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TOXICITY OF DIFFERENT GROUPS OF INSECTICIDES AND DETERMINATION OF RESISTANCE IN *Aedes aegypti* FROM DIFFERENT HABITATS

Sadia Abbas[1], Shabab Nasir[1,*], Muhammad Fakhar-e-Alam[2] and Malik Saadullah[3]

[1]Department of Zoology, Government College University, Faisalabad, Pakistan; [2]Department of Physics, Government College University, Faisalabad, Pakistan; [3]Department of Pharmaceutical Chemistry, Government College University, Faisalabad, Pakistan
[*]Corresponding author's e-mail: flourenceshabab@yahoo.com

A large number of insecticides are used for the control of agricultural pests as well as household pests, such as mosquitoes, cockroaches and house flies with the application of over and under doses in Punjab, Pakistan. Moreover, after the dengue epidemic that occurred during 2010, insecticides were sprayed in huge quantity at high doses in major cities of Punjab to control mosquitoes. This resulted in insecticidal resistance in mosquitoes. Mosquitocidal assays of larvae (in beakers) and adults (impregnated papers) were evaluated after 24 (hr.). The larvicidal LC_{50} value of temephos ranged from 0.007 to 0.416 ppm. In the case of adulticides, three groups of insecticides were applied by filter paper method and used against twelve different populations collected from urban, agricultural and industrial areas of Lahore (LHR), Rawalpindi (RWP), Sialkot (SKT) and Faisalabad (FSD). Pyrethroids demonstrated the lowest effective concentrations among the tested pesticides (organophosphates OP & carbamates). Among the pyrethroid group, deltamethrin was recorded as being the most toxic with LC_{50} (0.483–9.245 ppm), followed by cypermethrin (1.839 – 33.139 ppm) and permethrin (5.145 – 101.533 ppm). The chi-squared value showed no heterogeneity between all the experiments. Moreover, the obtained results indicated that the LHR population was highly resistant, followed by the RWP, SKT and FSD populations. In addition, the mosquito populations from agricultural areas were more resistant than those from urban and industrial areas. The biochemical analysis showed the elevated activity of enzymes (esterases, mixed-function oxidases, glutathione S-transferase and acetyl-cholinesterase) in resistant populations of mosquitoes. LHR population showed the maximum activity of enzymes, like esterase (0.54), mixed-function oxidase (0.72), glutathione S-transferase (0.16) and acetyl-cholinesterase (0.13) from agricultural areas. It was concluded that the injudicious application of chemicals in such areas caused the potential risk of resistance and resurgence of certain mosquitoes. Thus, further research is needed to identify health and environmental risks and to devise an effective programme through the use of selective and specific insecticides.

Keywords: *Aedes aegypti*, agricultural populations, industrial areas, insecticidal resistance, urban areas.

INTRODUCTION

Aedes mosquitoes are responsible for the spread of dengue, chikungunya, filarial diseases and Zika virus (Lambrechts *et al.*, 2010). Pakistan has a population of about 180 million inhabitants, of which 23.4% of the Pakistani population is at a high risk of contracting mosquito-borne diseases (WHO, 2006; Anonymous, 2016). Dengue fever is a severe infectious disease in several tropical and subtropical countries in Asia, Africa including the Americas, as it causes more illnesses than any other arboviral infection (WHO, 2006). Although *Aedes* mosquitoes were reported during the 1960s in Pakistan, dengue fever was reported for the first time in 1994 in Karachi, Pakistan, and then spread to other parts of the country, with *Ae. aegypti* being the major vector (Mohsin *et al.*, 2016). As most mosquito-borne diseases are viral, there is neither a proper vaccine nor treatment available for these diseases. The only solution is to manage the mosquito population. Although the quickest and easiest method for controlling mosquito populations is the use of insecticides but the risk of rapidly accelerated resistance is a drawback to reliance on a limited number of insecticides. Due to the development of resistance, vector-borne diseases are spreading and causing more problems for the world population, especially in Africa and South Asia (Strode *et al.*, 2014).

Insecticidal resistance was also described in *Anopheles* mosquitoes in Greece and Nigeria in 1951 and 1955, respectively (Brown, 1958). Resistance has been reported in relation to many group of insecticide, including microbial and insect growth regulators (IGRs) (Alsheikh *et al.*, 2016). Despite intense efforts and lengthy research investigation, the available information about the practical aspects of insecticidal resistance that would help in the adjustment of control practices according to specific requirements are not sufficient (Soko *et al.*, 2015). Therefore, the potential risk of

re-emergence of vector-borne diseases is severe in many cases where this issue is not present, vector-borne disease re-emergence is expected to threaten disease control. However, the critical analysis of the present knowledge regarding vector resistance (e.g., WHO resistance database and records of control programmes) shows that resistance is often not addressed in control strategies (Ranson *et al.*, 2011). In addition to insecticidal resistance, some other problems such as the availability of non-registered insecticides for public health use, unavailability of registered insecticides. This includes high prices at the time of epidemic, also contribute to the failure of vector control (Owusu *et al.*, 2015). Previous research studies have shown that many genetic, biological, environmental and operational factors contribute to the level and development of insecticidal resistance (Ranson *et al.*, 2011). For an insecticide to be banned due to its resistance, the level of resistance must be high enough that it would not help to decrease disease transmission through vector control. If the resistance level is low (i.e., <10%), then it will not affect mosquito control programmes, and only surveillance and monitoring will be sufficient (Raghavendra *et al.*, 2010).

Production of different enzymes in mosquitoes in response to different insecticides is also responsible for insecticidal resistance (Hemingway and Ranson, 2000). The over-production of esterases has also given rise to noticeable resistance in mosquitoes due to the use of organophosphate (OP) and carbamate groups of insecticides (Hemingway *et al.*, 2004). The increased activity of esterases, oxidases and glutathione S-transferase (GSTs) due to the application of different insecticides, such as permethrin, deltamethrin and cypermethrin, also increases the resistance level in mosquitoes (Pimsamarn *et al.*, 2009).

