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Cytochrome P450 Mono-Oxygenase and Resistance Phenotype in DDT and Deltamethrin-Resistant Anopheles gambiae (Diptera: Culicidae) and Culex quinquefasciatus in Kosofe, Lagos, Nigeria

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Vector Control, Pest Management, Resistance, Repellents

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Cytochrome P450 Mono-Oxygenase and Resistance Phenotype in DDT and Deltamethrin-Resistant *Anopheles gambiae* (Diptera: Culicidae) and *Culex quinquefasciatus* in Kosofe, Lagos, Nigeria

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Abstract

Pyrethroids and DDT are key insecticides in the control of malaria, yellow fever, and lymphatic filariasis vectors. Knockdown and metabolic resistance mechanisms have been proven to be important in determining the efficacy of insecticides. Here we investigated cytochrome P450 as a resistance mechanism in *Anopheles gambiae* Giles and *Culex quinquefasciatus* Say exposed to deltamethrin and DDT. Two- to three-days-old adult female mosquitoes were used for insecticide exposures and PBO synergistic assays using WHO standard guidelines, kits and test papers (DDT 4%, deltamethrin 0.05%, and PBO 4%). Polymerase chain reaction (PCR) assays were used for the identification of the species and for characterization of the *kdr* allele. Mortality at 24 h post-exposure was 18 and 17% in *An. gambiae* s.s. exposed to DDT and deltamethrin, respectively; 1 and 5% in *Cx. quinquefasciatus* exposed to DDT and deltamethrin respectively. Significant ($P < 0.01$) levels of susceptibility was recorded in mosquitoes pre-exposed to PBO, as KDT_{50} and 24 h of exposure ranged from 37.6 min to 663.4 min and 27 to 80%, respectively. Presence of a knockdown resistance allele was recorded in *An. gambiae* s.s., 22.5% for homozygote resistance and 7.5% for heterozygotes, while *Cx. quinquefasciatus* populations showed no *kdr* allele despite the high level of resistance to DDT and deltamethrin. Findings from this study indicated that cytochrome P450 mono-oxygenase expression is highly implicated in the resistance phenotype to DDT and pyrethroids in *An. gambiae* and *Cx. quinquefasciatus* in the study area.

Key words: cytochrome P450 mono-oxygenase, knockdown resistance, pyrethroid, DDT, mosquito

Mosquitoes are important vectors of several infectious diseases such as yellow fever, malaria, filariasis, dengue, and other arboviruses (Nkya et al. 2013, Wan-Norafikah et al. 2013, Okorie et al. 2014). In Nigeria, *Anopheles* and *Culex* mosquitoes have been reported to be important vectors of malaria and lymphatic filariasis (Okorie et al. 2014). Effective vector control, therefore, seems to be a more realistic means of preventing mosquito-borne diseases in that it protects individuals from infective mosquito bites (WHO 2007). The control of malaria and other mosquito-borne diseases highly depends on the use of insecticides. Long-lasting insecticidal nets (LLINs) and Indoor residual spray (IRS) are the main strategies for malaria vector control and interruption of malaria transmission (WHO 2006, 2011). Increased insecticidal intervention can result in mass killing of vector populations leading to the protection even of

those people in a community who are not directly covered by LLINs or IRS, ultimately reducing the capacity of mosquitoes to transmit malaria and other mosquito-borne diseases (Escamilla et al. 2017, WHO 2018). Indeed mosquito control has played a crucial role in the reduction of the morbidity and mortality rates due to malaria globally and also has been outlined to play a major role in the Global Technical Strategy for malaria 2016–2030 (WHO 2018). Increased usage of pesticides in agriculture and public health has led to mosquito resistance to the different classes of insecticides recommended for use by WHO (Brogdon and McAllister 1998, Corbel and N'Guessan 2013). Insecticide resistance has become a serious concern in all insect vectors of emerging diseases (Hemingway and Ranson 2000). Insecticide resistance can be mediated by changes in one or more genes, leading to the reduction in insecticide sensitivity

