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## Evaluation of insecticide resistance and biochemical mechanisms of *Culex pipiens* L. in four localities of east and middle mediterranean basin in Turkey

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### Abstract

West Nile virus (WNV) infections in Turkey and East part of Europe have become one of the most important vector-borne diseases. *Cx. pipiens* L. complex species is a main vector species of the West Nile virus in these areas. Vector control practice with different classes of insecticides is the most commonly used alternative for mosquito control. The aim of this survey was to measure three different classes (Organochlorine, Organophosphate and Pyrethroids) of insecticide effectiveness, effects of synergist and possible biochemical mechanisms of *Culex pipiens* L. in Turkey.

Bioassays results showed that permethrin and deltamethrin toxicity ranged from 50% (Mersin) to 84% (Huzurkent), whereas DDT toxicity was under 50% for all populations, except for Kapikaya (64%). In addition, Malathion toxicity ranged from 40% (Mersin) to 74% (Huzurkent). As a result of inhibitor assays, PBO and DEF increased the toxicity related to the insecticide classes. Biochemical assay results indicated that the MFO and NSEs (especially p-NPA) played an important role for DDT, malathion and pyrethroid resistance. GST activity assays implied that resistance was multifactorial for all tested populations.

**Keywords:** *Culex pipiens* L., insecticide resistance, biochemical resistance mechanisms, Turkey

### 1. Introduction

*Culex pipiens* L. complex presence is known in many tropical and subtropical countries and is a West Nile virus vector throughout the world. *Cx. pipiens* L. complex species is a main vector species of the West Nile virus in Turkey. In 2010, 47 West Nile virus (WNV) infections were detected and 10 patients died from the WNV infection [1].

The use of insecticide on larvae and adults is efficient strategy for mosquito-borne disease control [2]. However, mosquito control has a big problem with regard to the developing insecticide resistance [3]. Therefore, understanding the nature of resistance helps to create efficient strategies for mosquito control.

Insecticide resistance is defined with two mechanisms in mosquitoes: increasing rate of detoxification and target site changes [4, 5]. Mixed function oxidase, glutathione S-transferase (GST) and Non-specific esterases (NSEs) enzyme systems are important for insecticide metabolism. Enhancements of esterases, cyt p450 activities and their relation to insecticide resistance have been reported in different mosquito species [4, 6]. Pyrethroid resistance as a result of the increase in p450 oxidases has been reported in *Cx. pipiens quinquefasciatus* [7]. Expression of up-regulated GST gene has been reported in a permethrin resistant *Anopheles gambiae* Giles [8]. Elevations of the non-specific esterases and high insensitive Acetylcholinesterase (AChE) frequencies and their relation to insecticide resistance have been reported in Cuba for *Culex quinquefasciatus* Say strain [9].

Mixed function oxidase and hydrolases (NSEs) enzyme systems are impeded by Piperonyl butoxide (PBO) and S,S,S, tributylphosphorotrithioate (DEF) [10, 11]. Consequently, PBO and DEF have been used with insecticides as a synergist [12]. In Turkey, wide ranges of insecticides are used for different pest controls [13]. Although many insecticides have been used for many years, studies dealing with the development of resistance and resistance mechanisms have been carried out insufficiently for mosquito species in Turkey.

This study aims to evaluate insecticide resistance/susceptibility status and mechanisms in *Cx. pipiens* complex populations of Mersin (City Center, Huzurkent), Adana (Kapikaya) and Antalya (Aksu) in Turkey.

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**2. Material and methods**

**2.1. Mosquito strains**

Different life stage samples of *Cx. pipiens* L. complex were collected from four different localities (Mersin (City Center, Huzurkent), Adana (Kapikaya) and Antalya (Aksu)) in Turkey and were taken to the laboratory. All sample areas were chosen nearly 1 km around the agricultural production areas. The agricultural areas are selected particularly for sampling and the second criteria for choosing sampling sites were population

densities. All sampling sites are very important agricultural areas and products of these areas are distributed in Turkey and also exported to another country. Coordinates and general characteristics of study areas are given in Fig 1. The history of insecticide usage in the last ten years in sampling sites is given in Table 1. This data is compiled according to the insecticide markets data and during individual interviews with the collection sites residents.

