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■ RESEARCH ARTICLE

Detoxification Enzyme Levels that Might Cause Insecticide Resistance in *Aedes albopictus*

Nida Çildağ

Aydın Fen Lisesi, Girne, 2203. Sk. No:4, 09100 Efeler/Aydın, TURKEY; nidacildag@gmail.com
Mentor: Mrs. Sare Yavaşoğlu

ABSTRACT: *Aedes albopictus* (Skuse, 1895) (Diptera: Cullicidae) is an aggressive and invasive mosquito species. It has been shown that *Ae. albopictus*, which can be a vector in spreading some diseases such as zika, dengue, and chikungunya, develops resistance to some insecticides. This study investigated enzyme level alterations, considered among the physiological resistance mechanisms. Two groups which are *Ae. albopictus* samples that were collected from the city of Aydın in Turkey (study group) and insecticide-free and laboratory-grown *Ae. albopictus* samples (control group) were the subjects of the study. The acetylcholinesterase (AChE), esterase, oxidase, and glutathione-s-transferase (GST) enzyme levels and specific activity assays were carried out in both groups. All enzyme analyses were conducted according to World Health Organization's protocol, and protein determination was done by the Bradford method. In conclusion, the two groups had significant differences in all enzyme levels. This study, which revealed such enzyme differences in insecticide-resistant *Aedes albopictus*, might guide future genetic studies that will explain these enzyme differences and new studies for selecting insecticides that will not harm the environment and humans.

KEYWORDS: Biology, *Aedes albopictus*; Insecticide Resistance; Enzyme Analysis.

■ Introduction

Aedes albopictus, also known as the Asian Tiger Mosquito, is a blood-sucking mosquito of Far Eastern origin belonging to the Culicidae family. It is usually less than one centimeter long, with white stripes on its body and white spots on its legs.[1] As a consequence of custom goods' intercontinental transportation and improving transportation facilities in the 19th century and the first half of the 20th century, it was not limited to the Far East and spread to the islands in the west of the Indian Ocean and the east of the Pacific Ocean.[2] Today, *Ae. albopictus* has been reported in more than 20 European countries and is considered one of the world's fastest spreading and most vicious invasive species.[3-5] Over half of the world's population lives in regions where vector mosquitoes are widespread.

According to data from the World Health Organization (WHO), millions of people are infected with mosquito-borne diseases yearly.[6] In addition, *Ae. albopictus* can carry different organisms, such as viruses and bacteria, and spread diseases caused by these agents. For years, municipalities and health institutions have been trying to control vector mosquitoes because of the pathogens they carry to animals and humans. These control measures include physical, chemical, and biological methods. Because of their ease of accessibility and their immediate and long-lasting effects, synthetic chemical agents have been preferred for mosquito control. Today, most insecticides used in mosquito control are synthetic organic insecticides. Widespread use of insecticides has led pests to develop behavioral, morphological, and physiological resistance mechanisms. Physiological resistance mechanisms are divided into two groups: metabolic resistance through increased detoxification enzyme levels and target site mechanism involving voltage-gated sodium channel, acetylcholinesterase (AChE) mutations, and γ aminobutyric acid (GABA) receptor genes.[7] Three enzyme classes called cytochrome P450-dependent monooxygenases (mixed-function oxidase), glutathione-s-transferases, and carboxyl esterases are responsible for metabolic resistance.[8] Alterations in enzyme levels are among the physiological resistance mechanisms and play an essential role in resistance development mechanisms against insecticides. *Ae. albopictus* has also been shown to develop resistance to insecticides.[9]

In this project, the levels of acetylcholinesterase (AChE), esterase, oxidase, and glutathione-s-transferase (GST) enzymes, which may cause physiological resistance, were measured and specific activity determinations were performed in *Ae. albopictus* samples from Aydın province, a touristic province of Turkey connecting Asia and Europe (study group) and *Ae. albopictus* samples were grown in the laboratory with no contact with insecticides (control group), following the guidelines proposed by the World Health Organization (WHO).

