 doi:10.20944/preprints202206.0243.v1

Article

High Pyrethroid Resistance to Deltamethrin and DDT in Major Malaria Vector Anopheles gambiae s.l. from South-Western Nigeria is Probably Driven by Metabolic Resistance Mechanisms

Adedapo Adeogun[1,2], Ahmed Omotayo[2], Ayodele Babalola[2]*, Tosin Joseph[3], Oluwakemi Adesalu[2], Romoke Jimoh[2], Tolulope Oyeniyi[2], Samson Awolola[2], Olusola Ladokun[3]*

[1] Department of Biological Sciences, Lead City University, Ibadan, Oyo State, Nigeria; dapoadeogun@hotmail.com
[2] Public Health and Epidemiology Department, Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria; ayodelebabalola2011@gmail.com
[3] 3Department of Biochemistry, Lead City University, Ibadan, Oyo State, Nigeria; solajp@gmail.com
* Correspondence: ayodelebabalola2011@gmail.com; solajp@gmail.com

Abstract: Background: Insecticide resistance in Anopheles gambiae s.l. is a major challenge for malaria vector control in Nigeria. Both target-site insensitivity and metabolic resistance have been implicated in resistance process, with the latter receiving little attention in Nigeria. Therefore, we investigated metabolic enzyme activities in Anopheles gambiae s.l populations resistant to Deltamethrin and Diethyldichlorotriethylethane (DDT) in South-West Nigeria. **Methods**: Anopheles larvae were collected from Ibadan, Oyo and Badagry, Lagos. Adults were exposed to Deltamethrin and DDT using WHO method. Cohorts of populations were further exposed to Pyperonil Butoxide (PBO) and Deltamethrin. Insecticide-exposed and unexposed cohorts were examined for metabolic enzyme activities. Results were compared between exposed and unexposed samples ANOVA ($P<0.05$). **Results**: Mosquitoes were identified as An. gambiae (89%, Ibadan; 0%, Badagry) and An. coluzzii (11%, Ibadan; 100%, Badagry). The populations showed varied level of resistance to Deltamethrin (26%, Ibadan; 71%, Badagry) and DDT (2%, Ibadan; 44%, Badagry). Mortality to Deltamethrin increased from 26% to 64% (Ibadan) and 71% to 84% (Badagry) when populations were pre-exposed to PBO. Biochemical analysis revealed significant high levels ($P<0.05$) of cytochrome P450 and GST in exposed samples. **Conclusions**: Cytochrome P450 and GST are involved in Deltamethrin and DDT resistance in Anopheles gambiae s.l populations in South-West Nigeria.

Keywords: : Insecticide resistance; *Anopheles gambiae*; *Anopheles coluzzii*; Cytochrome P450; Gluthathione-S-Transferases

1. Introduction

Malaria remains one of the deadliest parasitic diseases which affect millions of people globally[1], particularly low immune response individuals such as children under five years old and pregnant women [2]. In sub-Saharan Africa, Nigeria contribute significantly to global cases and deaths due to malaria [2]. Nigeria currently accounts for the highest number of cases of malaria in the world, with an estimated 51 million cases and 207 000 deaths annually [3]. Prevalence of the causative parasite has been attributed mainly to the presence and abundance of its main vectors, Anopheles gambiae s.l. and Anopheles funestus s.l. [4].

Vector control efforts aimed at reducing the burden of the disease have largely depend on the use of Long-Lasting Insecticide Nets (LLINs) and Indoor Residual Spray (IRS).

 doi:10.20944/preprints202206.0243.v1

These strategies basically involve the use of insecticides from four classes of insecticides [5] and they remain one of the frontline and effective tools for controlling malaria [6]. There is widespread use of LLINs and IRS in sub-Saharan Africa and these have led to reduction in the incidence of the disease over the years [7]. However, mosquito populations have developed resistance to all insecticides recommended by WHO for malaria vector control and this has negatively impacted the progress recorded in previous years [8,9].

