). After the addition of 25 µl of 3% H_2O_2 in plates, the plates were incubated for 90 minutes at 25 ºC and absorbance readings were taken at 620 nm. For Esterases, 10 µl homogenate was taken in 96-well plates after centrifugation at 12000 g for one minute. Then, 200 µl of PNPA/Na (p-nitrophenyl acetate) phosphate in each well along with 24.75 ml of 50 mM sodium phosphate buffer at 7.4 pH and absorbance readings were taken at 540 nm. For glutathione-S-transferase, 20 µl of homogenate was mixed with 100 µl of CDNB (10 mg 1-chloro-2,4-dinitrobenezene, 5 ml acetone, 45 ml KPO4 buffer) and 100 µl of reduced glutathione. This was incubated for 5 minutes; absorbance was noted at 340 nm. Protein concentration was determined by adding 10 µl of mosquito homogenate to 200 µl of Protein dye (10 ml Bio-RadProtein Assay Dye Reagent diluted with 40 ml deionized water) and incubated for 5 minutes. An absorbance reading was taken at 595 nm (23).

Data computation and statistical analysis

The recorded data were analysed using SPSS for ANOVA, and means were separated by employing the Tukey HSD (honestly significant difference) test. Further, the data were subjected to PoloPC computer-based software to obtain Probit regression estimates and median lethal concentrations (LC_{50}) for each bioassay. After estimation of LC_{50}, the LAB and TDW strains showed the lowest values for each treatment, indicating the most susceptible strains among the tested group (TKW, JC and UAF). Therefore, lethal concentrations of these strains are further used to determine the resistance ratio (RR) of all field strains to all insecticides. The resistance ratio is obtained by dividing the LC value of the field strain by the corresponding LAB or TDW strain. However, the RR scoring criteria were: RR< 1 (susceptible), RR= 1–10 (very low resistance), RR= 10–20 (low resistance), RR= 20–50 (medium resistance), RR= 50–100 (high resistance), and RR> 100 (very high resistance) (24).

Results

Mortality data for all treatments revealed that the LAB strain was found very susceptible, followed by TDW, TKW and JC, while the UAF population ranked very resistant, showing the least mortality at 24, 48 and 72 h (Fig. 1). The results also exhibited variation in mean mortality with respect to exposure time and area. Minimum mortality was recorded after 24h and its mean value increased after 48 and 72 h. Despite the LAB strain, the TDW strain was found most sensitive, and the highest mortality counts were recorded after 24, 48 and 72 h, while minimum mortality was recorded in the UAF strain in the case of every insecticide used. Temephos was found to be more effective and potent, showing more mortality than alfa-cypermethrin. For temephos, the TDW mosquito strain revealed the highest mortality at 24, 48 and 72h exposure time (14.267, 15.600 and 17.133, respectively) followed by TKW (13.733, 15.067 and 16.400), JC (13.667, 13.667 and 14.800) and minimum mortality in the UAF strain (13.333, 14.400 and 15.400, respectively). Lambda cyhalothrin was recorded as more responsive and efficient in comparison with temephos and alfa-cypermethrin. The TDW strain exhibited the highest sensitivity at all exposure times (24, 48 and 72 h), followed by TKW, JC and UAF populations. The same trend has been recorded for deltamethrin and Bti. Compared with 2025 estimates, each field strain developed resistance against the tested insecticides (Fig. 2).

Toxicological bioassay compared responses of *Ae. aegypti* strains to different insecticides, and furthermore variations of different field strains were evaluated by taking LAB and TDW strains as standard in two consecutive years (Table 1 and 2). LC estimates exhibited that the LAB strain was susceptible as compared with the TDW collected strain. The

