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Research article

Assessment of susceptible *Culex quinquefasciatus* larvae in Indonesia to different insecticides through metabolic enzymes and the histopathological midgut

Rizal Subahar [a,*], Annisa Putri Aulia [b], Yulhasri Yulhasri [c], Ris Raihan Felim [b], Lisawati Susanto [a], Rawina Winita [a], Gulshan Fahmi El Bayani [d], Tilahun Adugna [e]

[a] Department of Parasitology, Faculty of Medicine, Universitas Indonesia, Depok, Indonesia
[b] Medical Doctor Program, Faculty of Medicine, Universitas Indonesia, Depok, Indonesia
[c] Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Depok, Indonesia
[d] Department of Medical Physiology and Biophysics, Faculty of Medicine, Universitas Indonesia, Depok, Indonesia
[e] Debre Tabor University, Ethiopia

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ABSTRACT

Filariasis and virus diseases that are transmitted by *Culex quinquefasciatus* are still a global health problem. Control of mosquito vectors with synthetic insecticides causes resistance to these mosquitoes to insecticides so that detection of susceptibility of the mosquito larval stage to insecticides is important for evaluating mosquito control programs. The aim of this study was to evaluate the susceptibility of wild-caught *Cx. quinquefasciatus* larvae in Jakarta, Indonesia, following exposure to temephos, malathion, cypermethrin, and deltamethrin; this was done by examining the detoxifying enzyme activities and histological damage to the larval midgut. *Cx. quinquefasciatus* larvae were collected from five fields in Jakarta and exposed for 24 h to temephos (1.25, 6.25, 31.25, and 156.25 ppm), malathion (0.5 ppm), cypermethrin (0.25 ppm), and deltamethrin (0.35 ppm). The larvae were then examined for acetylcholinesterase (AChE), glutathione S-transferase (GST), and oxidase activities using biochemical methods. Histological damage to the larval midgut was examined using routine histopathological methods and transmission electron microscopy (TEM). After 24 h, temephos and deltamethrin led to 100% mortality in the *Cx. quinquefasciatus* larvae. Temephos and malathion significantly inhibited the activity of AChE, while cypermethrin and deltamethrin significantly inhibited oxidase activity. Histologically, all insecticides damaged the larval midgut, as indicated by irregularities in the epithelial cell (ECs), microvilli (Mv), food boluses (FBs), peritrophic membranes (PMs), and cracked epithelial layers (Ep). The TEM findings confirmed that temephos and cypermethrin damage to the midgut ECs included damage to the cell membrane, nucleus, nucleoli, mitochondria, and other cell organelles. Overall, *Cx. quinquefasciatus* larvae in Jakarta were completely susceptible to temephos and deltamethrin. Synthetic insecticides may kill *Cx. quinquefasciatus* larvae through their actions on the metabolic enzyme activities and histopathological midgut.

1. Introduction

The *Culex quinquefasciatus* Say (Diptera: Culicidae) mosquito is an important mosquito vector that transmits several mosquito-borne diseases (MBDs), such as Bancroftian filariasis, Rift Valley Fever, West Nile Fever, Saint Louis encephalitis, and Zika (Lopes et al., 2019), which are distributed worldwide in tropical and subtropical countries. Since the Brazilian Zika outbreak, *Cx. quinquefasciatus* samples in urban environments have been detected as being infected with the Zika virus (ZIKV), suggesting participation in a new cycle of this emergent virus in some regions (Turell 2012; Eastwood et al., 2019; Lopes et al., 2019). Additionally, many reports have shown that *Cx. quinquefasciatus* transmits sylvatic arbovirus from birds, indicating that it may function as an important bridge between sylvatic arbovirus transmission from birds to humans in urban areas (Lopes et al., 2019; Gil et al., 2021; Pereira et al., 2020).

No vaccines are presently available for MBDs; therefore, mosquito control is the best way to reduce the transmission of MBDs to humans

* Corresponding author.
E-mail address: subaharizal@yahoo.com (R. Subahar).

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(WHO, 2011; Lopes et al., 2019). Many mosquito control programs rely on insecticides, so many studies have evaluated the susceptibility of *Cx. quinquefasciatus* larvae to insecticides (Boyer et al., 2006; DeLisi et al., 2017; Shemshadian et al., 2020). Similarly, the susceptibility status of the adult *Cx. quinquefasciatus* mosquitoes to insecticides is also important in these programs (Kalyanasundaran et al., 2003; Alves et al., 2011), as is the susceptibility of other mosquito species, such as *Aedes* spp. and *Anopheles* spp. (Demok et al., 2019; Korti et al., 2021). The methods used in mosquito control programs can include eliminating mosquito larval habitats, applying larvicides to kill mosquito larvae, or spraying insecticides from trucks or aircraft to kill adult mosquitoes (CDC, 2017; Fontoura et al., 2021; Wilke et al., 2021).

