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Original Article

Biochemical Mechanism of Insecticide Resistance in Malaria Vector, *Anopheles gambiae* s.l in Nigeria

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Abstract

Background: Malaria is a parasitic vector-borne disease endemic in the tropical and subtropical countries of the world. The aim of this study was to investigate the current activities of the detoxification enzymes in resistant and susceptible *Anopheles gambiae* s.l. in northern Nigeria.

Methods: *Anopheles* larvae were collected from northeast and northwestern Nigeria between Aug and Nov 2018. Biochemical analyses was carried out on the mosquitoes exposed to various insecticides (deltamethrin, DDT, bendiocarb, malathion) to measure and compare the enzymatic activities of the major detoxification enzymes (P_{450}, GSTs, Esterase).

Results: High levels of resistance was observed; DDT 37%-53% (95%, CI: 29-61), bendiocarb 44%-55% (CI: 39-60) and deltamethrin 74%-82% (CI: 70-86). However, these mosquitoes were found to be susceptible to malathion 99%-100% (CI: 98-100). The P450 and GSTs enzymes were found to be elevated in the resistant mosquitoes exposed to deltamethrin (1.0240±0.1902); (1.3088±1.2478), DDT (1.7703±1.4528); (1.7462±0.9418) and bendiocarb (1.1814±0.0918); (1.4479±1.0083) compared to the Kisumu strain (0.764±0.4226); (0.6508±0.6542), (0.3875±0.3482); (0.4072±0.4916) and (0.6672±0.3949); (0.7126±0.7259) at $P<0.05$. Similarly, the resistant mosquitoes expressed increased activity to esterase (0.7606±1.1477), (0.3269±1.1957) and (2.8203±0.6488) compared to their susceptible counterpart (0.6841±0.7597), (0.7032±0.5380) and (0.6398±0.4159) at $P<0.05$. The enzyme ratio was found to be: P450 (1.341, 4.568 and 1.77); GSTs (2.011, 4.288 and 2.031); Esterases (1.111, 0.469 and 4.408). One way Anova and single sample t-test were also conducted to determine the effect of the enzymes on the resistant and susceptible strains.

Conclusion: High level of insecticide resistance was observed with significant elevation of detoxification enzymes activities in the resistant mosquitoes.

Keywords: Detoxification enzymes; Resistant; Susceptible; *Anopheles gambiae*; Nigeria

Introduction

Malaria is a life-threatening parasitic vector-borne disease endemic in the tropical and subtropical countries of the world (1). Approximately 100 million cases of malaria and over 200,000 deaths are reported annually in Nigeria (2, 3). *Anopheles gambiae* and *An. funestus* species complexes are the main malaria vectors that transmit the malaria parasite in Africa (4-7).

Insecticide-treated nets (ITNs)/long-lasting insecticide-treated nets (LLINs) are the main control measures adopted in Nigeria for malaria vectors (8). Insecticide resistance is defined as "The ability in a population to tolerate doses of insecticide which would prove lethal to the majority of individuals in a normal population of the same species, developed as a result of selection pressure to the insecticide" (9).

The two most important mechanisms by which insects accomplish resistance are; metabolic pathway and target site insensitivity (8, 10-12). The metabolic pathway is activated when insects are exposed to toxic substances leading to either increase or decrease in the metabolism of the substance. Three major families of enzymes are involved in the detoxification of toxic substances in living organisms; Glutathione S-transferases (GSTs), carboxylesterase, and cytochrome P450 monooxygenase (P450s) (13). The GSTs are extensive group of detoxification enzymes that are cytosolic dimeric proteins with 2 domains, each containing two binding sites, the hydrophilic G site and the hydrophobic H site (14). Scientists have identified six different classes of GSTs in insects, which include Delta, Epsilon, Omega, Sigma, Theta and Zeta (15). The Delta and Epsilon are the two most important classes in insects due to the role they play in insecticide resistance to the major classes of insecticides (16). The GSTs confer resistance to the organophosphates by increasing GST detoxification rates through the O-dealkylation and O-dearylation pathways (17-19). The carboxylesterases are another enormous group of detoxification enzymes classified into A or B esterases based on substrate specificity for α or β-naphthyl acetate (20). They produce resistance by either sequestration or catalyzing the hydrolysis of carboxylic and phosphotriester bonds in a wide range of insecticides such as organophosphate, carbamates and pyrethroids (21-23).