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response of the LAB strain to different insecticides were recorded in 2024 (2.54×10^{-4} µL/L for alpha cypermethrin, 3.29×10^{-5} µL/L for temephos, 0.46×10^{-3} µL/L for lambda cyhalothrin, 1.21×10^{-1} µL/L for Bti and 3.43×10^{-5} µL/L for deltamethin) and 2025 (2.25×10^{-4} µL/L for alpha cyper, 2.48×10^{-4} µL/L for temephos, 1.09×10^{-3} µL/L for lambda cyhalothrin, 1.03×10^{-1} µL/L for Bti and 1.10×10^{-4} µL/L for deltamethin). In case of TDW strain, the LC estimates were; 1.01×10^{-3} µL/L for alpha cypermethrin, 5.45×10^{-5} µL/L for temephos, 1.05×10^{-3} µL/L for lambda cyhalothrin, 1.56×10^{-1} µL/L for Bti and 3.72×10^{-5} µL/L for deltamethin (for 2024), and 1.24×10^{-3} µL/L for alpha cyper, 4.68×10^{-4} µL/L for temephos, 2.47×10^{-3} µL/L for lambda cyhalothrin, 2.5×10^{-1} µL/L for Bti and 6.24×10^{-4} µL/L for deltamethin (for 2025).

In contrast to the LAB strain, the TDW strain revealed susceptibility to deltamethrin (RR= 1.085-fold) and a low-level resistance to alpha cypermethrin (RR= 4.213-fold) in 2024, while this resistance ratio increased from 1.887-fold for temephos to 5.511-fold for alpha cypermethrin in 2025. In 2024, RR values for UAF ranged from 3.6 to 17.7 relative to LAB; by 2025, these increased to 45–403, while these values were from 2.4 to 6.6 relative to TDW, which increased from 24 to 178 during 2025. While the lowest RR values were recorded for TKW relative to LAB (2.2 to 5.3) and TDW (1.2 to 1.7) during 2024.

Compared with the LAB strain in 2024, the trend of resistance in different strains (TKW, JC and UAF) exhibited susceptibility to lambda cyhalothrin, very low resistance to Bti, very low resistance to temephos, and very low to low levels of resistance to deltamethrin and alpha cypermethrin. While this level of resistance increased drastically in 2025, very low to moderate resistance to temephos and very low to very high levels of resistance to Bti, deltamethrin, alpha cypermethrin and lambda cyhalothrin.

When different strains were compared with the TDW strain a very low level of resistance trend to all insecticides have been estimated in 1st study year and this trend of resistance showed progressive increase (very low to moderate resistance to temephos, very low to high resistance to alpha cypermethrin and deltamethrin, very low to very high resistance to deltamethrin and low to high resistance to Bti) during 2nd study year.

Enzymatic characterization

Table 3 represents enzyme characterization of bioassayed *Ae. aegypti*. The data showed that the field population was collected from different locations in Faisalabad. The activity of different enzymes increased across different collection sites. These observations indicated that a high level of resistance was estimated in the UAF population, followed by JC and TKW. While the minimum level of resistance in the TDW population. The results also revealed a significant increase in all enzymes during the 2nd study year, indicating a sharp rise in resistance level.

Table 1. Toxicological response of *Aedes aegypti* strains to insecticides used in public health programs, Faisalabad, Pakistan in 2024

Treatment	Strain	N	LC_{50} (95% CI)	Probit line fitness					
				Slope (SE)	χ^2	df	P	RR (LAB)	RR (TDW)
Alfa cypermethrin	LAB	480	2.54×10^{-4} (5.43×10^{-6}–1.18×10^{-3})	0.35 (0.5)	1.27	4	0.63		
	TDW	460	1.01×10^{-3} (2.25×10^{-4}-2.92×10^{-2})	0.22 (0.03)	1.89	4	0.59	4.213	
	TKW	460	1.35×10^{-3} (2.97×10^{-4}-3.66×10^{-3})	0.215 (0.03)	1.03	4	0.79	5.315	1.262
	JC	460	4.25×10^{-3} (9.23×10^{-4}-1.18×10^{-2})	0.17 (0.02)	3.37	4	0.33	16.732	3.972
	UAF	480	4.5×10^{-3} (9.51×10^{-4}-1.31×10^{-2})	0.16 (0.02)	2.87	4	0.42	17.717	4.206