The synthetic insecticides that are frequently used in mosquito control programs have negative impacts on the environment, human health, and nontarget organisms and have developed insecticide-resistant mosquitoes, including *Cx. quinquefasciatus* (Mužinić and Želježić, 2018; Struelens and Silvie, 2020). Currently, many reports have shown that the larval and adult stages of *Cx. quinquefasciatus* sre resistant to synthetic insecticides (Lopes et al., 2019; Atyame et al., 2019; Nachaiwieng et al., 2021) because of knockdown resistance (*kdr)* gene mutations, such as L1014F, G1195S ace-1, and the cytochrome P450 genes CYP9J45, CYP9J40, and CYP6AA7 (Delannay et al., 2018; Silva Martins et al., 2019; Maestre-Serrano et al., 2020; Yoshimizu et al., 2020). These *kdr* gene mutations of *Cx. quinquefasciatus* have been found in several countries: Guadeloupe (Delannay et al., 2018), India (Rai et al., 2019), Thailand (Nachaiwieng et al., 2021), and Cameron (Talipouo et al., 2021).

Metabolic enzymes, which are known as detoxifying enzymes, are used to detect the underlying resistance mechanisms within insecticides that cannot be detected by bioassays (Brogdon 2014; Belmert et al., 2014). Detoxifying enzymes consisting of acetylcholinesterase (AChE), glutathione S transferase (GST), esterase, and cytochrome c oxidase (oxidase) have been applied to assess the susceptibility of mosquito species to different insecticides. They could also be applied to assess the susceptibility of mosquito larvae to insecticides (Boyer et al., 2006; Liu et al., 2020). Thus, metabolic enzymes are an important tool to assess the susceptibility of mosquito larvae and adults to insecticides.

Furthermore, insecticides are toxic agents for insects, so the histopathological midgut is a valuable tool to assess the toxicity of insecticides. For example, cypermethrin causes histological damage in the midgut of *Chironomus calligraphus* larvae (Lavarías et al., 2017). *Bacillus thuringiensis* (Bt), permethrin, tebufenozide, and thiamethoxam showed changes in the midgut of *Podisus nigrispinus*, the predatory bug, such as irregular epithelial architecture, cytoplasm vacuolization, and the release of cell fragments in the midgut lumen (Silva et al., 2021). Additionally, detoxifying enzymes are found in the midgut of the insect, which means that the midgut is damaged by insecticides associated with changes in detoxifying enzyme activity (Lavarías et al., 2017; Lu et al., 2019; Silva et al., 2021). For example, deltamethrin oxidative stress causes damage to the cells and metabolic enzyme changes (de Melo et al., 2018). Therefore, the histological alterations in the larval midgut structure caused by insecticide toxins could affect detoxifying enzyme activity (de Melo et al., 2018).

The current study was conducted in Jakarta, the capital city of Indonesia, because MBD cases such as malaria, dengue hemorrhagic fever (DHF), and filariasis are still found in Jakarta. For example, in 2019, in East Jakarta, there were 52 suspected cases of malaria, 8716 cases of DHF with an incidence rate of 83.0% per 100,000 population, 22 cases of chronic filariasis, and one new case of filariasis (Jakarta Provincial Health Office, 2019). In Jakarta, there are many breeding places for *Cx. quinquefasciatus* larvae around people's homes, such as sewers with dirty and foul-smelling water. By contrast, in Jakarta, an assessment of the metabolic enzymes and histological damage to the midgut to the susceptibility of *Cx. quinquefasciatus* larvae to temephos, malathion, cypermethrin, and deltamethrin has not been carried out so far.

The current study used temephos with the WHO standard kits (1.25, 6.25, 31.25, and 156.25 ppm), malathion (0.5 ppm), cypermethrin (0.25 ppm), and deltamethrin (0.35 ppm). Wild-caught *Cx. quinquefasciatus* larvae from Jakarta were exposed to these insecticides to evaluate their susceptibility (CDC, 2017). The metabolic enzymes of *Cx. quinquefasciatus* larvae from Jakarta were focused on changes in the activity of AChE, GST, and oxidase after exposure to these insecticides using a biochemical method (Brogdon, 2014; Belmert et al., 2014). The histological damage of the midgut of these larvae was examined by a routine histopathological method (de Lemos et al., 2018) and transmission electron microscopy (TEM) (Ma et al., 2017).

2. Methods

2.1. Ethical approval

The current study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No. KET-055/UN2.F1.D1.2/PDP/Riset-2/2019).

2.2. Insecticides

Malathion ($C_{10}H_{19}O_6PS_2$), cypermethrin ($C_{22}H_{19}Cl_2NO_3$) and deltamethrin ($C_{22}H_{19}Br_2NO_3$) were purchased from chemical shops in Jakarta. Malathion is registered in Indonesia as MEGATHION 1200 UL, PT Citra Sari Kimia, No. Reg. RI 0809120103771 and deltamethrin as DELFOX 25 EC, PT Indo Pest Biochem, No. Reg. RI 06090120093424. The present study also used cypermethrin (CYNOFF 50 EC, PT Bina Guna Kimia, No. Reg. RI 06090120124286). Temephos was purchased in liquid form from the Directorate of Vector-Borne and Zoonotic Diseases, Ministry of Health Indonesia, Jakarta, Indonesia.

2.3. Study design

Wild-caught *Cx. quinquefasciatus* larvae were collected from five fields in Jakarta: 1) Kampung Gedong (East Jakarta), 2) Johar Baru (Central Jakarta), 3) Marunda (North Jakarta), 4) Cengkareng (West Jakarta), and 5) Setu Babakan (South Jakarta), as shown in Figure 1. The larvae were collected from natural habitats such as polluted stagnant water bodies, sewers, or drains surrounding houses. All the specimens were subsequently identified at the Entomology Laboratory of the Department of Parasitology, Universitas Indonesia. Only the third and fourth instar larvae were used in the larval bioassays.