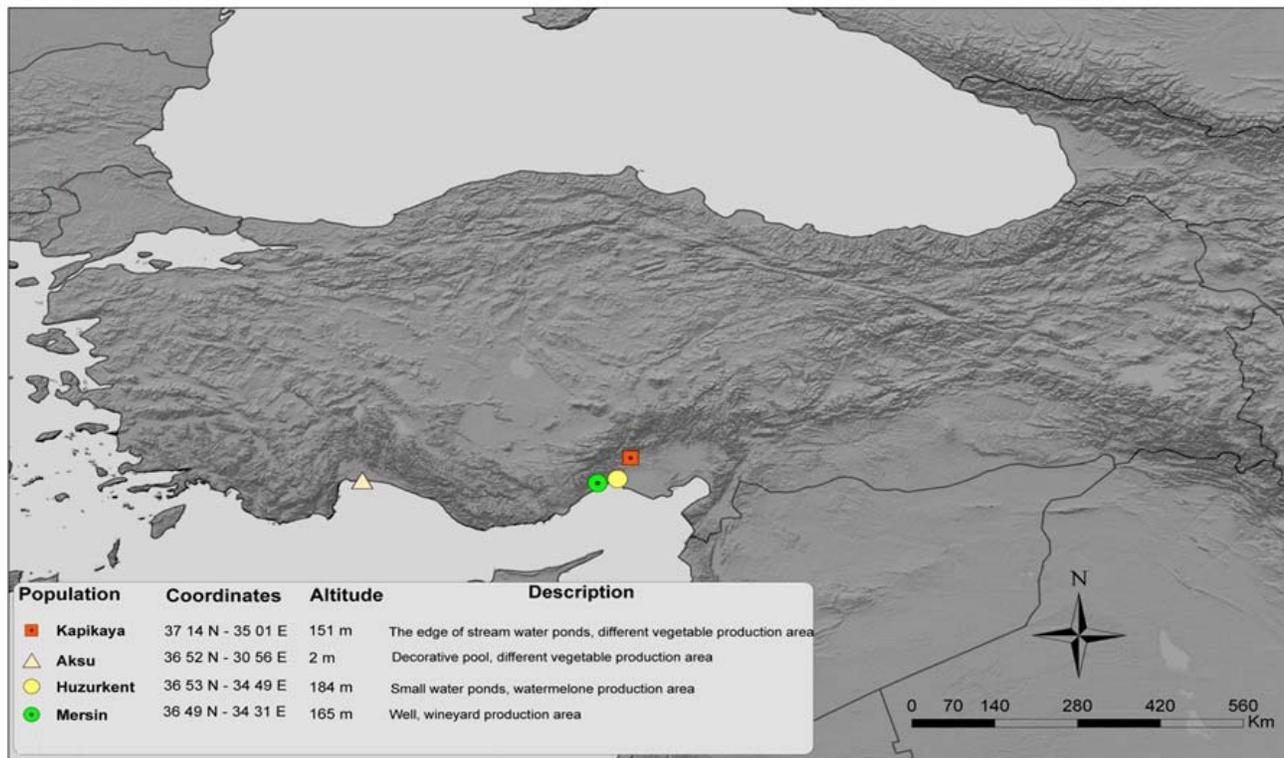


Fig 1: Collection sites

Table 1: *Culex pipiens* strains and the use of insecticides last ten years

	a.i /area	Kapikaya	Huzurkent	Aksu	Mersin
Larvicides	Temephos	X		X	X
	Bti	X	X	X	X
	Diffubenzuron			X	
	Aqnique			X	
Adulticides	Cypermethrin	X		X	X
	Alfa Cypermethrin		X	X	
	Deltamethrin	X	X	X	X
	Permethrin	X	X	X	X
	Resmethrin			X	
	D Phenothrin	X			
	Thiametoxam				
Agricultural	Fenthion		X	X	X
	Malathion	X	X	X	X
	Deltamethrin	X	X	X	X
	Alfa Cypermethrin		X	X	
	Carbaryl				

Colonies were maintained under 26 ± 2 °C, 70 ± 5% relative humidity and 14:10 hrs. light dark conditions. Live chickens were used as a source of blood meal two times a week. Plastic cups that contained deionized water were placed inside the

cages for oviposition. Eggs were transferred to fresh containers after oviposition, and Tetramin® fish food was provided for larval feeding. The strain Slab was used as the susceptible reference strain [14].