of an insect population (Soko et al. 2015). Malaria vector resistance to DDT and pyrethroid insecticides has been reported to be mainly caused by knockdown resistance (*kdr*; Martinez-Torres et al. 1998). Insecticide resistance in vectors may not only be gene based but can also be enzymatic which can be genetically or not genetically related (Hemingway et al. 2004, Corbel et al. 2007, Liu 2015). Increased enzymatic activities resulting in the detoxification of insecticide has been linked to cytochrome P450s mono-oxygenases (P450s), carboxylesterases (COEs), and glutathione-S-transferases (GSTs) (Chouaïbou et al. 2014, Scott et al. 2015, Soko et al. 2015). Synergists are nontoxic but are used in combination with insecticides to enhance potency and counter-measure insecticide resistance in insects, including mosquitoes. Piperonyl butoxide (PBO) is a synergist that inhibits cytochrome P450 enzymes within mosquitoes, and has been incorporated into pyrethroid-treated LLINs to form PBO-combination nets (Gleave et al. 2017). This study was aimed at assessing the impact of cytochrome P450 mono-oxygenases and *kdr* alleles on the insecticidal resistance phenotypes following exposure to DDT and deltamethrin by *Culex quinquefasciatus* Say and *Anopheles gambiae* Giles mosquitoes collected from the Kosofe Local Government Area of Lagos State.

Materials and Methods

Study Area and Sample Collection
Lagos State is one of the six states in southwestern Nigeria, and shares a boundary with Ogun State to the north and east, and with Republic of Benin to the west. The two main climatic seasons in Lagos are dry (November to March) and rainy (April to October), with rainfall of between 1,400 mm to 1,800 mm received by the state annually, and the temperature can be as high as 30 to 38°C. The state has a population of about 9.01 million people (6.44%) of 140.003 million of the Nigeria total population, according to the 2006 national population census (NPC). Lagos State has 20 Local Government Areas (LGAs), each LGA having varying amounts of urban, semi-urban and rural areas (Ayeni 2014). The study was carried out in Kosofe LGA of Lagos State, situated at 6°36′N, 3°25′E, and 36 meters' above sea level, where several man-made ponds created by construction work and a poor drainage system provide favorable breeding sites for mosquitoes that transmit diseases in the study area. *Anopheles* and *Culex* larval samples were collected between June 2017 and May 2018 in different communities of the LGA. Larval collections were obtained from different suitable mosquito breeding sites using a 350-ml dipper and transferred to well-labeled plastic containers before being transferred to the insectary for the emergence of adult mosquitoes. Adult mosquitoes were fed with 10% sugar solution.

Insecticide Susceptibility and Resistance Tests
The tests were performed using WHO test filter paper impregnated with the selected insecticides purchased from the Vector Control Research Unit (VCRU), University Sains Malaysia (http://www.inreskit.usm.my). Non-blood fed, 2- to 3-d-old female mosquitoes were exposed to DDT (4%) and deltamethrin (0.75%) using WHO insecticide-treated paper in groups of 25. Each experiment consisted of four replicates. The mosquitoes were exposed for an hour with the assay cylinders in a vertical position. Knockdown rates of mosquitoes were recorded at 10-min intervals for 1 h and then were transferred into tubes with untreated papers and allowed a 24-h recovery period after which mortality was recorded. All the bioassays were accompanied by negative controls, where 25 female

mosquitoes were exposed to insecticide-free plain papers in test tubes. The mosquitoes were supplied with a 10% sugar meal during the recovery period (WHO 2016).

PBO Synergist Assay
Synergist assays were conducted using 4% PBO impregnated papers. A total of 25 female *An. gambiae* and *Cx. quinquefasciatus* were exposed to PBO for 1 h using four replicates. Thereafter, PBO treated mosquitoes were exposed to either DDT (4%) or deltamethrin (0.05%) for another 1 h. In parallel with the treatment groups, two controls (one for PBO only) were run within the time of testing. Knockdown rates were taken at intervals for 60 min, thereafter mosquitoes were transferred and kept inside holding tubes for 24 h, and mortality was recorded (WHO 2016).

