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Effects of Synergist Piperonyl Butoxide (PBO) on Deltamethrin Tolerance in *Anopheles Gambiae sensu lato* Larvae from Mono Department in South-Western Benin, West Africa

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Abstract: ***The current study was aimed to study the effects of synergist piperonyl butoxide (PBO) on deltamethrin tolerance in Anopheles gambiae sensu lato larvae from mono department in south-western Benin, West Africa. Larvae and pupae were collected from March to July and August to November 2018 during the rainy season in the locations of Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa. Larval bioassays were performed on these collected Anopheles gambiae sensu lato larvae using deltamethrin as larvicide and piperonyl butoxide (PBO) as enzyme inhibitor or synergist. The results showed that mono-oxygenases played a role in Anopheles gambiae sensu lato larvae tolerance to deltamethrin.***

Keywords: deltamethrin, piperonyl butoxide, tolerance, malaria vectors, Benin

1. Introduction

Although malaria has been successfully eradicated in high income and many middle-income countries, the disease remains a major health problem in poor nations. This disease wreaks havoc in Africa, where the majority of the global malaria incidence (70% of clinical *Plasmodium falciparum* cases) is concentrated [1]. It was estimated that 18% of child deaths were directly attributable to the disease in this region in 2000 [2]. Malaria is ranked second after human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and accounts for an estimated 10% of the total disease burden on this continent [3]; a study showed that coinfection fuels the spread of both diseases and results in excess HIV infections and clinical malaria episodes [4]. In addition to its direct health impact, the disease imposes a huge economic burden on afflicted nations through high healthcare costs, missed days of work and school, reduced economic productivity and output, and low levels of foreign investment [5].

Pyrethroids are the only option for net treatment due to their relative safety for humans at low dosage, excito-repellent properties, rapid rate of knock-down and killing effects [6]. Resistance to this insecticide class is now widespread in the main malaria vectors *Anopheles gambiae s.l* from Benin [7]. Although public health use accounts for only a very small fraction of overall insecticide quantities applied, many vector species of public health importance have already developed resistance to one or more insecticides [8]. Although there are no short term solutions to vector resistance problems, it is important for programme managers to better understand resistance issues and to promote good practices in insecticide based vector control. It is essential to use public health insecticides in such a way that they are safe, effective, and affordable, while taking into account resistance management issues. Vector control programmes need to meet this condition in order to be effective and sustainable.

Several previous studies ([9]; [10]; [11]; [12]) have established the impact of several breeding sites ecogeographical, topographical, agricultural and other environmental indices on *Anopheles* larval diversity, abundance, and dynamics, as well as breeding sites productivity. Also, induction of detoxification enzymes by various environmental xenobiotics in many species of insects has been well documented [13].

Very few researches were published on deltamethrin tolerance in *Anopheles gambiae s.l.* larvae from mono department in south-western Benin. Therefore, there is a need to carry out new researches for this purpose.

The goal of this study was to explore the detoxification enzymes mechanisms conferring deltamethrin tolerance in *Anopheles gambiae s.l.* larvae in Benin.

2. Materials and Methods

Study area

The study area is located in Republic of Benin (West Africa) and includes the department of Mono. Mono department is located in the south-western Benin and the study was carried out more precisely in Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa districts. The choice of the study site took into account the economic activities of populations, their usual protection practices against mosquito bites, and peasant practices to control farming pests. These factors have an impact on resistance development in the local vector mosquitoes. We took them into account to determine the effects of synergist piperonyl

butoxide (PBO) on deltamethrin tolerance in *Anopheles gambiae s.l.* larvae from this department. Mono has a climate with four seasons, two rainy seasons (March to July and August to November) and two dry seasons (November to March and July to August). The temperature ranges from 25 to 30°C with the annual mean rainfall between 900 and 1100 mm.

Figure 1: Map of districts of mono department surveyed

Mosquito sampling

An. gambiae s.l. larvae were collected from March to July and August to November 2018 during the rainy season in Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa districts selected in south-western Benin. Larvae and pupae were collected in these locations within both padding and town using the dipping method on several breeding sites (brick pits, pools, marshes, streams, ditches, pits dug for plastering traditional huts, puddles of water, water pockets caused by the gutters). Once, larvae and pupae collected, they were then kept in labeled bottles related to the locations surveyed. Otherwise, larvae collected from multiple breeding sites were pooled together then re-distributed evenly in development trays containing tap water. Larvae were provided access to powdered TetraFin® fish food under insectary conditions of 25+/-2°C and 70 to 80% relative humidity at Department of Sciences and Agricultural Techniques located in Dogbo district in south-western Benin. *An. gambiae* Kisumu larvae, a reference susceptible strain was used as a control for the larval bioassays. All larval bioassays were conducted in the Laboratory of Applied Entomology and Vector Control of the Department of Sciences and Agricultural Techniques at 25+/-2°C and 70 to 80% relative humidity.