■ Materials and Methods

In this study, fieldwork was carried out in October 2021 at the cemetery in Güzelçamlı town of Kuşadası district in Aydın province, Turkey (Figure 1). Larvae of *Ae. albopictus* were collected from plastic containers, flower vases, and puddles on marble tombstones in the cemetery. Larval samples were gathered using larval scoops and transferred to the vector insect laboratory in plastic containers. Larvae were raised in an insectarium under standard conditions of 26-28°C, a 12:12 h photoperiod, and relative humidity of 70-80%. Larvae were raised in cages (30*30*30 cm) until adult F1 females were obtained. Morphological identification was performed using the identification keys.[10] Forty-five adult F1 females were considered in the study group. Fifty-one *Ae. albopictus* samples of the

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ditions for more than five years at the university where the study took place. These individuals were regularly tested for insecticide resistance and were known to be sensitive to insecticides.

Figure 1: Turkey, shown in red on the World Map, and the city of Aydın, where the samples were collected, shown in red on the map of Turkey.

1. Protein assay:

The study analyzed the samples for proteins by the Bradford method.[11] From the homogenates obtained from mosquitoes, 10 µL was transferred to 96-well microtiter plates in triplicates, and 300 µL of Bradford solution was added. For blinding, 10 µL of 50 mM sodium phosphate buffer in triplicates and 300 µL Bradford's solution were added to the wells. After five minutes of incubation, spectrophotometric measurements were performed at a wavelength of 590 nm.

1.1. Preparation of the protein standard graph:

Bovine serum albumin (BSA) was used as the protein standard. Initially, standard solutions were prepared using 0.9% NaCl (physiological saline) and diluted to a concentration between 50-1000 µg/mL. Then, 10 µL of these solutions were transferred to 96-well microtiter plates in triplicates for each standard concentration, and 300 µL of Bradford solution was added. For blinding, 10 µL of 0.9% NaCl and 300 µL of Bradford solution were added to the wells in triplicates. After five minutes, the standards were read at 590 nm wavelength, and linear regression analysis was performed to obtain a protein standard graph of absorbance versus concentration. Similarly, the equation y=0.0006x+0.0592 (Regression coefficient, R2=0.9867) obtained from plotting this standard graph was used to calculate the amount of protein in homogenates obtained from mosquitoes.

2. Nonspecific esterase activity quantification with three different substrates

2.1.Quantitative Assessment of Alpha and Beta Esterases Activity :

Alpha and beta esterase (α-esterase; β-esterase) activities were determined using the method proposed by WHO.[12] First, from each of the homogenates obtained from mosquitoes, 20 µL was taken in duplicates and transferred to 96-well microtiter plates, and then 200 µL of α-NA and β-NA solutions were added. Similarly, for blinding, 20 µL of 50 mM sodium phosphate buffer was added to the wells in duplicates. Then 200 µL of α-NA (alpha naphthyl acetate) and β-NA (beta naphthyl acetate) solutions were added. Next, this mixture was incubated at room temperature for 30 minutes, and then 50 µL of color reagent was added to inhibit enzyme-substrate interaction. This process resulted in a pink-purple color, and absorbances were read at 570 nm. The amount of product was calculated from the alpha and beta naphthol standard curves. One enzyme unit (EU) was expressed as the amount of enzyme that converted 1 µM of the substrate to the product in one minute under standard conditions. The enzyme and specific activities were calculated based on the formulas below.

Volume activity (U/mL)= Product concentration (µM) x Total volume (mL) / Incubation time (min) x Enzyme amount (mL)

Specific activity (U/mg protein)= Volume activity (U/mL) / (mg protein (mL)

2.2. Preparation of Alpha and Beta Naphthol Standard Graphs:

For calculation of α-esterase and β-esterase enzyme activities, firstly, 1 mM stock solutions of α-naphthol and β-naphthol, the products of these enzymes, were prepared. Then, intermediate solutions were obtained from these stock solutions at five different concentrations of 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, and 0.5 mM. 20 µL of these intermediate solutions were transferred to 96-well microtiter plates in triplicates, and 200 µL of 20 mM sodium phosphate buffer was added. This mixture was incubated at room temperature for 30 minutes. Then 50 µL of color reagent was added to inhibit enzyme-substrate interaction, and spectrophotometric analysis was performed at 570 nm. The formula for the standard graph curve obtained for the alpha naphthol product at known concentrations was calculated as y=2.233x+0.0042 (R2=0.9988), and the formula for the standard graph curve for the beta naphthol product was as y=2.093x-0.0153 (R2=0.9991). These formulas were used to determine the alpha-esterase and beta-esterase enzymes' specific activities.