Pakistan is generally an agricultural country, and more than 75% of its inhabitants live in rural areas and depend on agriculture for their livelihood. The majority of people living in villages are illiterate, and they do not know what kinds of insecticide should be used on crops and household pests. They are also unaware of the dosage and potency of insecticides to use. During 1954, 254 tonnes of formulated pesticides were imported into the country for the first time but this amount of pesticides increased to 665 tonnes in 1980 and to 61,229 tonnes in 2000, worth 0.154 billion $USD (Ahmad *et al.*, 2002). The main reason for insecticidal resistance in Punjab, Pakistan, is the use of agricultural insecticides (cypermethrin, deltamethrin, etc) against household pests, such as mosquitoes, in high doses. Moreover, after the dengue epidemic that occurred during 2010, insecticides were sprayed repeatedly and at high doses in major cities of Punjab to control mosquitoes. Therefore, the present study was undertaken to determine the insecticidal resistance of *Ae. aegypti* from different localities (urban, agricultural and industrial) to different insecticidal groups (OP, pyrethroids & carbamates).

MATERIALS AND METHODS

***Study sites and use of pesticides*:** Samples from larval populations were collected from urban, agricultural and industrial areas in the cities of Lahore, Rawalpindi, Sialkot and Faisalabad. These cities were selected due to high insecticidal usage for agriculture and household pest management: Faisalabad (31° 25′N and73° 5′E) is an industrial hub and the third most populous city of Pakistan, with agricultural activities occurring in the villages. People and the government have been using insecticides moderately here for mosquito control since 2011. Lahore (31° 34′N and74° 19′E) is the 2nd most populous city of Pakistan, and agricultural activities are mostly limited to the growing of fruits and vegetables. Huge amounts of pesticides have been used since the dengue epidemic in 2010. Rawalpindi (33° 37′N and73° 4′E) is a semi-arid city and is an entry point to the Punjab province. The government has been using a huge amount of insecticides for the control of mosquitoes since 2013. Sialkot (32° 29′N and74° 31′E) is known around the world due to the production of sports items, and people generally grow rice in this city.

In urban (only meant for residence of people) and industrial areas (specially designed for factories and business) people mostly use temephos for mosquito larvae control and cypermethrin, deltamethrin, lambda cyhalothrin, malathion and permethrin against household pests, including mosquitoes and fruit and vegetable pests. In agricultural areas (arable, under permanent crops or under permanent pastures) people use all groups of insecticides (OPs, pyrethroids, carbamates and IGRs) along with the above-mentioned insecticides.

***Experimental population*:** The collected larvae were kept in plastic trays under laboratory condition (26±2°C and 60±5% RH) and separated using siphon tubes. Newly emerged third/fourth instar larvae were separated and used for the larval bioassay. The remaining larvae were reared to the adult stage for culture establishment. Four- to five-d-old females were separated and used for the adult bioassay. The susceptible laboratory strain was collected from remote areas of Bahawalpur and then reared under laboratory conditions for more than 30 generations without any exposure to insecticides.

***Insecticide stock preparation*:** The different insecticides along with their trade names and the formulations used in this study were temephos (Abate 1 SG, BASF S.A., Brazil), fenitrothion (Fenitro 50 EC, Sinochem Ningbo Chemical Co. Ltd., China), malathion (Fyfanon 57 EC, Jaffer group, Pakistan), pirimiphos-methyl (Actellic 50 EC, Syngenta UK Limited, cypermethrin (Bulletin 10% EC, Ali Akbar Group, Pakistan), deltamethrin (Decis 2.5% EC, Bayer Pakistan (Pvt.) Ltd.), permethrin (Ambush 25% EC, Sara Pak Zist Co., Iran), and bendiocarb (Ficam 80% WP, Bayer CropScience Ltd., UK).

To assess the impact of resistance, the effectiveness of the insecticide treatments under simulated field conditions was evaluated in routine laboratory experiments using commercial products and the application techniques typically used in control programmes. These included the focal application of the larvicide temephos (1, 0.5, 0.25, 0.125, 0.06 and 0.03 ppm) and space-spray and residual treatments for adult control. For the latter, the products evaluated were the fenitrothion (2500, 1250, 625, 312.5, 156.25 and 78.1 ppm), malathion (1425, 712.5, 356.25, 178.13, 89 and 44.53 ppm), and pirimiphos-methyl (1000, 500, 250, 125, 62.5 and 31.25 ppm), the pyrethroids; cypermethrin (250, 125, 62.5, 31.25, 15.5 and 7.58 ppm), deltamethrin (62.5, 31.25, 15.62, 7.8, 3.9 and 1.95 ppm), and permethrin (625, 312.5, 156.25, 78.1, 39 and 19.5 ppm) and the carbamate bendiocarb (24,000, 12,000, 6000, 3000, 1500 and 750 ppm). These concentrations were selected in accordance with the concentrations used under field conditions.

***Larval bioassay*:** Sets of 20 (third/fourth instar) larvae were placed in glass beakers filled with 249 ml of distilled water and 1 ml of each concentration of temephos in water. Three replicates of 20 larvae were used for each concentration and the control trials. For temephos, the number of dead larvae due to lack of movement was recorded 24 h after the introduction of the larvae to the beakers.

***Adult bioassay*:** Sugar-fed three- to five-d-old female mosquitoes were tested. Sets of 25 adults were introduced to holding tubes before being exposed to insecticide-impregnated papers. Equal numbers of control tests were also carried out by exposing mosquitoes to insecticide-free papers. The experiment was replicated four times. After the pre-determined period of exposure (60 minutes), all mosquitoes were transferred to new tubes, provided with a 10% sugar solution and held for a 24 h recovery period. Thereafter, mortality was recorded, and the resistance status was determined according to WHO criteria: a population was considered susceptible if the mortality rate was 98- l00%. The possibility of resistance occurred at 80-97% mortality, and a population was considered resistant at <80% mortality.

The standard mortality bioassay techniques for various insecticides as suggested by the Insecticide Resistance Action Committee (IRAC) were used. All the precautions were undertaken while performing the bioassay experiments. The mortality data were corrected using Abbott's formula and then analysed with probit analysis (Finney, 1971) using statistical software Mini tab 17.

The strains showing the highest resistance factors (this value shows how much insecticide is required to provide equal control against a resistant strain compared to a susceptible one) were selected for enzyme studies. The following scale was used to categorize the populations collected from Sialkot, Rawalpindi, Lahore and Faisalabad on the basis of their resistance factors (RFs): Low <5, Moderate >5 and <10, High 10-50 and Extremely High >51 (Lima *et al.*, 2011).

