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MOLECULAR AND BIOASSAY-BASED ASSESSMENT OF TEMEPHOS RESISTANCE IN *AEDES* LARVAE IN PAKISTAN'S TWIN CITIES

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Abstract

Chemical insecticides are still essential for managing dengue and other arboviral disease vectors. With temephos, a neurotoxic organophosphate, being the most commonly used larvicide against Aedes larvae worldwide; larviciding is a crucial part of vector management programs. However, through a variety of mechanisms, the ongoing use of temephos has reduced its effectiveness by causing resistance to emerge in Aedes populations. The present study identifies the presence of the Ace-1 gene linked to organophosphate resistance and assesses Aedes larvae' susceptibility from specific areas of Rawalpindi and Islamabad, Pakistan, to temephos. A study was conducted on Aedes larvae collected from Rawalpindi and Islamabad between February and December 2024. The active ingredient temephos was used in the stock solution, and DNA extraction was performed manually using the SDS method. PCR amplification was used to identify the Ace-1 gene of Aedes, and gel electrophoresis was performed to confirm its presence. Statistical analysis was performed using probit analysis to determine Lethal Concentrations (LC) for the species. The results will inform future research and pest control strategies. According to the biological tests, Aedes larvae from the study sites showed resistance to temephos. Molecular analysis revealed that the Ace-1 gene, a marker for organophosphate resistance, was current. Aedes populations in Rawalpindi and Islamabad have developed a resistance to temephos, according to genetic study and bioassay data. These results highlight the necessity of using a variety of vector management techniques in order to successfully fight dengue and other Aedes-transmitted infections.

Keywords: *Temephos resistance, Aedes larvae, Dengue vector control, Molecular analysis, Ace-1 gene.*

Introduction

Mosquitoes represent a significant threat to public health due to their role in the transmission of various viruses and parasites, including dengue fever, malaria, Japanese encephalitis, and chikungunya. An estimated 2.5 billion individuals inhabit regions classified as high-risk, in between 50 to 100 million individuals suffering from dengue fever annually (Gupta *et al*., 2021). Numerous fatal illnesses, such as dengue, Zika, yellow fever, and chikungunya viruses, are spread by Aedes mosquitoes, specifically *Aedes aegypti* and *Aedes albopictus*(Bharati and Saha, 2021). These mosquitoes have spread to previously uninfected areas thanks to increased international trade and human mobility (Gupta*et al.*,2021).

Any of the four serotypes of dengue fever (DENV1-4) can cause widespread outbreaks, which are a major concern worldwide, especially in tropical and subtropical areas (Bharati and Saha, 2021). Dengue outbreaks have been common in Pakistan; the first case was reported in Karachi in 1994, and since there have been multiple significant epidemics, most recently in 2013 and especially in 2004–2005 (Rasheed*et al*.,2013). Aedes mosquitoes continue to pose a threat, particularly in urban areas like Rawalpindi and Islamabad, as evidenced by the rise in cases after 2010^{th}floods and the outbreaks that followed (Arslan *et al*., 2015).

Genetic studies have shown that *A. albopictus* is adaptable; it can breed in volatile habitats; and feeds on a variety of hosts, including humans and other animals. Some other characteristics have been observed that it spread in temperate climates where it is well adapted to cooler temperatures than the other Aedes species therefore remain active and breed (Egid *et al.,* 2022). Eradication and monitoring are still under process in several areas and the methods applied are environmental manipulation and biological control along with insect control. However, people have also resisted these methods due to various reasons leading to the need for integrated pest management to reduce impacts of this notorious Aedes mosquito species on public health (López-Solís *et al*.,2020).