The cytochrome P450 (monooxygenase) is one of the most comprehensive gene super-families present in all living organisms which play an integral role in the metabolism of endogenous and exogenous compounds such as steroids, fatty acids and xenobiotics (17, 24-26). Several P450 enzymes have been identified and are named as CYP followed by a number, a letter and a number respectively, for example, CYP6D1 (24). The CYP6D1 is over-produced in the house fly, *Musca domestica*, which results in high level of permethrin resistance, and a similar situation is seen with organophosphate resistance where CYP6A1 is also overproduced (15, 21). Elevated levels of monooxygenase have been reported from several species of mosquitoes, for example, CYP6Z1 was found to be associated with pyrethroids resistance in *An. gambiae* from Western Kenya (27), and CYP6P5 and CYP6AA2 have been copied from *An. minimus* resistant to deltamethrin in Thailand (26, 28).

Raised activities of detoxification enzymes have been reported in resistance populations of *An. gambiae* from Nigeria and *An. sinensis* from China (12, 29-32).

Obtaining the baseline information about the vector susceptibility and detection of resistance mechanisms are very important steps in the resistance management strategies (33-35). There are reports on the rising level of *An. gambiae* resistance to commonly used insecticides in Nigeria. However, very few studies have reported on the metabolic mechanism of resistance.

Therefore, this study aimed to investigate the current activities of the detoxification enzymes in resistant and susceptible *An. gambiae* s.l. mosquitoes exposed to some WHO recommended insecticides in northern Nigeria.

Methods

The inclusion criteria for the selection of the study area sampled included presence of vector control and irrigation activity, availability and high density of target species, high intensity of malaria transmission and paucity of data on biochemical mechanism of insecticide resistance to the principal malaria vector *An. gambiae* s.l.

Study Location

The study was conducted in three locations Yamaltu Deba (Gombe state), Auyo (Jigawa state) and Kumbotso (Kano state) within north-east and northwestern Nigeria between Aug and Nov, 2018 (Fig. 1).

Yamaltu Deba (10° 13' 0" N, 11° 23' 0" E) is one of the 11 Local Government Areas in Gombe State, Nigeria. It has a population of 255,248 and an area of 1,981 km² (36, 37).

Auyo (12°21'N, 9°59'E) is a locality in Jigawa State, northwestern Nigeria. The town is known for its irrigation activities in which rice and vegetables are produced. It has a total population of 132,001 with estimated landmass area of 512 km (10).

Kumbotso (11°53'17"N, 8°30'10"E) is situated in Kano state, northwestern Nigeria with a population of 409,500 and an area of 158 km² (9, 38).

