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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Smoke and ethanolic extract of *nicotiana tabacum* altered hippocampal histology and behaviour in mice

Adeniyi, Philip Adeyemi* and Ogundele, Olalekan Micheal

Department of Anatomy, Addiction and Neurotoxicity Unit, Afe Babalola University College of Medicine and Health Sciences, Ado-Ekiti, PMB 5454, Nigeria.

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The effects of tobacco use on human health are well known and are documented in scientific reports. When tobacco is smoked or chewed, nicotine is absorbed by the lungs and oral cavity and quickly moved into the bloodstream where it is distributed rapidly through the circulatory system to reach the brain and peripheral nervous system. Male and female adult mice (N=32) were used for this study. The animals were randomly divided in to four (4) groups, A, B, C and D, of eight (n=8) animals each. Group A animals were treated by oral administration of 10.72 mg of the extract, B 10.72 mg of the tobacco smoke exposure for 3 min (in a controlled air chamber), C were given 0.2 ml of normal saline and D were expose to equal weight (0.02 g) of cotton wool for 3 min, for 21 experimental days. The mice were sacrificed 4 h after the last administration and the brains excised, blotted, weighed and fixed in formol calcium for histological analysis, using Haematoxylin and Eosin. There was a significant decrease in the body weight, brain weight and relative brain weight in the treatment groups. The pyramidal and granular cell layers showed changes in cell count scores; significant at *p<0.05* when compared with the control. The results suggested that the consumption of *Nicotiana tabacum* leaves; either smoking or chewing may lead to alterations in cell count, brain weight and neurobehavioral patterns. Weight loss was also observed in the treatment and was found to be dependent on the route of administration.

Key words: Nicotine tobacco, hippocampus, memory, cell death.

INTRODUCTION

The role of the cholinergic systems has been established over the years in neuropsychiatry research. The cholinergic system constitutes part of the reward feedback mechanism of the brain and nervous system (Janes et al., 2014). This is characterized by the presenceof acetylcholine at the synapses (Wu et al., 2014). The cholinergic neurons are diffused in the brain and are excitatory in nature (Shinohara et al., 2014). This is also found at the neuromuscular junctions where it facilitates muscular contraction and mechanical activity in the body (Gallego et al., 2013). Other sub-classifications of the cholinergic receptors involves the nicotinic and muscarinic receptors located in glands, the cells of the gastro intestinal tract and the lungs (Chan et al., 2013).

The nicotinic receptors are the sites of activity of the nicotine and are widely distributed in the CNS and

*Corresponding author. E-mail: adephiladex.ng@gmail.com. Tel: +2348032922323.

neuromuscular junctions (Chan et al., 2013; Zhu et al., 2013). Cellular mechanism studies reveals that potentiation of nicotinic receptor by nicotine is in a manner similar to that of acetylcholine. Thus, nicotine is a cholinergic agonist (Sharma et al., 2014). Other structural analogues of nicotine and acetyl choline will potentiate the nicotinic cholinergic receptor (Cahill et al., 2014; Gannon et al., 2013).

Addictive properties of nicotine are linked directly with the positive reward system of the brain. This is because, nicotine can provide prolonged stimulation of the cholinergic receptors, keeping the brain and neuromuscular junctions active. Other behavioral changes includes profuse sweating, dilation of the pupil, restlessness and hyper activity (Smith and Chapman, 2014; Zwart et al., 2014). At the onset, efficiency is recorded in the activity of such individuals. In prolonged use (addiction), over stimulation of nicotinic cholinergic receptors leads to excitotoxicity and neuronal cell loss characterized by disorientations and other neuropsychiatric symptoms (Umene-Nakano et al., 2014).

The prolonged activity of nicotine in the synapses has been reported. The neuronal metabolic machinery is endowed with systems that are capable of regulating and reducing cholinergic stimulation of acetylcholine (Jain et al., 2013). Acetyl cholinesterase (AChE) often deactivates the cholinergic stimulation and breaking down acetyl choline into choline and acetate both at the synapse and neuromuscular junction. However, AChE is incapable of breaking down nicotine, thus a prolonged excitation at the cholinergic sites (Hashimoto et al., 2013).

Hippocampus is the structure that lies on the fringes of the medial aspect of each cerebral hemisphere (the limbic system) of the brain (Yin et al., 2014). Exposure to tobacco (nicotine) either from cigarettes and other forms of tobacco including cigars, pipe tobacco, snuff and chewing tobacco has been reported to be associated with alteration in the normal functions of the brain and the whole nervous system (Stephen, 1999; Charles, 2000; Anthony, 2002; NIDA, 2009A). Nicotine has been reported to be the highest and most toxic compound of aqueous extract of tobacco leaves (Sas, 1990; Leroy, 1999; Philip, 2002). However, therapeutic uses of nicotine include its use to aid smoking cessation and other nicotine addictions (Charles, 2000; NIDA, 2009A). Using a controlled amount of nicotine helps to reduce nicotine withdrawal symptoms when one attempts to quit the use of tobacco products (Charles, 2000; Adeniyi, 2007; NIDA, 2009A). Annually, about 5 million deaths is attributed to tobacco smoking contributing the second leading cause of mortality among adults worldwide (Aghaji, 2008; Uwakwe and Modebe, 2008). This frightening data attests to the death of about three million people in the year 2007 alone (WHO, Resolution, 1993; World Health Statistics, 2007), these findings and reports suggest the need for further experimental and clinical studies of the role of tobacco intake on the body systems, most especially the brain in particular and the aim of this study is to investigate the effects of both ethanolic and smoke extract of tobacco on the hippocampus of juvenile mice.

MATERIALS AND METHODS

Animal care

All experimental investigations were done in compliance with humane animal care standard outlined in the “Guide to the Care and Use of Animals in Research and Teaching”, as approved by the Institute of Laboratory Animal Resource, National Research Council, DHHS, Pub. No NIH 86 - 23 (1993).

The study was carried out using healthy juvenile mice of both sexes (18 - 25 g). The animals were kept under standard laboratory conditions (12 h light and 12 h darkness, temperature, humidity and ventilation). They were given standard rat diet, purchased from the same company, Bethel Feeds, Ilorin, Nigeria.

Extract preparation

The *Nicotiana tabacum* leaves pack was collected from Igboho, the northern part of Oyo State, Nigeria. Plant samples were authenticated at the Department of Plant Science, University of Ilorin, Nigeria. The leaves were air-dried at room temperature. 50 g of the blended leaves was dissolved in 500 ml of 70% alcohol for 24 h at room temperature. The filtrate was thereafter obtained from the solution using Whatman’s No 1 filter paper and evaporated to dryness in an air-dry oven at 40°C. The residue of the extract obtained in form of paste was stored in a capped bottle and kept in a desiccator (Carla et al., 1997; Adeniyi et al., 2010). The pH of the extract was determined to be 4.19 before concentration and 5.72 after concentration. This was done using a pH meter (pHs - 25 Model). The yielding rate of the tobacco extract was determined to be 41.35%.

Animal treatment

The animals were given the *N. tabacum* as shown in the Table 1.

The tobacco extract

This was given orally with the aid of an orogastric tube.

The tobacco smoke

This was administered by exposing the animals to dried *N. tabacum* leaves wrapped with 0.02 g of cotton wool in a burning chamber for three minutes [Burning time (BT); this was determined by allowing three of the *N. tabacum* leaves of known weight (10.72 mg) to burn and their average burning time was determined]. The administration was done for 21 days and 4 h after which mice from each group was sacrificed for analysis; while the rest were sacrificed by 7 days (a week) after the last administration, to study the withdrawal effects of the *N. tabacum* exposure on the animals.

Experimental design

Total of N=32 mice (n=16 males and n=16 females) were used for this study. The animal was randomly divided in to four (4) groups, A, B, C and D, of eight (n=8) animals each. Group A received 10.72

Table 1. Brain weight (g) and relative brain weight (RBW) changes in animals during the experimental period (mean ± SEM).