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Temephos	LAB	500	3.29×10^{-5} (3.77×10^{-6}–6.52×10^{-4})	0.124 (0.02)	0.72	4	0.78		
	TDW	520	5.45×10^{-5} (1.2×10^{-6}–3.4×10^{-4})	0.171 (0.03)	2.27	4	0.51	1.657	
	TKW	480	8.13×10^{-5} (1.8×10^{-6}-5.14×10^{-4})	0.152 (0.02)	0.43	4	0.93	2.471	1.492
	JC	460	1.17×10^{-4} (2×10^{-5}–8×10^{-4})	0.131 (0.02)	0.09	4	0.97	3.556	2.147
	UAF	500	1.33×10^{-4} (2.2×10^{-6}–9.15×10^{-4})	0.128 (0.02)	0.6	4	0.89	4.043	2.440
Lambda cyhalothrin	LAB	520	0.46×10^{-3} (2.55×10^{-4}–6.18×10^{-2})	0.331 (0.06)	1.41	4	0.51		
	TDW	480	1.05×10^{-3} (1.36×10^{-4}–6.69×10^{-3})	0.231 (0.04)	1.26	4	0.73	2.283	
	TKW	480	1.5×10^{-3} (9.02×10^{-5}–6.48×10^{-3})	0.209 (0.04)	1.33	4	0.72	3.261	1.429
	JC	460	2.43×10^{-3} (1.91×10^{-4}–9.79×10^{-3})	0.195 (0.03)	1.19	4	0.75	5.283	2.314
	UAF	500	4.69×10^{-3} (4.17×10^{-4} – 1.85×10^{-2})	0.171 (0.03)	0.149	4	0.98	10.196	4.467
Bti	LAB	520	1.21×10^{-1} (4.83×10^{-1} – 1.01)	0.412 (0.06)	1.05	4	0.58		
	TDW	480	1.56×10^{-1} (5.42×10^{-2} – 4.94×10^{-1})	0.142 (0.82)	1.42	4	0.70	1.289	
	TKW	480	2.7×10^{-1} (8.46×10^{-2} – 1.14)	0.126 (0.02)	1.01	4	0.79	2.231	1.731
	JC	460	7.28×10^{-1} (2.26×10^{-1} – 3.95)	0.128 (0.02)	1.39	4	0.70	6.017	4.667
	UAF	480	8.6×10^{-1} (3.05×10^{-1} – 3.64)	0.149 (0.02)	0.22	4	0.97	7.107	5.513
Deltame-thrin	LAB	500	3.43×10^{-5} (9.19×10^{-6} – 1.84×10^{-4})	0.207 (0.07)	1.44	4	0.41		
	TDW	480	3.72×10^{-5} (1.9×10^{-6} – 1.69×10^{-4})	0.208 (0.04)	0.92	4	0.82	1.085	
	TKW	480	5.61×10^{-5} (3.2×10^{-6} – 2.55×10^{-4})	0.183 (0.03)	0.37	4	0.94	1.636	1.508
	JC	460	6.83×10^{-5} (2.9×10^{-6} – 3.52×10^{-4})	0.156 (0.03)	0.89	4	0.82	1.991	1.836
	UAF	480	2.54×10^{-4} (1.76×10^{-5} – 1.04×10^{-3})	0.144 (0.03)	0.73	4	0.87	7.405	6.824

TKW= Theekriwala, TDW= Tidiwala, JC= Jinnah Colony, UAF= University of Agriculture, Faisalabad, UAF vs LAB for lambda-cyhalothrin RR= 17.7 → "low resistance"

Table 2. Toxicological response of *Aedes aegypti* strains to insecticides used in public health programs, Faisalabad, Pakistan in 2025

