INSECTICIDE RESISTANCE AND EFFECT OF PIPERONYL BUTOXIDE AS A SYNERGIST IN THREE STRAINS OF *Aedes aegypti* (Linn.) (Diptera: Culicidae) AGAINST INSECTICIDES PERMETHRIN, CYPERMETHRIN, AND D-ALLETHRIN

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UJI RESISTENSI DAN EFEK PIPERONYL BUTOXIDE SEBAGAI SINERGIS PADA TIGA STRAIN NYAMUK Aedes aegypti (Linn.) (Diptera: Culicidae) *TERHADAP INSEKTISIDA PERMETRIN, CYPERMETRIN, DAN D-ALLETRIN*

Abstrak. *Tiga strain nyamuk* Aedes aegypti *yang dipelihara di laboratorium dan dikoleksi dari lapangan diuji menggunakan tiga jenis insektisida dari golongan piretroid (permetrin, cypermetrin, dan d-alletrin) dengan metode bottle bioassay untuk mengetahui tingkatan dan mekanisme dari resistensi yang terjadi terhadap insektisida piretroid. Hasil penelitian menunjukkan bahwa ketiga strain yang diuji kemungkinan telah resisten terhadap ketiga insektisida tersebut, termasuk strain-strain yang telah dipelihara selama beberapa generasi di laboratorium, yang diindikasikan dengan nilai* LT_{90} *yang tinggi. Hasil ini mengindikasikan bahwa terdapat aktivitas MFO (Mixed-Function Oxidase) pada ketiga strain yang diuji, di mana mekanisme tersebut mungkin berperan dalam menimbulkan resistensi pada ketiga strain, meskipun diduga terdapat mekanisme lain yang juga ikut terlibat.*

Kata kunci: Aedes aegypti, cypermetrin, d-alletrin, permetrin, PBO, resistensi

INTRODUCTION

Aedes aegypti is the primary vector for dengue and dengue haemorrhagic fever in South East Asia, followed by *Aedes albopictus* as the secondary vector [1]. Mosquito control using synthetic chemical insecticides has created new problem; the resistance of mosquitoes to insecticides. Insecticides commonly used today are pyrethroids, and the resistance of mosquitoes to pyrethroids has become a common phenomenon. Some mosquito species in many parts of the world, including *Ae. aegypti* in Thailand, Indonesia, and Puerto Rico are already resistant to pyrethroids [2]. Research conducted by Butar Butar [3] and Arief [4] showed that *Ae. aegypti* collected in Bandung and other cities in West Java, Indonesia tend to be resistant to a variety of insecticides, including pyrethroids. Research on *A. aegypti* in Thailand showed that those mosquitoes which were resistant to pyrethroids were usually also resistant to DDT [5], which proves that resistance to pyrethroids could be the result of cross resistance.

To overcome resistance to pyrethroids, synergists are often used. Synergists are compounds which work by inhibiting the activities of detoxifying enzymes, such as DEF (S,S,S-tributyl phosphorothioate) that inhibits esterase enzymes and PBO (Piperonyl Butoxide) that inhibits MFO (Mixed-Function Oxidases) [6]. However, adding some synergists will only be effective if the resistance is due to biochemical mechanisms which involve the detoxifying enzymes [7]. It will not be effective if the mechanism is the target site insensitivity which involves resistant gene *kdr* [8]. This *kdr* type resistance has been found in the

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Ae. aegypti strain in Indonesia and Puerto Rico [2].

Our research describes the resistance level of three strains of *Ae. aegypti* from different locations, as well testing the mechanisms involved in the resistant strain. PBO was added as a synergist.

MATERIALS AND METHOD

Mosquito Strains. Three strains of *Ae. aegypti* were used in this research. Two strains were reared for several generations in the entomology laboratory at U.S. NAMRU-2 Jakarta (NAMRU-2 strain), one was from the Faculty of Veterinary Medicine Institut Pertanian Bogor (IPB strain), and one strain (ITB strain) was collected from the area surrounding the Institut Teknologi Bandung. All strains were maintained in the laboratory.

Insecticides. Pyrethroid insecticides were used in this research in the form of active ingredients. They were: permethrin 92 %, cypermethrin 92 %, and d-allethrin 93%, provided by PT Triman Sentosatama, Jakarta. Piperonyl butoxide (PBO) was used as the synergist and technical acetone was used as a solvent.

Bioassay Procedure. The method used in this study was a standard bottle bioassay developed by CDC in the U.S.[9] with little adjustment which uses time mortality as a parameter. Since the insecticides used were active ingredients, stock solutions had to be prepared by diluting the insecticide with acetone. To prepare a test bottle, 20 µL of the stock solution (containing 7500 mg permethrin/mL) was transferred by pipette into the bottle (about 315 mL in volume) and aceton was added to dilute the pesticide further. The bottle then was rolled over to spread the pesticide across the inner surface and then left open in order to evaporate the acetone and obtain a test bottle containing permethrin 150 µg/bottle. The same steps were carried out for other insecticides as well; the total amount of insecticide inside the test bottles for cypermethrin was 70 µg/bottle and d-allethrin 180 µg/bottle. These numbers were based on the results of preliminary testing performed previously. For tests using PBO, PBO was added to the test bottles in addition to the insecticide at a ratio of 4:1 (PBO: insecticides). As for control, only acetone was added to the control test bottles without any other ingredients.