2.3. Quantitative Assessment of pNPA Esterase Activity:

In addition to α-NA and β-NA, para nitro phenyl acetate (pNPA) was used as a substrate for esterase activity measurement, similarly following the guideline proposed by WHO for this method.[12] For this method, 100 mM of pNPA stock solution was prepared (obtained by dissolving 0.18115 g of pNPA in 10 mL acetonitrile) Then, the stock solution was diluted at 1:100 with 50 mM sodium phosphate buffer (pH 7.4) to produce the pNPA solution. Finally, 20 µL of the homogenates obtained from mosquitoes were transferred to 96-well microtiter plates in duplicates, and 200 µL of pNPA solution was added to each. Similarly, for blinding, 20 µL of 50 mM sodium phosphate buffer was added to the wells in duplicates, and 200 µL of pNPA solution was added. The absorbance value of this mixture was measured at 405 nm kinetically.

2.3.1. Preparation of the Para Nitro Phenol Standard Graph:

Firstly, a 10 mM stock solution was prepared from p-nitro phenol, the product of this enzyme, to calculate the p-esterase enzyme activity. Then, intermediate solutions were prepared from the stock solutions at five different concentrations ranging from 0.5 mM to 1 mM. Finally, 20 µL of each intermediate solution was transferred to 96-well microtiter plates with triplicates, and absorbance measurements were carried out at 405 nm wavelength. A standard graph for p-nitro phenol was generated using the absorbance values obtained. The formula of the standard graph was calculated as y= 0.9026x-0.1408 (R2=0.9834). Enzyme activity and specific activity were calculated based on this formula.

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3. Quantitative Assessment of Mixed Function Oxidases:

The WHO protocol was followed for determining oxidase activity.[12] First, 20 µL of each of the homogenates obtained from mosquitoes was taken as duplicates and transferred to 96-well microtiter plates, then 200 µL of TMBZ (3,3',5,5'-Tetramethyl-Benzidine Dihydrochloride) solution was added. Finally, 25 µL of 3% H2O2 was added to this mixture and left to incubate for 5 minutes. Similarly, for blinding, 20 µL of 50 mM sodium phosphate buffer was added to the wells in duplicates, and 200 µL of TMBZ solution and 25 µL of 3% H_2O_2 were added to each well. The absorbance value of this mixture at 620 nm was read spectrophotometrically.

3.1. Preparation of Cytochrome-C Standard Graph:

0.25 M (pH 5.0) Cytochrome-c was dissolved in sodium acetate buffer, and a stock solution containing 1 mg/ml was prepared. This stock solution was diluted 100-fold to obtain a new 10 µg/ml solution. Dilutions were made again from this intermediate solution to obtain solutions with concentrations ranging from 1-10 µg/ml, and their absorbances were read at 620 nm wavelength. The standard graph of cytochrome-c protein was derived using linear regression analysis and the absorbance values obtained for known concentrations. In the graph y=0.1126x-0.0885 (R2=0.9993) belonging to the standard curve of cytochrome-c, the x value gives the concentrations of the samples with absorbance values written in place of y. Using this graph, firstly, the amount of heme protein in micrograms was calculated for each sample. Then, this value was divided by the protein amount to calculate the amount of heme protein per mg of protein.

4. Quantitative Assessment of Glutathione-S-Transferase Activity:

The glutathione-s-transferase (GST) activity was measured according to the WHO method.[12] First, 10 µL of each homogenate obtained from mosquitoes was transferred to 96-well microtiter plates as duplicates and 200 µL of GSH/CDNB solution was added to each. Similarly, for blinding, 10 µL of 50 mM sodium phosphate buffer was added to the wells as duplicates and 200 µL of GSH/CDNB solution was added. Following a 20-minute incubation period, the absorbance values of this mixture were read spectrophotometrically at 340 nm wavelength. Glutathione-s-transferase enzyme activity was calculated using the following formula (extinction coefficient Ɛ=4.39 mM-1).12 Thus, specific GST activity was calculated in EU/mg.