Treatment	**Strain**	**N**	**LC_{50} (95% CI)**	**Probit line fitness**					
				Slope (SE)	**χ^2**	**df**	**P**	**RR (LAB)**	**RR (TDW)**
Alfa cyperme-thrin	LAB	520	2.25×10^{-4} (6.07×10^{-5} – 5.28×10^{-3})	0.271 (0.04)	0.57	4	0.68		
	TDW	480	1.24×10^{-3} (1.63×10^{-4} – 4.22×10^{-3})	0.168 (0.03)	4.09	4	0.25	5.511	
	TKW	500	2.33×10^{-3} (2.83×10^{-4} – 8.24×10^{-3})	0.146 (0.02)	0.18	4	0.98	10.356	1.879
	JC	480	1.72×10^{-2} (3.37×10^{-3} – 5.45×10^{-2})	0.129 (0.02)	1.23	4	0.74	76.444	13.871
	UAF	460	6.57×10^{-2} (1.74×10^{-2} – 2.17×10^{-1})	0.125 (0.02)	0.94	4	0.81	292.000	52.984
Temephos	LAB	520	2.48×10^{-4} (9.21×10^{-4} – 2.75×10^{-2})	0.376 (0.09)	1.04	4	0.76		
	TDW	500	4.68×10^{-4} (3.76×10^{-5} -1.91×10^{-3})	0.154 (0.03)	0.53	4	0.91	1.887	
	TKW	460	6.04×10^{-4} (2.07×10^{-5} -3.2×10^{-3})	0.118 (0.02)	0.64	4	0.88	2.435	1.291
	JC	480	3.16×10^{-3} (3.81×10^{-4} -1.13×10^{-2})	0.129 (0.02)	0.52	4	0.91	12.742	6.752
	UAF	520	1.13×10^{-2} (6.1×10^{-4} -6.03×10^{-2})	0.086 (0.02)	1.57	4	0.66	45.565	24.145
Lambda cyhalothrin	LAB	500	1.09×10^{-3} (4.73×10^{-3} -6.04×10^{-4})	0.133 (0.06)	1.22	4	0.43		
	TDW	460	2.47×10^{-3} (1.24×10^{-4} -1.19×10^{-2})	0.161 (0.03)	0.54	4	0.90	2.266	
	TKW	500	4.6×10^{-3} (8.75×10^{-5} – 2.89×10^{-2})	0.116 (0.03)	0.80	4	0.84	4.220	1.862
	JC	480	7.23×10^{-2} (1.01×10^{-2} – 2.49×10^{-1})	0.127 (0.02)	2.06	4	0.56	66.330	29.271
	UAF	520	4.39×10^{-1} (8.42×10^{-2} – 1.68)	0.109 (0.02)	1.07	4	0.78	402.75	177.73

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Table 2. Continued ...

Bti	LAB	500	1.03×10^{-1} (3.88×10^{-2} – 6.15×10^{-1})	0.153 (0.03)	0.86	4	0.65	2	3
	TDW	460	2.5×10^{-1} (9.32×10^{-2} -8.03× 10^{-1})	0.150 (0.02)	2.38	4	0.50	2.427	
	TKW	460	2.77 (5.69×10^{-1} – 62.93)	0.103 (0.02)	0.11	4	0.98	26.893	11.080
	JC	480	9.69 (1.80 – 309.29)	0.113 (0.02)	0.77	4	0.85	94.078	38.760
	UAF	500	15.222 (3.135 – 316.198)	0.133 (0.02)	0.58	4	0.90	147.786	60.888
Deltamethrin	LAB	480	1.10×10^{-4} (3.78×10^{-5} – 1.43×10^{-3})	0.119 (0.04)	1.22	4	0.72		
	TDW	500	6.24×10^{-4} (8.9×10^{-5} – 2.05×10^{-3})	0.156 (0.03)	1.38	4	0.71	5.672	
	TKW	460	2.9×10^{-3} (7.19×10^{-4} – 7.89×10^{-3})	0.157 (0.02)	1.11	4	0.77	26.364	4.648
	JC	480	5.51×10^{-3} (9.16×10^{-4} – 1.92×10^{-2})	0.117 (0.02)	0.346	4	0.95	50.090	8.831
	UAF	500	3.94×10^{-2} (7.54×10^{-3} – 2.39×10^{-1})	0.095 (0.02)	0.192	4	0.97	358.181	63.149

TKW= Theekriwala, TDW= Tidiwala, JC= Jinnah Colony, UAF= University of Agriculture, Faisalabad, UAF vs LAB for lambda-cyhalothrin RR= 402.75 → "very high resistance"

