

Effects of synergist ethacrynic acid (ETAA or EA) on dichlorodiphenyltrichloroethane (DDT) tolerance in *Culex quinquefasciatus* Say larvae from Klouekanmè district in Couffo department in south-western Benin, West Africa

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ABSTRACT

Background: Mosquito control programs are now threatened by the selection of mosquito populations resistant to the chemical insecticides. Thus, alternative vector control methods are necessary.

Material and Methods: Larvae and pupae were collected from April to July 2024 during the great rainy season in Klouekanmè district surveyed. Larval bioassays were performed on these collected *Culex quinquefasciatus* larvae using dichlorodiphenyltrichloroethane (DDT) as larvicide and ethacrynic acid (ETAA or EA) as enzyme inhibitor or synergist.

Results: The results showed that glutathione S-transferases (GSTs) may play a role in *Culex quinquefasciatus* larvae resistance to DDT in Klouekanmey.

Conclusion: Glutathione S-transferases played a role in *Culex quinquefasciatus* larvae tolerance to DDT in Couffo department in south-western Benin.

KEYWORDS: DDT, ethacrynic acid, tolerance, lymphatic filariasis vector, Republic of Benin

1. INTRODUCTION

Arboviruses are viruses transmitted to vertebrate hosts by arthropods (e.g., ticks and mosquitoes), causing diseases that range from asymptomatic to life-threatening. Some of the most recognized arboviruses impacting public health systems are the dengue virus (DENV), chikungunya virus (CHIKV), and zika virus (ZIKV). These viruses are transmitted by mosquito vectors, particularly of the *Aedes* genus, in the American continent; the main vector is *Ae. aegypti* (Linnaeus, 1762). Over the years, mosquitoes have been creating many health issues in different parts of the world, such as Africa, Canada, Thailand, America, Srilanka and Pakistan (1). Some of these issues are spread of diseases to humans such as dengue, encephalitis, filariasis, malaria etc. which could result to deaths (2). Mosquito affects the socioeconomic status of many nations and in so many cases; it acts against human by causing allergy including local skin reaction (3).

Mosquitoes are relatively small flying insects measuring about 3mm-6mm in length, although some species can be small as 2mm-3mm, while others may be as long as 10mm. Mosquitoes have long slender wings and are usually among flies in having small scales over most of the wing veins. Mosquitoes are group into 39 genera and 135 sub-genera (4). Mosquitoes are also widely spread due to their high adaptability, higher reproductive rate. Mosquito lay their eggs in stagnant waters, pods, ditches, gutters (5).

Different utilizations of mosquito insecticides face several serious problems such as negative impact on non-target organisms especially human rather than the environment. The number of resistant-insecticide mosquito populations is developed and increased that extremely threat on crop protection, vector control and human health worldwide (6,7) and more specifically Saud Arabia (8,9).

Very few researches were published on organochlorine tolerance in *Culex quinquefasciatus* larvae from Couffo 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 organochlorine tolerance in *Culex quinquefasciatus* larvae in south-western Benin.

2. MATERIAL AND METHODS

Study area

The study area is located in Republic of Benin (West Africa) and includes the department of Couffo. Couffo department is located in the south-western Benin and the study was carried out more precisely in Klouekanmè 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 Ethacrynic acid (ETAA or EA) on dichlorodiphenyltrichloroethane (DDT) tolerance in *Culex quinquefasciatus* larvae from this department. Couffo 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.

