International Journal of Innovation Sciences and Research
Vol.10, No, 05, pp.1651-1654, May, 2021

Available online at http://www.ijisr.com

RESEARCH ARTICLE

EFFECTS OF BOTH SYNERGISTS S.S.S-TRIBUTYLPHOSPHOROTRITHIOATE (DEF) AND PIPERONYL BUTOXIDE (PBO) ON PERMETHRIN TOLERANCE IN *ANOPHELES GAMBIAE S.L.* LARVAE FROM ATHIÉMÉ DISTRICT IN MONO DEPARTMENT IN SOUTH-WESTERN BENIN, WEST AFRICA

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Received 17th March, 2021; Accepted 10th April, 2021; Published 30th May, 2021

ABSTRACT

The current study was aimed to study the effects of both synergists S.S.S-tributylphosphorotrithioate (DEF) and piperonyl butoxide (PBO) on permethrin tolerance in *Anopheles gambiae s.l.* larvae from Athiémé district in 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 Athiémé district. Larval bioassays were performed on these collected *Anopheles gambiae s.l.* larvae using permethrin as larvicide and S.S.S-tributylphosphorotrithioate (DEF) and piperonyl butoxide (PBO) as enzymes inhibitors or synergists. The results showed that both esterase and mono-oxygenase enzymes played a role in *Anopheles gambiae s.l.* larvae tolerance to permethrin in Athiémé district.

Key words: Permethrin, S.S.S-Tributylphosphorotrithioate, Piperonyl Butoxide, Tolerance, Malaria Vectors, Benin.

INTRODUCTION

Malaria is a severe public health problem, causing an estimated 225 million disease cases and 781,000 deaths per year (WHO, 2010). Most victims are children under five years old living in sub-Saharan Africa (WHO, 2010). Malaria is transmitted by *Anopheles* mosquitoes, and because there is currently no vaccine available, vector control is one of the most important means of malaria prevention. In most cases, this vector control is carried out through the use of insecticide treated materials or indoor residual spraying. The enormous toll that malaria takes on the world's poorest and most vulnerable populations require all proven and cost-effective interventions be deployed to battle this scourge. Malaria control efforts are driven by the persisting health and economic burden of the disease. However, restricted impact on disease transmission of the current patient management and preventive strategies (e.g., insecticide treated nets [ITNs]) limits progress toward internationally set malaria goals and targets and poverty reduction (United Nations Millennium Project, 2005). In West Africa, the resistance of *Anopheles gambiae s.l.* to the four major classes of insecticides available for public health has been reported (Chandre *et al*., 1999; Djogbenou *et al*., 2008; Diabate *et al*., 2004).

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Several previous studies (Mwangagngi *et al*., 2010; Animut *et al*., 2012; Imbahale *et al*., 2011; Mala *et al*., 2011) 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 (David *et al*., 2013). Very few researches were published on permethrin tolerance in *Anopheles gambiae s.l.* larvae from Athiémé district in 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 permethrin tolerance in *Anopheles gambiae s.l.* larvae in Benin.

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 Athiémé district. 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 both synergists S.S.S-tributylphosphorotrithioate (DEF) and piperonyl butoxide (PBO) on permethrin tolerance in *Anopheles gambiae s.l.* larvae from this district of the

department. Mono has a climate with four seasons, two rainy seasons (March-July and August-November) and two dry seasons (November-March and July-August). The temperature ranges from 25 to 30°C with the annual mean rainfall between 900 and 1100 mm.

Figure 1. Map of Republic of Benin showing Athieme district surveyed

MOSQUITO SAMPLING

An. gambiae s.l. larvae were collected from March-July and August-November 2018 during the rainy season in Athiémé district selected in south-western Benin. Larvae and pupae were collected in this district 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 localities 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 (WHO, 2005). The volume of stock solution was 20 ml of 1%, obtained by weighing 200 mg of permethrin 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 permethrin 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 permethrin 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 (WHO, 2005). 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 permethrin, each of four replicates (WHO, 2005).

BIOCHEMICAL ASSAYS USING SYNERGIST: The presence of metabolic-based resistance mechanisms was investigated by exposing larvae to enzyme inhibitors prior to bioassays with permethrin. For that, tested samples that showed high tolerance to permethrin in *Anopheles gambiae s.l.* larvae from Athiémé district surveyed, were exposed to the effects of two synergists: S.S.S-tributylphosphorotrithioate (DEF) (125 µg per test cup), which inhibits esterase activity; and piperonyl butoxide (PBO) (400 µg per test cup), which inhibits oxidase activity. These two synergists were used separately and in combination.