The application of insecticides, including the organophosphate temephos, to Aedes mosquito larvae is central to current vector control techniques. But in many areas, resistance to temephos has developed as a result of its frequent and extensive use, making it more difficult to manage mosquito populations and stop the spread of dengue (Adhikari and Khanikor, 2021). Temephos paralyses and kills mosquitoes by inhibiting acetylcholinesterase, an enzyme essential to their nervous system (Knutsson, 2016). Tragically, resistance has been noted, which lessens its efficacy and calls for different approaches (Al Nazawi, 2019; Pulkrabkova*et al*., 2023). Insecticides resistance makes managing diseases like dengue fever, Zika, and chikungunya difficult at the operational level. The rapid rate of resistance development has been demonstrated by certain research that suggest Aedes have developed resistance to a substantial degree in at least five generations of their species. This goes to support that management needs to be constantly monitored and adjustments made to the existing approaches. Cross-resistance is one of the main limitations in insecticide applications. This information might help in acquiring perception of mechanisms of temephos resistance and possibility of cross-resistance in order to

develop proper and long term and sustainable vector control strategies (Davila-Barboza *et al*., 2024a).

The present study assessed the development of resistance to temephos on dengue larvae especially by focusing on key indicators like the Ace-1 gene, and its impact on dengue management strategies, emphasizing the need for continuous monitoring and insecticide adjustment.

Materials and methods

Study Area and Sampling

In Rawalpindi and Islamabad, Aedes larvae were collected for the study during the moths of February and December 2024. Taramri Chowk, Park Road, Saddar, Kuri Road, and Gerry Office are among the well-known locations where the larvae were discovered. The larvae were brought to the lab for additional analysis after being directly removed from clean water sources.

Preparation of Stock solution

The stock solution was prepared using the active ingredient temephos, which was purchased from Multi-Linkx Enterprises, Islamabad. The stock solution was obtained by dissolving the 7g active temephos in 500 ml of distilled water. The solution was dissolved properly with the help of a magnetic stirrer for about 40-45 minutes. As a result, an effective stock solution was obtained.

Dose-Response Assays

The dose-response assays were performed using the protocols provided by the WHO (Davila-Barboza *et al*., 2024b).The tests were performed with late third instar/early fourth instar larvae in which various concentrations of temephos, *i.e*. 0.01 mg/l, 0.013 mg/l, 0.019 mg/l, 0.025 mg/l, 0.028 mg/l, 0.034 mg/l, 0.04 mg/l, and 0.0425 mg/l were tested against the larvae. For each concentration, larvae were transferred to the disposable cups. Each cup contains 20 larvae of *Aedes*. For control, the cup only contained distilled water, and no insecticide was added to it. The mortality rate was recorded after 24 hours of exposure, and the larvae that remained alive were regarded as resistant.

DNA Extraction

DNA was extracted from larvae that were alive after exposure to temephos. DNA extraction was done manually using the SDS (Sodium Dodecyl Sulfate) method. The method is elaborated below in the Table 1.

Table 1. SDS method used for DNA extraction of the *Aedes*.

Isolation step	**SDS Method**	
	Reagents	**Centrifugation / Incubation time**
Lysis	mosquito larvae +100 µL lysis buffer + 5 µL proteinase K	1h (+ 55°C)
	5 µL RNase A	20 min
	25:24:1 phenol+ chloroform + Isoamyl alcohol	10 min (+55°C)
		10 min 10,000 rpm
	24:1 chloroform+ Isoamyl alcohol	10 min 10,000 rpm
Precipitation-ion	0.2 volume 5M NaCl + 2.5 volume ethanol 100%	-20°C One hour
		25 min 12,000 rpm
Drying		15-30 min
Wash	500 µL ethanol 70%	10 min 10,000 rpm
Elution	100 µL T-10 Buffer	

DNA Quantification

By evaluating the DNA content, the concentration and purity of DNA were evaluated. The Nanodrop apparatus was also used to measure the concentration and purity of the DNA. This instrument is used in laboratories that measure the amounts of proteins, RNA, and DNA as well as the purity of samples in 1-2µL quantities. This is particularly useful in molecular biology because of its precision, rapidity, and ease of use (García-Alegría *et al*., 2020).

PCR Amplifications

In order to identify the Aedes Ace-1 gene, the isolated DNA was amplified using Polymerase Chain Reaction (PCR). For PCR, the primers listed in Table 2 were used.

Table 2. Primers used for the PCR.