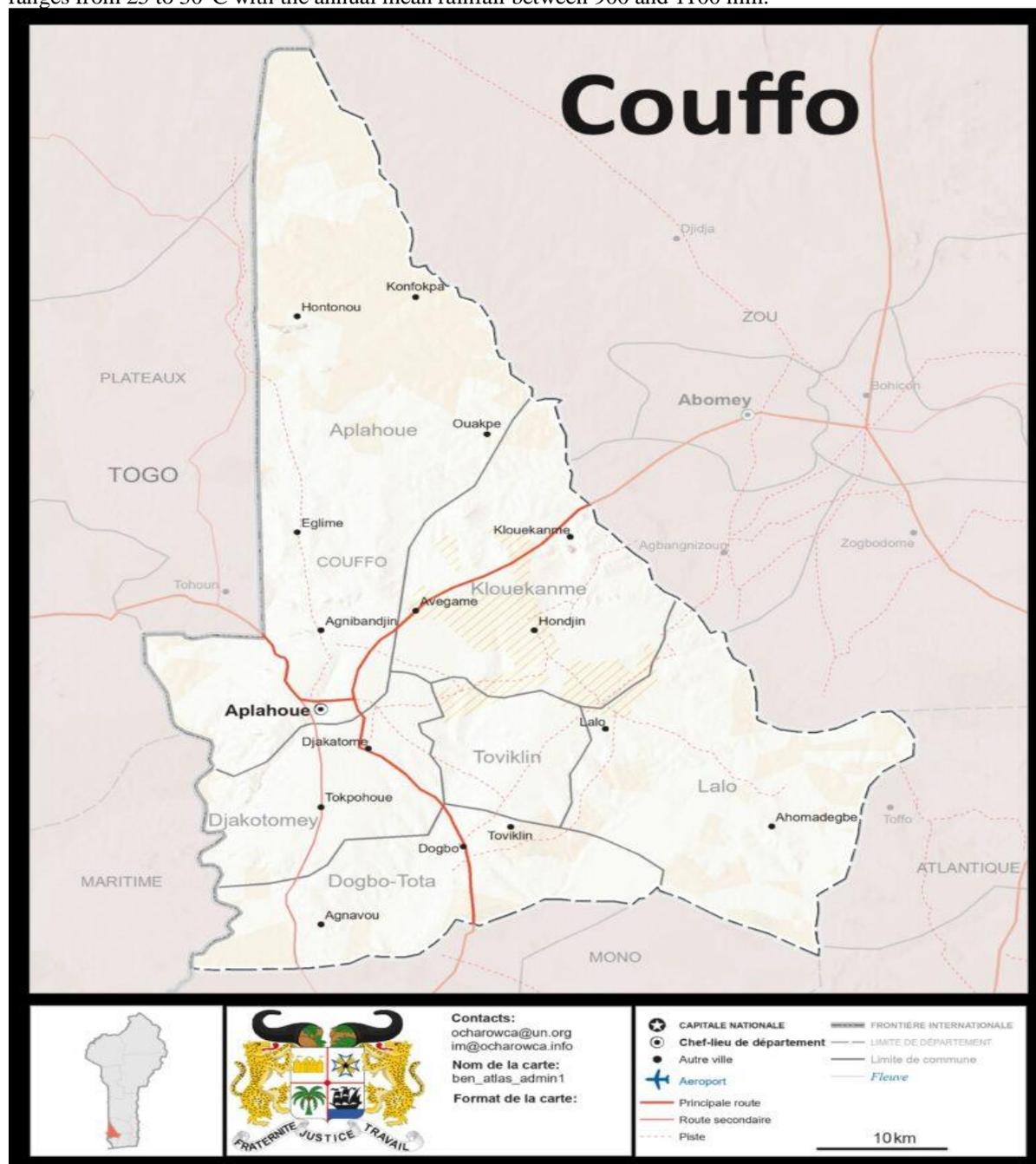


Figure 1: Map of Republic of Benin showing Klouekanmèy district surveyed

Mosquito sampling

Culex quinquefasciatus larvae were collected from April to July 2024 during the great rainy season in Klouekanmè 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 Pluridisciplinary Researches of Technical Teaching (LaRPET) 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 (10). The volume of stock solution was 20 ml of 1%, obtained by weighing 200 mg of dichlorodiphenyltrichloroethane (DDT) 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 shaken vigorously to dissolve or disperse the dichlorodiphenyltrichloroethane 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 dichlorodiphenyltrichloroethane 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 (10).

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 hours 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 dichlorodiphenyltrichloroethane, each of four replicates (10).

Biochemical assays using synergist

The presence of metabolic-based resistance mechanisms was investigated by exposing larvae to enzyme inhibitor prior to bioassays with DDT. For that, as all tested samples showed high tolerance to DDT in *Culex quinquefasciatus* larvae from Klouekanmè, they were exposed to the effects of synergist: Ethacrynic acid (ETAA or EA) (80 µg per test cup), which inhibits glutathione S-transferases 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 were 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 $P < 0.05$). To appreciate the effects of synergist ETAA on *Culex quinquefasciatus* larvae tolerance to DDT, 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 *Culex quinquefasciatus* larvae from Klouekanmè district in Couffo department were highly resistant to DDT (see Resistance ratios RR50 and RR95).

Table 1: Determination of Lethal Concentrations LC50 and LC95.

Populations	LC50 (mg/l)	LC95 (mg/l)	RR50	RR95
Kisumu (Control)	0.0176	0.0553	–	–
Klouekanmè	0.9900	4.210	56.25	76.13

The analysis of tables 2 and 3 showed that the underlying mechanism of the resistance pattern observed in this population was explored using a synergist assay. The synergist assay with ethacrynic acid (ETAA or EA), an inhibitor of glutathione S-transferases (GSTs), indicated that this enzyme family plays a role in this high DDT resistance observed in *Culex quinquefasciatus* larvae from Klouekanmè.

