World Journal of Biology Pharmacy and Health Sciences
eISSN: 2582-5542
Cross Ref DOI: 10.30574/wjbphs
Journal homepage: https://wjbphs.com/

(RESEARCH ARTICLE)

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Anopheles gambiae s. l. larval control: An important method for malaria control

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World Journal of Biology Pharmacy and Health Sciences, 2021, 06(03), 027–034

Publication history: Received on 01 May 2021; revised on 12 June 2021; accepted on 15 June 2021

Article DOI: https://doi.org/10.30574/wjbphs.2021.6.3.0043

Abstract

The current study aimed to investigate on the control tools against larvae and adults of *Anopheles gambiae s. l.* and then explore the detoxification enzymes mechanisms conferring permethrin tolerance in *Anopheles gambiae s. l.* larvae in Benin. Larvae and pupae were collected from March to July and August to November 2018 during the rainy season in Bopa district in Mono department in south-western Benin, West Africa. Larval bioassays were performed on these collected *Anopheles gambiae s. l.* larvae using permethrin as larvicide and synergist piperonyl butoxide (PBO) as enzyme inhibitor or synergist. WHO susceptibility tests were also conducted on adult unfed female mosquitoes aged 3-5 days old with impregnated papers of permethrin (0.75%). The results showed that malaria elimination in Benin needs integrated control. Both larvae or pupae and adults malaria vectors must be controlled.

Keywords: Permethrin; Piperonyl butoxide; Larval bioassay; WHO susceptibility test; Malaria vectors; Benin

1. Introduction

Mosquitoes transmit numerous human and animal pathogens and chemical insecticides are widely employed in their control. However, the success of control programs is now threatened as the repeated exposure of mosquito populations to chemical insecticides has led to the selection of mutations conferring an increased resistance to these insecticides [1]. So, the efficacy of these insecticides is influenced by the mosquitoes' history of past exposure. Long-term exposure to a toxicant will eventually select for mutations conferring a level of resistance to that toxicant and indeed, insecticide-resistant populations of mosquitoes are now threatening the success of control programmes.

Since mosquito breeding sites are found in various environments ranging from farmlands to sites of industrial activities, deliberate human intervention at controlling mosquito populations may not be the only contribution to the factors affecting larval growth and development. For instance, some of the most active breeding sites for mosquitoes are located around farmlands where various agro-allied chemicals such as fertilizers, pesticides and herbicides, are applied to enrich soil and control agricultural pests and diseases [2,3]. Active mosquito breeding sites have also been reported in areas polluted by industrial effluents, rotting vegetation, human faeces, cow urine, as well as oil and grease mostly in urban centers [4,5]. However, most *Anopheles* mosquitoes traditionally breeds in clear, clean and apparently less contaminated surroundings usually around human habitation. The factors governing the choice of water bodies by female mosquitoes to lay their eggs for breeding is still poorly understood and the knowledge of the mechanisms behind habitat selection by most species of mosquitoes is still at its infancy [6]. What is clear though is that mosquito breeding sites are located in a variety of environments including contaminated and uncontaminated environments. Thus, larval

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productivity, development, and survival may be attributed to degree of contamination, possible priming and selectivity for chemical tolerance and cross extended insecticide resistance [2-7].

Several previous studies [8-11] 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 [12].

Very few researches were published on permethrin tolerance in *Anopheles gambiae s. l.* larvae from Bopa 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 investigate on the control tools against larvae and adults of *Anopheles gambiae s. l.* and then explore the detoxification enzymes mechanisms conferring permethrin tolerance in *Anopheles gambiae s. l.* larvae in Benin.