Group	Day 21		Day 28	
	BWT	RBW (%)	BWT	RBW (%)
A	0.5172*±0.0112	2.57	0.3786*±0.0209	1.89
B	0.4121±0.0121	1.93	0.3667±0.0072	1.66
C	0.3434±0.0122	1.61	0.3080±0.0066	1.28
D	0.03623±0.0212	1.52	0.3480 ±0.0370	1.42

*Significantly different from control mice (P<0.05).

mg of orally administered extract, B 10.72 mg of the tobacco smoke exposure for 3 min. The animals in Group C were given 0.2 ml of normal saline, while the treatment Group D was expose to equal weight (0.02 g) of cotton wool for 3 min, for 21 days.

Neurobehavioural observations

The neurobehavioural analysis was done at 0800 h of the day using Elevated Plus Maze (EPM) to study the locomotion, exploration and motor coordination in both the treated and control animals. The results are show in Figure 3.

Animal sacrifice

After administration, the mice (four from each group) were sacrificed by cervical dislocation at days 21 and 28 of the treatment and their brains were excised, blotted with filter paper and the wet weights were taken and recorded, and brains were quickly transferred to a specimen bottle containing 10% formol calcium and fixed for two days (Bancroft and Stevens, 1990). Thereafter, the hippocampus was excised to process for histological analysis and the wet weights of the brain and volume was recorded for analysis. The brain volume was determined by liquid (water) displacement method and recorded in millimeter cube (mm^3).

Histology

The brains are fixed in 10% formol calcium, hippocampus were excised and processed for Haematoxylin and Eosin (H&E) and Cresyl Fast Violet (CFV) staining technique (Bancroft and Stevens, 1990). The slices of 5 µ were sectioned with the Letiz rotary microtome. The sections were mounted and examined with the light microscope. Image acquisition was done using the Cameroscope 5.5 connected to a computer interface and mounted on the Olympus binocular research microscope.

Morphometry

The pyramidal (PCL) and granular (GCL) cell layers thickness were measured using the method of W.H.O (1991) and Ofusori et al. (2008) in which an occulometer (10X10 grid lines) was inserted into the microscope and focused through stained slides.

Statistical analysis

The data were expressed as means ± standard error of mean (SEM). The data were statistically evaluated with SPSS software version 14.0 software. One-way analysis of variance (ANOVA) at significant level $2\alpha = 0.05$ were used.

RESULTS

Physical examination

There were no significant changes in the skin colour and arrangement; the colour of their eyes was normal as compared to the control groups. Also, the gross anatomy of the brain of the experimental appeared normal as compared to the control groups.

Weight changes

The average weight gain recorded for treatment group during the experimental period was reduced during the first 14 days in group A and B as compare to C and D. However, all the treatment groups recorded an increase in average weight during the 7 withdrawal days.

Neurobehavioral parameters

The general behaviour of the animals was comparatively normal. However, rate of head deeding (HD), stretching (S), quadrate duration (QD) and transition (T) were significantly ($p<0.05$) different between experimental groups and the control groups (Figures 1 to 4).

Brain weight (BWT) changes

The average brain weight recorded for treatment group during the experimental period reduced during the 7 days of withdrawal (Table 1).

Relative brain weight (RBW) changes

The RBW changed between the experimental groups; those in group A had the highest RBW as compared to C and as in group B as compared to D after 21 days of

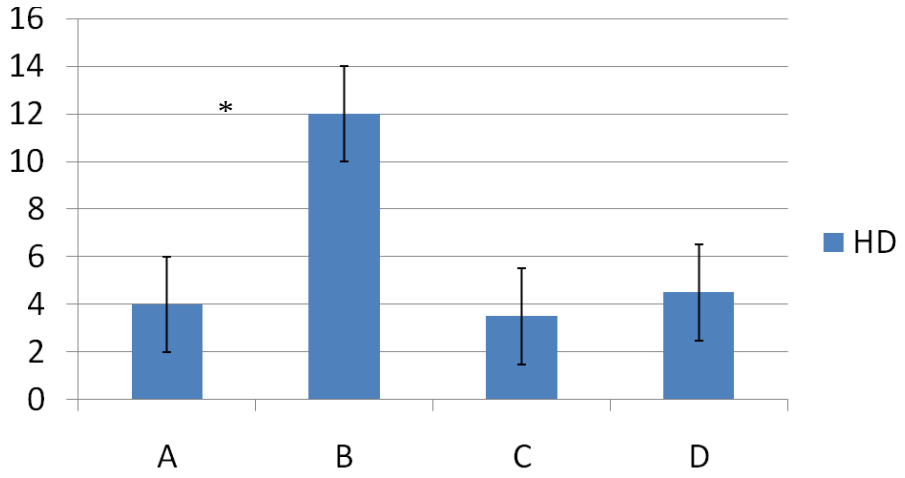

Figure 1. Head dipping (HD) of mice after 21 days of *Nicotiana tabacum* exposure.

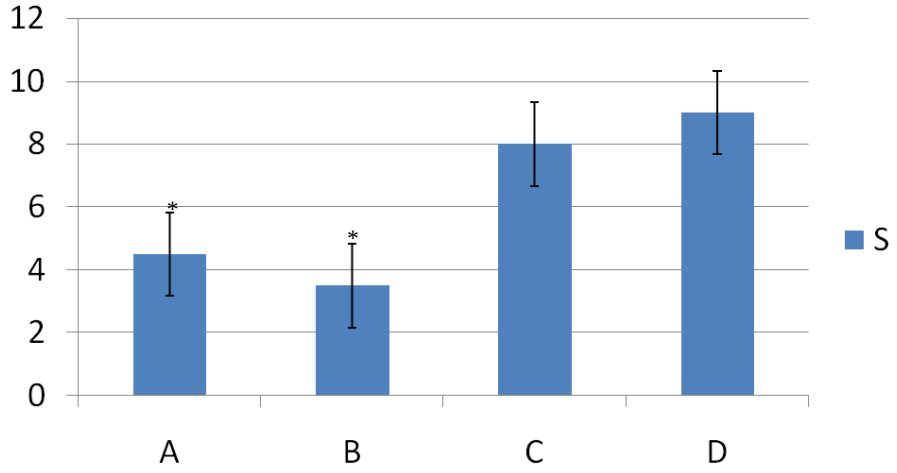

Figure 2. Stretching attempt (S) of mice after 21 days of *Nicotiana tabacum* exposure.

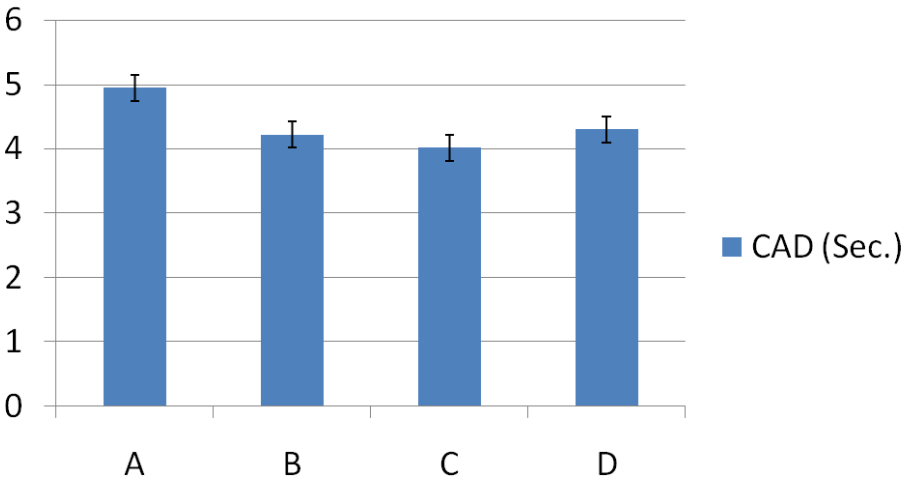

Figure 3. Close arm duration (CAD) in seconds of mice after 21 days of *Nicotiana tabacum* exposure.