All strains identified morphologically with regards to Harbach [15], Darsie and Samanidou [16] and checked by autogenous or anatoogenous behavior for var. pipiens or molestus. After checking oviposition behaviour, ten individuals were taken from the cages randomly and tested molecularly with regards to Smith and Fonseca [17].

**2.2. Chemicals**

Test insecticides impregnated on filter papers (DDT, permethrin, deltamethrin and malathion) were obtained from the WHO (World Health Organization). PBO and DEF were purchased from Sigma Aldrich® Company.

**2.3. Resistance tests**

Tests were performed on F<sub>2</sub> and F<sub>3</sub> laboratory generations in order to avoid misleading results related to the inbreeding. Resistance tests were conducted by using adult mosquitoes and standard concentrations of permethrin, deltamethrin, malathion and DDT were applied in accordance with WHO [18]. Tests were performed with 50 individuals and 2<sup>nd</sup> replicates were

performed after two days period first test period. Mortalities were recorded after 24 hrs. Insecticide resistance status was assessed according to WHO (Mortality range 98 – 100% was susceptible, 80–97% possible resistant, and < 80% resistant) [18].

S,S,S, tributylphosphorotrithioate (DEF) and piperonyl butoxide (PBO) synergistic effects were investigated by using DEF and PBO-impregnated papers for 1 hr. before resistance tests. The maximum sublethal concentrations of (5 mg/L) DEF and (2 mg/L) PBO were used in impregnated paper for all insecticide test groups. 10 millilitre acetone impregnated papers were used for the control group. One hour application period was conducted as in resistance assay. A total of 1200 3-5-day-old non-blood-fed adult females from four localities were analyzed. Three hundred adult females were tested for all populations (100 with PBO, 100 with DEF and 100 without synergists).

#### 2.4. Possible Resistance mechanisms assays

All biochemical tests were performed according to World Health Organization test procedures except MFO level [19] and MFO levels were determined by the amount of heme according to the Brogdon *et al.* [20] in order to understand the biochemical basis of resistance. Nunc maxisorp® flat-bottom 96 well plates were used to measure the absorbance which was recorded through microplate reader (Power Wave® XS).

Tests were conducted as two replicates. In total, 285 adult females from five localities (Kapikaya 60, Huzurkent 67, Serik 70, Mersin 48 and control 40) were analyzed.

Samples of *Culex pipiens* L. complex were randomly selected from cages and immobilized by CO<sub>2</sub> exposure. Each specimen was crushed in sodium phosphate buffer (50 mM, pH 7.2) on ice, and was centrifuged at 10000 g for 10 min at +4°C.

$\alpha$  - naphthol acetate,  $\beta$  - naphthol acetate and p-nitrophenol acetate (p - NPA) were used to measure NSEs activity. MFO levels were determined by the amount of heme. Glutathione S - Transferase levels were measured by GSH / CDNB conversion rate.

Supernatant protein quantities were identified to measure enzyme activity for each sample. 10  $\mu$ l of supernatant was used for this purpose. Three hundred microlitre of Bradford dye reagent was added on supernatant and the endpoint absorbance was read at 595 nm. Protein quantities were assessed according to Bradford [21].

#### 2.5. Statistical analysis

Data were not normally distributed ( $P < 0.05$ ). Thus, the differences of enzyme activity among populations were statistically assessed with a Kruskal - Wallis test. Correlation analysis was performed to find association between enzyme levels and survival rates.

### 3. Results

#### 3.1. Resistance/Susceptibility

All samples showed anautogenous behavior. Molecular analysis gave 610 bp fragments on agarose gel electrophoresis. All strains identified *Cx. pipiens pipiens* according to the autogenous/ anautogenous tests and molecular tests.

According to the bioassay results, all tested populations showed resistance to DDT. Synergistic applications increased the mortality rates. However, PBO synergism effect was found to be more than DEF for DDT and pyrethroid group insecticides (Table 2).