2. Material and methods

2.1. **Study area**

The study area is located in Republic of Benin (West Africa) and includes the department of Mono. Mono department is

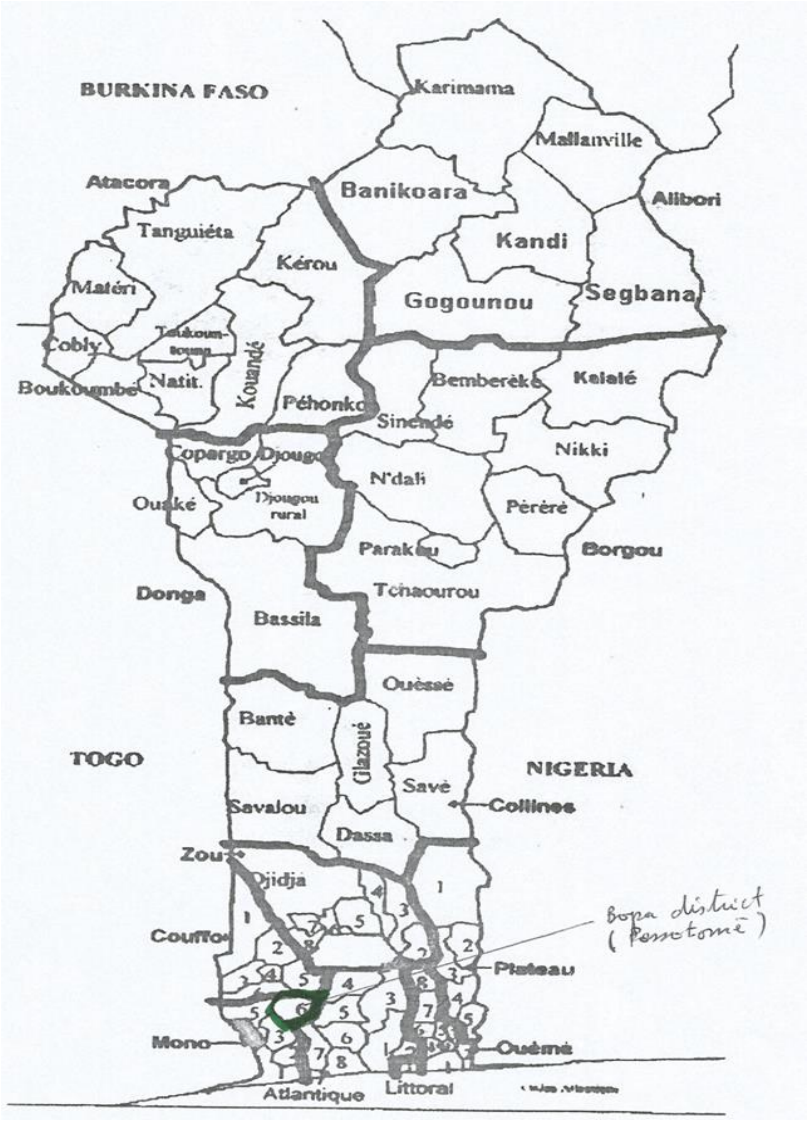

Figure 1 Map of Republic of Benin showing district surveyed (Source: Council Africa, 2006)

located in the south-western Benin and the study was carried out more precisely in Bopa 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 synergist 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 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.

2.2. **Mosquito sampling**

An. gambiae s. l. larvae were collected from March to July and August to November 2018 during the rainy season in Bopa 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. 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°C +/- 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°C +/- 2°C and 70% to 80% relative humidity. Regarding the susceptibility tests performing, collected samples (larvae and pupae) from Bopa district were reared up to adult emergence at the insectary. *An. gambiae* Kisumu, a reference susceptible strain was used as a control for the bioassay tests. We used Kisumu more precisely to confirm the quality of treated or impregnated papers. Susceptibility tests were done following WHO protocol on unfed females mosquitoes aged 3-5 days old reared from larval and pupal collections. All WHO susceptibility tests were conducted in the Laboratory of Applied Entomology and Vector Control of the Department of Sciences and Agricultural Techniques at 25°C +/- 2°C and 70% to 80% relative humidity.

2.3. **Preparation of stock solutions or suspensions and test concentrations**

Stock solutions and serial dilutions were prepared following the protocol described in WHO guidelines [13]. 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.

2.4. **Larval 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 [13].

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 incapables 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 [13].

2.5. **Biochemical assays using synergist**

The presence of metabolic-based resistance mechanisms was investigated by exposing larvae to enzyme inhibitor prior to bioassays with permethrin. For that, tested samples that showed high tolerance to permethrin in *Anopheles gambiae s. l*. larvae from Bopa district 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.