***Biochemical analysis*:** For enzymatic estimation, thirty mosquito larvaewere washed thoroughly with distilled water and the adhering water was removed by using bloating paper. The larvae were homogenized using ice-cold sodium phosphate buffer (20mM. pH 7.0) with the help of Teflon hand homogenizer. Thereafter, the homogenate was centrifuged at 8000×g and 4°C for 20 minutes and supernatant was used for the estimation of Esterases or Phosphatases. Solutions and glassware used for homogenization were kept at 4°C prior to use, and the homogenates were held on ice until used for various assays.

The dosing of acetylcholinesterase (AChE) was made in two distinct 96-well plates, to determine activity of AChE with *propoxur* inhibitor. 25 µl of Homogenates was added in plates:145 µl of Triton/Na phosphate (5 ml of 100% TritonX-100 in 50 ml of 1M sodium phosphate buffer at pH 7.8 and 455 ml of distilled water), and 10 µl of DTNB/Na phosphate (10 mM DTNB in 100 mM sodium phosphate buffer at pH 7.0). In the AChE plates, 10 mM acetylcholine iodide in water was added to each well in the absence of propoxur as a substrate. The plates were incubated for one hour at room temperature, protected from light and read at 405 nm. For Mixed Function Oxidase, 20 µl homogenate was added: 60 µl of 90 mM Potassium phosphate buffer (final pH adjusted to 7.2), 200 µl of Na acetate/TMBZ working solution (0.012g of 3,3,5,5 tetramethyl benzidine dihydrochloride in 6 ml of methanol and 18 ml of 250 mM sodium acetate buffer at pH 5.0), and 25µl of 3% hydrogen peroxide (H2O). The plates were incubated for 90 minutes at room temperature, protected from light and subsequently read at 620 nm.

For Esterases, the homogenates were centrifuged at 12,000 g for 60 seconds and 10 µl were taken in duplicate and placed in 96-well plates. The Following was added to each well: 200 µl of PNPA/Na phosphate working solution prepared by adding 100 mM PNPA in acetonitrile (0.01815 g of PNPA in 1 ml of acetonitrile) to 24.75 ml 50 mM sodium phosphate Buffer at pH 7.4 (50 ml of 1 M sodium phosphate Buffer at pH 7.4 in 950 ml of distilled water). This assay aims to estimate the reaction of kinetics. Therefore, the absorbance variation represented by the amount of substrate consumed was measured through nine readings at 405 nm, every 15 seconds (Li *et al.*, 2007).

***Statistical analysis*:** Means were calculated in Excel from mortality data. These means were further analysed with probit regression analysis using the computer-based software Polo-PC 2002. Resistance ratios (LC_{50} of resistant strain / LC_{50} of susceptible strain) were also calculated with respect to the corresponding susceptible populations.

RESULTS

***Larval bioassay*:** Third instar larvae of *Ae. aegypti* were subjected to temephos, and 50 percent mortality for lethal concentrations of 0.007 to 0.416 ppmwas recorded after 24 h

in susceptible strain and field populations, such as LHR, RWP, SKT and FSD. The fiducial limits ranged between 0.001–0.292 and 0.014–0.831 among all populations, and these populations showed up to 58.43-fold variation. The obtained results from the urban areas indicated that the LHR population was the most resistant (59.45-fold), followed by the RWP (21.14-fold), SKT (8.98-fold) and FSD populations (4.83-fold). The mosquito populations from agricultural and industrial areas in LHR were also the most resistant (55.47- & 58.43-fold, respectively), whereas those from FSD were the least (4.00- & 4.71-fold, respectively), as shown in Table 1. Among the entire mosquito populations from the different areas, those from the LHR showed the highest LC_{50} value, and the lowest were from the FSD city, whereas those from the other two cities were intermediate between the values of LHR and FSD. These results indicate that the LHR mosquito populations were more resistant than the others due to the greater and longer (approximately 8 years) use of temephos in the LHR city, followed by RWP (5 years of application).

***Adult bioassay*:** This bioassay included three groups of insecticides: synthetic pyrethroids, OPs and carbamates. The synthetic pyrethroids included deltamethrin, cypermethrin and permethrin. Deltamethrin-impregnated paper was found to be more highly toxic and potent (0.483 – 8.375) in adults of *Ae. aegypti* in comparison with cypermethrin (1.839 – 22.572) and permethrin (5.145 – 96.747). The obtained results also showed that deltamethrin was the most effective against mosquitoes from all selected locations (Table 3b), followed by cypermethrin and permethrin, but the resistance ratios were in the order of FSD < SKT < RWP < LHR, as shown in Table 2a-c. Mosquitoes collected from the different areas of the LHR city showed the highest resistance (12.27- to 18.02-fold) against cypermethrin, followed by those from RWP (5.85 to 7.83- fold), as shown in Table 2a. The mosquito populations collected from the FSD were the most susceptible, with low LC_{50} values (7.26 – 12.039 ppm), as shown in Table 2c.

The resistance of adults was examined with the OPs group of insecticides, including fenitrothion, malathion and pirimiphos-methyl. Pirimiphos-methyl was found to be highly toxic, killing 50 percent of the adult populations from agricultural areas (the SS, FSD, SKT, RWP, LHR populations had LC_{50} values of 12.933, 58.457, 83.418, 207.704 and 318.798 ppm, respectively), followed by malathion (the SS, FSD, SKT, RWP, LHR populations had LC_{50} values of 21.962, 106.076, 132.650, 431.992 and 630.529 ppm, respectively) and fenitrothion (the SS, FSD, SKT, RWP, LHR populations had LC_{50} values of 38.17, 87.028, 121.381, 324.063 and 611.483 ppm, respectively), 24 h after exposure to impregnated papers, as shown in Table 3a-c. In the case of fenitrothion, the LHR mosquito populations from agricultural, urban and industrial areas showed 16.02, 14.32 and 12.89-fold resistance, respectively, as shown in Table 3a. In the case of malathion, the highest LC_{50} value was noted from the LHR city (630.529 ppm), and lowest value was found in mosquitoes from industrial areas of the FSD city (66.325 ppm), as shown in Table 3b. In the organophosphate (OPs) group, pirimiphos-methyl was highly toxic, so it showed lower LC_{50} values than the other insecticides of this group, as shown in Table 3c.