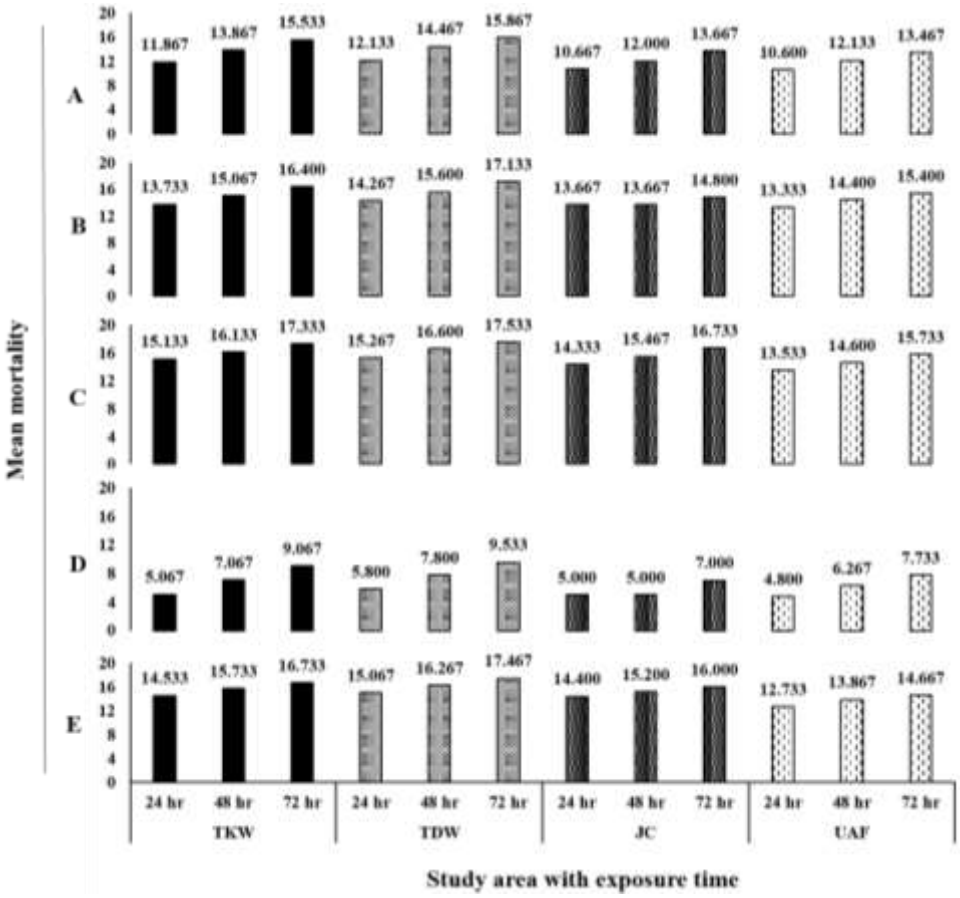

Fig. 1. Mortality data (%) of *Aedes aegypti* exposed to different insecticides at 24, 48 and 72 hours across multiple locations in Faisalabad, Pakistan in 2024

A= Alpha cypermethrin, B= Temephos, C= Lambda cyhalothrin, D= Bti, E= Deltamethrin, TKW= Theekriwala, TDW= Tidiwala, JC= Jina colony, UAF= University of Agriculture, Faisalabad