DNA Extraction
A whole mosquito from the insecticide susceptibility bioassay test was homogenized in a 1.5-ml micro-tube using 100 µl of grinding buffer with the aid of a plastic pestle. 20 µl proteinase K was added to the homogenized mosquito, the samples were incubated at 65°C in a dry bath for 30 min. Thereafter, the samples were placed on ice for 30 min and then centrifuged at 14,000 × g for 15 min. The supernatants were then transferred to sterile micro-tubes (1.5 ml); 200 µl ice cold (100%) ethanol was added and kept at room temperature for 5 min. The mixture was centrifuged for 20 min at 14,000 × g and supernatant was carefully discarded. The pellet was washed carefully with 200 µl of 70% ice cold ethanol, followed by 100% ice cold ethanol. The tubes were dried using a speed-vac. The DNA was re-suspended in 100 µl of Tris-EDTA buffer (Collins et al. 1987).

Molecular Identification of *An. gambiae* and *Cx. pipiens* mosquitoes complex
Molecular identification of mosquito samples was carried out according to the method described by (Collins et al. 1987) for *An. gambiae,* four primers including, ME (TGACCAACCCACTCCCTTGA), AR (AAGTGTCCTTCTCCATCCTA), QD (CAGACCAAGAT GGTTAGTAT), UN A (GTGTGCCCCTTCCTCGATGT), GA (CTGGTTTGGTCGGCACGTTT) were used, this was done to identify sibling species of the *An. gambiae s.s.* complex.

For *Culex*; Three primers, ACEquin (5′-CCTTCTTGAATGGC TGTGGCA-3′), ACEpip(GGAAACAACGACGTATGTACT-3′ and B1246s (5′TGGAGCCTCCTCTTCACGG-3′) were used to amplify a 274 bp diagnostic fragment of the extracted DNA according to (Smith and Fonseca 2004). This was done to identify two members of *Culex pipiens* complex which are; *Culex quinquefasciatus* Say (Diptera: Culicidae) and *Culex pipiens* L. Gel electrophoresis was used to analyze the amplified fragments using 1.5% agarose gels and were visualized by ethidium bromide staining under Ultra Violet light (UV light).

Detection of *kdr* Mutation
kdr assay were carried out as described by (Martinez-Torres et al. 1998). For the PCR assays, primers used were: Agd1(5′-ATAGATTCCCCGACCATG-3′), Agd2(5′AGACAAGGATGATGA ACC-3′), Agd3(5′-AATTTGCATTACTTACGACA-3′) and Agd4(5′-CTGTAGTGATAGGAAATTTA-3′). The PCR conditions included initial denaturation at 95°C for 5 min, then 40 cycles at 95°C for 1 min, 48°C for 2 min, 72°C for 2 min. Final extension was at 72°C for 10 min, and hold at 10°C.

The PCR reactions were conducted by adding 12.5 μl of PCR master mix containing 1×PCR buffer which is made up of 1.5 mM MgCl$_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, one unit of Taq polymerase, and 1 μl of genomic DNA into each 20 μl tube. 10 μl of PCR product with 1ul of loading buffer and 10 μl of standard markers per gel were loaded into each well of the prepared agarose gels. Gel electrophoresis was used to analyze the amplified fragments using 1.5% agarose gels and were visualized by ethidium bromide staining under UV light.

Data Analysis

Insecticide susceptibility was based on the criteria that 98–100% mortality of mosquito indicates susceptibility, 80–97% mortality implies potential resistance that needs to be confirmed via biochemical assays, and <80% mortality implies resistance (WHO 2016). Regression probit analysis was used to compute KDT$_{50}$ and KDT$_{95}$. Chi-square analysis was used to compare percentage mortality between insecticide only and PBO + insecticide. All data analyses were computed using Microsoft Excel version 2016 and IBM SPSS Statistics 23.