2.4. Larval bioassay

A larval bioassay was carried out following WHO protocols (WHO, 2005). In the control group, 25 wild-caught *Cx. quinquefasciatus* larvae were exposed only to tap water in a 200 mL plastic cup. In the treatment groups, 25 *Cx. quinquefasciatus* larvae per 200 mL plastic cup were exposed to temephos using WHO standard kits (1.25, 6.25, 31.25, and 156.25 ppm), malathion (0.5 ppm), cypermethrin (0.25 ppm), and deltamethrin (0.35 ppm) concentrations by modifying the temephos concentration of the WHO standard kits (WHO, 2005; Abai et al., 2017). All larval bioassays were carried out in five replicates. After 24 h of exposure, dead larvae were recorded.

2.5. Biochemical assays

In the current study, 250 *Cx. quinquefasciatus* larvae were used to examine detoxifying enzyme activity, with 50 larvae from the control, temephos, malathion, cypermethrin, and deltamethrin treatments. The activities of AChE, GST, and oxidase were investigated as part of the detoxifying enzyme analysis.

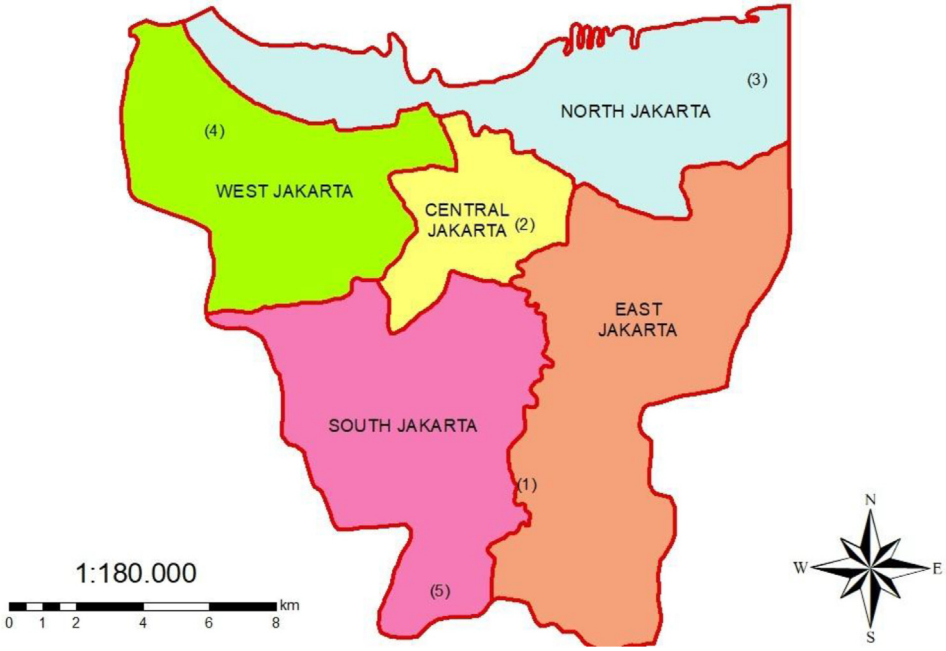

Figure 1. Collection sites of *Cx. quinquefasciatus* larvae in Jakarta. Ujung Gedong, East Jakarta (1), Johar Baru, Central Jakarta (2), Marunda North Jakarta (3), Cengkareng, West Jakarta (4), and Situ Babakan, South Jakarta (5).

2.5.1. AChE assay

The enzyme activity assays were used to analyze AChE activity, as previously described (Brogdon, 2014; Belmert et al., 2014). Dead *Cx. quinquefasciatus* larvae were collected from larval bioassays and homogenized in 1 mL of 0.25 M KPO4 (pH 7.2). At room temperature, 100 μL aliquots of the test sample homogenates were loaded into triplicate ELISA microplate wells. Similarly, 100 μL of the control group was added to triplicate microplate wells. Acetylcholine iodide (ACTH) and 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were then added to every well (100 μL of each per well) and the absorbance at 414 nm was immediately read with an ELISA reader (T_0) and again after 10 min (T_{10}). The unit of AChE activity was absorbance per minute, or Abs/min. The ELISA reader was made in Finland by Thermo Fisher Scientific™ cat # 51119000.

2.5.2. Glutathione S-transferase assay

The enzyme activity assays were used to analyze GST activity, as previously described (Brogdon 2014; Belmert et al., 2014). In the present study, dead *Cx. quinquefasciatus* larvae were collected from larval bioassays and homogenized in 1.0 mL 0.25 M KPO_4 (pH 7.2). Triplicate 100 μL aliquots of each homogenate were loaded into ELISA microplate wells at room temperature; similar wells were prepared using 100 μL of the control group. Aliquots (100 μL) of the reduced glutathione solution (Sigma G4251) and 1-chloro-2,4′- dinitrobenzene (cDNB) were added, and the plates were read immediately at 340 nm (T_0) with an ELISA reader and again at 5 min (T_5). The unit of GST activity was absorbance per minute, or Abs/min.