Table 1. LC_{50} values and RRs determined for susceptible strain and field populations of *Ae. aegypti* after 24 hr. of exposure to temephos.

Population	LC_{50}(ppm)	Fiducial limit*	Equation	χ^2	RR[$]	P value[Ψ]
Urban Area						
SS	0.007	(0.001–0.014) a	0.40x+1.99	2.09	1.00	0.71
FSD	0.034	(0.013–0.054) ab	0.22x+0.95	1.45	4.83	0.69
SKT	0.063	(0.029–0.091) bc	0.27x+0.69	0.32	8.98	0.86
RWP	0.148	(0.082–0.238) cd	0.21x+0.39	1.89	21.14	0.52
LHR	0.416	(0.29 –0.831) e	0.22x+0.23	0.46	59.45	0.91
Agricultural Area						
SS	0.007	(0.001–0.014) a	0.40x+1.99	2.09	1.00	0.71
FSD	0.028	(0.010–0.041) ab	0.25x+0.92	1.35	4.00	0.72
SKT	0.053	(0.022–0.079) bc	0.25x+0.72	0.24	7.54	0.66
RWP	0.137	(0.078–0.212) cd	0.20x+0.42	2.07	19.65	0.35
LHR	0.388	(0.269–0.799) e	0.23x + 0.19	0.45	55.47	0.82
Industrial Area						
SS	0.007	(0.001–0.014) a	0.40x+1.99	2.09	1.00	0.71
FSD	0.033	(0.012–0.056) ab	0.26x+0.91	1.55	4.71	0.81
SKT	0.057	(0.027–0.090) bc	0.25x+0.73	0.54	8.14	0.96
RWP	0.147	(0.084–0.241) cd	0.21x+0.40	2.87	21.00	0.57
LHR	0.409	(0.262–0.820) e	0.23x+0.21	0.57	58.43	0.96

SS= Susceptible Strain; FSD= Faisalabad; SKT= Sialkot; RWP= Rawalpindi; LHR= Lahore. *Different letters in the same column indicate significant differences due to non-overlapping 95% confidence intervals. [$]Resistance ratio calculated by dividing the LC_{50} values of the different populations by that of the susceptible laboratory population. [Ψ]P-value was calculated for goodness of fit, and a heterogeneity factor was applied in the calculation of fiducial limits.

Table 2a. LC_{50} values and RRs determined in susceptible strain and field populations of *Ae. aegypti* after 24 hr. of exposure to cypermethrin (synthetic pyrethroid insecticide group).

Population	LC_{50}(ppm)	Fiducial limit*	Equation	χ^2	RR[$]	P value[Ψ]
		Urban Area				
SS	1.839	(0.417–3.916) a	0.46x–0.48	2.68	1.00	0.72
FSD	2.52	(0.655–5.191) ab	0.31x–0.28	1.63	1.37	0.80
SKT	3.778	(0.908–7.747) abc	0.25x–0.34	1.92	2.05	0.75
RWP	10.265	(2.229–20.083) bcd	0.18x–0.42	0.94	5.85	0.91
LHR	22.572	(11.110–36.115) de	0.21x–0.68	0.14	12.27	0.99
		Agricultural Area				
SS	1.839	(0.417–3.916) a	0.46x-0.48	2.68	1.00	0.72
FSD	3.641	(0.874–5.986) ab	0.29x–0.23	2.17	1.98	0.70
SKT	6.087	(1.018–8.471) abc	0.22x–0.28	2.66	3.31	0.61
RWP	13.369	(3.289–22.081) bcd	0.17x–0.39	1.21	7.27	0.87
LHR	33.065	(13.12–37.211) de	0.19x–0.51	1.28	17.98	0.86
		Industrial Area				
SS	1.839	(0.417–3.916) a	0.46x-0.48	2.68	1.00	0.72
FSD	2.611	(0.872–6.194) ab	0.30x–0.27	2.19	1.42	0.70
SKT	4.579	(1.308–8.471) abc	0.24x–0.32	2.22	2.49	0.69
RWP	14.399	(3.821–21.993) bcd	0.19x–0.40	1.09	7.83	0.89
LHR	33.139	(13.118–39.107) de	0.21x–0.58	1.27	18.02	0.86

Table 2b. LC_{50} values and RRs determined in susceptible strain and field populations of *Ae. aegypti* after 24 hr. of exposure to deltamethrin (synthetic pyrethroid insecticide group).

Population	LC_{50}(ppm)	Fiducial limit*	Equation	χ^2	RR[$]	P value[Ψ]
		Urban Area				
SS	0.483	(0.138–0.956) a	0.42x+0.30	2.28	1.00	0.76
FSD	0.639	(0.159–1.332) ab	0.30x+0.13	2.17	1.32	0.70
SKT	1.118	(0.305–2.188) abc	0.25x–0.02	2.66	2.31	0.61
RWP	3.174	(0.884–5.858) bcd	0.18x–0.21	1.21	6.57	0.87
LHR	8.375	(5.354–12.301) de	0.26x–0.56	1.28	17.34	0.86
		Agricultural Area				
SS	0.483	(0.138–0.956) a	0.42x+0.30	2.28	1.00	0.76
FSD	0.976	(0.163–1.423) ab	0.31x+0.15	2.10	2.02	0.68
SKT	1.502	(0.389–2.192) abc	0.24x–0.02	2.06	3.11	0.60
RWP	3.559	(0.896–6.038) bcd	0.19x–0.23	1.11	7.37	0.72
LHR	9.245	(6.129–13.311) de	0.24x–0.52	1.08	19.14	0.06
		Industrial Area				
SS	0.483	(0.138–0.956) a	0.42x+0.30	2.28	1.00	0.76
FSD	0.927	(0.161–1.348) ab	0.29x+0.12	2.07	1.92	0.50
SKT	1.348	(0.567–2.587) abc	0.26x–0.03	2.12	2.79	0.21
RWP	3.077	(0.904–6.808) bcd	0.18x–0.22	1.01	6.37	0.67
LHR	8.708	(5.584–13.362) de	0.25x–0.50	1.02	18.03	0.26

Table 2c. LC_{50} values and RRs determined in susceptible strain and field populations of *Ae. aegypti* after 24 hr. of exposure to permethrin (synthetic pyrethroid insecticide group).