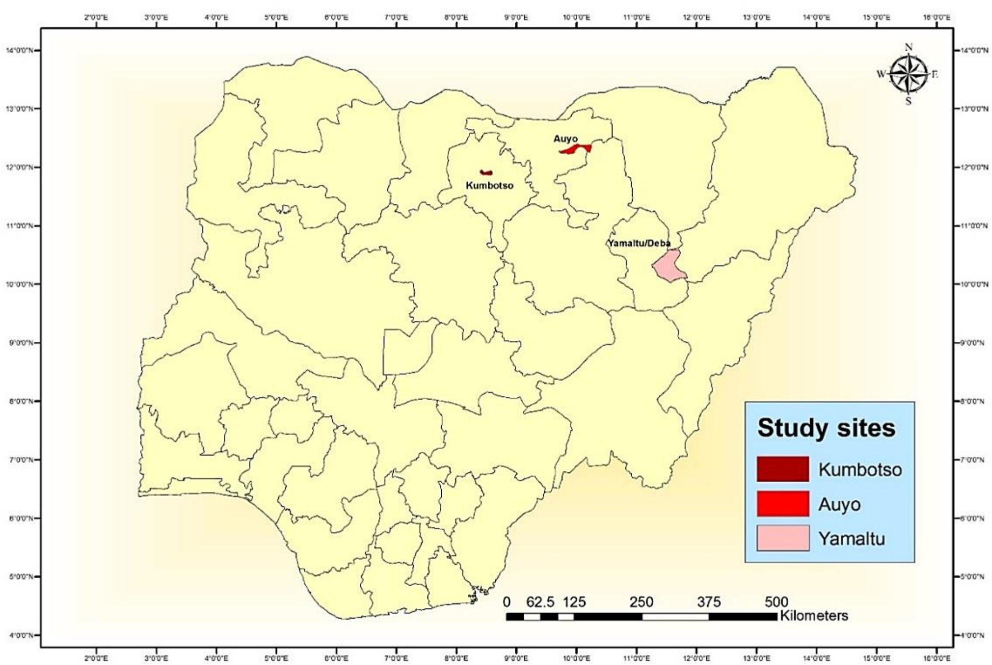

Fig. 1: Map showing the geographical locations of the study sites in Nigeria, 2018

Study Sample

Dipping method was used to collect field strain larvae samples from different breeding places in the study sites (8, 35) to provide laboratory stock of mosquitoes. The Kisumu, an *An. gambiae* laboratory susceptible strain was originated from Kenya in 1953 and was kept in the insectary (39).

WHO Susceptibility Test

Adult susceptibility test was conducted according to the recent WHO bioassay guideline (34).

Enzyme Analyses

The protocol of WHO/WHOPES 2008 (23) was used to perform the biochemical assays. Although it might not be based on the cited reference as it emphasises using mosquito specimens unexposed to insecticides, we exposed our specimens to insecticide before biochemical assays, to compare the levels of detoxifying enzymes in live and dead mosquitoes. A total of 288 mosquitoes from the study locations were used during the assay (Table 1) and the activity of detoxification enzymes mainly glutathione S-transferases (GSTs), esterase and P450s were measured.

Table 1: Specific activities of detoxification enzymes (Mean ± SD) in *Anopheles gambiae* mosquitoes exposed to insecticides collected from the study locations in Nigeria 2018

Insecticides	*No. Tested*	*GSTs (Mean ± SD) (μmol/min/mg protein)*	*Enzyme Ratio (ER)*	*Esterase (Mean ± SD) (μmol/min/mg protein)*	*Enzyme Ratio (ER)*	*P450 (Mean ± SD) (p450/mg protein)*	*Enzyme Ratio (ER)*
Deltamethrin R [a]	48	1.3088±1.2478	2.011	0.7606±1.1477	1.111	1.0240±0.1902	1.341
Deltamethrin S [a]	48	0.6508±0.6542	1	0.6841±0.7597	1	0.764±0.4226	1
DDT R [b]	48	1.7462±0.9418	4.288	0.3269±1.1957	0.465	1.7703±1.4528	4.568
DDT S [b]	48	0.4072±0.4916	1	0.7032±0.5380	1	0.3875±0.3482	1
Bendiocarb R [c]	48	1.4479±1.0083	2.031	2.8203±0.6488	4.408	1.1814±0.0918	1.77
Bendiocarb S [c]	48	0.7126±0.7259	1	0.6398±0.4159	1	0.6672±0.3949	1

R: resistant, S: susceptible
Values with similar superscripts indicate significant difference (P<0.05) when the groups were compared.