2.6. **Testing insecticide susceptibility**

Females *An. gambiae s. l.* were exposed to WHO diagnostic dosage of permethrin 0.75% according to the WHO protocol [14]. Thus, an aspirator was used to introduce 20 to 25 unfed female mosquitoes into six WHO holding tubes (four tests and two controls) that contained untreated papers. They were then gently blown into the exposure tubes containing the insecticide impregnated papers. After one-hour exposure, mosquitoes were transferred back into holding tubes and provided with cotton wool moistened with a 10% honey solution. The number of mosquitoes "knocked down" at every five minutes and mortalities at 24 hours were recorded following the WHO protocol [14]. The choice of permethrin was justified by OlysetNet distribution made free recently throughout the entire country by Beninese National Malaria Control Programme to increase coverage of long-lasting insecticidal nets (LLINs).

2.7. **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 from Bopa 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%.

The resistance status of the used mosquito sample was determined according to the WHO criteria [14] as follows:

- Mortality rates between 98%-100% indicate full susceptibility
- Mortality rates between 90%-97% indicate possible resistance
- Mortality rates < 90%, the population is considered resistant to the tested insecticides

Abbott's formula was not used in this study for the correction of mortality rates in the test-tubes because the mortality rates in all controls was always less than 5% [15]. Analysis using Fisher's exact test and test of proportion was performed on the data sets gathered from the location surveyed to compare control means against larvae and adults of *Anopheles gambiae s. l.* and assess the insecticide resistance status of tested *An. gambiae s. l.* populations from Bopa.

3. Results

3.1. **Control tools against malaria vectors in Benin**

The table 1 shows the different control tools used against malaria vectors in Benin. These tools concerned those used against *An. gambiae* adult vectors and those used against *An. gambiae* larvae or pupae.

Table 1 Control tools against malaria vectors in Benin

Control tools against *An. gambiae* adult vectors	**Control tools against *An. Gambiae* larvae or pupae**
Long Lasting Insecticidal Nets (LLINs)	Larvicide spraying in breading sites
Indoor Residual Spraying (IRS)	Use of larvivorous fishes against larvae of *Anopheles gambiae*
Household insecticide spraying	Hygiene around houses
Use of insect repellents	Physical destruction of breading sites:
Use of Human Landing Catches (HLC)	Destruction of brick pits
Use of Window traps	Destruction of pools
And so on	Destruction of marshes
	Destruction of streams
	Destruction of ditches
	Destruction of pits dug for plastering traditional huts
	Destruction of puddles of water
	Destruction of water pockets caused by the gutters
	And so on

3.2. **Susceptibility status to permethrin in adult *An. gambiae s. l.***

The analysis of table 2 showed that An. *gambiae s. l.* adult populations from Bopa were resistant to permethrin.

Table 2 Susceptibility status to permethrin in adult *An. gambiae s. l.*

Populations	**Number tested**	**% Mortality**	**Resistance status**
Kisumu (Control)	100	100	S
Bopa	100	85	R

S: Susceptible; R: Resistant

3.3. **Susceptibility status to permethrin in *An. gambiae s. l.* larvae**

The analysis of table 3 showed that *An. gambiae s. l.* larvae from Bopa were also resistant to permethrin (see Resistance ratios RR50 and RR95).

Table 3 Susceptibility status to permethrin in *An. gambiae s. l.* larvae

Strains	**LC50 (mg/l)**	**LC95 (mg/l)**	**RR50**	**RR95**
Kisumu	0.002	0.004	_	_
Bopa	0.052	0.18	26	45

3.4. **Determination of lethal concentrations LC50 and LC95 and Synergism ratios SR50 and SR95 of *An. gambiae s. l.* larvae from Bopa to Permethrin + PBO**

The analysis of table 4 showed that Synergism Ratio (SR50) (before addition of PBO/after addition of PBO) was high (5.77). In the same way, the Synergism Ratio (SR95) (before addition of PBO/after addition of PBO) was also high (6.66).