Gene	Primer	Sequences	Primer Length	Product Length	Reference
Ace-1	Forward	5'- GAAAGAGGAGGGTGTCACA- 3'	20	215bp	Resti *et al*., 2022
	Reverse	3'-CATATCGCTGGGCAAACTC-5'	20	215bp	Resti *et al*., 2022

A 20µL final volume was used for PCR amplification, which included 10µL of Master mix, 2µL of DNA, 4µL of nuclease-free water, 2µL of Forward primer, and 2µL of Reverse primer. The following were the settings for the thermal cycler's amplification: After five minutes of initial denaturation at 94°C, there were thirty-five cycles of denaturation, annealing, and extension. In a gradient thermocycler, annealing was done at 55, 57, 58.8, and 61.8 degrees Celsius. A final extension was done for 10 minutes at 72 degrees Celsius (Table 3).

Table 3. Conditions for PCR of *Ace-1* gene.

Temperature	PCR step	Duration	No. of cycles
94° C	Initial denaturation	5 min	1
95° C	Cyclic denaturation	45 sec	35
55°C, 57°C, 58.8°C, 61.8 °C	Annealing	30 sec	
72° C	Cyclic extension	30 sec	
72° C	Final extension	10 min	1

Gel Electrophoresis

For interpreting the gradient PCR result to check the presence of the *Ace-1* gene, gel electrophoresis was performed. For this 1.5 % agarose gel was used (Table 4).

Table 4. Gel electrophoresis requirements.

Materials	Volume
Running TAE Buffer	Volume estimated according to gel tank (1X)
TAE buffer	50 mL (1X)
Agarose	1.5 % (0.75 g for 50 mL TAE)
Ethidium bromide	3 µl
Loading dye	1 µl (6X)
PCR product (DNA)	5 µl
Ladder	3 µl

Agarose (0.75 g) was dissolved in 1X 50 ml TAE buffer in a glass flask and was placed in a microwave for heating to dissolve. After heating, immediately 3 µl Ethidium bromide was added to a glass flask (having a solution of Agarose + TAE), gently mixed, and poured into a gel casting tray (that has a comb), leaving it at room temperature to solidify the gel (Table 5).

Table 5. Gel electrophoresis conditions.

Gel conc.	Voltage	Current	Time
1.5 %	90 V	500 mA	35 min

Statistical Analysis

Probit Analysis

A specific type of regression analysis called probit analysis is used on variables that have just one of two possible outcomes (positive or negative), or binomial response variables. The procedure is performed by transforming the concentration-response curve to a straight line, which is analyzed either by the least squares or maximum likelihood regression (Sá *et al*., 2019). Using specialized software, probit analysis was performed, plotting the corresponding (or logarithmic) concentration values on the x-axis and detection probabilities on the y-axis. This type of analysis is usually done to determine the Lethal Concentrations (LC).

Lethal Concentration (LC)

Lethal Concentration (LC) refers to the concentration of the products/chemicals in water that is fatal for the population of a particular species. This is usually performed to determine mortality at a proper concentration, for example, LC50 kills 50% of the population we are testing and LC98 kills 98%.

Results

Temephos Bioassays

Dose-response assays were performed to determine the lethal concentrations of the *Aedes* larval population from the different areas of Islamabad and Rawalpindi. The assays were carried out on the late 3rd and early 4th instar *Aedes* larvae. Different concentrations of temephos were used to find lethal concentration values. The concentrations range from 0.1 mg/l to 0.045 mg/l. The results of the various concentrations are given in Table 6.

Table 6. Results of Concentrations used for temephos assay.

Concentration (mg/l)	Larvae Exposed	Alive Larvae	Dead Larvae	Mortality (%)
0.01	20	19	1	5
0.013	20	17	3	15
0.019	20	13	7	35
0.025	20	9	11	55
0.028	20	7	13	65
0.034	20	5	15	75
0.04	20	2	18	90
0.045	20	0	20	100

The mortality increased as concentrations increased. At 0.01mg/l, we only observed 5% mortality but as the concentration increased to 0.045 mg/l, the mortality rate also increased to 100%.