Esterase Assay

General esterase activity was measured using naphthyl acetate in a reaction mixture containing 20 μl of the homogenate and 200 μl of naphthyl acetate solution (120 μl of 30 mM alpha- or beta-naphthyl acetate dissolved in 12 ml 0.02 M phosphate buffer pH 7.2), respectively. The enzyme hydrolysis paranitrophenyl acetate to acetate forming a yellow color that maximally absorbs light at 570 nm wavelength. After incubating the mixtures at room temperature for 15 min, 50 μl of fast blue solution (0.023 g fast blue dissolved in 2.25 ml distilled water and 5.25 ml of 5% SDS .1 M sodium phosphate buffer pH 7) was added to each microplate well. After another incubation for 5 min at room temperature, the absorbance was measured at 570 nm.

Cytochrome P_{450} (Monooxygenase) Assay

The P_{450} measurement followed Safi et al. (39) method. The monooxygenase catalyze the reduction of hydrogen peroxide and oxidation of tetramethyl benzidine (TMBZ) to form water and oxidized blue color TMBZ which absorbs light at 450 nm wavelength. The reaction mixture in each well consisted of 20 μl of the homogenate, 80 μl of 0.0625 M potassium phosphate buffer pH 7.2, 200 μl of 3,3',5,5' TMBZ solution (0.01 g TMBZ dissolved in 5 ml methanol plus 15 ml of 0.25 M sodium acetate buffer pH 5.0) and 25 μl of 3% hydrogen peroxide. The absorbance was measured at 450 nm as an endpoint after incubating the plate at room temperature for 2 hours.

Glutathione S transferase (GST) Assay

The GSTs measurement also followed Safi et al. (40) method. Ten microliter of the homogenate was mixed with 200 ul reduced glutathione plus 1-chloro-2,4-dinitrobenzene (CDNB) solution (10 mM reduced glutathione dissolved in 0.1 M phosphate buffer pH 6.5 and 3 mM CDNB originally dissolved in methanol). The absorbance was measured at 340 nm for 5 min.

Data Analysis

Microsoft office excel, version 2003 was used to create charts, calculate the standard deviation, sort and clean the data. While SPSS version 16 was used to calculate the means of the variable using the Chi X^2 and Student's t-test. Moreover, one way ANOVA was conducted to compare the effect of the detoxification enzymes on the resistant and susceptible strains. Enzyme ratios (ER) were calculated by dividing the mean activities or content of the enzymes in the resistant with those of the susceptible mosquitoes. The Beer–Lambert law (41) was used to convert the absorbance, optical density, (OD) into the actual activity for GST and esterases while a standard curve of cytochrome C was used to obtain a crude estimate of the amount of monooxygenase present. The Beer's equation states that: $A=\varepsilon lc$ Where: ε, is the molar attenuation coefficient or absorptivity of the attenuating species; l, is the optical path length; c, is the concentration of the attenuating species. A standard protein curve (42) with straight-line equation (y=mx+c; y=absorbance, x=BSA concentration, m=gradient (0.7079), c=intercept (0.0058)) was used to calculate the unknown protein concentration of the mosquitoes.

Ethics approval

Ethical approval number: IR.TUMS.SPH.REC.1397.150 from Tehran University of Medical Sciences.

Results

Bioassay

A. gambiae mortality 24 h post-exposure to Malathion was found to be 99%-100% (CI: 98-100). Mortality for deltamethrin was 74%-82% (CI: 70-86), bendiocarb 44%-55% (CI: 39-60) and DDT 37%-53% (CI: 29-61), respectively (Fig. 2A-C). According to the knock-down effect, malathion showed a high knock-down effect, after 1 h 100% (SE=0.0), 92% (SE=0.19) and 88% (SE=0.23) of the *An. gambiae* were knocked-down across the study sites (Fig. 2A-C). Deltamethrin, DDT and bendiocarb showed a lower knock-down effect after one hour of between 16%-56% (SE=0.26-0.36) across the study locations (Fig. 2A-C).