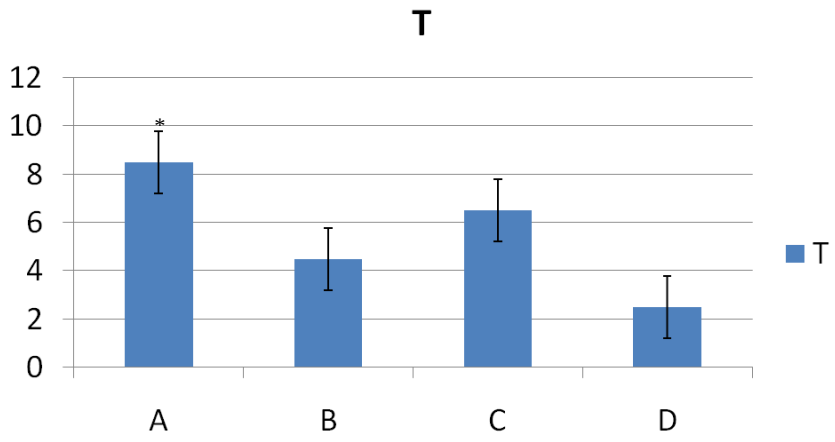

Figure 4. Transition (T) of mice after 21 days of *Nicotiana tabacum* exposure.

Table 2. Brain volume (ml) changes in animals during the experimental period (mean ± SEM).

Group	Day 21	Day 28	Percentage brain volume changes (%)
A	4.0±0.00	3.5±0.50*	(12.50)
B	4.0±0.00	4.0±0.00	0.00
C	4.0±0.00	4.0±0.00	0.00
D	4.0±0.00	4.0±0.00	0.00

*Significantly different from control mice (P<0.05).

treatment and this is dose dependent (Table 1).

Brain volume (BRV) changes

The volume of brain of the animals was relatively the same in both experimental and control groups. Although, there was slight (12.5%) increase in the brain volume in group A, there brain volume changes were relatively the same across the groups (Table 2).

Hippocampal neurohistology (Plate 1)

Cell body stain intensity

The cell bodies are more densely stained in the experimental groups in a dose dependent manner as compared to the control groups and the architectural arrangement appeared normal.

Vaculations

There are more vaculations in the experimental groups as compared to the control group C.

Cell population

The population of the neural cells (pyramidal cells) appeared to be more in the experimental groups as compared to the control groups in dose dependent pattern.

Pyramidal cell layer

This appeared uniformly normal with cell bodies of experimental group densely stained as compared to the control group (Table 3).

Granule cell layer

This appeared uniformly normal with cell bodies of experimental groups densely stained as compared to the control group (Table 3).

DISCUSSION

The observed reduction in weight gain of the animals in the experiment may implicate nicotine in tobacco plant

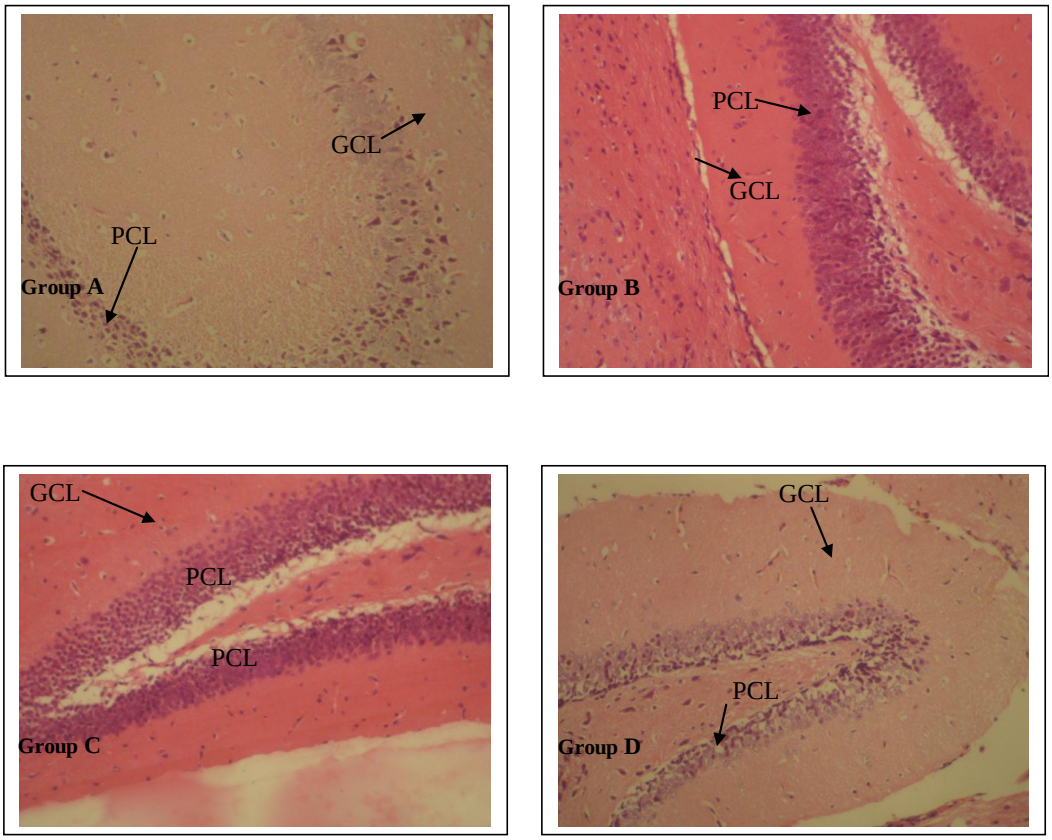

Plate 1. Hippocampus (H & E) at day 21: Magnification 480x. PCL: Pyramidal cell layer; GCL: granular cell layer.

Table 3. Hippocampal histometry analysis changes in animals at day 21 of tobacco exposure (mean ± SEM).

Group	PCL (X 10^{-3} mm)	GCL(X 10^{-3} mm)
A	5.2000±0.6633	7.3333*±1.4530
B	6.8000±0.9695	10.5000*±1.3229
C	6.0000±0.4083	9.2500±1.1087
D	5.4000±0.4000	9.8000±1.2806

*Significantly different from control mice (P<0.05).

use as reported by Chen et al. (2005) and this may be associated with reduction in food intake by the tobacco users. Also, the brain weight after administration and withdrawal were significant between the mice in group A as compared to those in groups C and D ($p<0.05$), but those in group A have the highest relative brain weight (RBW) as compare to C ($p<0.05$) and as in group B as compare to D ($p<0.05$) after 21 days of treatment and this is dose dependent. This may account for the shift in the carbohydrate metabolic pathway due to stress induced by the activities of nicotine in the brain of the animals. The observed increase in locomotor activities in the mice (Figure 1 to 4) in the treated groups as compared to the control groups reflect the possibility of tobacco administered to elevate anxiety characteristics in the treated groups which may probably explain the reason for increased cell density observed in the treated groups as compared the control groups (Adeniyi et al., 2010). Long term behavioral effects and molecular changes in brain mechanisms are resultant from nicotine exposure either through smoke or tobacco chewing. This is often evident and prominent in the adolescence humans (Romano et al., 2013). Depression is a characteristic long term behavior that may result from nicotine addiction (van der Meer et al., 2013). Long term smokers are more likely to suffer negative mood changes after nicotine withdrawals, thus contributing to their high morbidity and mortality from smoking related illness.

Several biochemical changes will often precede the structural changes observed in the hippocampus of the treated groups. The pattern of such changes might differ from the effect of nicotine exposure on the cortex owing to the effect of neurogenesis in the hippocampus. Our findings also suggests that nicotine administration, although was indifferent on gross brain structure, created a wide variation in behavior and memory. Considering the duration of our experiment, the effect of the treatment on long term memory cannot be elucidated. However, short

term memory processing is also associated with the hippocampus. The findings suggested that movement and cognition was reduced in the treatment group. This was also evident from the histological analysis with a reduction in cell count observed in the nicotine treatment group.

Comparative histology for the nicotine treatment groups A and B shows that tobacco smoke gave a more detrimental effect versus aqueous extract. To improve the model for studying nicotine dependence, we have employed the use of oral (aqueous) and smoke administered nicotine in tobacco.