In some vector populations, resistance to two or more of the four classes of insecticides recommended for public health use has been reported[5, 10, 11]. Two main strategies; Target site mutation and metabolic enzymes activities have been largely implicated in resistance development. Target-site mutations, widely referred to as 'kdr' (knockdown resistance) mutations, are the most understood resistance mechanism found in insects resistant to pyrethroids and DDT [12]. These changes in target sites are associated with reduction in knockdown effect of the insecticide [11]. Recent data have suggested alteration in metabolic enzymatic activities as the most important mechanism in resistance development [13]. Enzymatic metabolic resistance can be through increased detoxification or increased activities of certain enzymes that help in reducing the effects of the applied insecticides [14-16]. Furthermore, the simultaneous presence of different resistance mechanisms also confers cross-resistance to many insecticides used in public health [17] and this can be a major setback to the success of insecticide-based vector control programs [18].

Efforts at characterizing resistance in Nigeria has largely focused on the frequencies of target-site mutations with little attention paid to metabolic resistance mechanisms [19, 20]. However, management of insecticide resistance can only be done with sound knowledge of extent and spread of insecticide resistance in malaria vectors [8] coupled with in-depth understanding of resistance mechanisms of local vector populations. This can be achieved through consistent monitoring of type and spread of resistance mechanisms. Therefore, we investigated metabolic resistance mechanisms in populations of Anopheles gambiae s.l resistant to Deltamethrin and DDT in South-West, Nigeria.

2. Materials and Methods

Study Site

The study was conducted in New Garage situated in an urban community in Ibadan, Oyo state, (Latitude 7.331425, Longitude 3.858757) and Marina road situated in a semi-urban community in Badagry, Lagos State (Latitude 6.414167, Longitude 2.878056), South-West, Nigeria (Figure 1). The climate in South-West Nigeria is equatorial, notably with dry and wet seasons with relatively high humidity and average daily temperature ranging between 25°C (77.0°F) and 35°C (95.0°F) throughout the year.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 17 June 2022 doi:10.20944/preprints202206.0243.v1