Biochemical

Overall, 288 mosquitoes were used during the biochemical assay (Table 1). The P450 (p450/mg protein) enzyme was found to be elevated in the resistant mosquitoes exposed to deltamethrin (1.0240±0.1902), DDT (1.7703±1.4528) and bendiocarb (1.1814±0.0918) compared to the susceptible mosquitoes exposed to the same insecticides (0.764±0.4226), (0.3875±0.3482) and (0.6672±0.3949) and was statistically significant at $P<0.05$ (Table 1).

The activity of GSTs (µmol/min/mg protein) was also found to be elevated in the resistant mosquitoes exposed to deltamethrin (1.3088±1.2478), DDT (1.7462±0.9418) and bendiocarb (1.4479±1.0083) compared to the susceptible mosquitoes exposed to the same insecticides (0.6508±0.6542), (0.4072±0.4916) and (0.7126±0.7259) and was statistically significant at $P<0.05$ (Table 1). Similarly, the resistant mosquitoes expressed increased activity to esterase (µmol/min/mg protein) in deltamethrin (0.7606±1.1477), DDT (0.3269±1.1957) and bendiocarb (2.8203±0.6488) compared to their susceptible counterpart (0.6841±0.7597), (0.7032±0.5380) and (0.6398±0.4159) at $P<0.05$ (Table 1).

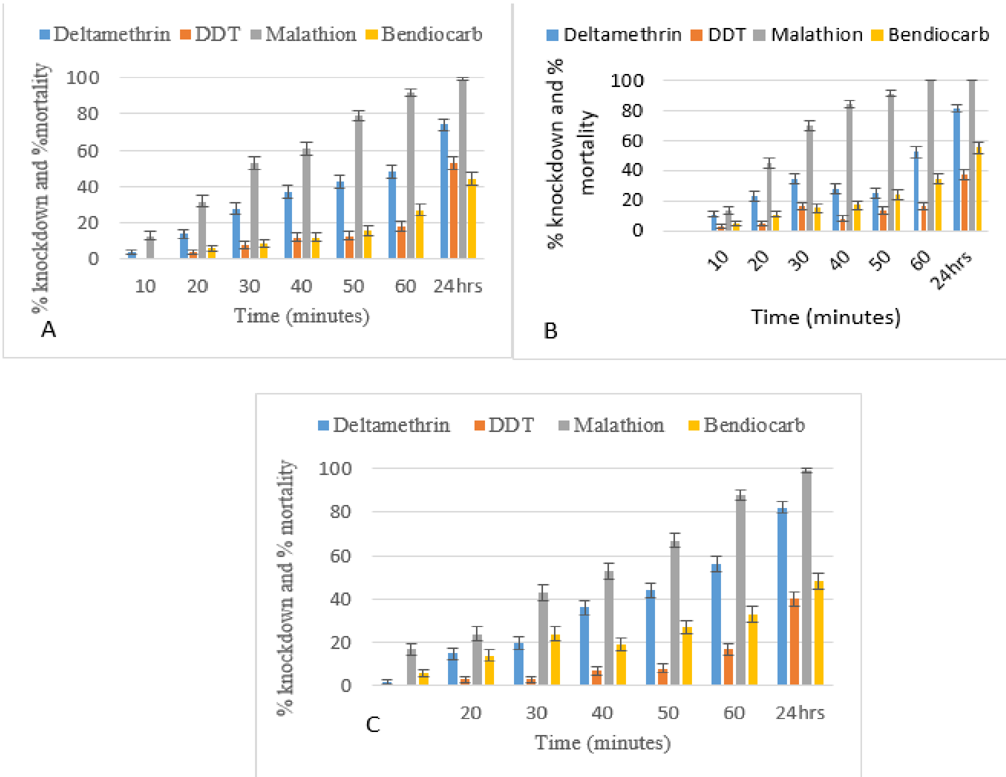

Fig. 2: Knockdown profile (10-60 mins) of *An. gambiae* s.l. from: (A) Yamaltu Deba (Gombe state), (B) Auyo (Jigawa state) and (C) Kumbotso (Kano state) Nigeria, 2018