Results

Results from WHO insecticide resistance bioassays showed that both *An. gambiae* and *Cx. quinquefasciatus* in Kosofe LGA are resistant to DDT (4%) and deltamethrin (0.05%) insecticides. Percentage mortalities after 24 h was 18 and 17% for *An. gambiae*, and 1 and 5% for *Cx. quinquefasciatus* to DDT and deltamethrin, respectively. *An. gambiae* exposed to deltamethrin had the highest percentage knockdown after 60 min with 51%, while *Cx. quinquefasciatus* exposed to both DDT and deltamethrin had the lowest percentages of 1%. PBO synergistic assays for both DDT and deltamethrin showed significant increases ($P > 0.01$) in the 24 h percentage mortality for *Cx. quinquefasciatus* and *An. gambiae*. KDT$_{50}$ and KDT$_{95}$ for PBO plus assayed insecticides was lower than that of insecticide only, the lowest KDT$_{50}$ of 37.6 min and 59.6 min recorded in PBO + deltamethrin for both *Cx. quinquefasciatus* and *An. gambiae*, respectively (Table 1). *kdr* allele frequencies of 27.5% was recorded in the *An. gambiae* resistant to DDT and deltamethrin in this study, 22.2% for homozygote resistant (RR) and 6.9% for heterozygote resistant (RS). No *kdr* alleles were detected in *Cx. quinquefasciatus* resistant to DDT and deltamethrin (Table 2). Figures 1 and 2 compare the percentage progressive knockdown of resistant populations of *An. gambiae* and *Cx. quinquefasciatus* to DDT and deltamethrin with that of PBO plus insecticide assays. All except deltamethrin and PBO + deltamethrin for *An. gambiae* showed higher knockdown for the 60-min exposure period, and the final mortality after the 24-h holding period for deltamethrin was reduced compared to what was recorded for the 60-min exposure period.

Discussion

Increasing mosquito resistance to DDT and pyrethroid insecticides could be detrimental to the Roll Back Malaria (RBM), Sustainable Development Goals (SDGs), and WHO Action and Investment against Malaria (AIM) goal of reducing malaria incidence and mortality rate by 90% in the year 2030 (WHO 2015, 2017a, 2017b), and also the control of other mosquito-borne diseases. In this study, high levels of resistance were recorded in *An. gambiae* and *Cx. quinquefasciatus* to DDT and deltamethrin. Similarly, mosquitoes resistant to DDT and pyrethroid insecticides have been widely reported in southern Nigeria (Awolola et al. 2009, Oduola et al. 2010). Indiscriminate use of pyrethroids has led to ineffectiveness of vector control activities as a result of selection pressure. Mosquitoes that are already resistant to DDT used for agriculture or public heath purposes can easily develop resistance to pyrethroid due to cross resistance (Wan-Norafikah et al. 2013, Dadzie et al. 2017). Resistance to DDT and pyrethroids is of major concern, especially in malaria vector control considering that IRS and LLINs are the main vector control tools.

Results in this study showed that the *kdr* mutation was present in *An. gambiae* with homozygote (RR) and heterozygote (RS) alleles present in resistant populations, similar to previous reports in West Africa (Awolola et al. 2007, 2009; Corbel et al. 2007). The *kdr* allele frequency in DDT and permethrin resistant mosquitoes in this study significantly departs from the Hardy–Weinberg expectation at the 95% confidence level, and this may be related to heterozygote deficits. Target site protein mutations are likely the best-understood resistance mechanism found in insects to pyrethroids and DDT (Nkya et al. 2013). These changes in target sites are widely referred to as '*kdr*' mutations because they have been associated with the reduction in the knockdown effect of the insecticide.