Table 4 Lethal concentrations LC50 and LC95 and Synergism ratios SR50 and SR95 of *An. gambiae s. l*. larvae from Bopa to Permethrin + PBO

Strain	Enzyme inhibitor	LC50 (mg/l)	LC95 (mg/l)	SR50	SR95
Bopa	Without Synergist	0.052	0.18	_	_
	With PBO	0.009	0.027	5. 77	6.66

4. Discussion

The table 1 shows the control tools used against malaria vectors in Benin. The analysis of this table shows that Long-Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS) are the most use cornerstones of malaria prevention more precisely in malaria adult vector control. In fact, Long-Lasting Insecticidal Nets (LLINs) are frequently distributed by National Malaria Control Program (NMCP) in the country. In addition, from 2008 to 2010, the National Malaria Control Program (NMCP) had undertaken under financial support of the PMI (President's Malaria Initiative) of U.S. Government a full coverage of IRS in no-flood zones in the Ouémé region in southern Benin, coupled with the distribution of LLINs in flood zones. IRS was not implemented in the flood zone because of the presence of water bodies, which could be at risk of contamination by insecticides. For that reason, Long-Lasting Insecticidal Nets (LLINs) (Permanet 2.0) were distributed to households, with particular attention to children under-five and pregnant women in October 2008 and May 2009. The IRS campaign coupled with the distribution of LLINs in Ouémé region was successful [16]. National Malaria Control Program (NMCP) had also implemented Indoor Residual Spraying (IRS) campaign under financial support of the PMI (President's Malaria Initiative) of U.S. Government using bendiocarb in the north of the country more precisely in Atacora department in 2011 and with pyrimiphos-methyl in 2013. OlysetNets were also distributed free by the NMCP in July 2011 across the entire country. In addition to these cornerstones of malaria prevention, Household insecticide spraying, Human Landing Catches (HLC) and Window traps were also used. People also used insect repellents in Ouémé and Atacora department as across the entire country. The distribution of Long-Lasting Insecticidal Nets (LLINs) by NMCP was continuous in the country. The analysis of table 1 also showed that one of the important tools for malaria control is *An. gambiae s. l.* larvae or pupae control. This control can be done by larvicide spraying in breading sites, by physical destruction of breading sites (destruction of brick pits, pools, marshes, streams, ditches, pits dug for plastering traditional huts, puddles of water, water pockets caused by the gutters), by Hygiene around houses and also by using larvivorous fishes against larvae of *Anopheles gambiae* (biological control). But very few researches were conducted in the country on the use of larvivorous fishes against larvae of *Anopheles gambiae*. It is worth mentioning that when these *An. gambiae s. l.* larvae or pupae would be destroyed; no adult emergence will be observed and therefore there will have no mosquito bites or no malaria transmission.

The analysis of table 2 showed that *An. gambiae s. l.* adult populations from Bopa were resistant to permethrin. In addition, the table 3 showed that *An. gambiae s. l.* larvae from Bopa were also resistant to permethrin (see Resistance ratios RR50 and RR95). So, the resistance is a hereditary phenomenon. The permethrin resistance observed in *An. gambiae* Bopa 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 Bopa district. These xenobiotics available in larval breeding sites in Bopa district 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 in Cotonou district in southern Benin [17].

The analysis of table 4 showed that the hereditary phenomenon of the resistance observed in Bopa district can be due to metabolic mechanism like overproduction of detoxifying enzymes activity. In fact, 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 Bopa district. So, the resistance is a hereditary phenomenon which can be due to metabolic mechanism like overproduction of detoxifying enzymes activity. But, it is also dynamic phenomenon which can be influenced by many factors such as mosquito sex, mosquito age and its physiological status [17-19].

5. Conclusion

Malaria elimination in Benin needs integrated control. Both larvae or pupae and adult malaria vectors must be controlled. *Anopheles gambiae* larval control is an important method for malaria control. Unfortunaly, this important disease control method meets threats as some breeding sites are not easy to reach. This integrated control must involve the control of the parasite *Plasmodium falciparum* too.

Compliance with ethical standards

Acknowledgments

I would like to thank people from localities surveyed who had helped us in mosquito collection. I would also like to thank KOUASSI Prisca for technical assistance in laboratory during the current study.

Disclosure of conflict of interest

I declare there is no conflict of interest.

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