All populations showed resistance to permethrin. It is apparent from the bioassay results that the effect of DEF application on increasing mortality rate was lower than PBO for Kapikaya, Huzurkent, and Mersin populations (Table 2).

Kapikaya, Aksu and Mersin populations were resistant to deltamethrin (Mortality rates under 80%). Huzurkent population was at surveillance category (Mortality rates between 80–97%). Mortality rates increased with the use of PBO and DEF, but the effect of PBO on mortality rate was higher than that of DEF for deltamethrin as well as for permethrin (Table 2).

Malathion test results revealed that mortality rates were fewer than 80% in all populations. DEF application increased mortality rates nearly up to 90% except in Mersin population. PBO application increased mortality rates ranging from 4% (Huzurkent) to 24% (Aksu). These rates were lower than DEF application.

**Table 2:** Toxicity (%) of DDT, Malathion, permethrin, and deltamethrin with and without PBO/DEF to *Culex pipiens* L. populations

Insecticides	Populations			
	Kapikaya	Huzurkent	Aksu	Mersin
DDT 4%	64	37	46	35
DDT+PBO	72	91	84	88
DDT+DEF	67	75	66	70
Permethrin 0,75%	58	66	74	70
Permethrin+PBO	100	94	96	85
Permethrin+DEF	74	72	86	80
Deltamethrin 0,05%	73	84	62	67
Deltamethrin+PBO	94	100	98	82
Deltamethrin+DEF	86	90	71	75
Malathion 5%	63	74	57	40
Malathion+PBO	78	78	81	57
Malathion+DEF	100	94	89	70

**Note:** Experiments were performed using 2 replicates and each replicate contained 50 female mosquitoes. Percentage mortality includes sum up of two replicates.

#### 3.2. Biochemical Analysis

Activity results of three possible enzymatic systems related to the resistance are summarized in Table 3.

**Table 3:** Mean enzymatic activity levels in *Culex pipiens* L. populations (mean $\pm$ S.D.)

Population	MFO nmole product/min/mg protein	NSE $\mu$ mole pNPA/min/mg protein	NSE nmole $\alpha$ naphthol/min/mg protein	NSE nmole $\beta$ naphthol/min/mg protein	GST $\mu$ mole CDNB/min/mg protein
Kapikaya	0.00160 $\pm$ 0.0008*	0.32 $\pm$ 0.29*	0.00035 $\pm$ 0.00041*	0.00037 $\pm$ 0.00041*	0.423 $\pm$ 0.203*
Huzurkent	0.00160 $\pm$ 0.00140*	0.43 $\pm$ 0.35*	0.00053 $\pm$ 0.00092*	0.00047 $\pm$ 0.00082*	0.423 $\pm$ 0.201*
Aksu	0.00170 $\pm$ 0.00130*	0.45 $\pm$ 0.41*	0.00100 $\pm$ 0.00140*	0.00081 $\pm$ 0.00100*	0.636 $\pm$ 0.422*
Mersin	0.00018 $\pm$ 0.00004*	1.20 $\pm$ 0.64*	0.00005 $\pm$ 0.000008§	0.00004 $\pm$ 0.0000064§	0.748 $\pm$ 0.416*
Control	0.00005 $\pm$ 0.000014	0.11 $\pm$ 0.055	0.00012 $\pm$ 0.000030	0.00010 $\pm$ 0.000021	0.140 $\pm$ 0.052

NSEs activities of the samples were detected by using three different substrates ( $\alpha$ ,  $\beta$  NA and  $p$  - NPA). The enzyme activity with  $p$  - NPA ranged from  $0.32 \pm 0.29 \mu\text{mol } p$  - NPA/min/mg protein (Kapikaya) to  $1.20 \pm 0.64 \mu\text{mol } p$  - NPA/min/mg protein (Mersin) in all tested populations (Table 3). There was a significant difference between control and tested populations. Additionally, the difference was also significant ( $p < 0.05$ ) between Mersin and the rest of the populations.