2.5.3. Oxidase assay

The enzyme activity assays were used to analyze oxidase activity, as previously described (Brogdon, 2014; Belmert et al., 2014). The dead *Cx. quinquefasciatus* larvae were collected from larval bioassays and homogenized with 1000 μL 0.25 M KPO_4 (pH 7.2). The following positive controls were also prepared: (i) 1:55 (22 μL stock, μL 1.2 mL KPO_4 buffer) and (ii) 1:110 (11 μL cytochrome stock, 1.2 mL KPO_4 buffer). Triplicate aliquots (100 μL) of the test sample homogenates were added to the ELISA microplate wells, and 100 μL KPO4 was added to the negative and positive control wells. The Cytochrome-C positive control (Cytochrome-C bovine heart) was added (100 μL), followed by a 200 μL TMBZ solution. One drop of 3% hydrogen peroxide (H_2O_2) was added to each well and incubated for 5 min. The plates were immediately read (T_0) at 620 nm with an ELISA reader. The unit of oxidase activity was absorbance per minute or Abs/min.

2.6. Histopathological examination

A routine histopathological technique was employed as previously described (de Lemos et al., 2018). In total, 150 dead *Cx. quinquefasciatus* larvae were examined; these consisted of 25 larvae from the control group and 125 larvae from the treatment groups (25 larvae from each insecticide treatment). All of these specimens were fixed with 10% formalin and then dehydrated using a series of increasing alcohol concentrations (70%, 80%, 90%, 95%, and 100%). Afterward, the specimens were embedded in xylene 1, xylene 2, and xylene 3 solutions and paraffin blocks. The blocks were cut to a thickness of 5 μm using a manual microtome (Model 320, No. 17664, USA) and feather microtome blades (Feather, S35, Japan). Finally, the sections were stained with hematoxylin and eosin, and the stained specimens were observed under a light microscope and imaged using a digital microscopic mounted camera (Zeiss Axiocam ERC 5s, Germany).

2.7. Transmission electron scanning (TEM) study

The present study used TEM to evaluate damage to the midgut cells, including the cell membrane, mitochondria, and others. The samples were processed according to Ma et al. (2017) with a slight modification of the fixation liquid. The whole bodies of the treated *Cx. quinquefasciatus* larvae were pre-fixed in 2.5% glutaraldehyde at 4 °C for a minimum of 2 days and then washed three times with cacodylate buffer for 15 min each time. The samples were fixed in 2% osmium tetroxide and 2.5% $K_3Fe(CN)_6$ in the buffer for 2 h before being rinsed in cacodylate buffer, as described in the previous step. The samples were then dehydrated in an ethanol series in ascending order (30%, 50%, 70%, 80%, 90%, and 100%) for 15 min each. After dehydration, the samples were infiltrated using absolute ethanol and propylene oxide in specific ratios (2:1, 1:1, 1:2, v/v) for 30 min each. The samples were embedded in Spurr resin. The prepared samples were cut using an ultramicrotome (Leica UC6, Wetzlar, Germany) and observed using TEM (JEOL JEM 1010, Japan).

2.8. Data analysis

The Statistical Package for Social Science (SPPS) version 26.0 was used to analyze the data. The susceptibility response of *Cx. quinquefasciatus* larvae to the insecticides was determined based on criteria previously described (WHO, 2016). A 100% death rate indicated that *Cx. quinquefasciatus* larvae were completely susceptible to the insecticide, a 99–90% death rate indicated moderate susceptibility, and a <90% death rate indicated low susceptibility (WHO, 2016). Wilcoxon Signed Rank tests were used to evaluate AChE (0 min–10 min), GST (0 min–5 min), and oxidase (0 min–5 min) activities (Rosner et al., 2006). The Kruskal-Wallis H test was used to determine whether there was a significant difference in midgut damage because of exposure to the insecticide tested (Fan et al., 2011).

3. Results

Table 1 shows the MBD cases, mosquito species in the mosquito control programs, the use of insecticides, and the natural mosquito breeding sites in Jakarta. In all areas of Jakarta, DHF cases were more common than malaria and filariasis cases. Therefore, mosquito eradication activities in Jakarta have been focused on eradicating *Ae. aegypti* and *Ae. albopictus*, which are key DHF vectors, whereas no eradication programs are conducted for other mosquitoes, such as *Cx. quinquefasciatus* and *Anopheles* spp. Furthermore, cypermethrin has been actively used to control DHF vectors in Jakarta.

The natural breeding sites for *Anopheles* spp. were located only in the northern parts of Jakarta, whereas *Aedes* spp. and *Cx. quinquefasciatus* were found in all five of the study areas (Table 1). Figure 2 shows a natural breeding site for *Cx. quinquefasciatus* larvae: a sewer in front of dirty homes that had no running water, and large amounts of garbage.

3.1. Mortality of Cx. quinquefasciatus larvae

In the current study, as many as 4375 *Cx. quinquefasciatus* larvae were successfully examined at five sampling sites in Jakarta. Temephos and deltamethrin caused 100% mortality for *Cx. quinquefasciatus* larvae at all sampling sites. The malathion and cypermethrin groups and mortality rate of *Cx. quinquefasciatus* larvae ranged from 90.4% to 100% at 0.5 and 0.25 ppm, but a 100 % mortality rate was found in East Jakarta and North Jakarta. The overall results show that *Cx. quinquefasciatus* larvae in Jakarta were completely susceptible to temephos and deltamethrin (Table 2).