Population	LC_{50} (ppm)	Fiducial limit*	Equation	χ^2	RR[$]	P value[Ψ]
		Urban Area				
SS	5.145	(1.553–9.979) a	0.42x-0.68	2.09	1.00	0.71
FSD	7.266	(1.838–14.94) ab	0.28x–0.57	2.19	1.41	0.70
SKT	13.354	(4.034–25.092) bc	0.25x–0.67	2.22	2.59	0.69
RWP	37.73	(11.016–69.032) bcd	0.17x–0.65	1.09	7.33	0.89
LHR	96.747	(69.918–486.622) e	0.25x–1.18	1.27	18.8	0.86
		Agricultural Area				
SS	5.145	(1.553–9.979) a	0.42x-0.68	2.09	1.00	0.71
FSD	12.039	(2.847–16.93) ab	0.29x–0.58	2.17	2.34	0.70
SKT	20.631	(5.185–27.095) bc	0.26x–0.69	2.66	4.01	0.61
RWP	41.520	(19.012–76.029) bcd	0.16x–0.62	1.21	8.07	0.87
LHR	101.533	(78.92–498.719) e	0.23x–1.20	1.28	19.74	0.86
		Industrial Area				
SS	5.145	(1.553–9.979) a	0.42x-0.68	2.09	1.00	0.71
FSD	10.187	(2.389–15.79) ab	0.27x–0.53	2.09	1.98	0.60
SKT	15.486	(5.734–27.019) bc	0.27x–0.70	2.02	3.01	0.59
RWP	42.857	(16.012–73.032) bcd	0.15x–0.59	0.99	8.33	0.38
LHR	101.254	(79.908–496.612) e	0.23x–1.09	0.87	19.68	0.26

SS= Susceptible Strain; FSD= Faisalabad; SKT= Sialkot; RWP= Rawalpindi; LHR= Lahore. *Different letters in the same column indicate significant differences due to non-overlapping 95% confidence intervals. [$]Resistance ratio calculated by dividing the LC_{50} values of the different populations by that of the susceptible laboratory population. [Ψ]P-value was calculated for goodness of fit, and a heterogeneity factor was applied in the calculation of fiducial limits.

Table 3a. LC_{50} values and RRs determined in susceptible strain and field populations of *Ae. aegypti* 24 hr. after exposure to fenitrothion (organophosphate insecticide group).

Population	LC_{50} (ppm)	Fiducial limit*	Equation	χ^2	RR[$]	P value[Ψ]
		Urban Area				
SS	38.17	(16.273–63.961) a	0.41x–1.48	2.79	1	0.61
FSD	60.407	(23.477–103.962) b	0.29x–1.22	0.58	1.58	0.96
SKT	90.951	(39.383–153.188) bc	0.27x–1.23	1.63	2.38	0.80
RWP	278.469	(139.732–457.52) cd	0.20x–1.15	0.77	7.29	0.94
LHR	546.599	(373.557–836.19) de	0.27x–1.71	1.24	14.32	0.87
		Agricultural Area				
SS	38.17	(16.27 –63.961) a	0.41x–1.48	2.79	1	0.61
FSD	87.028	(34.454–123.987) b	0.28x–1.18	0.53	2.28	0.89
SKT	121.381	(61.357–174.16) bc	0.27x–1.03	1.03	3.18	0.75
RWP	324.063	(186.78–507.51) cd	0.21x–1.12	0.69	8.49	0.92
LHR	611.483	(423.56–893.15) de	0.25x–1.32	0.99	16.02	0.78
		Industrial Area				
SS	38.17	(16.273–63.961) a	0.41x–1.48	2.79	1	0.61
FSD	45.041	(18.482–93.298) b	0.25x–1.19	0.48	1.18	0.86
SKT	79.394	(31.354–124.14) bc	0.26x–1.20	1.51	2.08	0.78
RWP	247.723	(109.70–412.23) cd	0.19x–1.09	0.56	6.49	0.89
LHR	492.011	(323.53–782.10) de	0.29x–1.92	1.03	12.89	0.81

Table 3b. LC_{50} values and RRs determined for susceptible strain and field populations of *Ae. aegypti* after 24 hr. of exposure to malathion (organophosphate insecticide group).

Population	LC_{50} (ppm)	Fiducial limit*	Equation	χ^2	RR[$]	P value[Ψ]
		Urban Area				
SS	21.962	(7.698–39.805) a	0.33x–1.02	3.47	1.00	0.48
FSD	82.439	(47.92–119.428) b	0.32x–1.42	1.62	3.75	0.80
SKT	118.239	(71.493–170.285) bc	0.29x–1.40	2.23	5.38	0.69
RWP	396.955	(254.501–715.472) d	0.23x–1.37	0.05	18.07	0.99
LHR	587.883	(378.11–1160.58) de	0.24x–1.54	0.21	26.77	0.99
		Agricultural Area				
SS	21.962	(7.698–39.805) a	0.33x–1.02	3.47	1.00	0.48
FSD	106.076	(62.89–159.784) b	0.29x–1.35	1.22	4.83	0.78
SKT	132.650	(89.492–187.278) bc	0.28x–1.39	1.97	6.04	0.64
RWP	431.992	(298.489–789.489) d	0.19x–1.07	0.02	19.67	0.89
LHR	630.529	(483.13–1234.78) de	0.14x–1.04	0.01	28.71	0.87
		Industrial Area				
SS	21.962	(7.698–39.805) a	0.33x–1.02	3.47	1.00	0.48
FSD	66.325	(41.82–106.434) b	0.33x–1.49	1.02	3.02	0.68
SKT	107.175	(68.478–154.278) bc	0.25x–1.01	2.01	4.88	0.59
RWP	361.714	(229.132–678.453) d	0.22x–1.30	0.04	16.47	0.92
LHR	536.532	(367.109–798.51) de	0.25x–1.48	0.11	24.43	0.94

Table 3c. LC_{50} values and RRs determined for susceptible strain and field populations of *Ae. aegypti* after 24 hr. of exposure topirimiphos-methyl (organophosphate group).