Statistical Analysis

Probit regression analysis and Microsoft Excel were used for statistical evaluation of the dataset.

Probit Analysis

Based on probit analysis, lethal concentration values were determined. The results of the Probit analysis are shown in Figure 1.


RWP/lab, 2024, F4 - temephos

Y = A + BX
A = 12,5167136 B = 4,576328275
L1b (sup.) 5,024290603
L2b (inf.) 4,128365946

	# mortos	# expostos
controle:	0	80

OK! #VALUE! som chi^2 0,131 p 1,0000

mg/L mort. Controle % 0

concentração	# mortos:	# expostos:	% mort.	Abbott	Probit	log concentração	mortes esperadas	chi 2
0,01	1	20	5,0	5,0	3,36	-2,00	1	0,00
0,013	3	20	15,0	15,0	3,96	-1,89	3	0,05
0,019	7	20	35,0	35,0	4,61	-1,72	7	0,00
0,025	11	20	55,0	55,0	5,13	-1,60	11	0,02
0,028	13	20	65,0	65,0	5,39	-1,55	13	0,00
0,034	15	20	75,0	75,0	5,67	-1,47	16	0,03
0,04	18	20	90,0	90,0	6,28	-1,40	17	0,02
0,045	20	20	100,0	100,0	0,00	0,00	0	0,00
				0	0,00		0,00	0,00
				0	0,00		0,00	0,00
				0	0,00		0,00	0,00

CL		i.c. 95%		
50	0,02278	0,01844	< LC <	0,02813
90	0,04340	0,02989	< LC <	0,06303
95	0,05211	0,03346	< LC <	0,08116
98	0,06401	0,03783	< LC <	0,10832
99	0,07342	0,04099	< LC <	0,13151

Figure 1: Results of the Probit Analysis.

Lethal Concentrations (LC)

We obtained an LC50 for temephos at **0.02278 mg/l** (CI95 = 0.0184 – 0.0281) and for LC98 to kill 98% of the *Aedes* population **0.06401 mg/l** concentration of temephos is needed. Concerning

the toxicant concentration, a higher LC value denotes a larger number of organism fatalities. These values are shown in table 7.

Table 7. Lethal concentration values of temephos against *Aedes* larvae.

Lethal Concentration (LC) value	Temephos Concentration (mg/l)	Confidence Interval (CI) (95%)
50	0.02278	0.01844 - 0.02813
90	0.04340	0.02989 - 0.06303
95	0.05211	0.03346 - 0.08116
98	0.06401	0.03783 - 0.10832
99	0.07342	0.04099 - 0.13151

Log Regression Graph

Mortality and log concentration values are plotted and shown in Figure 2, *i.e.*, given below.