Some 10 to 15 adult mosquitoes were placed into the test bottle, which was then closed. Then, the knockdown time (KT) and mortality time (LT/Lethal Time) were observed and determined. Knockdown time was determined by counting the number of mosquitoes knocked down at 5 minutes intervals until all the mosquitoes in the bottle had been affected. The time needed for all mosquitoes inside the test bottle to fall to the bottom was termed as the knockdown time (KT_{100}). Mortality time (LT) was determined by counting the number of mosquitoes that died every hour for a maximum of 5 hours, or until all mosquitoes in the test bottle were dead. Mosquitoes were considered dead if they did not show any movement when stimulated.

Data Analysis. Data gained from the bioassay were analyzed using probit analysis to obtain the LT_{90} and KT_{100} values. For the synergist effect, SRs (Synergist Ratios) were counted by dividing LT_{90}s for bioassays without a synergist to LT_{90}s with a synergist [10]. SR values were said to be significant if the statistic tests using T-test showed significant difference between LT_{90} values with and without the PBO. To compare the susceptibility levels between the three strains, statistic tests using ANOVA were conducted.

RESULTS AND DISCUSSION

Resistance Tests and Resistance Mechanisms

LT_{90} values in all three strains were high, indicating that these strains were probably resistant to d-allethrin (Table 1). The strain with the highest LT_{90} values was ITB; therefore, this strain was probably the most resistant to d-allethrin compared to other strains, while the most susceptible strain was the IPB strain. Both the NAMRU-2 and IPB strains, which were assumed not to have been exposed to any insecticides, were also apparently resistant to d-allethrin. The reason for this result is unknown, but it is possible that mosquitoes from US NAMRU-2 and IPB had been exposed to at least one type of insecticides from the class of pyrethroids or DDT, which lead to cross-resistance. Another possibility is that the strains had undergone some change in gene composition whilst being reared in the laboratory. A study of some strains of *Blatella germanica* showed that rearing in the laboratory for several generations could increase resistant gene frequency, which would lead to an increase in the resistance ratio of the population, especially if the resistant individuals had at least the same or higher reproductive potential [(8)].

In the NAMRU-2 and ITB strains, the addition of PBO tended to reduce the LT_{90}s, which meant that the mortality rate increased (SR > 1). In the ITB strain, the significantly increased mortality rate indicated that MFO activities might be involved in the occurrence of resistance, while in the NAMRU-2 strain, the increase was not significant. It was probable that the strain was resistant but by different mechanisms. The reduction of LT_{90} values, which was not significantly different, could not prove that the strain was resistant, because even in a susceptible strain, MFO activities also occurred, although only at a low level, so the addition of PBO could reduce the mortality time of the strain, even if only slightly [(8)].

Table 1. Mortality Time (LT_{90}) for d-Alletrin

Strain	Without PBO		With PBO		SR
	n	LT_{90} (hour)	n	LT_{90} (hour)	
NAMRU	95	13.55^b	67	11.43^a	1.19
IPB	71	9.55^c	61	14.51^b	0.66*
ITB	10	43.38^a	10	15.23^b	2.85*

- Means within column followed by the same letter are not significantly different (p > 0.05)
- * shows SR values that significantly different (p < 0.05)
- n = number of mosquitoes tested

In the IPB strain, the mortality rate decreased significantly after the addition of PBO. In some cases, the use of synergists at the same time as the application of insecticide could inhibit the penetration of the insecticide through the cuticle, therefore reducing the amount of insecticide entering the insect's body [7], the result of which was that the toxicity effect would also be reduced. However, it could not be concluded whether this strain is resistant or not, because although the LT_{90} for this strain was high, it was relatively low when compared to other strains and the use of PBO did not reduce the LT_{90} value.

The results of the bioassay with permethrin showed that the LT_{90} for all three strains was high (Table 2). The ITB strain were collected directly from the field and were assumed to have been exposed to many insecticides. However, they were apparently the most susceptible compared to other strains, while the NAMRU-2 strain was the most resistant. This result was supported by the results of studies by Butar Butar [3] and Arief [4] which showed that the NAMRU-2 strain were already resistant to permethrin.

The addition of PBO to the ITB and IPB strains only slightly reduced the LT_{90} values, while in the NAMRU-2 strain the reduction was significant (SR = 1,72). This result showed that MFO were involved in causing resistance to permethrin in the NAMRU-2 strain, while in the ITB and IPB strains, the MFO were only slightly involved and there were other mechanisms or the resistance levels were low.