Preparation of stock solutions or suspensions and test concentrations

Stock solutions and serial dilutions were prepared following the protocol described in WHO guidelines [14]. The volume of stock solution was 20 ml of 1%, obtained by weighing 200 mg of deltamethrin and adding 20 ml solvent to it. It was kept in a screw-cap vial, with aluminium foil over the mouth of the vial. Then, it was shacked vigorously to dissolve or disperse the deltamethrin in the solvent. The stock solution was then serially diluted (ten-fold) in ethanol (2 ml solution to 18 ml solvent). Test concentrations were then obtained by adding 0.1–1.0 ml (100–1000 µl) of the appropriate dilution to 100 ml or 200 ml distilled water.

Bioassays

Initially, the mosquito larvae were exposed to a wide range of test concentrations of deltamethrin and a control to find out the activity range of the larvicide under test. After determining the mortality of larvae in this wide range of concentrations, a narrower range (of 4-5 concentrations, yielding between 10% and 95% mortality in 24h or 48h) was used to determine LC50 and LC90 values [14].

Batches of 25 third or fourth instar larvae were transferred by means of strainers, screen loops or droppers to small disposable test cups or vessels, each containing 100-200 ml of water. Small, unhealthy or damaged larvae were removed and replaced. The depth of the water in the cups or vessels was remained between 5 cm and 10 cm; deeper levels may cause undue mortality.

The appropriate volume of dilution was added to 100 ml or 200 ml water in the cups to obtain the desired target dosage, starting with the lowest concentration. Four replicates were set up for each concentration and an equal number of

controls were set up simultaneously with tap water, to which 1 ml alcohol was added. Each test was run three times on different days. For long exposures, larval food was added to each test cup, particularly if high mortality was noted in control. The test containers were held at 25-28°C and preferably a photoperiod of 12h light followed by 12h dark (12 L: 12 D).

After 24 h exposure, larval mortality was recorded. Moribund larvae were counted and added to dead larvae for calculating percentage mortality. Dead larvae were those that could not be induced to move when they were probed with a needle in the siphon or the cervical region. Moribund larvae were those incapable of rising to the surface or not showing the characteristic diving reaction when the water was disturbed. The results were recorded on the result form, where the LC50 and LC90 values, and slope and heterogeneity analysis were also noted. The form was accommodated three separate tests of six concentrations of deltamethrin, each of four replicates [14].

Biochemical assays using synergist

The presence of metabolic-based resistance mechanisms was investigated by exposing larvae to enzyme inhibitor prior to bioassays with deltamethrin. For that, tested samples that showed high tolerance to deltamethrin in *Anopheles gambiae s.l.* larvae from locations surveyed, were exposed to the effects of synergist: piperonyl butoxide (PBO) (400 µg per test cup), which inhibits oxidase activity. The test allowed us to compare the obtained percentage of dead larvae before the addition of the synergist to that obtained after the addition of the synergist.

Data analysis

Data from all replicates were pooled for analysis. LC50 and LC90 values were calculated from a log dosage-probit mortality regression line using computer software programs. Bioassays were repeated at least three times, using new solutions or suspensions and different batches of larvae each time. Standard deviation or confidence intervals of the means of LC50 values vere calculated and recorded on a form. A test series was valid if the relative standard deviation (or coefficient of variation) was less than 25% or if confidence limits of LC50 overlap (significant level at $P < 0.05$). Abbott's formula was not used in this study for the correction of mortality rates in test cups because the mortality rates in all controls was always less than 5% [15]. To appreciate the effects of synergist PBO on *Anopheles gambiae s.l.* larvae tolerance to deltamethrin, we used a Kruskal-Wallis test. LC50 and LC90 values were estimated using SPSS version 16.0 (SPSS Inc., Chicago, IL). The significance level was set at 5%.

3. Results

The analysis of table 1 showed that all tested larvae or strains from mono department were resistant to Deltamethrin (see Resistance ratios RR50 and RR95). After the addition of synergist PBO in test cups, the LC50 values obtained with larvae from Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa which were 12.7 µg per liter, 13.9 µg per liter, 11.1 µg per liter, 13.9 µg per liter, 11.8 µg per liter and 14.2 µg per liter respectively were slightly lower than those obtained with Deltamethrin alone which were 15.7 µg per liter, 15.9 µg per liter, 16.1 µg per liter, 15.9 µg per liter, 15.8 µg per liter and 16.2 µg per liter respectively. The Synergism Ratios (SR50) (before addition of PBO/after addition of PBO) were 1.23, 1.14, 1.45, 1.14, 1.33 and 1.14 respectively (Table 2).