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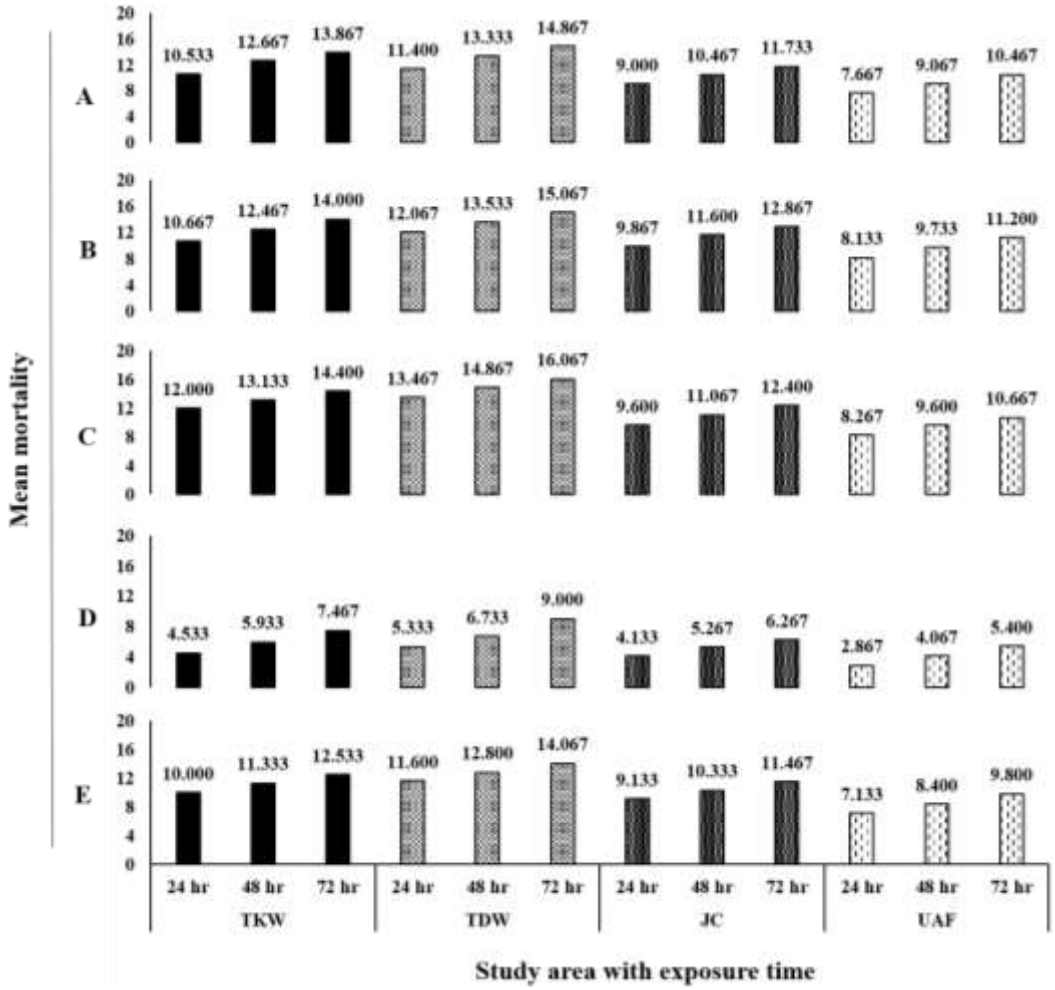

Fig. 2. Mortality data (%) of *Aedes aegypti* exposed to different insecticides at 24, 48 and 72 hours across multiple locations in Faisalabad, Pakistan in 2025

A= Alpha cypermethrin, B= Temephos, C= Lambda cyhalothrin, D= Bti, E= Deltamethrin, TKW= Theekriwala, TDW= Tidiwala, JC= Jina colony, UAF= University of Agriculture, Faisalabad

Table 3. Spectrum of enzymes involved in resistance of *Aedes aegypti* collected from Faisalabad, Pakistan in 2024 and 2025

Population	Glutathione-S-transferase	Mixed function Oxidases	Esterase	Acetylcholinesterase
TDW	0.132 ± 0.03^{C}	0.513 ± 0.02^{C}	0.238 ± 0.01^{D}	0.072 ± 0.01^{C}
	$(0.139 \pm 0.02)^{D}$	$(0.533 \pm 0.03)^{C}$	$(0.251 \pm 0.02)^{D}$	$(0.081 \pm 0.02)^{C}$
TKW	0.141 ± 0.02^{BC}	0.548 ± 0.05^{B}	0.271 ± 0.04^{C}	0.084 ± 0.02^{B}
	$(0.154 \pm 0.01)^{C}$	$(0.557 \pm 0.04)^{B}$	$(0.283 \pm 0.03)^{C}$	$(0.089 \pm 0.01)^{B}$
JC	0.152 ± 0.03^{B}	0.586 ± 0.06^{AB}	0.322 ± 0.03^{B}	0.095 ± 0.06^{AB}
	$(0.163 \pm 0.02)^{B}$	$(0.591 \pm 0.04)^{AB}$	$(0.349 \pm 0.03)^{B}$	$(0.106 \pm 0.02)^{AB}$
UAF	0.173 ± 0.05^{A}	0.597 ± 0.07^{A}	0.406 ± 0.02^{A}	0.101 ± 0.03^{A}
	$(0.182 \pm 0.02)^{A}$	$(0.611 \pm 0.05)^{A}$	$(0.421 \pm 0.02)^{A}$	$(0.112 \pm 0.02)^{A}$