No *kdr* mutation was found in *Cx. quinquefasciatus* resistant to DDT and deltamethrin, though some previous reports in different parts of Africa have reported *kdr* mutations in DDT and pyrethroid resistant *Cx. quinquefasciatus* in Benin Republic (Yadouléton et al. 2015), Zanizibar (Jones et al. 2012), and *Cx. pipiens* in Morocco (Tmimi et al. 2018).

The study showed that PBO synergist significantly suppresses the resistance of *An. gambiae* and *Cx. quinquefasciatus* to DDT and deltamethrin. The combination of PBO and deltamethrin achieved faster knockdown rates compared to PBO plus DDT, as KDT$_{50}$ of 37.6 min and 59.6 min was recorded for *Cx. quinquefasciatus* and *An. gambiae* s.s., respectively. Similar to several previous studies from around the world, PBO has significantly increased the susceptibility of resistant mosquitoes, e.g., *An. gambiae* s.s. showed increased susceptibility to pyrethroid insecticides in Ghana (Dadzie et al. 2017) and Cote d'Ivoire (Chouaïbou et al. 2014), increased susceptibility to DDT and permethrin in *An. gambiae* in Benin Republic (Aïzoun et al. 2014), increased susceptibility of *Cx. quinquefasciatus* larvae to permethrin

Table 1. Knockdown times and percentage mortality of *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes exposed to DDT and deltamethrin in Kosofe LGA, Lagos State

Mosquito species	Insecticide	Number exposed (*N*)	KDT$_{50}$ (min)	KDT$_{95}$ (min)	Mortality (%) after 24 h	*P* value	Status
An. gambiae s.s.	DDT	100	663.4	14219.1	18	0.003	Resistant
	PBO + DDT	100	144.1	4015.3	41		
	Deltamethtrin	100	91.8	563	17	0.000	Resistant
	PBO + deltamethrin	100	59.6	144.1	80		
Cx. quinquefasciatus	DDT	100	679	3,719	1	0.000	Resistant
	PBO + DDT	100	170.8	2132.2	27		
	Deltamethrin	100	183	726	5	0.00	Resistant
	PBO + deltamethrin	100	37.6	198.7	57		

Table 2. Frequency of *kdr* allele and genotype in *Anopheles gambiae* s.s. and *Culex quinqufasciatus* mosquitoes exposed to DDT and deltamethrin in Kosofe LGA, Lagos State

Mosquito species	Insecticides	Number (*N*)	*kdr* genotype (*N*)		Allele frequency *F* (%)	H–W *P* value
			RR	RS		
An. gambiae s.s.	DDT	36	7	3	0.000	0.257
	Deltamethrin	36	9	2		
	Total	72	16 (22.2%)	5 (6.9%)		
Cx. quinquefasciatus	DDT	36	-	-		0
	Deltamethrin	36	-	-		
	Total	72	-	-		