In the same way, the LC95 values obtained with larvae from Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa which were 44.5 µg per liter, 48.5 µg per liter, 38.5 µg per liter, 48.5 µg per liter, 41.3 µg per liter and 49.6 µg per liter respectively were lower than those obtained with Deltamethrin alone which were 54.4 µg per liter, 54.9 µg per liter, 55.1 µg per liter, 54.9 µg per liter, 54.6 µg per liter and 55.6 µg per liter respectively. The Synergism Ratios (SR95) (before addition of PBO/after addition of PBO) were 1.22, 1.13, 1.43, 1.13, 1.32 and 1.12 respectively (Table 3).

Table 1: Determination of Lethal Concentrations LC50 and LC95

Strain	LC50 (mg/l)	LC95(mg/l)	RR50	RR95
Kisumu	0.0116	0.0465	_	_
Athiémè	0.0157	0.0544	1.35	1.16
Grand Popo	0.0159	0.0549	1.37	1.18
Comè	0.0161	0.0551	1.38	1.18
Lokossa	0.0159	0.0549	1.37	1.18
Houéyogbé	0.0158	0.0546	1.36	1.18
Bopa	0.0162	0.0556	1.39	1.18

Table 2: Determination of Lethal Concentrations LC50 of *An. gambiae s.l.* larvae to Deltamethrin with and without PBO and Synergism ration SR50

Strain	Without PBO		With PBO		Synergism Ratio (SR50)
	Number tested	LC50 (mg/l)	Number tested	LC50 (mg/l)	
Athiémè	25	0.0157	25	0.0127	1.23
Grand Popo	25	0.0159	25	0.0139	1.14
Comè	25	0.0161	25	0.0111	1.45
Lokossa	25	0.0159	25	0.0139	1.14
Houéyogbé	25	0.0158	25	0.0118	1.33
Bopa	25	0.0162	25	0.0142	1.14

Table 3: Determination of Lethal Concentrations LC95 of *An. gambiae s.l.* larvae to Deltamethrin with and without PBO and Synergism ration SR95

Strain	Without PBO		With PBO		Synergism Ratio (SR95)
	Number tested	LC95 (mg/l)	Number tested	LC95 (mg/l)	
Athiémè	25	0.0544	25	0.0445	1.22
Grand Popo	25	0.0549	25	0.0485	1.13
Comè	25	0.0551	25	0.0385	1.43
Lokossa	25	0.0549	25	0.0485	1.13
Houéyogbé	25	0.0546	25	0.0413	1.32
Bopa	25	0.0556	25	0.0496	1.12

4. Discussion

All tested larvae or strains from mono department were resistant to Deltamethrin. Deltamethrin resistance in *An. gambiae s.l* Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa may be explained by the use of household insecticide and availability of xenobiotics for larval breading sites in these areas. They were one of the

possible factors selected for pyrethroid resistance in *An. gambiae s.l* from these locations.

A previous study carried out by Aïzoun *et al.* [16] on deltamethrin resistance in adult *An. gambiae* from three main ecological settings in Benin showed that *An. gambiae* Dassa-Zoumè populations collected in a rice growing area located in the middle part of the country were resistant to this product. *An. gambiae* populations were also collected in Allada located in the south part of the country in a cereal growing area (maize, ground-nut and so on) and were also resistant to this product. *An. gambiae* populations from Parakou resistance level to deltamethrin required further investigations before its resistance status clarification. This previous study also showed that L1014F *kdr* mutation was found in *An. gambiae s.s.* Parakou, Dassa-Zoumè and Allada at various allelic frequencies. But, no metabolic-based resistance was involved in the very high *kdr* allelic frequency observed in *An. gambiae s.s.* Dassa-Zoumè populations in this study.

The underlying mechanism of the resistance pattern observed in all tested larvae was explored using a synergist assay. The synergist assay with PBO, an inhibitor of Cytochrome P450 monooxygenases, indicated that this enzyme family may play a role in the deltamethrin resistance observed in larvae from Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa. So, mono-oxygenases may play a role in *An. gambiae s.l.* larvae from mono department resistance to Deltamethrin otherwise oxidases may be implicated in the resistance of larvae to Deltamethrin in these strains. Aïzoun *et al.* [17] have already reported on the implication of mono-oxygenases in resistance of adult *Anopheles gambiae* to pyrethroids in Misserete district in southern Benin. Metabolic resistance to pyrethroids is usually associated with increased cytochrome P450 activity [18]. The involvement of P450 enzymes in pyrethroid resistance in the Kenyan population of *Anopheles gambiae* was also demonstrated using the P450 inhibitor, piperonyl butoxide (PBO) [19].

5. Conclusion

Mono-oxygenases played a role in *Anopheles gambiae s.l.* larvae tolerance to deltamethrin. However, further studies using a microarray approach followed by quantitative real-time RT-PCR validation are need to identify detoxification genes putatively involved in metabolic resistance. This will improve the implementation and management of future control programs against this important malaria vector particularly in Benin and in Africa in general.

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