Conclusion

Above all, from all the changes observed from comparisons between the experimental and control groups, it is safe to conclude that the administration of tobacco leaves smoke and extract can result in body and brain weight loss, distorted carbohydrate metabolism and alterations in locomotor activities.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Dynamics of insecticide resistance and exploring biochemical mechanisms involved in pyrethroids and dichlorodiphenyltrichloroethane (DDT) cross-resistance in *Anopheles gambiae s.l* populations from Benin, West Africa

Nazaire Aïzoun[1,2]*, Roseric Azondekon[1,3], Rock Aïkpon[1,2], Rodrigue Anagonou[1,2], Virgile Gnanguenon[1,2] and Martin Akogbéto[1,2]

[1]Centre de Recherche Entomologique de Cotonou (CREC), 06 BP 2604, Cotonou, Bénin.
[2]Faculté des Sciences et Techniques, Université d'Abomey Calavi, Calavi, Bénin.
[3]University of Massachusetts Amherst, Amherst, Massachusetts, USA.

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As a result of the free-insecticide treated net distribution by National Malaria Control Programme (NMCP) in July 2011 throughout the entire country, it is useful to investigate the dynamics of insecticide resistance in *Anopheles gambiae* from Benin and the metabolic resistance mechanisms involved in this evolution of resistance. Larvae and pupae of *A. gambiae s.l.* mosquitoes were collected from the breeding sites in Littoral, Ouemé and Zou departments. Centers for Disease Control and Prevention (CDC), bioassays were performed with permethrin (21.5 µg per bottle) and dichlorodiphenyltrichloroethane (DDT) (100 µg per bottle) whereas World Health Organization (WHO) susceptibility tests were performed with lambdacyhalothrin (0.05%). CDC biochemical assays using synergists piperonyl butoxide (PBO) and ethacrynic acid (ETAA) were also carried out. *A. gambiae* Akron, Suru-léré and Bamè populations were resistant to permethrin and DDT in 2008 and 2013. *A. gambiae* Akron and Suru-léré were resistant to lambdacyhalothrin in 2013. *A. gambiae* Akron were susceptible to this product in 2008 whereas lambdacyhalothrin resistance status in *A. gambiae* Suru-léré populations in 2008 required further investigation. Mono-oxygenases were involved in resistance of *A. gambiae* Suru-léré to pyrethroids whereas glutathione S-transferases (GSTs) may play a role in *A. gambiae* Akron and Suru-léré resistant to DDT. This study shows that metabolic resistance conferred by detoxifying enzymes is an indication of phenotypic resistance to both DDT and pyrethroids in Southern Benin.

Key words: Dynamics, piperonyl butoxide, ethacrynic acid, insecticide, vectors, resistance.

INTRODUCTION

Malaria is a major health problem in Benin where it is the main cause of morbidity and mortality particularly among children under five and pregnant women (Djouaka et al., 2011). In this country and across Africa, malaria control relies heavily on vector control through the use of insecticide-treated nets (ITN) and indoor residual spraying (IRS) (Djouaka et al., 2011).

The evolution of insecticide-resistant mosquito strains

is an increasing problem and one of the major obstacles for the malaria vectors control. In West Africa, *Anopheles gambiae* resistance to the four major classes of insecticides available for public health has been reported (Djogbenou et al., 2008; Hunt et al., 2011; Dabiré et al., 2009).

Malaria vector resistance to insecticides in Benin is conferred by two main mechanisms: (1) alterations at site of action in the sodium channel, viz the *kdr* mutations and (2) an increase of detoxification and/or metabolism through high levels of multi-function oxidases (MFOs), non-specific esterases (NSEs) and glutathione S-transferases (Corbel et al., 2007; Djogbénou et al., 2009; Djègbé et al., 2011; Aïzoun et al., 2013a, 2014a). Although, in this country, very few studies have shown the involvement of glutathione S-transferases (GSTs) in *A. gambiae* resistant to DDT. In addition, the Beninese National Malaria Control Programme has implemented large-scale and free distribution of LLINs (OlysetNets) since July 2011 throughout the entire country to increase coverage of LLINs. It is crucial that information on susceptibility to the main insecticides used in public health in Benin and the underlying mechanisms are investigated. This will properly inform control programs of the most suitable insecticides to use and facilitate the design of appropriate resistance management strategies.

Corbel et al. (2007) have reported on multiple insecticide resistance mechanisms in *A. gambiae* from Ladji, a peripheral location of Cotonou district. It was useful to check if these resistance mechanisms were also present in other *A. gambiae s.l.* populations from the urban locations of Cotonou district such as Suru-léré location. In addition, according to Djouaka et al. (2008), *A. gambiae* Akron populations were resistant to permethrin and both target site mechanism (*kdr*W) and metabolic mechanism (cytochrome P450 genes) were involved. However, these authors also mentioned in their paper that there were several additional potential resistance mechanisms which need further investigation.

The main goal of this study was to investigate insecticide resistance evolution in *A. gambiae* Akron, Suru-léré and Bamè populations and explore the involvement of the detoxifying enzymes in the insecticide phenotype resistance in *A. gambiae* Akron and Suru-léré populations from southern Benin by using two classical synergists.

METHODOLOGY

Study area

The study was carried out in the South and Central part of Benin, more precisely in Suru-léré, in the Cotonou district of Littoral department, in Akron, in the Porto-Novo district of Ouemé department and in Bamè, in the Zangnanado district of Zou department (Figure 1). The choice of the study sites was as a result of the economic activities of populations, their usual protection practices against mosquito bites, the LLINs (long-lasting insecticidal nets, OlysetNets) distribution recently in these locations and peasant practices to control farming pests. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors. Cotonou is characterized by a tropical coastal guinean climate with two rainy seasons (April-July and September-November). The mean annual rainfall is over 1,500 mm. Ouemé has a climate with two rainy seasons (March-July and September-November). The temperature ranges from 25 to 30°C with the annual mean rainfall between 900 and 1,500 mm. The central part of the country is characterized by a sudano-guinean climate with an average rainfall of 1,000 mm per year.

Mosquito sampling

A. gambiae s.l. mosquitoes were collected during the rainy seasons (March-July and September-November 2008 and March-July 2013) in the vegetable growing area of Akron located in the Porto-Novo district selected in southern Benin. *A. gambiae s.l.* mosquitoes were also collected during the rainy seasons (April-July and September-November 2008 and April-July 2013) across Suru-léré, an urban locality in the Cotonou district also selected in southern Benin and in Bamè, a rice growing area in the Zangnanado district selected in the central part of Benin. Larvae and pupae were collected on breeding sites using the dipping method. They were then kept in separated labeled bottles related to each locality. The samples were reared up to adult emergence at the Centre de Recherche Entomologique de Cotonou (CREC), Benin insectary. *A. gambiae* Kisumu, a reference susceptible strain was used as a control for the bioassay tests. Susceptibility tests were done following both WHO and CDC protocols on unfed female mosquitoes aged two to five days old, reared from the larval and pupal collections. Each *A. gambiae s.l.* sample was separated into two batches: batch 1 was used for susceptibility tests following the WHO protocol and batch 2 for CDC susceptibility tests. All susceptibility tests were conducted in the CREC laboratory at 25±2°C and 70 to 80% relative humidity.

Testing insecticide susceptibility

WHO protocol

Females *A. gambiae* aged 2 to 5 days old were exposed to WHO diagnostic dosage with lambdacyhalothrin 0.05% according to the WHO protocol (WHO, 1998) in 2008 and 2013. Thus, an aspirator was used to introduce 20 to 25 unfed female mosquitoes into five WHO holding tubes (four tests and one control) 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 60 min and mortalities at 24 h were recorded following the WHO protocol (WHO, 1998). We used lambdacyhalothrin, an insecticide of same class as permethrin, as well as to assess cross-resistance with permethrin in localities surveyed.

*Corresponding author. E-mail: aizoun.nazaire@yahoo.fr. Tel: (229) 95317939. Fax: (229) 21308860.