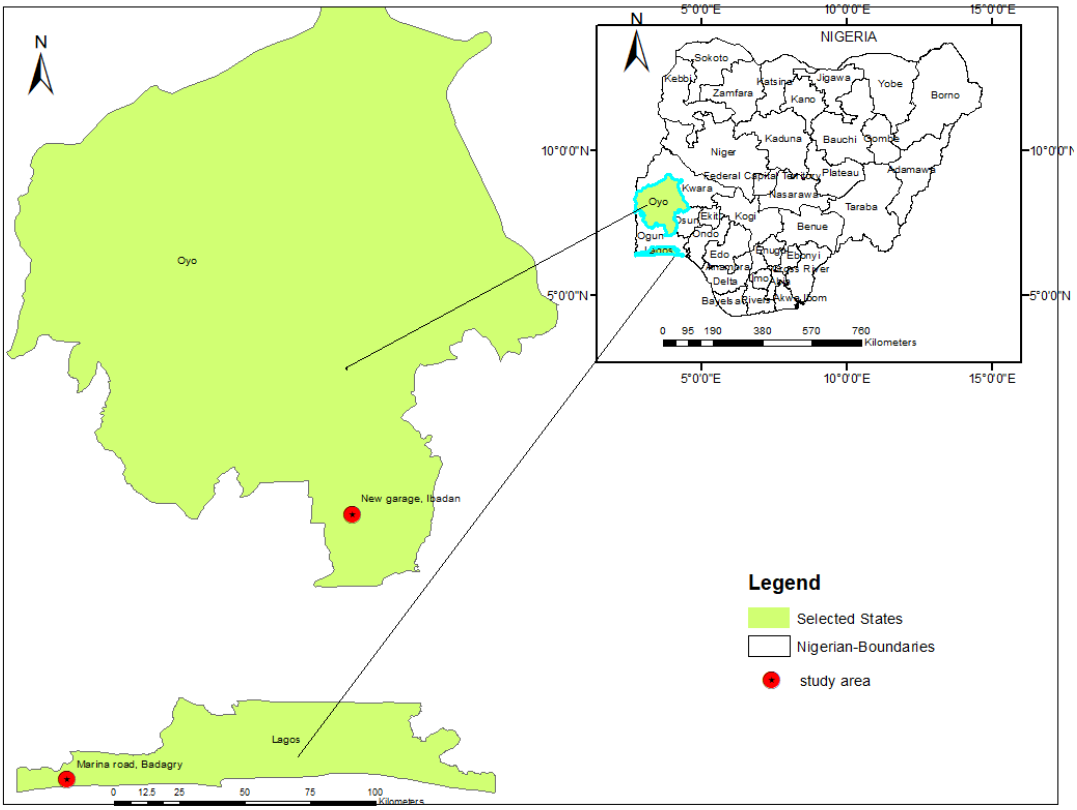

Figure 1: Study area where mosquito samples were collected

Mosquito collection and rearing

Anopheles larvae and pupae were collected from ditches and puddles using standard procedures [21]. They were then taken to the insectarium and transferred into larval bowls, suitably labelled and reared to adult at the insectary of Molecular Entomology and Vector Control Unit, Public Health and Epidemiology Division, Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria (NIMR). Emerged adults were transferred to cages and provided with 10% sugar solution.

Insecticide susceptibility test

Susceptibility tests were carried out using WHO standard protocol [22] by exposing 25 female adult mosquitoes of 3-5 days old in 4 replicates to 0.05% Deltamethrin and 4% DDT impregnated papers. Controls using 25 mosquitoes in two replicates were also used. Mosquitoes knocked down were recorded at 10, 15, 20, 30, 40, 50 and 60minutes and mortality was determined after 24hours.

Determination of insecticide resistance mechanism using synergist

Cohorts of sampled populations were further exposed to synergist PBO and then Deltamethrin to detect resistant mechanisms according to WHO criteria [22]. A total of 100 identified adult mosquitoes were first exposed to synergist PBO for one hour after which the samples were further exposed to Deltamethrin for another one hour. Mosquitoes knocked down were recorded at 10, 15, 20, 30, 40, 50 and 60minutes and mortality was determined after 24hours.

 doi:10.20944/preprints202206.0243.v1

Morphological and PCR Identification of samples

Mosquitoes collected were identified morphologically using identification keys provided by [23]. Molecular identification was done using PCR in line with the methods of [24, 25].

Metabolic enzymes activity analysis

The microtitre plate method was used to determine enzyme levels in mosquito populations (WHO, 1998). Twenty-five (25) mosquitoes that survived exposure to Deltamethrin and DDT insecticides were assayed for elevated esterase, cytochrome P450, glutathione-S-transferase (GST) and protein. Equal number of samples were analyzed from unexposed mosquitoes and the results compared between exposed and unexposed cohorts. All samples used for enzyme analysis were homogenized individually in 1.5ml eppendorff tubes containing 200µl of distilled water on ice. 25µl of homogenate per individual sample was kept in freezer at -200C. Esterase, cytochrome P450 and GST activities were determined and interpreted using standard procedures [26].

Statistical analysis of data

Mortality was determined after 24 hours and correction of percentage mortality with Abbott's formula was not necessary as mortality in all controls were below 5%. Resistance status was determined according to WHO criteria (WHO, 2013); mortality between 98-100% indicates susceptibility, mortality between 90-97% suggests resistance, while mortality values below 90% indicates resistance. KDT50 and KDT95 values were determined using Probit regression analysis. Results (Mean ± Standard Error of Mean) for metabolic enzyme activities for exposed survivors and unexposed samples were compared using one-way analysis of variance with p-values set at 0.05. All statistical analysis were performed using SPSS version 23.0 (SPSS IBM Inc.).

3. Results

3.1. Mosquito population used in the study

A total of 600 female Anopheles mosquitoes were used for the study. All mosquitoes were morphologically identified as members of Anopheles gambiae s.l. and further molecular analysis identified Anopheles gambiae (89% in Ibadan; 0% in Badagry) and An. coluzzii (11% in Ibadan; 100% in Badagry).