The NSEs activity not only fluctuated among populations by using  $\alpha$ ,  $\beta$  naphthyl acetate, but also showed that the values of  $\alpha$  esterases were nearly similar to those of  $\beta$  esterases in its own populations (Table 3). The tested populations showed higher activities than the control group except the Mersin

#### 4. Discussion

Different degrees of resistance to DDT, permethrin, deltamethrin and malathion for four populations of *Cx. pipiens* L in Turkey were detected in this study. Susceptibility studies on rural populations of mosquitoes may be important in terms of showing different selection pressure on resistance evolution. All collection points are situated in immediate agricultural areas. Therefore, this study is significant since it indicates the selection pressure related to insecticide usage with different purposes. These insecticides have been used in sampling sites in last ten years for mosquito control/or agriculture (Table 1). DDT had been used in many years until the 1980s and then forbidden by the Turkish Ministry of Health. DDT toxicity was lower than that of other experimented insecticides and mortality rates ranged from 20 to 64%. Although the use of DDT has been banned since 1980s, DDT resistance was persistent in all of the tested populations. Persistency of resistance for DDT after stopping the use of it has been reported both in other regions of Turkey and in other countries for *Aedes aegypti* and *Anopheles maculipennis* complex species [22, 23, 24]. Brengues *et al.* [25] reported that the cross resistance related to the novel mutation of the voltage gated sodium channel for *Aedes aegypti*. DDT resistance may be ensured that this situation in our strains. PBO and DEF experiments increased the toxicity of DDT, the result of which was supported by the enzymatic studies. MFO,  $\alpha$  and  $\beta$  esterases levels were higher in the majority of the populations compared to the control group. Nevertheless, the levels of  $\alpha$  and  $\beta$  esterases were significantly lower in the Mersin population than the control group. High GST levels indicated high DDT dehydrochlorinase activity. High DDT dehydrochlorinase activity was reported in many studies related to the DDT resistance in mosquito species [25, 26, 27]. Some authors also reported the highly produced DDT dehydrochlorinase and  $p$ -NPA conversion rate for NSEs levels in DDT and Pyrethroid resistant *Anopheles* species in Turkey [24, 28, 29] and our results support this situation. Correlation analysis showed that the negative correlation between DDT mortality and GST levels ( $-0.52$   $p < 0.05$ ) and  $p$ -NPA levels ( $-0.57$   $p < 0.05$ ).

Studied populations' was categorized as resistant to pyrethroid according to the WHO [18]. Additionally, PBO and DEF experiments indicated that monooxygenase and esterases played an important role for permethrin and deltamethrin dissimilation. PBO application increased the toxicity to a greater extent compared to DEF. This result implied that MFO-mediated resistance to pyrethroids was more predominant than NSEs in all the tested populations. The biochemical tests verified the bioassay tests. As far as majority

population of which enzyme activity was significantly lowers ( $p < 0.05$ ).

The activity levels of MFO were significantly higher than the control group for all the tested populations ( $p < 0.05$ ) (Table 3). They were nearly in the same range in Kapikaya, Huzurkent and Aksu populations within themselves, and did not show any significant differences from one another ( $p < 0.05$ ).

The GST levels were higher in all the tested populations compared to the control group ( $p < 0.05$ ) (Table 3). Aksu and Mersin populations showed nearly five times higher activity than the control group. Similarly, Kapikaya and Huzurkent populations showed three times higher activity than the control group

of the tested populations are concerned, all the tested enzyme levels were found to be higher than those of control group. These results implied that all of the biochemical mechanisms may be effective on resistance in all tested populations. Deltamethrin has been used all of the collection areas for both agriculturally and mosquito control. Permethrin usage profile just only limited the mosquito control purposes but permethrin based aerosol formulation usage was higher than other formulations in the areas according to the individual interview. These situations may be influenced pyrethroid group insecticides resistance. Cross resistance between organochlorines and pyrethroids either *kdr* type resistance or biochemical resistance mechanism may be affect high pyrethroid resistance. Correlation results were supported this situation and gave additional information about the pyrethroid group insecticides together with enzyme inhibitors (PBO and DEF). Especially  $p$  - NPA and GST correlation results showed negative correlation between deltamethrin resistance ( $-0,36$   $p$  - NPA,  $-0,09$  GST) but this situation was not found for permethrin.