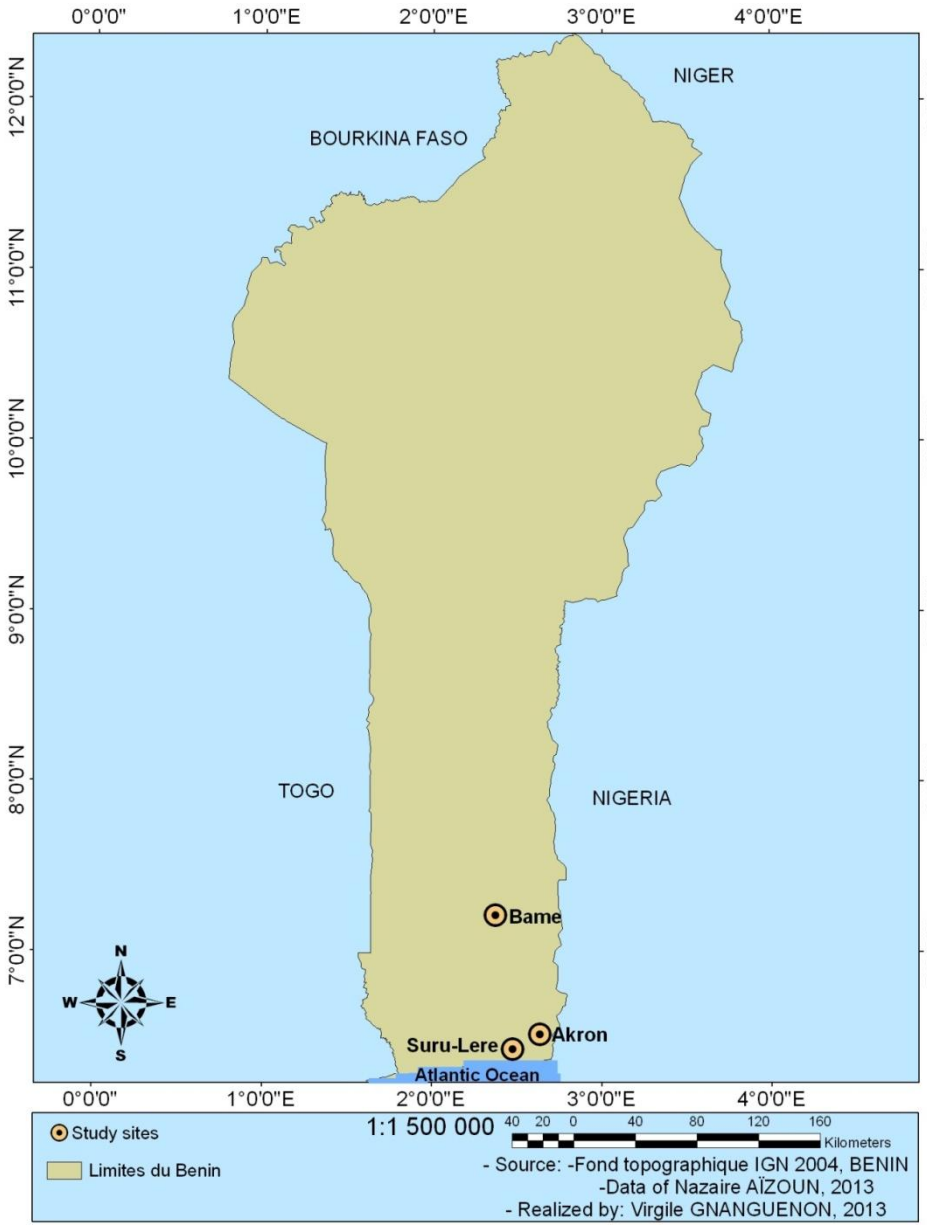

Figure 1. Map of the study area.

CDC protocol

The principle of the CDC bottle bioassay is to determine the time it takes an insecticide to penetrate an arthropod, traverse its intervening tissues, get to the target site, and act on that site relative to a susceptible control. Anything that prevents or delays the compound from achieving its objective of killing the arthropods contributes to resistance.

Diagnostic doses that were applied in the current study were the doses recommended by CDC (Brogdon and Chan, 2010). These doses were checked on the *A. gambiae* Kisumu susceptible reference strain before being applied to field populations. For *A. gambiae s.l.*, the diagnostic dose of 21.5 µg per bottle for permethrin was used for the diagnostic exposure time of 30 min, whereas the diagnostic dose of 100 µg per bottle for DDT was used for a diagnostic exposure time of 45 min. The choice of permethrin was justified by the insecticide used on OlysetNets that were distributed free by the NMCP in July 2011 across the entire country. DDT was tested because of its intensive use in the past as well as to assess cross-resistance with permethrin in localities surveyed.

The solutions were prepared and the bottles coated according to the CDC protocol (Brogdon and Chan, 2010). Fifteen to twenty unfed female mosquitoes aged 2-5 days old were introduced into four 250 ml Wheaton bottles coated with insecticide and one control bottle coated with acetone only. The number of dead or alive mosquitoes was monitored at different time intervals (10, 20, 30, 40, 50 and 60 min) in 2008 and (15, 30, 35, 40, 45, 60, 75, 90, 105 and 120 min) in 2013. This allowed us to determine the percentage of total mortality (Y axis) against the exposure time (X axis) for all replicates using a linear scale.