Table 1. Description of mosquito-borne disease cases, mosquito control, usage of insecticides, household insecticides, electric mosquito trap, and natural breeding place in Jakarta.

No	Description	DKI Jakarta				
		1	2	3	4	5
1	**VBD cases (in 2019)**					
	Malaria	27	2	5	7	11
	DHF	3014	491	922	2305	1975
	Filariasis	17	4	0	2	0
2	**Mosquito species in mosquito control programs**					
	Anopheles spp	No	No	No	No	No
	Aedes spp	Active	Active	Active	Active	Active
	Cx. quinquefasciatus	No	No	No	No	No
3	**Usage of Insecticides**					
	Pyrethroids	Active	Active	Active	Active	Active
	Organochlorine	No	No	No	No	No
	Organophosphate	No	No	No	No	No
	Carbamate	No	No	No	No	No
4	**Natural breeding place**					
	Anopheles spp	-	-	+	-	-
	Aedes spp	+	+	+	+	+
	Cx. quinquefasciatus	+	+	+	+	+

Note: 1 = east, 2 = central, 3 = north, 4 = west, 5 = south. Natural breeding places + = available, - = not available.

Figure 2. Representation of a breeding place of *Cx. quinquefasciatus* in East Jakarta.

3.2. Detoxifying enzyme activity

Table 3 shows the detoxifying enzyme activity of *Cx. quinquefasciatus* larvae that were treated with insecticides. In the control group, AChE activity showed increased level at 0 min to 10 min. Temephos and malathion significantly inhibited AChE activity ($p < 0.05$), while cypermethrin and deltamethrin significantly increased AChE activity ($p < 0.05$). The control, temephos, cypermethrin, and deltamethrin groups showed significantly increased GST activity ($p < 0.05$). GST activity was the highest in cypermethrin followed by temephos, malathion, and deltamethrin. The control group showed increased oxidase activity. Temephos and malathion caused significantly increased oxidase activity ($p < 0.05$). By contrast, cypermethrin and deltamethrin significantly decreased oxidase activity ($p < 0.05$).

3.3. Histological damage to the midgut

Table 4 shows a total of 40 *Cx. quinquefasciatus* larvae used to assess the histological midgut; temephos (n = 10), malathion (n = 10), cypermethrin

Table 2. The mortality rate of *Cx. quinquefasciatus* larvae after exposure to insecticides.

Insecticides	Concent.	Locations for collecting *Cx.quinquefasciatus* larvae				
	(ppm)	1	2	3	4	5
Temephos	1.25	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)
	6.25	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)
	31.25	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)
	156.25	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)
Malathion	0.5	100% (125/125)	98.4% (123/125)	99.2% (124/125)	96.8% (121/125)	99.2% (124/125)
Cypermethrin	0.25	99.2% (124/125)	99.2% (124/125)	100% (125/125)	98.4% (123/125)	90.4% (122/125)
Deltamethrin	0.35	100% (125/125)	100% (125/125)	100% (125/125)	100% (125/125)	97.6% (125/125)

Note: 1 = East, 2 = central, 3 = north, 4 = west, 5 = south.

Table 3. The detoxification enzyme activity of *Cx. quinquefasciatus* larvae after being exposed to insecticides.

Insecticides	N	Enzyme	Mean of Absorbance		Activity	Wilcoxon Test	
			T0	T5 0r T10		Z	Sig. (2-Tailed)
Temephos	50	AChE	0.364 ± 0.006	0.340 ± 0.026	Decrease	-8.690	0.000
		GST	0.325 ± 0.004	0.341 ± 0.001	Increase	-8.815	0.000
		Oxidase	0.176 ± 0.009	0.201 ± 0.003	Increase	-8.713	0.000
Malathion	50	AChE	0.410 ± 0.006	0.357 ± 0.048	Decrease	-8.690	0.000
		GST	0.296 ± 0.001	0.305 ± 0.002	Increase	-8.745	0.000
		Oxidase	0.212 ± 0.003	0.245 ± 0.003	Increase	-8.701	0.000
Cypermethrin	50	AChE	1.309 ± 0.008	1.750 ± 0.048	Increase	-2.738	0.006
		GST	0.378 ± 0.004	0.477 ± 0.004	Increase	-8.718	0.000
		Oxidase	0.240 ± 0.002	0.228 ± 0.014	Decrease	-8.701	0.000
Deltamethrin	50	AChE	0.619 ± 0.131	0.900 ± 0.135	Increase	-8.697	0.000
		GST	0.299 ± 0.003	0.309 ± 0.001	Increase	-8.713	0.000
		oxidase	0.216 ± 0.001	0.208 ± 0.006	Decrease	-8.719	0.000

(n = 10), and deltamethrin (n = 10). In the control group, the midgut of the *Cx. quinquefasciatus* larvae was normally shaped; the FB (food bolus), PM (peritrophic membrane), Ep (epithelial layer), Mv (microvilli), and EC (epithelial cell) exhibited normal structures. The Ep consisted of the single-layered epithelium and columnar, goblet, and degenerative cells, and it was distributed throughout the anterior and posterior regions of the midgut. The Ep was limited internally by a fine PM and was provided by the Mv (Figure 3A and 3B).