Elevated levels of MFO, GST and NSEs have been known to enhance the tolerance to insecticides [20]. Many authors reported that elevated activity of MFO, NSEs and GST in different insect species and mosquitoes is connected with DDT and Pyrethroid resistance [30, 31, 32, 33]. Rodriguez *et al.* [22] and Polson *et al.* [34] showed various degrees of resistance to pyrethroids in some *Aedes aegypti* populations, and reported elevated esterase and GST levels associated with the resistance. Tantley *et al.* [7] reported PBO effects and increasing in part of the toxicity for permethrin on *Cx. pipiens* mosquitoes, and showed the relationship with the  $p450$  oxidases. Brogdon *et al.* [35] reported more synergized effects of PBO on permethrin resistance than those of DEF in Guatemalan *Anopheles albimanus*. Our results to PBO showed more synergized effects than DEF for permethrin and deltamethrin resistance in Turkish *Cx. pipiens* L. populations, and thus supported the aforementioned studies related to PBO.

It was shown that there was a high resistance to malathion in all populations. PBO and DEF applications increased the toxicity, and indicated that malathion resistance was related to MFO and NSEs. Esterase-based resistance to malathion was found to be more predominant than MFO in all tested populations. Moreover, biochemical analysis supported the results, and NSEs levels were higher than control group for all populations. High carboxylesterase level in mosquitoes related to the malathion resistance is one of the main biochemical mechanisms [36, 37]. NSEs activity is biochemically determined with different substrates. Three of the substrates are used ( $\alpha$  and  $\beta$  naphthyl acetate and paranitrophenyl acetate) to determine NSEs activity. Malathion carboxylesterases act

preferably on the *p* - NPA, therefore this substrate is chosen for identification of NSEs level [38]. The tests with *p* - NPA demonstrated high malathion carboxylesterase activity for all tested populations.  $\alpha$  and  $\beta$  naphthyl acetate assays showed the same situation except for the Mersin population.  $\alpha$  and  $\beta$  esterases activity in Mersin population had the lowest level, whereas *p* - NPA activity had the highest levels in all of the tested populations and control group. Correlation results supported this situation and high negative correlation (-0.69  $p < 0.05$ ) was found between mortality and *p* - NPA levels. It was shown negative correlation between mortality and GST levels (-0.15  $p < 0.15$ ) and this situation showed that GST acting on malathion resistance. In *Culex tarsalis* malathion carboxylesterases band were shown in starch gel electrophoresis, but there is no correlation between the bioassay and the carboxylesterase activity when naphthyl acetate is used as a substrate [39]. Some authors reported high esterase levels for malathion resistance after the selection of *Cx. pipiens* L. complex species [9, 40], which was also shown herein. Malathion has generally been used on agricultural pests since 1980s in Turkey. Therefore detected high malathion resistance may be related to insecticide usage with agricultural purposes. Kasap *et al.* [41] and Akiner *et al.* [24] reported the same situation for *An. maculipennis* complex species in Turkey.

The control of the house mosquito (*Cx. pipiens* L.) is dependent on chemical insecticide application. Insecticide resistance is the main problem for vector control programs and it also leads to financial losses and environmental contamination. In this study, high resistance to different classes of insecticides was determined. According to the results, (I) DDT resistance was still high, (II) pyrethroid and malathion resistance showed the same situation for all populations, but (III) the toxicity of the insecticides was lower than that of DDT.

This study is very significant in many ways, as it points out the genetic mechanisms, which cause resistance to DDT, are still being kept in all populations during the time which the analyses were carried out. Both DDT and pyrethroids are acting on the same site [42]. Therefore pyrethroid resistance could be explained with selection pressure of insecticide usage. Insecticide usage profile supported this situation and deltamethrin, permethrin have been used all of the sampling sites in the last ten years. Additionally, DDT and pyrethroids are metabolized by the same systems (MFO, esterases and GST) [22, 30, 32]. Since all collection points are situated in immediate agricultural areas, populations may have been/be affected by the pesticide usage with agricultural purposes. In this condition, resistance mechanisms may evolve fast under the pressure of these insecticides.

West Nile infections will be a more severe problem in Europe and in the related areas in the future. Therefore, there is a need for more detailed monitoring studies about tendency of resistance mechanisms to prevent public health globally. Although this study gives detailed information about the resistance status of *Cx. pipiens* L. populations in Turkey, more detailed genetic analyses are still needed.

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