Table 1. Determination of lethal concentrations LC50 and LC95

Strain	LC50 (mg/l)	LC95(mg/l)	RR50	RR95
Kisumu	0.003	0.005	_	_
Athiémé	0.063	0.22	21	44

Table 2. Determination of lethal concentrations LC50 and LC95 and Synergism ratios SR50 and SR95 of *An. gambiae s.l.* larvae from Athiémé to Permethrin + PBO and Permethrin + DEF and Permethrin + PBO + DEF

Strain	Enzyme inhibitor	LC50 (mg/l)	LC95(mg/l)	SR50	SR95
Athiémé	Without Synergist	0.063	0.22	_	_
	With PBO	0.015	0.049	4.2	4.48
	With DEF	0.028	0.088	2. 25	2.5
	With PBO +DEF	0.013	0.043	4.84	5.11

The test allowed us to compare the obtained percentages of dead larvae before the addition of the synergist (s) to those obtained after the addition of the synergist (s).

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% (Abbott, 1987). To appreciate the effects of synergists PBO and DEF on *Anopheles gambiae s.l.* larvae from Athiémé tolerance to permethrin, 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%.

RESULTS AND DISCUSSION

The analysis of table 1 showed that *An. gambiae s.l.* larvae from Athiémé district in mono department were highly resistant to permethrin (see Resistance ratios RR50 and RR95). The permethrin resistance observed in *An. gambiae* Athiémé populations may be due to the presence of several environmental pollutants and pesticide residues from the neighbouring farms. This locality is crossed by the Athiémé Lake's streams. These streams sweep and converge these environmental pollutants and pesticide residues in Athiémé locality. These xenobiotics available in larval breeding sites in Athiémé may be one of the possible factors selecting for pyrethroid resistance in *An. gambiae* populations in this locality. A similar pattern was already observed with *An. gambiae* Ladji permethrin resistant populations (Aïzoun *et al.*, 2014a). The analysis of table 2 showed that the underlying mechanism of the resistance pattern observed in this population was explored using a synergist assay. The synergist assay with PBO, an inhibitor of Cytochrome P450 monooxygenases, indicated that this enzyme family plays a role in this high permethrin resistance observed in *An. gambiae s.l.* larvae from Athiémé. In the same way, the synergist assay with DEF, an inhibitor of esterase, indicated that this enzyme family also plays a role in this high permethrin resistance observed in Athiémé. So, metabolic resistance conferred by detoxifying enzymes such as cytochrome P450 mono-oxygenases and esterases were found to play a role in *An. gambiae s.l.* larvae Athiémé permethrin resistant populations. In addition, after the addition of both synergists PBO and DEF on *An. gambiae s.l.* larvae from Athiémé, we have not still obtained fully susceptibility. These results showed that there were other resistance mechanisms which were not synergizable by PBO and DEF. In southern Benin, Corbel *et al.* (2007) have already reported on multiple insecticide resistance mechanisms in *An. gambiae* Ladji populations. Among these mechanisms, there were mixed function oxidase (MFO) and -esterase with the presence of *Kdr* at high frequency (80%). However, even if the Leu-Phe *kdr* mutation is the most important resistance mechanism in these *An. gambiae* Ladji populations, metabolic resistance conferred by detoxifying enzymes is also an indication of phenotypic resistance to permethrin. Previous studies showed that metabolic resistance mechanisms have been identified in adult vector populations for all major classes of insecticides currently used for vector control in Benin, including organophosphates, carbamates, pyrethroids and DDT (Aïzoun *et al.*, 2013, Aïzoun *et al.*, 2014a, 2014b, 2014c). There is a need to overcome these resistance mechanisms by promoting for example Long Lasting Insecticidal Nets (LLINs) impregnated with synergists. But, these nets would not be effective in areas where Leu-Phe *kdr* mutation is the most important resistance mechanism.

CONCLUSION

Both esterase and mono-oxygenase enzymes played a role in *Anopheles gambiae s.l.* larvae tolerance to permethrin in Athiémé district. These enzymes were implicated as mechanisms of pyrethroid resistance in *An. gambiae s.l.* from mono department in south-western Benin. 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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