The findings show that the Ep, Mv, PM, and FB were seriously damaged by all insecticides (temephos, malathion, cypermethrin, and deltamethrin). Histologically, the EC and Mv were irregularly shaped, small, or shrunken, the PM was fractured or damaged, and the FB showed cracks (Figure 4).

Table 4 shows the percentage of histopathological alterations in the midgut. The highest damage to the midgut was found in the EC (90–100%), followed by Mv (30–50%), PM (10–20%), and FB (10%). Additionally, the Kruskal-Wallis H test shows that there were significant differences in damage to the EC, Mv, PM, and FB in all tested insecticides ($p < 0.05$) (Table 4).

3.4. Transmission electron microscopy (TEM) study

The current study focused on the ultrastructural EC of the Ep in the midgut of *Cx. quinquefasciatus* larvae induced by malathion and deltamethrin. As seen in Figure 5A and 5B, the ECs of the Ep in the larval midgut were damaged by malathion and deltamethrin respectively. The treatment of malathion and deltamethrin lead to that the Mv, nuclei (N), and nucleoli in the ECs becoming ruptured so that EC structures such as lysosome, centriole, microtubule, Golgi apparatus, nuclear membrane, mitochondrion, cell membrane, cytoplasm, ribosome, and endoplasmic reticulum were ruptured and disappeared (Figure 5A and 5B). The findings demonstrate that malathion and deltamethrin destroyed the EC.

Table 4. Percentage of histopathological alterations in the midgut of *Cx. quinquefasciatus* larvae.

Insecticide	n	EC	Mv	PM	FB	Kruskal-Wallis Tests	
						Chi-square	Sig
Temephos	10	100% (10/10)	40% (4/10)	20% (2/10)	10% (1/10)	65.081	0.000
Malathion	10	90% (9/10)	30% (3/10)	20% (2/10)	10% (1/10)		
Cypermethrin	10	100% (10/10)	40% (4/10)	10% (1/10)	10% (1/10)		
Deltamethrin	10	100% (10/10)	50% (5/10)	20% (2/10)	10% (1/10)		

EC = epithelial cell, Mv = microvilli, PM = peritrophic membrane, FB = food bolus.

4. Discussion

The findings indicate that the wild-caught *Cx. quinquefasciatus* larvae in Jakarta, Indonesia, were fully susceptible to temephos and deltamethrin. In contrast, the larvae showed only moderate susceptibility to malathion and cypermethrin. To the best of our knowledge, this study is the first to report the susceptibility of *Cx. quinquefasciatus* to temephos and deltamethrin in Jakarta, and our findings confirm that both temephos and deltamethrin are still effective insecticides for controlling *Cx. quinquefasciatus* populations here. Furthermore, these insecticides killed *Cx. quinquefasciatus* larvae; therefore, the number of emerging adult *Cx. quinquefasciatus* mosquitoes in natural habitats would be expected to decrease (Schorkopf et al., 2016).

The complete susceptibility of *Cx. quinquefasciatus* larvae in Jakarta to temephos and deltamethrin could be attributed to the rare use of these insecticides in the control of *Cx. quinquefasciatus* populations. In Jakarta and other large cities in Indonesia, *Cx. quinquefasciatus* mosquitoes are not prioritized in mosquito control programs, whereas the control of dengue vectors, such as *Ae. aegypti* and *Al. albopictus*, is a priority (Ministry of Health, 2017; Jakarta Provincial Health Office, 2019). For instance, in Jakarta, cypermethrin is routinely used to control *Ae. aegypti* and *Ae. albopictus* under the regional regulations of the Jakarta government (Jakarta Provincial Health Office, 2019). Thus, our results suggest that health workers and government officials in Jakarta should include *Cx. quinquefasciatus* in their vector control programs.

Metabolic enzymes, such as AChE, GST, and oxidase, are very important tools to assess the susceptibility of mosquito larvae to insecticides and for detecting the underlying resistance mechanisms that could not be detected by bioassays (Brogdon 2014; DeLisi et al., 2017). For instance, *Cx. quinquefasciatus* larvae resistant to lambda-cyhalothrin and temephos showed higher metabolic enzyme activities such as AChE, esterase, and glutathione reductase (Muthusamy and Shivakumar 2015; DeLisi et al., 2017). The findings show that susceptible *Cx. quinquefasciatus* larvae in Jakarta to insecticides, temephos, and deltamethrin had decreased AChE and oxidase activities (Table 3). This phenomenon of increased esterase and AChE activity can be caused by the G1195 mutations in the gene *ace-1* which encodes the acetylcholinesterase enzyme. The G1195 mutations were found in *Cx. quinquefasciatus* populations (Amorim et al., 2013).