The enzyme ratio was also calculated and found to be: P450 (1.341, 4.568 and 1.77); GSTs (2.011, 4.288 and 2.031); Esterases (1.111, 0.469 and 4.408) for Gombe, Auyo and Kumbotso (Fig. 3) respectively.

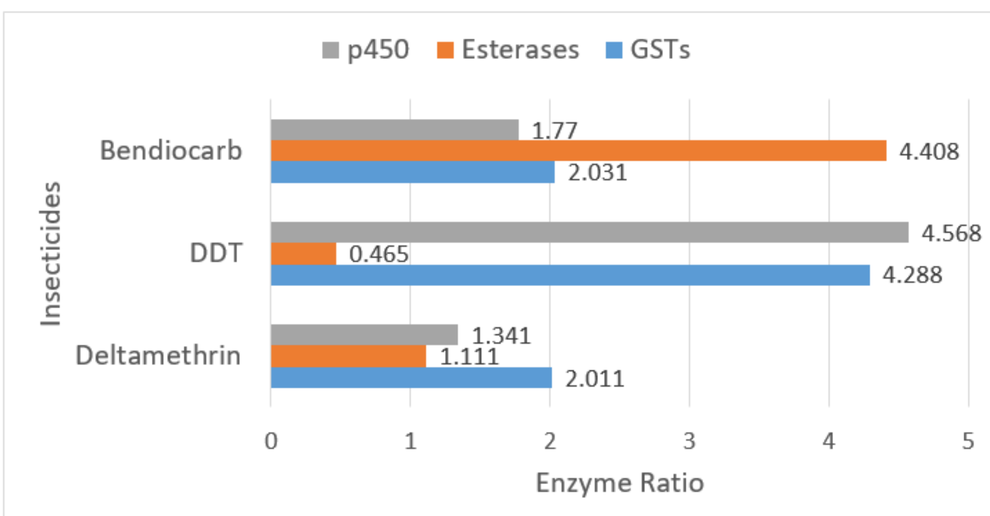

Fig. 3: Activities and enzyme rations of the main detoxification enzymes exposed to 3 insecticides from: (A) Yamaltu Deba (Gombe state), (B) Auyo (Jigawa state) and (C) Kumbotso (Kano state) Nigeria, 2018

A one-way Anova was conducted to compare the effect of the detoxification enzymes on the resistant and susceptible strains and there was a significant effect at the $P<0.05$ level for the 3 enzymes; P450: [f (1, 94) =235, *P*: 0.000], GSTs: [f (1, 94) = 17.5, *P*: 0.000], Esterase: [f (1, 94) = 35.1, *P*: 0.000]. Post hoc multivariate comparison using the Tukey HSD test indicated that the means for the resistant strains from the detoxification enzymes (m=4.20, SD=1.30) was significantly different from the susceptible strains.

A single sample *t*-test was also conducted to determine if a statistically significant difference existed between the detoxification enzyme values and the exposed mosquitoes. Statistically, significant association was found among the 3 different detoxification enzymes. P450: (m = 1.58, SD=0.0506) *t* (96)=31.3, 0.000. GSTs: (m=1.47, SD=0.501) *t* (96) =28.68, 0.000. Esterase: (m=1.53, SD: 0.501) *t* (96)=29.91, 0.000.