Population	LC_{50} (ppm)	Fiducial limit*	Equation	χ^2	RR[$]	P value[Ψ]
		Urban Area				
SS	12.933	(5.765–21.118) a	0.50x–1.29	2.06	1.00	0.61
FSD	45.028	(25.384–66.046) b	0.34x–1.31	0.58	3.48	0.96
SKT	67.077	(40.138–96.185) bc	0.31x–1.33	1.63	5.18	0.80
RWP	193.579	(124.799–307.76) d	0.24x–1.27	0.77	14.96	0.94
LHR	291.715	(197.703–483.08) de	0.26x–1.49	0.24	22.55	0.87
		Agricultural Area				
SS	12.933	(5.765–21.118) a	0.50x–1.29	2.06	1.00	0.48
FSD	58.457	(26.395–69.098) b	0.37x–1.28	1.62	4.52	0.80
SKT	83.418	(47.187–98.895) bc	0.30x–1.29	2.23	6.45	0.69
RWP	207.704	(143.758–317.78) d	0.26x–1.38	0.05	16.06	0.99
LHR	318.798	(201.702–497.08) de	0.24x–1.34	0.21	24.65	0.99
		Industrial Area				
SS	12.933	(5.765–21.118) a	0.50x–1.29	2.06	1.00	0.72
FSD	38.540	(24.356–67.123) b	0.32x–1.28	1.39	2.98	0.84
SKT	60.138	(34.145–86.185) bc	0.30x–1.31	2.36	4.65	0.66
RWP	175.371	(114.756–297.81) d	0.23x–1.20	0.44	13.56	0.97
LHR	258.919	(167.701–471.02) de	0.25x–1.42	0.48	20.02	0.97

SS= Susceptible Strain; FSD= Faisalabad; SKT= Sialkot; RWP= Rawalpindi; LHR= Lahore. *Different letters in the same column indicate significant differences due to non-overlapping 95% confidence intervals. [$]Resistance ratio calculated by dividing the LC_{50} values of the different populations by that of the susceptible laboratory population. [Ψ]P-value was calculated for goodness of fit, and a heterogeneity factor was applied in the calculation of fiducial limits.

The carbamate group, which included the bendiocarb insecticide, showed a LC_{50} range of 74.38 – 6089.35 ppm, with a fiducial limit of between 6.419 – 4379.09 and 223.303 – 8955.46 ppm(Table 4). The data also showed 81.86-fold variation in susceptibility across all populations. The obtained results indicate that the LHR population was the most resistant (81.86-fold), followed by the RWP (42.16-fold), SKT (13.36-fold) and FSD populations (7.36-fold).

In addition, the results from the biochemical analysis indicate that the field populations of *Ae. aegypti* collected from FSD, SKT, RWP and LHR showed the elevated activity of all enzymes. These values clearly indicate that resistance occurs

Table 4. LC_{50} values and RRs determined in susceptible strain and field populations of *Ae. aegypti* after 24 hr. of exposure to bendiocarb (carbamate insecticide group).

Population	LC_{50}(ppm)	Fiducial limit*	Equation	χ^2	RR[$]	P value[Ψ]
		Urban Area				
SS	74.38	(6.419–223.303) a	0.20x-0.314	1.83	1.00	0.76
FSD	567.66	(212.229–990.446) ab	0.29x–1.85	0.20	7.63	0.99
SKT	993.677	(422.804–1631.27) bc	0.26x–1.81	0.45	13.36	0.97
RWP	3136.47	(1963.4–4658.01) d	0.26x–2.09	1.11	42.16	0.89
LHR	6089.35	(4379.09–8955.46) de	0.21x–1.82	0.46	81.86	0.97
		Agricultural Area				
SS	74.38	(6.419–223.303) a	0.20x-0.314	1.83	1.00	0.56
FSD	713.304	(223.30–993.45) ab	0.26x–1.79	0.19	9.59	0.91
SKT	1114.96	(461.81–1682.29) bc	0.26x–1.82	0.39	14.99	0.92
RWP	3347.84	(1973.5–4708.02) d	0.25x–2.11	1.01	45.01	0.81
LHR	6263.79	(4299.07–8895.48) de	0.22x–1.83	0.41	83.81	0.87
		Industrial Area				
SS	74.38	(6.419–223.303) a	0.20x-0.314	1.83	1.00	0.71
FSD	523.64	(215.23–995.45) ab	0.28x–1.82	0.14	7.04	0.89
SKT	978.84	(429.80–1639.28) bc	0.25x–1.79	0.34	13.16	0.90
RWP	3123.22	(1983.4–4698.01) d	0.26x–2.10	1.02	41.99	0.78
LHR	6029.24	(4397.08–8994.36) de	0.22x–1.84	0.36	81.06	0.77

SS= Susceptible Strain; FSD= Faisalabad; SKT= Sialkot; RWP= Rawalpindi; LHR= Lahore. *Different letters in the same column indicate significant differences due to non-overlapping 95% confidence intervals. [$]Resistance ratio calculated by dividing the LC_{50} values of the different populations by that of the susceptible laboratory population. [Ψ]P-value was calculated for goodness of fit, and a heterogeneity factor was applied in the calculation of fiducial limits.

Table 5. Activities of different enzymes in *Ae. Aegypti*.

Population	Esterase	Mixed-function oxidases	Glutathione S-transferase	Acetyl- cholinesterase
		Urban Areas		
SS	0.20±0.01	0.48 ±0.01	0.11±0.01	0.04±0.01
FSD	0.23±0.01	0.50±0.03	0.12±0.03	0.06±0.00
SKT	0.26±0.01	0.53±0.02	0.11±0.01	0.08±0.01
RWP	0.32±0.01	0.57±0.01	0.13±0.02	0.09±0.01
LHR	0.40±0.01	0.59±0.02	0.14±0.03	0.10±0.02
		Agricultural Areas		
SS	0.20±0.01	0.48±0.01	0.11±0.01	0.04±0.01
FSD	0.33±0.02	0.54±0.02	0.13±0.01	0.07±0.01
SKT	0.42±0.02	0.63±0.02	0.12±0.02	0.09±0.01
RWP	0.45±0.03	0.67±0.03	0.14±0.02	0.11±0.01
LHR	0.54±0.02	0.72±0.02	0.16±0.01	0.13±0.01
		Industrial Areas		
SS	0.20±0.01	0.48±0.01	0.11±0.01	0.04±0.01
FSD	0.25±0.01	0.49±0.01	0.12±0.01	0.07±0.00
SKT	0.29±0.02	0.54±0.02	0.12±0.02	0.08±0.01
RWP	0.33±0.02	0.56±0.02	0.13±0.02	0.10±0.01
LHR	0.44±0.02	0.58±0.03	0.15±0.02	0.11±0.01

SS= Susceptible Strain; FSD= Faisalabad; SKT= Sialkot; RWP= Rawalpindi; LHR= Lahore

in all the selected areas. However, the LHR population showed the maximum activity of enzymes, like esterase (0.54, 0.40 & 0.29), mixed-function oxidase (0.72, 0.59 & 0.54), glutathione S-transferase (0.16, 0.14 & 0.12) and acetyl-cholinesterase (0.13, 0.10 & 0.08) from agricultural, urban and industrial areas respectively indicating a high level of resistance, followed by RWP, SKT and FSD. In general, the highest levels of elevation were also recorded in the populations that were collected from agricultural areas of the studied cities (Table 5).