Volume activity (U/mL)= Absorbance x Total volume (mL) / E (mM-1) x Total enzyme volume (mL) x I (cm) x t (d)

Specific activity (U/mg protein)= Volume activity (U/mL) / mg protein (mL)

5. Quantitative Assessment of Acetylcholinesterase Activity:

Acetylcholinesterase activity was determined following the method proposed by WHO.[12] Firstly, 25 µL of the homogenate obtained from mosquitoes was transferred to 96-well microtiter plates as duplicates. Then, 145 µL of 1% Triton-X-100 solution was added to each of them. Then, 10 µL of previously obtained 0.01 M DTNB solution was added. Finally, 25 µL of propoxur-free ASCHI solution was added to one well, and 25 µL of propoxur-containing ASCHI solution to the other. Similarly, for blinding, 25 µL of 50 mM sodium phosphate buffer was added to the wells in duplicates, followed by 145 µL of 1% Triton-X-100 solution, 10 µL of DTNB solution, and finally, 25 µL of ASCHI solution without propoxur in one well and 25 µL of ASCHI solution with propoxur in the other. Following a one-hour incubation at room temperature, the absorbance values of this mixture at 405 nm wavelength were read spectrophotometrically. With the absorbance values obtained, the percentage of inhibition in acetylcholinesterase levels of the individuals exposed to propoxur was calculated with the following formula:

Residual activity: (Absorbance with Propoxur/Absorbance without Propoxur)/100)-100).

Statistical Analysis:

Biochemical analysis data were compared for enzymatic differences between populations using Kruskal-Wallis non-parametric test (Statistica 7.0).

Results

Ae. albopictus samples known to be resistant[9] to DDT and permethrin and gathered from Güzelçamlı town were taken as the study group. *Ae. albopictus* samples grown in the laboratory and not in contact with insecticides were considered the control group. The study group involved 45 and the control group 51 *Ae. Albopictus* samples. The median values of Alpha esterase (EU/mg protein), Beta esterase (EU/mg protein), pNPA (EU/mg protein), AChE (% activity), Oxidase (ug cyt-c/mg protein), and GST (EU/mg protein)were 0.3072, 0.5097, 3.1254, 36.1702, 7.7270, and 0.0473, in the study group, respectively, whereas, 0.07930; 0.20164; 2.08586; 8.16326; 3.69209; 0.0023, in the control group, respectively (Table 1).

Table 1: Enzyme levels and activity measurement values in the study and control groups

	Nonspecific esterase activity with different substrates											
	α-Esterase EU/mg		β-esterase EU/mg		pNPAEU/mg		AChE (% activity)		Oxidase (ug cyt-c/mg)		GST EU/mg	
	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max
Study Group (n=45)	0.3072	0.10-2.36	0.5097	0.20-4.37	3.1254	0.72-20.1	36.1702	9.70-52.64	7.7270	1.84-43.15	0.0473	0.006-0.60
Control Group (n=51)	0.0793	0.01-0.26	0.2016	0.05-0.41	2.0858	0.45-3.11	8.1632	1.48-37.59	3.6920	1.04-61.30	0.0023	0.0004-0.015

In the statistical comparison between the median enzyme levels in the study and control groups, significant differences were observed between the median values of all enzymes ($p < 0.005$). The results are shown in Figures 2-7.

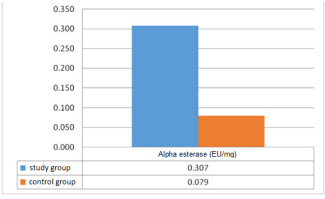

Figure 2: Alpha esterase

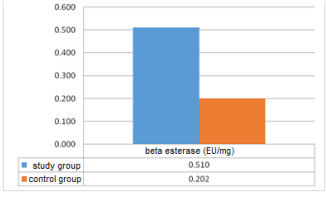

Figure 3: Beta esterase

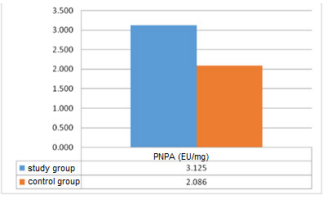

Figure 4: Para nitro phenyl acetate (PNPA)