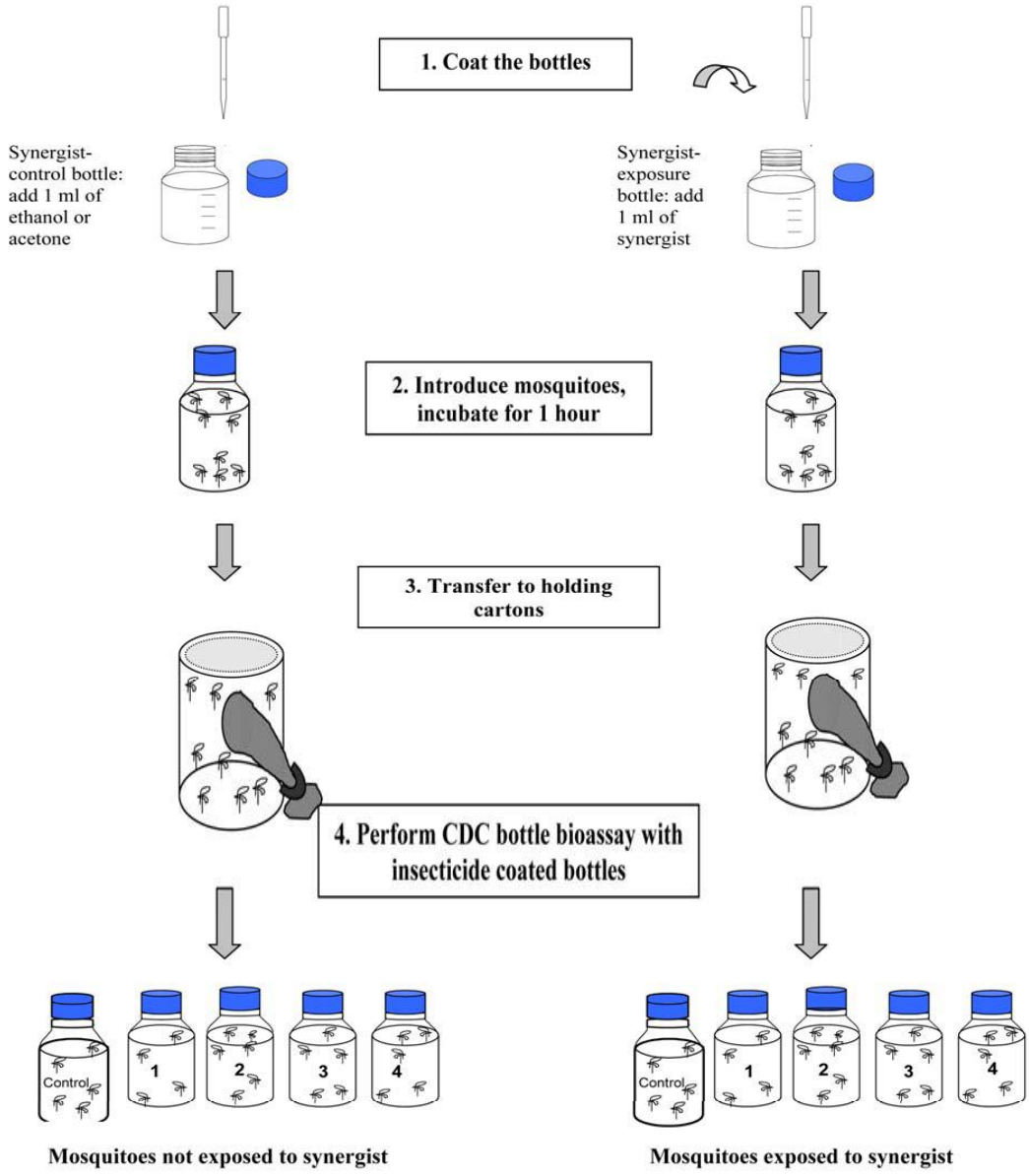

Figure 2. Diagram of the CDC bottle bioassay with synergists (CDC guideline, 2010).

Biochemical assays using synergists

Synergists were used according to the protocol described by CDC (Brogdon and McAllister, 1998; Brogdon and Chan, 2010) following the procedure outlined in Figure 2. Samples that showed high resistance to permethrin and DDT in 2013 in Suru-léré from the Cotonou district were exposed to the effect of synergist: PBO (piperonyl butoxide) (400 µg/bottle), which inhibits oxidase activity and to the effect of synergist: ETAA or EA (ethacrynic acid) (80 µg/bottle), which inhibits glutathione S-transferases activity, respectively. Samples that showed high resistance to DDT in 2013 in Akron from the Porto-Novo district were also exposed to the effect of synergist: ETAA or EA (80 µg/bottle). These two synergists were used separately.

Approximately 125 mosquitoes were used for each synergist assay. The number of dead or alive mosquitoes was monitored at different time intervals (0, 15, 30, 35, 40, 45, 60, 75, 90, 105 and 120 min). This test allowed us to compare the obtained percentages of dead mosquitoes (Y axis) against time (X axis) before the addition of the synergist to those obtained after the addition of the synergist (Figure 2).

Data analysis

The resistance status of mosquito samples from batch 1 was determined according to the latest WHO criteria (WHO, 2013) as follows:

1. Mortality rates between 98-100% indicate full susceptibility.
2. Mortality rates between 90-97% require further investigation.
3. Mortality rates < 90%, the population is considered resistant to

the tested insecticides.

The resistance status of mosquito samples from batch 2 was determined according to the CDC criteria (Brogdon and McAllister, 1998; Brogdon and Chan, 2010). The susceptibility thresholds at the diagnostic time of 30 min for pyrethroids and 45 min for organochlorines are:

1. Mortality rate = 100%: the population is fully susceptible.
2. Mortality rate < 100%: 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 either the test-tubes or test-bottles because the mortality rates in all controls was always less than 5% (Abbott, 1987).

To appreciate the effects of synergist PBO on *A. gambiae* Suru-léré populations resistant to permethrin and the effects of synergist ETAA or EA on *A. gambiae* Akron and Suru-léré populations resistant to DDT, we used a Kruskal-Wallis test. The knockdown times for 50 and 95% of tested mosquitoes (kdt50 and kdt95) were estimated using SPSS version 16.0 (SPSS Inc., Chicago, IL). The significance level was set at 5%. The software R-2.15.2. (R Development Core Team, 2011) was used for the statistical analysis.

RESULTS

Evolution of *Anopheles gambiae* resistance to insecticides in Suru-léré, Bamè and Akron locations from 2008 to 2013

The Kisumu strain (control) confirmed its susceptibility status as a reference strain. All female mosquitoes of *A. gambiae* Kisumu that were exposed to CDC bottles treated with permethrin 21.5 µg per bottle, DDT 100 µg per bottle in 2008 and in 2013, were dead and none of them could fly after 30 and 45 min, which represent the susceptibility threshold times or diagnostic times clearly defined by the CDC protocol. These results confirmed that this strain was fully susceptible to these products in 2008 and 2013. In similar way, all female mosquitoes of *A. gambiae* Kisumu that were exposed to WHO impregnated paper with lambdacyhalothrin 0.05% in 2008 and in 2013 were dead and none of them could fly after 24 h mortality recording required by WHO. These results also confirmed that this strain was fully susceptible to this product in 2008 and 2013 (Table 1).

Regarding field collected *A. gambiae* populations, a proportion of the *A. gambiae* Akron, Suru-léré and Bamè populations 24.17% in 2008 and 75% in 2013, 13% in 2008 and 17.55% in 2013, 23.3% in 2008 and 5.67% in 2013 respectively continued to fly again in the bottles following 30 min exposure to CDC bottles treated with permethrin 21.5 µg/bottle. In addition, a large proportion of the same populations: 87.79% in 2008 and 100% in 2013, 96.06% in 2008, 91.43% in 2013, 65.28% in 2008 and 87.18% in 2013 respectively also continued to fly again in the bottles following 45 min exposure to CDC bottles treated with DDT. This confirmed that *A. gambiae* Akron, Suru-léré and Bamè populations were resistant to both products. Female mosquitoes of *A. gambiae* Akron and Suru-léré that were exposed to WHO impregnated paper with lambdacyhalothrin 0.05% in 2013 were resistant to this product. *A. gambiae* Akron populations were susceptible to lambdacyhalothrin 0.05% in 2008 whereas *A. gambiae* Suru-léré populations resistance level to this product in 2008 required further investigation. No *A. gambiae* Bamè population was exposed to WHO impregnated paper with lambdacyhalothrin 0.05% in 2008 and in 2013 because it was difficult to collect a sufficient number of larvae and pupae of *A. gambiae* mosquitoes during these years (during our survey period) in rice growing area of Bamè (Table 1).

Effects of synergist PBO on *A. gambiae* Suru-léré population resistant to permethrin in 2013

The analysis of Table 2 shows that after the addition of synergist PBO in CDC bottles treated with permethrin, the KdT50 value obtained with *A. gambiae* Suru-léré populations was 10.63 min. This value was lower than the one obtained with permethrin alone which was 25.61 min. A similar pattern was observed with KdT95 value obtained with these same populations which was 42.94 min after the addition of synergist PBO. This value was also lower than the one obtained with permethrin alone which was 120.07 min. Synergist ratio (SR) (before addition of PBO/after addition of PBO) was 2.40 for KdT50 whereas SR (before addition of PBO/after addition of PBO) of these same populations was 2.79 for KdT95.

The analysis of Figure 3 shows that after the addition of synergist PBO to permethrin 21.5 µg/bottle, the percentage of dead mosquitoes from Suru-léré is slightly higher than the one obtained with permethrin alone. The use of PBO synergist in bottles treated with permethrin 21.5 µg/bottle did not eliminate permethrin resistance, but reduced the level, in point of fact that the mortality rate increased from 82.45% to 90.90% ($P>0.05$). These results show that mono-oxygenases may play a little role in *A. gambiae* Suru-léré resistant to permethrin.

Effects of synergist ETAA or EA on *A. gambiae* Suru-léré populations resistant to DDT in 2013

The analysis of Table 3 shows that after the addition of synergist EA in CDC bottles treated with DDT, the KdT50 value obtained with *A. gambiae* Suru-léré populations was 211.51 min. This value was higher than the one obtained with DDT alone which was 136.14 min. A similar pattern was observed with KdT95 value obtained with these same populations which was 398.41 min after the addition of synergist EA. This value was also higher than the one obtained with DDT alone which was 226.50 min. SR (before addition of EA/after addition of EA) was 0.64

Table 1. Mortality of *A. gambiae* from the localities of Akron, Suru-léré and Bamè after one hour exposure in 2008 and two hours exposure in 2013 to CDC bottles treated with permethrin (21.5 µg/bottle) and DDT (100 µg/bottle) and after one hour exposure to WHO impregnated paper with lambdacyhalothrin 0.05% in 2008 and in 2013.