DISCUSSION

This study confirmed the presence of resistance against all groups of insecticides in *Ae. aegypti* for the first time in Punjab, Pakistan. These groups of insecticides have extensively been used for the control of agricultural pests in Punjab (Ahmad *et al.*, 2007).

Aedes aegypti has become a major household mosquito that infects humans with its bite. Its superior adaptability and fitness have made this mosquito more threatening than other species. In Brazil (Obando *et al.*, 2015) and Saudi Arabia (Alsheikh *et al.*, 2016), larvicidal tests were conducted with temephos along with IGRs including diflubenzuron and methoprene. Farmers recorded resistance ratios of 3.6 and 2.5 in 2011 and 2012, respectively, and the latter indicated higher levels of resistance to temephos compared with resistance to diflubenzuron and methoprene in Saudi Arabia. Arslan *et al.* (2015) also noticed a higher resistance ratio in field-collected populations from Rawalpindi compared to mosquitoes that were reared in the laboratory to be resistant to temephos. These studies are consistent with our findings showing that exposure to temephos resulted in LC_{50} values in susceptible strain (SS) and field populations of *Ae. Aegypti* collected from different parts of Faisalabad (FSD), Sialkot (SKT), Rawalpindi (RWP) and Lahore (LHR) were 0.005 to 0.409 ppm, with fiducial limits ranging from 0.001 – 0.262 to 0.013 – 0.937. The LHR population was found to be highly resistant. The present study showed the presence of resistance in *Ae. aegypti* to all groups of insecticides, which might be due to the involvement of different resistance mechanisms. These mechanisms in mosquitoes have been widely studied in past years (Hemingway and Ranson, 2000). The over-production of esterases has also given rise to noticeable resistance in mosquitoes due to the use of organophosphate (OPs) and carbamate groups of insecticides (Hemingway *et al.*, 2004). Our study also showed elevated levels of esterases in resistant mosquito populations compared to the susceptible population. The increased activity of esterases, oxidases and glutathione S-transferase (GSTs) due to the application of different insecticides, such as permethrin, deltamethrin and cypermethrin, also increases the resistance level in mosquitoes (Pimsamarn *et al.*, 2009). These results are in consonance with our study, in which we noticed elevated levels of esterases, oxidases and glutathione S-transferase in resistant populations when compared to the susceptible population. In Africa, high levels of resistance to DDT, pyrethroids, malathion, and deltamethrin-treated net material were detected in *Cx. quinquefasciatus*. These authors further submitted mosquitoes to biochemical analysis, showing up-regulated glutathione S-transferases (GSTs), ct-esterases, and β-esterases. In Lebanon, insecticide resistance in *Culex* mosquitoes (Osta *et al.*, 2012) was tested using OPs by capturing *Cx. pipiens* from 25 villages, which were then assessed based on carboxylesterase for resistance to OPs using an enzymatic assay.

Spectrum changes in enzymes after insecticide application was also studied by previous researchers in different mosquito species (Nkya *et al.*, 2013; Liu, 2015). These investigations are in line with ours because in the areas where the continuous application of insecticide was conducted, the concentrations of enzymes such as cholinesterases, esterases and mixed-function oxidases were elevated up to 2-3 times than susceptible strain. This disturbance in the levels of different enzymes occurred due to the application of OPs, pyrethroid and carbamate insecticides.

Earlier studies (Pimsamarn *et al.*, 2009; Owusu *et al.*, 2015; Alsheikh *et al.*, 2016) were conducted in different parts of the world with the same objective to record the resistance levels in mosquitoes and further recommend to the respective authorities the most suitable and feasible vector management strategies. The results were variable in all parts of the world. However, the continuous use of one group of insecticides resulted in problems of resistance, which needs to be properly addressed. If the groups of insecticides were alternated, then the efficiency would definitely be improved. The same phenomenon of repeated application was found in the LHR and RWP areas. This is why the resistance level was recorded at a higher level in comparison with the SKT and FSD areas. However, the spraying of insecticides was a more common activity at LHR and RWP than at SKT and FSD. All the tested insecticides resulted in higher mortality in the SKT and FSD populations.

Insecticide resistance studies in the field as well as under laboratory conditions (Thomas and Read, 2016) have shown that mosquitoes are resistant to the pyrethroid group of insecticides, which are commercially available in markets. New approaches, such as the development new products with novel modes of action, must be evaluated under standardized conditions and using bioassay processes. On one hand, the products must have the desired effectiveness, whereas on the other hand, they must be risk free, environmentally friendly, cost-effective and easily available. Neverthless, the intensive use of insecticides has resulted in insecticidal resistance in many mosquito vectors to such a degree that their control has become a challenge in many cases.

In Punjab, Pakistan, government officials and the common people have been using insecticides for mosquito control that were used for years for pest control in agriculture (Akogbeto *et al.*, 2006). In this context, some vector ecologists have hypothesized that the indiscriminate use of pesticides in agriculture is the main reason for resistance in mosquitoes (Diabate *et al.*, 2002). One of the key threats that may affect future mosquito control strategies is the production of cross resistance between two different groups of insecticides, such as OPs (fenitrothion, malathion and pirimiphos-methyl) and pyrethroids (deltamethrin, cypermethrin and permethrin) (Brogdon and Barber, 1990). The extensive use of these

insecticides throughout the province will pose a potential threat to future control strategies against mosquitoes.