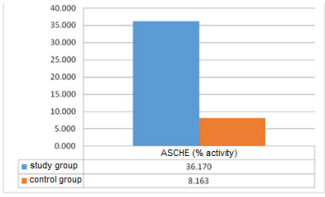

Figure 5: Acetylcholinesterase (ASCHE)

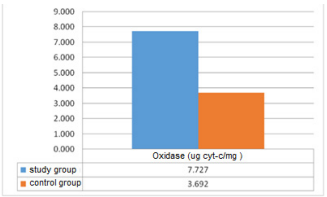

Figure 6: Oxidase

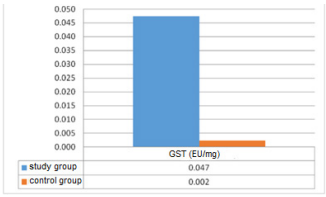

Figure 3: Glutathione-S-Transferase (GST)

Discussion

The success rate of mosquito control is dependent on numerous factors. Among these, the most significant ones are the efficiency of insecticides used and the response of the targeted mosquito against insecticides. In addition, the resistance of mosquitos against insecticides can become a significant problem regarding their control. In such situations, the type of insecticide with the potential to be administered can be identified through the vector's insecticide resistance analysis. The widespread insecticide use in mosquito control results in failure due to the development of insecticide resistance. An efficient insecticide resistance management and regular monitoring of insecticide resistance status are essential for preventing resistance development. Otherwise, we may be disappointed both economically and also regarding the ecosystem's balance, such as animals, humans, and plants, and we may even face unwanted damage.

Ae. albopictus, a mosquito rapidly spreading throughout the world, can cause viral infections and allergic reactions. Dengue, chikungunya, yellow fever, zika virus infection, La crosse encephalitis, and microfilariae infections are significant diseases that *Ae. albopictus* can cause as a vector.[13] With their strong antigenic structures, Saliva proteins of *Ae. albopictus* cause allergic reactions regardless of exposure method.[14] Mosquitoes are attempted to be controlled with organophosphorus, carbamates, and pyrethroid-group chemical insecticides.

On the other hand, the development of resistance against insecticides has been shown in *Ae. albopictus*.[9] Mosquitoes can develop resistance to insecticides through behavioral or physiological adaptation. Physiological resistance mechanisms are divided into two groups: metabolic resistance through increased levels of detoxifying enzymes (esterase, glutathione transferase, mixed-function oxidase) and target site mechanisms involving voltage-gated sodium channel (VGSC), acetylcholinesterase (AChE) mutations, and γ aminobutyric acid (GABA) receptor genes.[7] Increased expression of the gene encoding the primary enzyme responsible for xenobiotic metabolism is the mosquitoes' most common resistance type. Alterations in trans-acting regulatory elements, changes duplications, or gene amplifications in the promoter region of the gene encoding the detoxification enzyme can increase the detoxification enzymes excessively.[15,16] Due to this excessive increase, insecticides can be metabolized with no toxic effect in resistant insects.

In this study, the specific activities of the non-specific esterase group with three different substrates (alpha naphthol, beta naphthol, and pNPA) glutathione-s-transferase enzymes, the percentage inhibition rates of acetylcholinesterase enzyme, and the amount of cytochrome protein to express oxidase activity were calculated in adult *Ae. albopictus* females that were insecticide resistant and laboratory-grown control group females that were considered sensitive to all insecticides. Significant differences were observed between the study and the control groups regarding the levels of enzymes investigated. The insecticide resistance in various vector mosquito species of our region and the underlying biochemical and molecular mechanisms have been previously reported.[17] It was identified that there was no study on biochemical enzyme analysis in *Ae. albopictus*, which has been observed in our region in recent years. This study is the first regional research on analyzing enzymes that may cause insecticide resistance in *Ae. albopictus*. Since this research revealed enzyme differences in insecticide-resistant *Ae. albopictus* in our region, which is suitable for many mosquito species and the pathogens they carry due to its climatic, geographical, and geological characteristics: it may lead to new insecticides that will not harm the environment and humans and to genetic studies that can explain the mechanisms of differences in these enzymes.

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Authors

Nida Çildağ is a 17-years-old high school student at Aydın Fen Lisesi. She is interested in biology and genetics. This project was selected for the regional project competition.