H–W is the probability of the exact test for goodness of fit to Hardy–Weinberg equilibrium; *P* significant at <0.05.
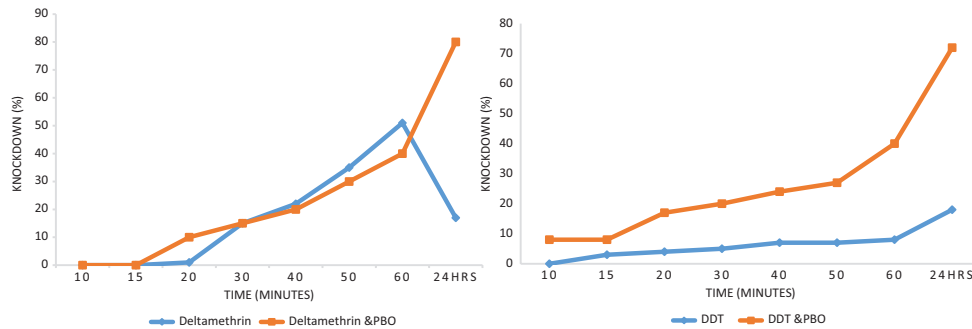
Fig. 1. Comparison between percentage knockdown of *An. gambiae* s.s. exposed to insecticides and PBO + insecticide.
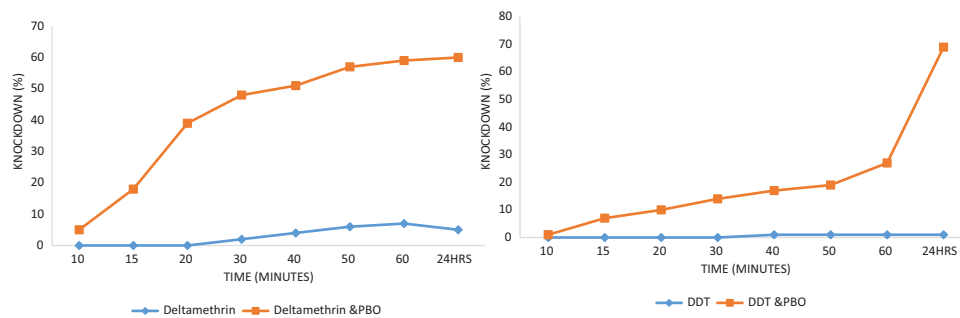
Fig. 2. Comparison between percentage knockdown of *Cx. quinquefasciatus* exposed to insecticides and PBO + insecticide.

in Malaysia (Wan-Norafikah et al. 2013), and *Anopheles*, *Culex*, and *Aedes* larvae in India (Vijayan et al. 2007, Fakoorziba et al. 2009). PBO is one of the chemical synergists available, which when used in combination with insecticides acts by inhibiting enzymes in the natural metabolic defense system of insects. PBO is a well-known inhibitor of cytochrome P450 mono-oxygenases (Hemingway and Ranson 2000, Gleave et al. 2017, Protopopoff et al. 2018). In recent study in Kagera, Tanzania, a malaria endemic area that has also recorded insecticide resistance to pyrethroids, vector control measures where PBO synergist was incorporated in pyrethroid used for LLINs and IRS showed a reduction in the incidence and transmission of malaria compared to areas where pyrethroid control measures were deployed without PBO synergist (Protopopoff et al. 2018).

The results from the PBO synergistic assay carried in the study indicated that metabolic enzymes could largely be responsible for the resistance phenotype observed in the mosquito populations, as higher percentage progressive knockdown and 24-h mortality was recorded in *An. gambiae* and *Cx. quinquefasciatus*. Target site and metabolic resistance mechanisms are primarily responsible for the resistance

phenotype in *An. Gambiae*, but mainly metabolic resistance is evident in *Cx. quinquefasciatus*. Similarly, a study from Chad in Central Africa has reported the cytochrome P450 gene (CYP6P4) to be responsible for high pyrethroid resistance in *kdr*-free *Anopheles arabiensis* Patton (Ibrahim et al. 2016). Multiple resistance mechanisms involving target site mutations and enzyme detoxification have reported in DDT and pyrethrois resistant *Cx. quinquefasciatus* and *An. gambiae* (Corbel et al. 2007, Awolola et al. 2009, Delannay et al. 2018).

There is a need for further investigation into other possible resistance mechanisms, especially in *Cx. quinquefasciatus*, as in our study area 100% mortality was not recorded even in the PBO-synergized assay. Other voltage sensitive sodium channel (*vssc*) mutations such as an L1014C mutation have been reported in *Cx. pipiens molestus* in China and a V1016G mutation in Saudi Arabia (Scott et al. 2015). Also the role of other detoxifying enzymes, especially esterases, in DDT and pyrethroid resistance need to be investigated. Pyrethroid and DDT insecticide resistance could be detrimental to the control of malaria and other mosquito-borne diseases control, Therefore, proper insecticide resistance management is urgently needed and the use of

PBO synergist plus pyrethroid and DDT should be encouraged in LLINs and IRS to enhance the effectiveness of mosquito control.

Acknowledgments

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