***Conclusions*:** The injudicious application of chemicals in an area have caused the risk of resistance and the reappearance and resurgence of certain mosquitoes. However, agricultural areas were found to be generally more prone to the risk of resistance than urban and industrial areas. Chemicals such as OPs, pyrethroids and carbamates are commonly available in the marketplace. However, consumers use one type of chemical each time. This action has caused risks of resistance that were further confirmed and noticed while conducting bioassays and biochemical analysis of mosquitoes. Therefore, it is recommended to use physical methods (source reduction) and mechanical devices, such as screening (on windows and vents), mosquito nets, mosquito magnet and mosquito killers, to combat the problem of mosquitoes.

REFERENCES

Ahmad, I., M.A. Khan, M.H. Soomro and H. Waibel. 2002. Pesticides hazards for health and environment. Farming Outlook October-December; pp.14-16.

Ahmad, M., I. Arif and M. Ahmad. 2007. Occurrence of insecticide resistance in field populations of *Spodopteralitura* (Lepidoptera: Noctuidae) in Pakistan. Crop Prot. 26:807-811.

Akogbeto, M., R.F.D. Jouaka and D.A. Kindé-Gazard. 2006. Screening of pesticide residues in soil and water samples from agricultural settings. Mal. J. 5:22-31.

Alsheikh, A.A., W.S. Mohammed, E.M. Noureldin, O.M. Daffalla, Y.A. Shrwani, K.J. Hobani, F.A. Alsheikh, M.H. Alzahrani and A.A. Binsaeed. 2016. Studies on *Aedes aegypti* resistance to some insecticides in the Jazan district, Saudi Arabia. J. Egypt. Soc. Parasitol.46:209-216.

Anonymous, 2016. Top ten house pests. Available online at https://www.rentokil.com/insects/insect-borne-diseases.

Arslan, A., M.U. Mukhtar, S. Mushtaq, A.B. Zakki, M. Hammad and A. Bhatti. 2015. Comparison of susceptibility status of laboratory and field populations of *Aedes aegypti* against temephos in Rawalpindi. J. Entomol. Zool. Stud. 3:374-378.

Brogdon, W.G. and A.M. Barber. 1990. Fenitrothion-deltamethrin cross-resistance conferred by esterases in Guatemalan *Anopheles albimanus*. Pestic. Biochem. Physiol. 3:130-139.

Brown, A.W.A. 1958. Insecticide Resistance in Arthropods. World Health Organization, Geneva.

Diabate, A., T. Baldet, F. Chandre, T.R. Guiguemde, P. Guillet, J. Hemingway and J.M. Hougard. 2002. First report of the kdr mutation in *Anopheles gambiae* from Burkina Faso, West Africa. Parasitologia 44:157-158.

Finney, D.J. 1971. Probit analysis. Cambridge University Press, Cambridge, UK.

Hemingway, J. and H. Ranson. 2000. Insecticides resistance in insect vectors of human disease. Ann. Rev. Entomol. 45:371-391.

Lambrechts, L., T.W. Scott and D.J. Gubler. 2010. Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. PLoSNegl. Trop. Dis.4:e646.

Li, X., M.A. Schuler and M.R. Berenbaum. 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Ann Rev Entomol. 52:231-53.

Lima, E.P., M.H. Paiva, A.P. de Araújo, E.V. da Silva, U.M. da Silva, L.N. de Oliverita, A.E. Santana, C.N. Barbosa, C.C. de Paiva Neto, M.O. Goulart, C.S. Wilding, C.F. Ayres and M.A. de Melo Santos. 2011. Insecticide resistance in *Aedes aegypti* populations from Ceará, Brazil. Parasit. Vectors 4:1-12.

Liu, N. 2015. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. Ann. Rev. Entomol. 60:537-559.

Mohsin, M., S.I. Naz, I.K. Khan, A. Jabeen, H. Bilal, R. Ahmad, Y. Alshamrani, E.I,M, Khater and E. Tambo. 2016. Susceptibility status of *Aedes aegypti and Aedes albopictus* against insecticides at Eastern Punjab, Pakistan. Int. J. Mosq. Res. 3:41-46.

Nkya, T.E., I. Akhouayri, W. Kisinza and J.P. David. 2013. Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects. Insect Biochem. Mol. Biol. 43:407-416.

Obando, O.A.A., B.A. Dalla, J.E. Duque and A.N. Mario. 2015. Insecticide resistance and genetic variability in natural populations of *Aedes* (Stegomyia) *aegypti* (Diptera: Culicidae) form Colombia. Zoologia 32:14-22.

Osta, M.A., Z.J. Rizk, P. Labbe, M. Weill and K. Knio. 2012. Insecticide resistance to organophosphates in *Culex pipiens* complex from Lebanon. Parasit. Vectors 5:132.

Owusu, H.N., D. Jancaryova, D. Malone and P. Müller. 2015. Comparability between insecticide resistance bioassays for mosquito vectors: time to review current methodology? Parasit. Vectors 8:357.

Pimsamarn, S., W. Sornpeng, S. Akksilp, P. Paeporn and M. Limpawitthayakul. 2009. Detection of insecticide resistance in *Aedes aegypti* to organophosphate and synthetic pyrethroid compounds in the north-east of Thailand. Dengue Bull. 33:194-202.

Raghavendra, K., T.K. Barik and T. Adak. 2010. Development of larval thermotolerance and its impact on adult susceptibility to malathion insecticide and *Plasmodium vivax* infection in *Anopheles stephensi*. Parasitol. Res. 107:1291–1297.

Ranson, H., R. N'Guessan, J. Lines, N. Moiroux, Z. Nkuni and V. Corbel. 2011. Pyrethroid resistance in African

anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol. 27:91–98.

Soko, W., M.J. Chimbari and S. Mukaratirwa. 2015. Insecticide resistance in malaria-transmitting mosquitoes in Zimbabwe: a review. Infect. Dis. Poverty 4:1-12.

Strode, C., S. Donegan, P. Garner, A.A. Enayati and J. Hemingway. 2014. The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African anopheline mosquitoes: systematic review and meta-analysis. PLoS Med. 11:e1001619.

Thomas, M.B. and A.F. Read. 2016. The threat (or not) of insecticide resistance for malaria control. Proc. Nati. Acad. Sci. 113:8900-8902.

WHO. 2006. World Malaria Report 2006. WHO Press, World Health Organization, Geneva Switzerland.