AChE is an important enzyme for reducing acetylcholine in the nervous systems of humans and animals. When AChE activity is inhibited by insecticides, it results in numerous acetylcholine deposits in nerve cells, leading to paralysis and dead cells. Temephos and malathion belong to

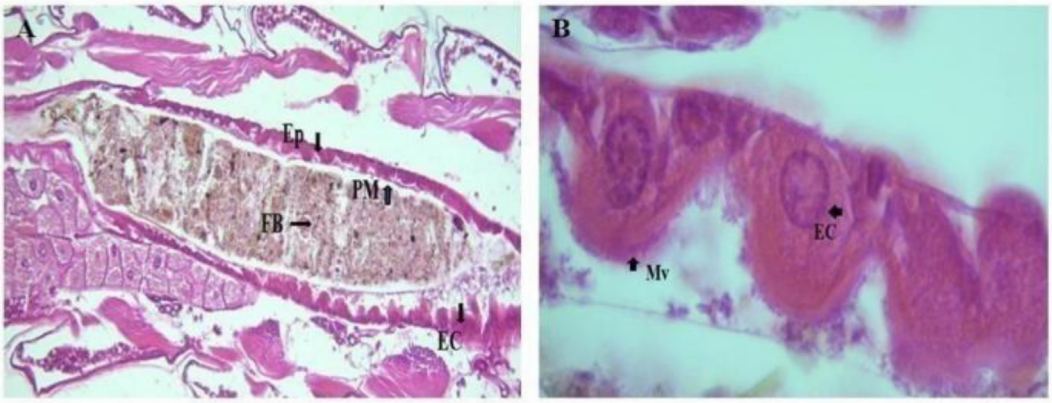

Figure 3. A and B. The midgut of a healthy *Cx. quinquefasciatus* larva stained with H&E. A = 10x magnification, B = 100x magnification. FB = food bolus, PM = peritrophic membrane, Ep = epithelial layer, EC = epithelial cell, Mv = microvilli.

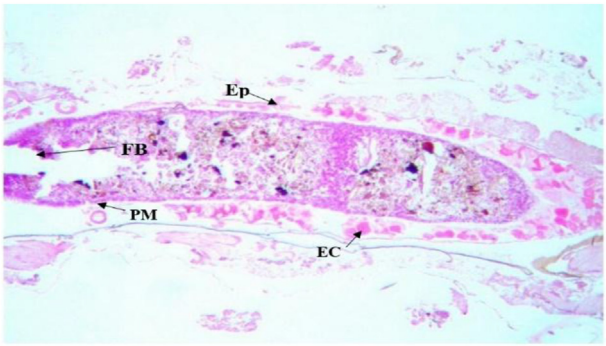

Figure 4. Representation of the histopathological midgut damage of *Cx. quinquefasciatus* larvae after being treated by insecticide, stained with H&E, 10x magnification. FB = food bolus, Ep = epithelial layer, EC = epithelial cell.

the organophosphate group, which has the target of inhibiting AChE activity (Jayaraj et al., 2016). The findings from the current study align with those of Alvarez et al. (2014), who reported *Ae. aegypti* larvae susceptible to temephos did not show overexpression of AChE activity. However, increased AChE activity was found in insecticide-resistant *Cx. quinquefasciatus* larvae. For example, Muthusamy and Shivakumar (2015) reported that *Cx. quinquefasciatus* larvae, resistance to lambda-cyhalothrin and temephos were associated with increased AChE activity.

GST is an important enzyme in the detoxification pathway in almost all organisms, acting as phase II detoxifying enzymes, conjugating glutathione to the products of metabolism or xenobiotics, thereby increasing their solubility and aiding in their excretion from the cell. GSTs benefit cells by protecting them from the harmful effects of oxidative stress, cell signaling pathways, intracellular transport, and several biosynthetic pathways (Ranson and Hemingway 2005; Tripathy and Kar 2015; Lushchak et al., 2018). The findings show that temephos, malathion, cypermethrin, and deltamethrin significantly increased GST activity in *Cx. quinquefasciatus* larvae in Jakarta ($p < 0.05$), protecting them from the toxic effects of insecticides. This study is supported by Xu et al. (2015), who reported that GST SIGSTe1 may play an important role in the gut of *Spodoptera litura* to protect the insect from the toxic effects of these compounds and heavy metals. Another study showed that increased GST activity was found among *Lygus lineolaris* (Hemiptera: Miridae) susceptible to insecticides (Fleming et al., 2016). GST activities in insects caused by insecticides are regulated by GST genes such as LdGSTe2a, LdGSTe2b, LdGSTo5, and LdGSTt1 in *Leptinotarsa decemlineata* (Han et al., 2016) and in the cabbage white butterfly, *Pieris rapae*, such as PrGSTe3, PrGSTs1, PrGSTs2, and PrGSTs4 (Liu et al., 2017).

Oxidase is an important enzyme used to assess the susceptibility status of mosquitoes to all insecticides (Brogdon, 2014). Cytochrome c oxidase (COX) is known as the terminal enzyme of the electron transport system (ETS) and utilizes most of the oxygen delivered to cells via the cardiorespiratory system, hence causing animals to breathe (Little et al., 2018). The findings show that cypermethrin and deltamethrin significantly