The results for the bioassay with cypermethrin showed that all three strains were resistant to this insecticide (Table 3). The LT_{90} values were high and the addition of PBO increased the mortality rate (reduced LT_{90} values) significantly for all three strains, indicating that resistance depends on MFO activity.

As can be seen in Figure 1, all three strains were apparently resistant to more than one pyrethroid, as indicated by relatively high LT_{90} values and the reduction of LT_{90}s after addition of PBO. It is possible that in all three strains, crossresistance between pyrethroids with similar modes of action has been developed, as commonly happens [11].

Table 2. Mortality Rate (LT_{90}) for Permethrin

Strain	Without PBO		With PBO		SR
	n	LT_{90} (hour)	n	LT_{90} (hour)	
NAMRU	86	23.21^{a}	65	13.50^{a}	1.72*
IPB	69	10.68^{b}	67	9.72^{ab}	1.10
ITB	10	9.30^{b}	12	8.14^{b}	1.14

- Means within column followed by the same letter are not significantly different ($p > 0.05$)
- * shows SR values that significantly different ($p < 0.05$)
- n = number of mosquitoes tested

Table 3. Mortality Rate (LT_{90}) for Cypermethrin

Strain	Without PBO		With PBO		SR
	n	LT_{90} (hour)	n	LT_{90} (hour)	
NAMRU	87	30.27[a]	68	11.22[a]	2.70*
IPB	67	12.54[c]	65	7.62[ab]	1.65*
ITB	10	24.00[d]	10	1.10[b]	21.22*

- Means within column followed by the same letter are not significantly different ($p > 0.05$)
- * shows SR values that significantly different ($p < 0.05$)
- n = number of mosquitoes tested

Table 4. The Average Knockdown Rate (KT/ Knockdown Time)

Insecticides	Strain	Without PBO		With PBO	
		n	KT_{100} (Min.)	n	KT_{100} (Min.)
D-Allethrin	NAMRU*	95	8.99	67	5.45
	IPB	71	5.00	61	5.00
	ITB	10	5.00	10	5.00
Permethrin	NAMRU*	86	9.82	65	8.23
	IPB*	69	8.04	67	6.12
	ITB	10	7.50	12	8.33
Cypermethrin	NAMRU	87	9.02	68	9.26
	*IPB**	67	9.10	65	5.00
	ITB*	10	10.00	10	7.00

- * shows the reduction of KT_{100} after the addition of PBO
- n = number of mosquitoes tested

However, the exact level of resistance of the strains tested could not be measured because there was no standard strain to be compared to. NAMRU-2 and IPB strain, which were supposed to be susceptible, turned out to be resistant as well. Nonetheless, the relatively high values of LT_{90} (before the addition of PBO), mostly after more than 10 hours, showed the possibility of resistance, although the addition of PBO sometimes only slightly lowered the LT_{90} values.

In most cases, the addition of PBO did not completely lower the LT_{90}s, and the LT_{90} values remained high, except for the ITB strain with cypermethrin. This indicated the involvement of other resistance mechanisms in addition to MFO, which meant that multiple resistance has occurred and that the mechanisms had developed independently for each insecticide.

Knockdown Effect

The Knockdown Effect is a characteristic of pyrethroids. It happens immediately after the insects are exposed to pyrethroids [12]. Therefore, if the time needed for insects to be knocked down increases, it indicates that the insects may be resistant to the insecticide [8]. When insects are exposed to pyrethroids, they fall down but will not die immediately. For susceptible insects, they will eventually die. But for resistant insects, after they are knocked down for a while, they will recover and soon be able to fly again after the pyrethroids entering their bodies are detoxified by their metabolism[8]. Since the insects in this method were continuously exposed to insecticides, they had no chance to recover, so it is not known if they were actually able to. In this study, the addition of PBO together with the application of insecticide tended to increase the knockdown rate for all three strains (Table 4), except in some cases where the knockdown rate was less or did not change. However, the results could not be concluded because there might be other factors to consider, such as the behavior of the mosquitoes [6]. According to Lee *et al.*, [6], test animals that made contact more often with the surface of the test bottle would get more insecticides than animals that tended to fly or stand still on the surface; therefore, the knockdown effect would occur faster.

Pest Management and Resistance Problems

Based on the results of this study, we can see that different strains collected from different areas can develop resistance to different insecticides at different levels. These differences can be associated with the history of the use of insecticide compounds in these areas respectively.

Knowledge regarding the level and mechanisms of resistance occurring in a pest population is very important for integrated pest control, in order to decide which control method is effective, efficient, and won't encourage further resistance [13].

Moreover, to obtain accurate information about resistance in a population, a pure susceptible mosquito strain that has never been exposed to any insecticides is needed as the standard of comparison. In this study, the resistance status of the mosquitoes cannot be concluded because the standard mosquito populations from NAMRU-2 and IPB that should be susceptible are apparently already resistant to the insecticide tested.

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