*Figures in parentheses indicate 2024 enzyme spectrum
Different superscript letters denote significant differences ($P < 0.05$) between the respective groups

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Discussion

The present study highlights a clear pattern of insecticide resistance in the dengue vector, *Aedes aegypti*. Although deltamethrin exhibited comparatively higher larvicidal efficacy, the overall trend revealed that mortality was higher in rural populations and consistently lower in urban populations, indicating the development of resistance in mosquitoes exposed to repeated chemical treatments. This pattern strongly suggests that continuous and indiscriminate use of the same group of insecticides, particularly in urban settings, has accelerated resistance evolution. Similar findings have been reported globally, where *Aedes aegypti* populations showed substantial resistance to pyrethroids such as permethrin and deltamethrin, often reflected by low mortality rates, whereas certain compounds like malathion remained highly effective in specific regions (14).

A notable strength of this study is the use of dual baseline strains (LAB and TDW) for resistance ratio estimation, which allows a more robust and context-dependent interpretation of resistance intensity across field populations. The comparison between these two baselines revealed that resistance ratios were consistently higher when referenced against the LAB strain, while comparatively lower but still substantial when standardized against the TDW strain, highlighting how baseline selection can influence perceived resistance magnitude. This dual-reference approach provides a more nuanced understanding of resistance dynamics, as LAB strains often represent long-established laboratory susceptibility, whereas TDW may better reflect local field-adapted genetic backgrounds. Such comparative baselines improve the reliability of resistance surveillance and help to avoid over- or underestimation of operational resistance levels in vector populations. This aspect of our study is similar to that of Haris et al. (25), who also studied pyrethroid resistance in *Aedes aegypti* by using two susceptible strains and studied resistance ratios.

Resistance to organophosphate insecticides, particularly temephos, has also been widely documented. Studies conducted in diverse ecological regions, including the Amazon and Peru, reported considerable resistance levels, even after the discontinuation of temephos application, indicating the persistence of resistance traits in mosquito populations (10, 26). In Iran, dose-response analysis showed that *Ae. aegypti* larvae were resistant to temephos (13). Furthermore, our findings are in agreement with those of Carrera et al. (27), who also reported resistance development in *Ae. aegypti* against deltamethrin, lambda-cyhalothrin and permethrin. They also noted significant differences in the levels of enzymatic activity of esterase, MFO and GST. Increasing resistance trends over time have also been documented, with a 6-fold increase in resistance observed in 2018 and an 11-fold increase by 2020, highlighting the rapid evolution of resistance in mosquito populations against temephos and deltamethrin (28). This observed change is most likely due to a combination of a true biological increase in resistance driven by intensified pyrethroid selection pressure and natural year-to-year sampling variability across localities, rather than a statistical artifact alone. The consistent rise across multiple sites in 2025 supports a real resistance escalation trend. This trend was also studied by Khan (29), who also noted a rise in resistance in *Aedes* mosquitoes against pyrethroids from different cities of Punjab, Pakistan. Similarly, the exceptionally high resistance level observed in the UAF strain in 2025, particularly the 402-fold resistance to lambda-cyhalothrin, represents a critical and alarming shift in local mosquito susceptibility. Such an extreme resistance ratio strongly indicates intense and sustained selection pressure, likely driven by repeated and widespread use of pyrethroid-based interventions in the

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region, leading to the enrichment of resistant alleles such as knockdown resistance (kdr) mutations and enhanced metabolic detoxification mechanisms (30). This magnitude of resistance suggests that lambda-cyhalothrin has become largely ineffective for operational control in this strain, raising serious concerns for vector control programs relying on pyrethroid-based strategies. The finding also highlights the urgent need for resistance management strategies, including insecticide rotation, integration of non-pyrethroid classes, and strengthened surveillance to delay further escalation of resistance in Faisalabad mosquito populations (31).

Additional studies have reported extremely high resistance levels across multiple insecticide classes. For instance, Rehman et al. (13) and Khan et al. (15) observed that *Aedes aegypti* populations exhibited very high resistance to permethrin, with mortality ranging from 0 % to 14.8%, along with elevated resistance ratios for chlorpyrifos (RR= 1174), fenitrothion (RR= 428), deltamethrin (RR= 316), chlorfenapyr (RR= 225) and α-cypermethrin. These findings clearly demonstrate the widespread and multi-insecticide resistance present in this species. In contrast, some studies have shown that rural mosquito populations remain relatively susceptible, particularly to pyrethroids (> 98% mortality), although emerging resistance to organophosphates has also been noted (32).