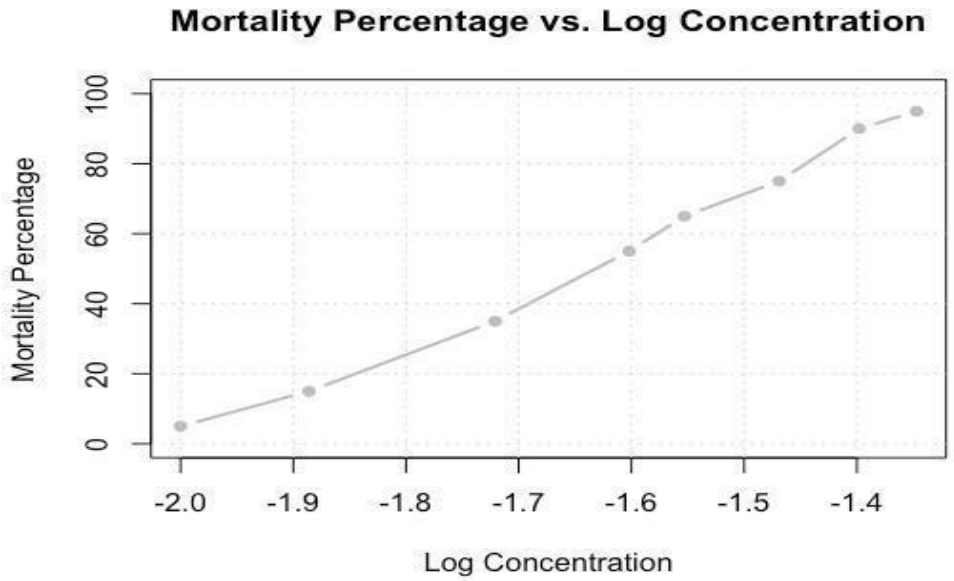

Figure 2. Log Regression graph, Mortality is shown on the y-axis while log concentrations are shown on x-axis.

PCR Amplification Results

Amplification of *the Ace*-1 gene was performed through a Polymerase Chain Reaction and visualized through a 1.5% gel. We saw that a band of 215 bp size was amplified as shown in figure 3. The optimum annealing temperature for amplification was 58.8 ºC (Lane B) and 61.4 ºC (Lane C). It shows that *the Ace*-1 gene is present in the tested samples.

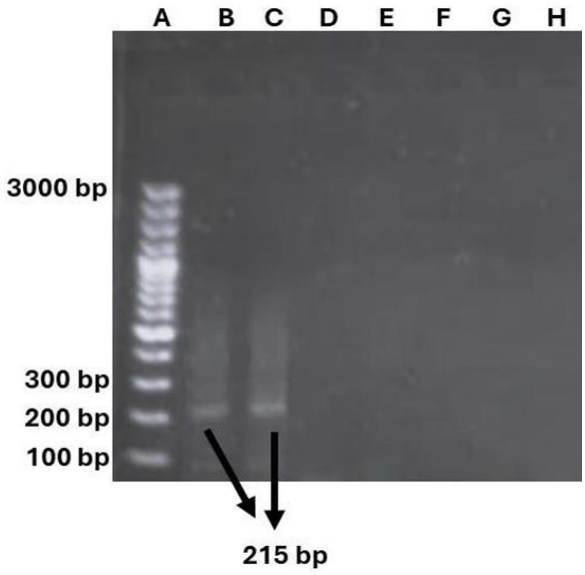

Figure 3. Gel Electrograph of PCR reaction of *Ace-1* gene. Lane A contains molecular marker (100bp), lane B shows sample amplified at 58.8ºC and lane C shows sample amplified at annealing temperature 61 ºC

Discussion

The present study was performed to evaluate the *Aedes'* resistance against temephos collected from various areas of the twin cities, Islamabad and Rawalpindi. The larvae were collected from 6th Road (Rawalpindi) and Park Road, Sector G-7, Gerry office, Kuri Road, and Taramri Chowk (Islamabad). Temephos is a neurotoxic larvicide that is widely employed in Integrated Vector Management programs to control the *Aedes* larvae (Peláez *et al*., 2018). The insecticides are frequently used without any knowledge of the risk of resistance selection due to the temephos in the field (Dusfour *et al*., 2019). To study the effectiveness of temephos, we treated the larvae of *Aedes* with varying concentration levels. When the larvae were exposed to temephos for 24 hours, most of the larvae exhibited a high level of resistance against temephos. This may be because temephos is commonly used and there is inadequate information about which concentrations this chemical is effective in. The other reason may be that the *Aedes* have developed various types of mutations against temephos to render them ineffectiveArslan *et al.*,(2015). Temephos resistance has been reported in the research carried out in Lahore, Pakistan, suggesting that there is a threat to control the *Aedes* vector population. The study found metabolic resistance mechanisms and target-site alterations in *Aedes* species from Lahore, confirming that organophosphate resistance is prevalent throughout Pakistan (Rahman *et al*., 2021). In Southeast Asia, similar work is done to identify the temephos resistance in the larvae of *Aedes* species. They also said that the temephos is not effective against the *Aedes* larvae in these smaller concentrations (Arslan *et al.,* 2015).