Locality	Year	Insecticide	Number tested	%Mortality	Resistance status
Kisumu (control)	2008	Permethrin	200	100	S
	2008	DDT	200	100	S
	2008	Lambdacyhalothrin	200	100	S
	2013	Permethrin	37	100	S
	2013	DDT	35	100	S
	2013	Lambdacyhalothrin	53	100	S
Akron	2008	Permethrin	84	75.83	R
	2008	DDT	79	12.21	R
	2008	Lambdacyhalothrin	182	98	S
	2013	Permethrin	64	25	R
	2013	DDT	48	0	R
	2013	Lambdacyhalothrin	54	42.59	R
Suru-léré	2008	Permethrin	456	87	R
	2008	DDT	366	3.94	R
	2008	Lambdacyhalothrin	162	95.9	r
	2013	Permethrin	57	82.45	R
	2013	DDT	70	8.57	R
	2013	Lambdacyhalothrin	92	7.6	R
Bamè	2008	Permethrin	91	76.7	R
	2008	DDT	61	34.72	R
	2008	Lambdacyhalothrin	-	-	-
	2013	Permethrin	53	94.33	R
	2013	DDT	39	12.82	R
	2013	Lambdacyhalothrin	-	-	-

S: susceptible; r: require further investigation; R: resistant.

Table 2. Knockdown time, KdT50 and KdT95 (minutes) of *Anopheles gambiae* Suru-léré populations to permethrin and permethrin + PBO.

Population	Without PBO		With PBO			Without PBO		With PBO		
	N. tested	KDT50 (min)	N. tested	KDT50 (min)	SR	N. tested	KDT95 (min)	N. tested	KDT95 (min)	SR
Suru-léré	125	25.61	125	10.63	2.40	125	120.07	125	42.94	2.79

N. tested: number tested.

for KdT50 whereas it was 0.56 for KdT95.

The analysis of Figure 4 shows that after the addition of synergist EA to DDT 100 µg/bottle, the percentage of dead mosquitoes from Suru-léré is slightly higher than the one obtained with DDT alone. The use of synergist EA in bottles treated with DDT 100 µg/bottle did not eliminate DDT resistance, and the mortality rate increased from 08.57 to 14.70% (P>0.05). These results show that GSTs may play a little role in *A. gambiae* Suru-léré resistance to DDT.

Effects of synergist ETAA or EA on *A. gambiae* Akron populations resistant to DDT in 2013

The analysis of Table 4 shows that after the addition of

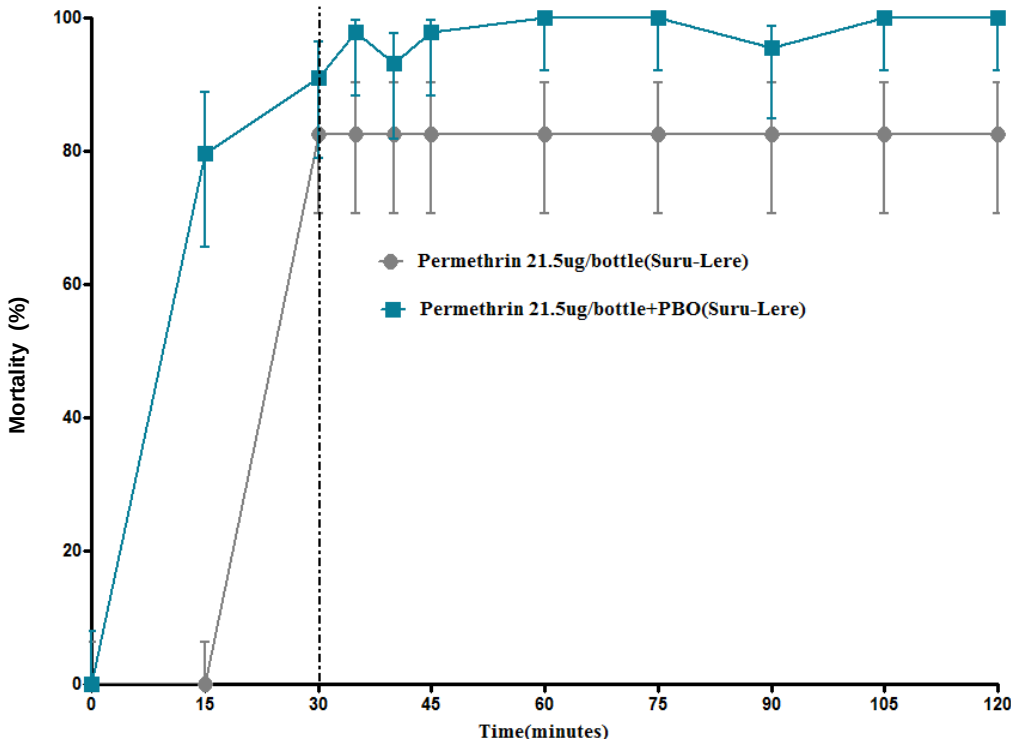

Figure 3. Implication of mono-oxygenases in resistance of *A. gambiae s.l.* to pyrethroid in Suru-léré locality.

Table 3. Knockdown time, KdT50 (minutes) and Knockdown Time KdT95 (minutes) of *Anopheles gambiae* Suru-léré populations to DDT and DDT + ETAA.

Population	Without ETAA		With ETAA			Without ETAA		With ETAA		
	N. tested	KDT50 (min)	N. tested	KDT50 (min)	SR	N. tested	KDT95 (min)	N. tested	KDT95 (min)	SR
Suru-léré	125	136.14	125	211.51	0.64	125	226.50	125	398.41	0.56

N. tested: number tested.

synergist EA in CDC bottles treated with DDT, the KdT50 value obtained with *A. gambiae* Akron populations was 62.22 min. This value was lower than the one obtained with DDT alone which was 81.78 min. A similar pattern was observed with KdT95 value obtained with these same populations which was 100.67 min after the addition of synergist EA. This value was slightly lower than the one obtained with DDT alone which was 101.99 min. SR (before addition of EA/after addition of EA) was 1.31 for KdT50 whereas it was 1.01 for KdT95.

The analysis of Figure 5 shows that after the addition of synergist EA to DDT 100 µg/bottle, the percentage of dead mosquitoes from Akron is higher than the one obtained with DDT alone. The use of synergist EA in bottles treated with DDT 100 µg/bottle did not eliminate DDT resistance, and the mortality rate increased from 0 to 18.18% ($P<0.05$). These results show that GSTs may play a role in *A. gambiae* Akron resistance to DDT.

DISCUSSION

A. gambiae Akron, Suru-léré and Bamè populations were resistant to both permethrin and DDT in 2008 and in 2013. In addition, higher mortality rates were observed with permethrin as compared to DDT in all *A. gambiae* tested populations in 2008 and in 2013 and may be explained by the presence of an additional resistance mechanism in Benin (e.g. “Leu-Ser” mutation) which might confer higher resistance to DDT than to permethrin (Ranson et al., 2000; Martinez-Torres et al., 1999). Djègbé et al. (2011) have recently showed a first evidence of the presence of *L1014S kdr* mutation in very few *A. gambiae* mosquitoes from West Africa.