Discussion

The mosquitoes from the study locations were found to be highly resistant to DDT, bendiocarb and moderately resistant to deltamethrin. However, these mosquitoes were susceptible to only malathion (Fig. 2A-C). Bendiocarb showed very high level of resistance across all the study sites (Fig. 2A-C). This finding agrees with studies from Nigeria and Ghana (5, 40, 43). The high carbamate resistance recorded in this study despite the cross-resistance with organophosphates, to which the *An. gambiae* mosquitoes were highly susceptible may have resulted from the elevated levels of esterase, reported to be a primary mechanism involved in organophosphate and carbamate resistance (44). This study reports moderate level of resistance to the deltamethrin (Fig. 2A-C). This finding is in agreement with studies conducted in Nigeria where they reported mortality of 78%-83% (10, 43). However, other studies within and outside Nigeria disagree with our finding (4, 5, 12, 42). The marginal resistance observed with deltamethrin in this study from all the locations indicates that detoxification enzymes alone might not be conferring resistance to pyrethroids; other mechanisms such as the kdr may be playing a significant role (10). DDT shows very high level of resistance (Fig. 2A-C). This finding is in agreement with previously conducted studies in Nigeria (4, 5, 8, 10, 11, 45). Malathion was the only insecticide susceptible in accordance with studies from different regions within and outside Nigeria (4, 10, 41, 46, 47).

Deltamethrin showed low knockdown effect after 24 h exposure (Fig. 2A-C). This finding agrees with previous studies reported (32). Similarly, bendiocarb and DDT recorded very low knockdown after 24 h exposure (Fig. 2A-C) This agrees with studies conducted in Nigeria and other parts of the world (4, 10, 32, 41, 43, 44). However, Habibu and colleagues reported very high knockdown to DDT (8).

The biochemical analysis of the metabolic detoxifying enzymes revealed elevation of the enzymes in the resistant mosquitoes compared to the susceptible populations across all the study locations. Taken together, these results suggest that high levels of detoxification enzymes do affect the susceptibility patterns of *An. gambiae* mosquitoes in the study area. Specifically, our results suggest that high levels of these enzymes reduce the susceptibility of mosquitoes to insecticides and confer resistant to them. Based on the results of the biochemical assay, deltamethrin insecticide showed high metabolic enzyme activities in the following order of increase GSTs>p450>esterases while for the DDT it was p450>GSTs>esterase. As esterases are not involved in DDT resistance, the levels of esterases in the susceptible strain are almost twice the level in the DDT resistance mosquitoes and the reduction is because of the fitness cost. It means mosquitoes overproduce only enzymes that are critical and more important for their survival and conserve energy than producing other enzymes that are not very important to their survival in the face of insecticide being used. Therefore, in this case, because GSTs and p450 are more important in DDT resistance than esterases, the mosquitoes here over produced GSTs and p450 but it down regulated the production of esterases. In the bendiocarb resistance, we have raised enzyme ratio for esterase followed by GSTs and then p450. This is very correct, because in bendiocarb resistance the order of importance of these enzymes are esterases followed by GSTs and then p450. Therefore, esterases has the highest activity, which clearly explains the mechanism of resistance to bendiocarb. This finding agrees with previous studies conducted in Kano and Jigawa in northern Nigeria, where increased activity was seen with GSTs, esterase and monooxygenase in resistant populations of *An. gambiae* exposed to DDT, bendiocarb and deltamethrin (12, 31, 32). Similarly, some studies conducted in Cote d'lvoire and Dakar reported increased levels of monooxygenase, GSTs and esterase (30, 48). Other studies from China and Afghanistan, also reported raised activities of detoxification enzymes (29, 39).

Conclusion

Very high level of insecticide resistance was observed in this study with significant elevation of the detoxification enzymes activities both in the resistant and susceptible mosquitoes. Therefore, a recommendation to the malaria program in Nigeria is that it can adopt the control programme using Malathion since the programme cannot use deltamethrin or bendiocarb, DDT is out of the picture since it's not recommended for IRS in Nigeria. We also recommend considering the distribution of PBO nets where we observed high content of p450 in Anopheles populations.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that they have no competing interest.

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