Our study's molecular analysis corroborated the existence of the Ace-1 gene, which is a key mechanism by which *Aedes* mosquitoes acquire resistance to temephos and encodes a mutated acetylcholinesterase enzyme. This is consistent with the findings of Davila-Barboza *et al*.,

(2024); Dusfour *et al*., (2019) who found identical resistance markers in field populations in Mexico and Southeast Asia, respectively. The resistance to temephos has been well documented on a global scale. Nationwide monitoring measures were put in place after Grisales *et al*., (2013) discovered moderate to high resistance in several urban locations in Colombia. Similarly, Ace-1 mutations were crucial in the widespread resistance observed in over 30 dengue-endemic regions in PeruPalomino *et al.*, (2022).Studies carried out in Saudi Arabia Al Nazawi, (2019) and India Muthusamy and Shivakumar, (2015) also demonstrate the increasing inefficiency of temephos in controlling Aedes because of genetic changes in the vector. Understanding that ecological and operational factors influence resistance in addition to genetic changes is essential. As mentioned by Spadar *et al.*, (2024) and Sá *et al.*, (2019) selection pressure may result from differences in local temephos administration techniques, water container use, and environmental conditions. Furthermore, even though they are useful, laboratory bioassays may not accurately represent resistance at the field level, especially in populations exposed to a variety of climatic and chemical factors.

Reducing mosquito breeding grounds and delaying the evolution of resistance are further benefits of environmental management, especially the removal of stagnant water sources (Udayanga *et al*., 2019).The study of temephos resistance can be affected by the different limitations that can influence the study results. Genetic diversity in the *Aedes* population can be a crucial challenge as it can cause different types of resistance mechanisms that make it difficult to generalize the results across the different regions and species. It is reported that resistance can be influenced by local environmental factors and the historical use of insecticide exposure, which results in different resistance phenomena even within populations that are nearly located (Spadar *et al*., 2024). Nevertheless, such limitations at the methodological level can constitute a definite factor within the process. For instance, laboratory experiments may not produce the right outcomes like field experiments because of variations in factors such as temperature and humidity, among others. Therefore, the laboratory strains of the *Aedes* cannot represent the full processes of the resistance of the wild populations, and as a result, the results cannot be meaningful. The sample size and the biasness in the sampling methods can also become a challenge and will not allow an arrival at definite conclusions (Diniz *et al*., 2014). Another drawback associated with the excessive use of temephos is the development of resistance towards the insecticide and the production of possible ecological effects. Thus, it can stress that besides purely chemical control one should also pay attention to biological and environmental aspects as well (Udayanga *et al*., 2019).

By examining its mechanisms, detecting genetic alterations, and assessing its effects on Aedes ha bitats, future research should concentrate on comprehending and preventing temephos resistance. It's also critical to look at other pesticides and concentrate on biological control methods like sust ainable surveillance systems.

This will enable effective regulation and rapid reaction to Aedes larvae. Integrated Vector Management (IVM) can also play an important role in reducing temephos resistance management by promoting the rational use of temephos and mixing different control methods to

minimize the dependence on a single strategy which may help reduce or delay temephos resistance (Roiz *et al*., 2022). There should also be work done on using alternative strategies like using *Bacillus thuringiensis israelensis* (Bti) as an alternative tool because studies have shown that *Bti* does not induce cross-resistance to temephos which makes it a valuable tool in integrated vector management (IVM) strategies (Sá *et al*., 2019). By lowering breeding grounds and larval habitats, source management in particular, the removal of containers can be extremely important in controlling temephos resistance. This strategy reduces the number of mosquitoes and decreases the selection pressure that riggers resistance mechanisms.

Conclusion

Existing vector control methods are facing a serious challenge as the *Aedes aegypti* and *Aedes albopictus* populations in Rawalpindi and Islamabad have become resistant to the widely used larvicide, temephos. According to the study, existing programs should incorporate alternative control approaches, improve resistance management measures, and conduct routine monitoring of pesticide susceptibility. A change to integrated vector management (IVM) strategies, which incorporate biological, chemical, environmental, and educational elements, provides a framework for addressing vector resistance that is more adaptable and sustainable.

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