Biological control agents such as *Bacillus thuringiensis israelensis* (Bti) have generally maintained low resistance levels, making them promising alternatives for vector control. Koou et al. (33) reported that although *Aedes aegypti* larvae were largely sensitive to temephos, early signs of resistance were emerging, while Bti remained effective. Similarly, Paris et al. (34) and Silva et al. (35) found that larvae were still susceptible to Bti, with low resistance ratios (RR ≈ 2.8) and moderate mortality rates. The results of Asgarian et al. (36) showed that both the wettable powder and suspension formulations of Bti showed 100% mortality of *Ae. aegypti* larvae in laboratory studies at the highest concentration. During the semi-field phase, the wettable powder formulation demonstrated residual activity lasting 2 to 14 days.

Although detoxifying enzymes such as esterases, monooxygenases, and glutathione S-transferases are often associated with resistance mechanisms, some studies have reported minimal changes in their activity, suggesting that resistance in *Aedes aegypti* is complex and may involve multiple mechanisms. Overall, the present findings, together with previous reports, emphasize the urgent need for integrated resistance management strategies, including insecticide rotation and the incorporation of biological control agents, to effectively manage the growing problem of resistance in dengue mosquito populations.

Being an agricultural country, different insecticides, including pyrethroids, are extensively used against different crop pests on the one hand and are commonly used for Urban pests in houses on the other hand in Pakistan. This might be a major reason for pyrethroid resistance in *Ae. aegypti* mosquitoes (15). Other studies conducted in the semi-arid region of Pakistan indicated insecticide (deltamethrin and bifenthrin) resistance against *Aedes* mosquitoes (14, 37). Hence, this present scenario of pyrethroid resistance may be due to cross-resistance because of the use of different classes of insecticides at a time or increased selection pressure of pyrethroids due to their extensive use in agriculture and household purposes.

We acknowledge several limitations in the present study that should be considered when interpreting the findings. First, the use of single-generation laboratory rearing may have led to an underestimation of true field resistance, as it reduces exposure to natural environmental pressures and multi-generational insecticide selection that shape resistance dynamics in wild populations. Second, the use of rat blood feeding instead of human blood sources may introduce variability in mosquito feeding efficiency, digestion, and physiologi-

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cal response, potentially influencing susceptibility outcomes and bioassay performance. Additionally, laboratory conditions cannot fully replicate field heterogeneity in temperature, host availability and insecticide exposure, which may further affect resistance expression. Despite these constraints, the standardized laboratory approach was essential to ensure experimental control and comparability across strains. These limitations are now explicitly acknowledged in the revised manuscript in line with recommended vector resistance monitoring guidelines.

Conclusions

As Pakistan is an agricultural country and most of its population is illiterate (about 50%), they are using insecticides blindly. Due to the intensive blind use of chemical insecticides, mosquito vectors are rapidly developing resistance against most of them. This study documents rapid progression of insecticide resistance in *Ae. aegypti* across different habitats in Punjab between 2024 and 2025. The Tidiwala (TDW) strain, collected from an area with minimal insecticide use, provides a valuable regional susceptible baseline. Alarmingly, the UAF strain showed very high resistance (RR> 100) to lambda-cyhalothrin and deltamethrin by 2025, and moderate resistance to temephos. Bti remained relatively effective (RR= 60.9 for UAF vs. TDW), suggesting it may be a viable rotational partner. Integrated vector management-including insecticide rotation, Bti use and non-chemical methods (for example, source reduction)-is urgently needed to preserve the efficacy of remaining susceptible compounds. Continued resistance monitoring with expanded geographic coverage is recommended.

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Ethical consideration

This study was carried out according to the guidelines provided by the ‘Ethical Committee of the Faculty of Biological Sciences’, Government College University, Faisalabad, Pakistan, with approval number Ref. No. GCUF/ERC/3011 dated 12-01-2024.

Conflicts of interest statement

The authors declare that there is no conflict of interest.

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