Table 2: Determination of Lethal Concentrations LC50 of *Culex quinquefasciatus* larvae to DDT with and without ETAA and Synergism ration SR50

Population	Without ETAA		With ETAA		Synergism Ratio (SR50)
	Number tested	LC50 (mg/l)	Number tested	LC50 (mg/l)	
Klouekanmè	25	0.9900	25	0.0990	10.00

Table 3: Determination of Lethal Concentrations LC95 of *Culex quinquefasciatus* larvae to DDT with and without ETAA and Synergism ration SR95

Population	Without ETAA		With ETAA		Synergism Ratio (SR95)
	Number tested	LC95 (mg/l)	Number tested	LC95 (mg/l)	
Klouekanmè	25	4.210	25	0.3901	10.79

The analysis of table 4 showed that there are more advantages than disadvantages in the use of synergist

Table 4: Advantages and disadvantages of the use of synergist

Advantages	Disadvantages
Synergist contributes significantly to improve the efficacy of insecticides, particularly when problems of resistance have arisen.	Increased toxicity to non-target organisms (humans, bees) (is the main disadvantage)
Synergist inhibits detoxifying enzymes or increases target sensitivity, allowing lower doses and overcoming resistance.	
Synergist has to be exploited in vector-borne disease management programs for intention of resistance management in insects.	

Synergist can be used on mosquito's nets to improve bed's net effectiveness against mosquito's bites.	
Synergist can be used on insecticide for Indoor Residual Spraying programs in vector-borne disease control.	
The use of synergists reduces the quantity of insecticide to be used.	

4. DISCUSSION

In the current study, *Culex quinquefasciatus* larvae from Klouekanmè district in Couffo department were highly resistant to DDT. In addition, the underlying mechanism of the resistance pattern observed in this population was explored using a synergist assay. The synergist assay with ethacrynic acid (ETAA or EA), an inhibitor of glutathione S-transferases (GSTs), indicated that this enzyme family played a role in this high DDT resistance observed in *Culex quinquefasciatus* larvae from Klouekanmè. These results corroborated with those obtained by Lopes *et al* (11) who had studied the insecticide resistance in *Culex quinquefasciatus* Say, 1823 in Brazil. In their study, they had shown that *Culex quinquefasciatus* was resistant to dichlorodiphenyltrichloroethane (DDT). In addition, Aïzoun (12) had studied the effects of synergist ethacrynic acid (ETAA or EA) on dichlorodiphenyltrichloroethane (DDT) tolerance in *Anopheles gambiae* s.l. larvae from mono department in south-western Benin, West Africa. For that, 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* s.l. larvae using dichlorodiphenyltrichloroethane (DDT) as larvicide and ethacrynic acid (ETAA or EA) as enzyme inhibitor or synergist. The results showed that glutathione S-transferases (GSTs) may play a role in *Anopheles gambiae* s.l. larvae from Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa resistance to DDT.

Previous studies conducted in others departments in the country on adults also showed the involvement of GSTs in *An. gambiae* s.l. populations from Suru-léré and Akron resistance to DDT (13) and in *An. gambiae* s.l. populations from Ladji resistance to DDT (14). But GSTs may play no role in *An. gambiae* s.l. populations from Parakou and Bohicon resistance to DDT (15). They may also play no role in *An. gambiae* s.l. populations from Sèkandji resistance to DDT (14). 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 (16), the result of which was that the toxicity effect would also be reduced. Regarding the advantages and disadvantages of the use of synergists, it is important to notice that there are more advantages than disadvantages in this use. In fact, as advantages: Synergist contributes significantly to improve the efficacy of insecticides, particularly when problems of resistance have arisen. Synergist inhibits detoxifying enzymes or increases target sensitivity, allowing lower doses and overcoming resistance. Synergist has to be exploited in vector-borne disease management programs for intention of resistance management in insects. Synergist can be used on mosquito's nets to improve bed's net effectiveness against mosquito's bites. Synergist can be used on insecticide for Indoor Residual Spraying programs in vector-borne disease control. The use of synergists reduces the quantity of insecticide to be used. However, as disadvantages: Increased toxicity to non-target organisms (humans, bees) is the main disadvantage.

5. CONCLUSION

Glutathione S-transferases played a role in *Culex quinquefasciatus* larvae tolerance to organochlorine in Couffo 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 lymphatic filariasis vector particularly in Benin and in Africa in general.

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