A. gambiae Akron and Suru-léré populations were resistant to DDT in 2008 and still remained resistant to the same product in 2013. According to Akogbeto and Yakoubou (1999), the emergence of DDT resistance,

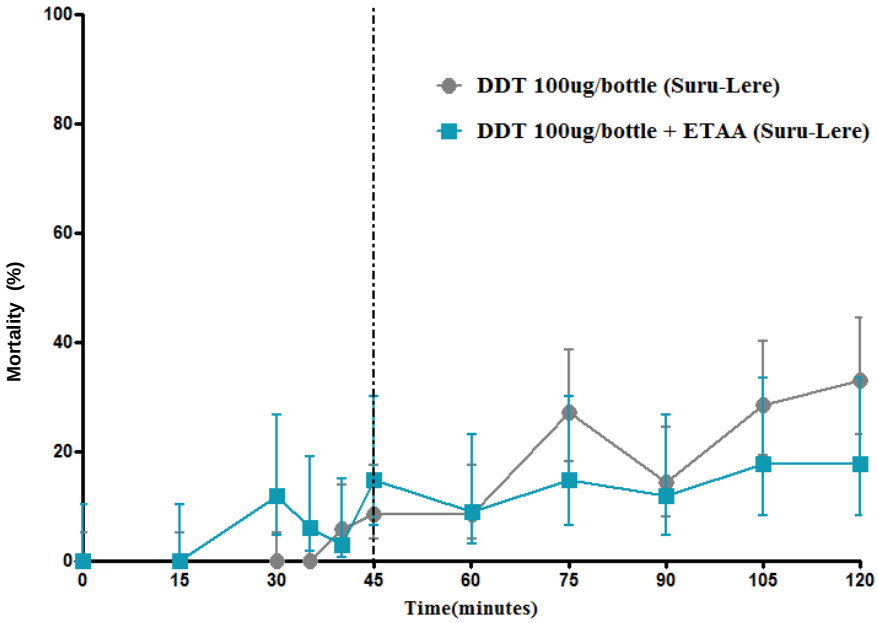

Figure 4. Implication of glutathione *S*-transferases in resistance of *A. gambiae s.l.* to organochlorine in Suru-léré locality.

Table 4. Knockdown time, KdT50 and KdT95 (minutes) of *Anopheles gambiae* Akron populations to DDT and DDT + ETAA.

Population	Without ETAA		With ETAA			Without ETAA		With ETAA		
	N. tested	KDT50 (min)	N. tested	KDT50 (min)	SR	N. tested	KDT95 (min)	N. tested	KDT95 (min)	SR
Akron	125	81.78	125	62.22	1.31	125	101.99	125	100.67	1.01

N. tested: number tested.

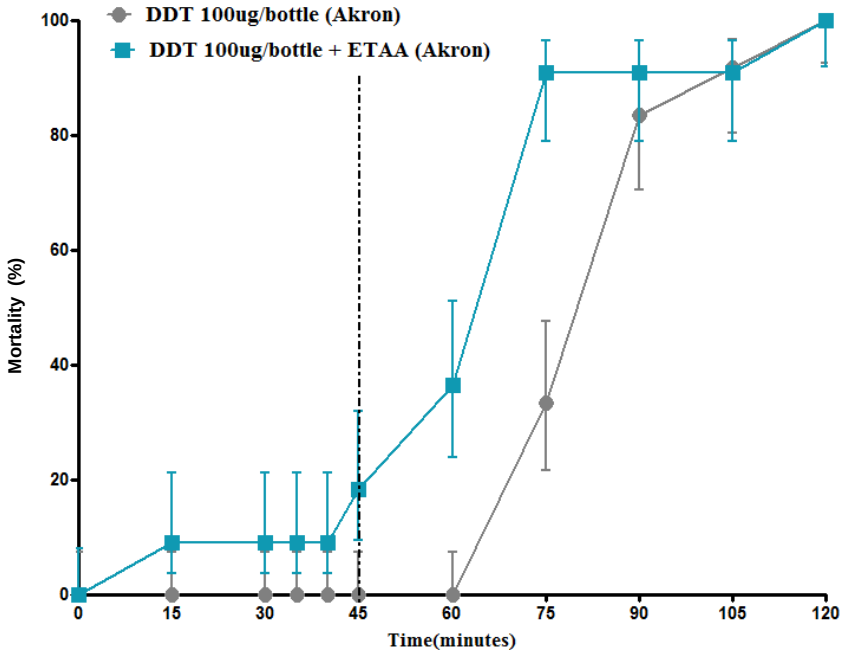

Figure 5. Implication of glutathione *S*-transferases in resistance of *A. gambiae s.l.* to organochlorine in Akron locality.

recorded in *A. gambiae* from meridian regions, was related to two phenomena: the massive use of DDT and dieldrin for house-spraying applications in southern villages from 1953 to 1960 during WHO programmes of malaria eradication and the massive use of organochlorine in agricultural settings during the 1950s (OMS, 1976). *A. gambiae* Akron and Suru-léré populations were resistant to lambdacyhalothrin in 2013. In addition to the lambdacyhalothrin resistance observed in *A. gambiae* Suru-léré populations in 2013; these populations were also resistant to permethrin.

Pyrethroid resistance in *A. gambiae* Suru-léré may be explained by increased use of household insecticide and availability of xenobiotics for larval breeding sites in the urban area. They were one of the possible factors selected for pyrethroid resistance in *A. gambiae* in urban areas (Aïzoun et al., 2014b). The underlying mechanism of resistance patterns observed in *A. gambiae* Suru-léré populations in 2013 was explored through a synergist assay. The synergist assay with PBO, an inhibitor of Cytochrome P450 mono-oxygenases, indicated that this enzyme family plays a little role in the permethrin resistance observed in Suru-léré. Indeed, the mortality rate to permethrin has slightly varied when mosquitoes are exposed to PBO. The use of synergist PBO to overcome permethrin resistance in *A. gambiae* Suru-léré showed that this synergist has partially inhibited mono-oxygenase activity and therefore slightly improved permethrin effectiveness in this *A. gambiae* population. In southern Benin, a recent study carried out by Aïzoun et al. (2013a), in Seme district precisely in Agbalilame locality have already suggested an implication of mono-oxygenases in resistance of *A. gambiae s.l.* to pyrethroids in Ouemé department. In this same department, another recent study, carried out by Aïzoun et al. (2013b), in Misserete district also suggested an implication of mono-oxygenases in resistance of *A. gambiae s.l.* to pyrethroids.

A. gambiae Suru-léré populations were resistant to DDT in 2008 and still remained resistant to the same product in 2013. The synergist assay with ETAA, an inhibitor of Glutathione S-transferases, indicated that this enzyme family also plays a little role in this high DDT resistance observed in Suru-léré. Indeed, the mortality rate to DDT was shown to vary slightly when mosquitoes were exposed to EA. The use of synergist ETAA to overcome DDT resistance in *A. gambiae* Suru-léré showed that this synergist has partially inhibited Glutathione S-transferases activity and therefore slightly improved DDT effectiveness in this *A. gambiae* population.

A. gambiae Akron populations were resistant to DDT in 2008 and still remained resistant to the same product in 2013 with an increase in the resistance level. The underlying mechanism of the resistance pattern observed in this population in 2013 was explored using a synergist assay. The synergist assay with ETAA, an inhibitor of glutathione S-transferases, indicated that this enzyme family also plays a role in this high DDT resistance observed in Akron. Indeed, the mortality rate to DDT was shown to vary significantly when mosquitoes were exposed to EA. The use of synergist ETAA to overcome DDT resistance in *A. gambiae* Akron showed that this synergist has inhibited Glutathione S-transferases activity and therefore improved DDT effectiveness in this *A. gambiae* population. So, in addition to the metabolic mechanism (cytochrome P450 genes) involved in *A. gambiae* Akron permethrin resistant (Djouaka et al., 2008), glutathione S-transferases may also play a role in *A. gambiae* Akron resistant to DDT. Thus, our study confirmed the study conducted by Djouaka et al. (2008) who reported on multiple insecticide resistance mechanisms in *A. gambiae* from Akron with emphasize on several additional potential resistance mechanisms which need further investigation.

Conclusion

This study shows that *A. gambiae s.l.* populations from central part and southern Benin were resistant to both DDT and pyrethroids. The resistance level of these populations to these products follows a dynamic process. Metabolic resistance conferred by detoxifying enzymes is an indication of phenotypic resistance to both DDT and pyrethroids in southern Benin. CDC biochemical assays using synergist are a key, quantitative, and reliable method in detection of metabolic mechanisms involved in the insecticide phenotype resistance in mosquito populations, malaria vectors.

Conflict of interests

The author(s) have not declared any conflict of interests.

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