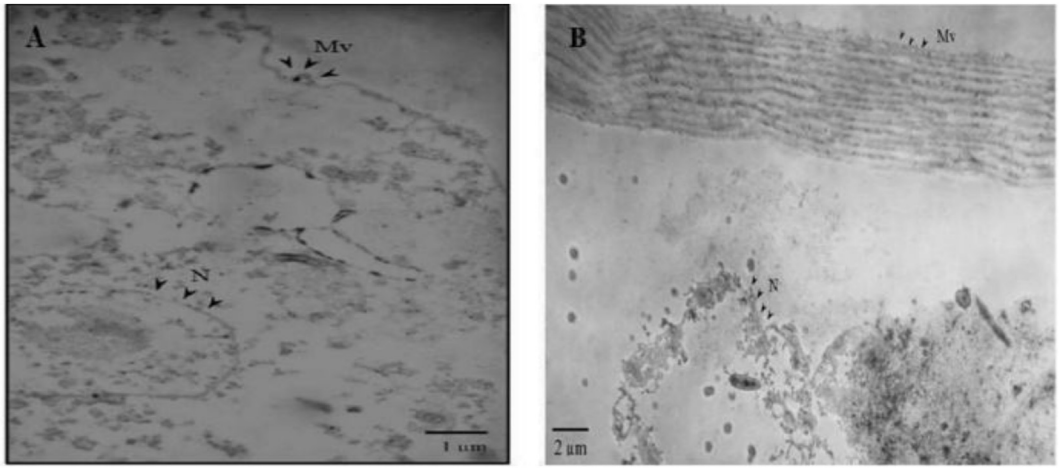

Figure 5. A and B. Ultrastructural ECs in the Ep of the *Cx, quinquefasciatus* midgut larvae induced by malathion (7A) and deltamethrin (7B). Transmission electron microscopy (TEM), N = nucleus, Mv = microvilli.

inhibited the oxidase activities of *Cx. quinquefasciatus* larvae in Jakarta ($p < 0.05$). However, mosquito resistance to insecticides showed elevated oxidase activity. For instance, insecticide-resistant *Ae. aegypti* significantly increased oxidase activity (Little et al., 2018; Fernando et al., 2020; Contreras-Perera et al., 2020).

The present study has shown that all tested insecticides caused histological alterations in all midgut parts of *Cx. quinquefasciatus* larvae, such as FB, PM, Ep, Mv, and EC. The histological damage of the midgut consisted of cracks in the FB, and PM and an irregular shape of the Ep, Mv, and ECs. Statistically, there was a significant difference in EC damage to other parts of the midgut in all tested insecticides (Table 4). In addition, the results of the TEM observations show that there was damage to midgut ECs in the form of damage to cell membranes, mitochondria, nucleolus membranes, and other cell organelles (Figure 5A and 5B). Here, susceptible *Cx. quinquefasciatus* larvae were found to have serious histological damage to their midgut, which was destroyed by low concentrations of insecticides. The findings are supported by de Melo et al. (2008), who reported cytopathological alterations observed in susceptible larvae of *Cx. quinquefasciatus* treated with a lethal concentration of toxin included breakdown of the endoplasmic reticulum, mitochondrial swelling, microvillar disruption, and vacuolization. Additionally, changes in the histological midgut of *Cx. quinquefasciatus* could be attributed to the reactive oxygen species (ROS)-mediated oxidative stress of insecticides. For instance, deltamethrin causes oxidative stress, resulting in an increase in ROS and cell damage (Chargui et al., 2012; Lu et al., 2019). Thus, histopathological midguts could be a valuable tool for assessing the susceptibility of *Cx. quinquefasciatus* larvae to insecticides (de Melo et al., 2008; de Melo et al., 2018; Lu et al., 2019; Lavarías et al., 2017).

Numerous reports have shown that the histological damage of the midgut caused by insecticides is associated with changes in detoxifying enzyme activities (Lavarías et al., 2017; Lu et al., 2019; Silva et al., 2021). For instance, deltamethrin oxidative stress causes damage to the cells and metabolic enzyme changes (de Melo et al., 2018). The histological alterations in the larval midgut structure caused by insecticide toxins could affect the activity of detoxifying enzymes (de Melo et al., 2018). This phenomenon indicates that insecticides have widely harmful effects on susceptible *Cx. quinquefasciatus* larvae in Jakarta, leading to their death.

The present study has several limitations in assessing the susceptibility of wild-caught *Cx. quinquefasciatus* larvae in Jakarta to different insecticides. First, the female mosquitoes of *Cx. quinquefasciatus* from Jakarta have not been investigated because of the susceptibility status of female mosquitoes of *Ae. aegypti* and *Cx. quinquefasciatus*, which have been studied in Indonesia (Bangs et al., 1993; Hamid et al., 2017). Second, the current study has not shown relationship between the histological damage of the midgut larvae and detoxifying enzyme activities of *Cx. quinquefasciatus* larvae. Biomarkers of oxidative stress such as malondialdehyde (MDA) and protein carbonyl (PCO) levels, are not included in the present study (Pirinccioglu et al., 2010). Third, there was no study of synergistic effect between the studied insecticides and natural products on *Cx. quinquefasciatus* larvae. Finally, no study on the effects of insecticides among animals such as rats in Jakarta has been conducted.

5. Conclusion

The present study has shown that wild-caught *Cx quinquefasciatus* larvae from Jakarta, Indonesia, are completely susceptible to temephos and deltamethrin but moderately susceptible to malathion and cypermethrin. The underlying susceptible mechanisms involved inhibited AChE and oxidase activities and the histological damage of the midgut, cracks in the FB, PM, and Ep, and the irregular shape of the EC and Mv. Metabolic enzymes and the histopathological midgut could be valuable tools for assessing the susceptibility status of *Cx. quinquefasciatus* larvae to insecticides.

Declarations

Author contribution statement

Rizal Subahar: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Annisa Putri Aulia and Ris Raihan Felim: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yulhasri Yulhasri: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Lisawati Susanto, Rawina Winita, Guslan Fahmi El Bayani and Tihalun Adugna: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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