3.2. Susceptibility studies

Mosquito population from Ibadan was highly resistant to Deltamethrin with 24 hours mortality of 26%. Also, the mortality observed in DDT (2%) was extremely low (Table 1). Result of synergist assay showed increase in mortality values from 26% to 64% for Deltamethrin (Figure 2), however, the numbers of mosquitoes knockdown after 60 mins of exposure to Deltamethrin (80%) and PBO+Deltamethrin (87%) were in close range.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 17 June 2022 doi:10.20944/preprints202206.0243.v1

Table 1. Twenty-four hours post exposure mortality of Anopheles gambiae s.l. mosquitoes from Ibadan, Oyo exposed to diagnostic doses of Deltamethrin and DDT

Insecticide	No Exposed	KDT_{50} (min) 95% cl	KDT_{95} (min) 95% cl	Knockdown at 60mins	Mortality (%)	Susceptibility status
DDT (4%)	100	916.82 -	5582.96 -	2	2	Resistant
Deltamethrin (0.05%)	100	23.67 (21.07–26.35)	142.46 (108.09–210.68)	80	26	Resistant
PBO+ Deltamethrin	100	27.76 (24.27–31.57)	77.09 (61.65–109.67)	87	64	Resistant

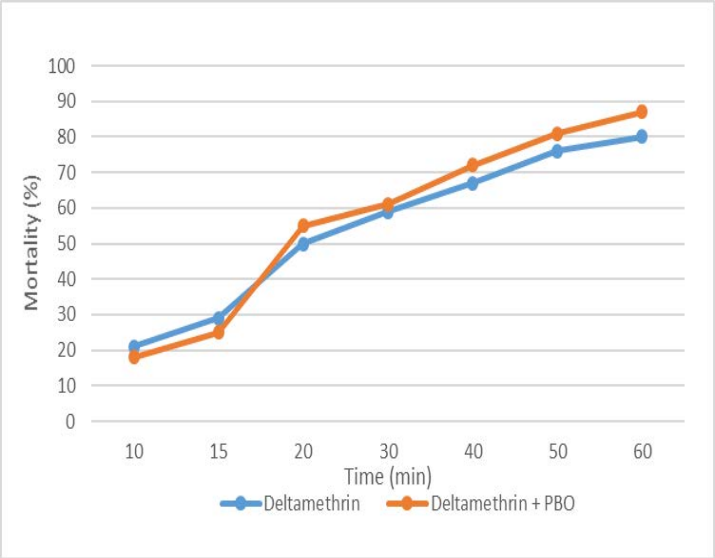

Figure 2. Percentage knockdown of Anopheles gambiae s.l from Ibadan, Oyo exposed to Deltamethrin only and Deltamethrin + PBO for 60 mins

Table 2 shows 24-hours post exposure mortality values of Anopheles gambiae s.l from Badagry, Lagos to Deltamethrin and DDT. Unlike the result obtained for population from Ibadan, mortality to DDT was 44% while mortality to Deltamethrin was 71%. When cohorts of same population were further exposed to PBO and deltamethrin, mortality to deltamethrin increased from 71% to 84% (Figure 3).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 17 June 2022 doi:10.20944/preprints202206.0243.v1

Table 2. Twenty-four hours post exposure mortality of Anopheles gambiae s.l. mosquitoes from Badagry, Lagos exposed to diagnostic doses of Deltamethrin and DDT

Insecticide	No Exposed	KDT_{50} (min) 95% cl	KDT_{95} (min) 95% cl	Knockdown at 60mins	Mortality (%)	Susceptibility status
DDT (4%)	100	74.15 (64.08- 92.08)	257.66 (179.44-457.27)	36	44	Resistant
Deltamethrin (0.05%)	100	44.87 (41.84– 48.56)	117.55 (99.32–147.54)	64	71	Resistant
PBO+Deltamethrin	100	37.30 (34.80– 40.16)	104.83 (89.73–128.27)	78	84	Resistant

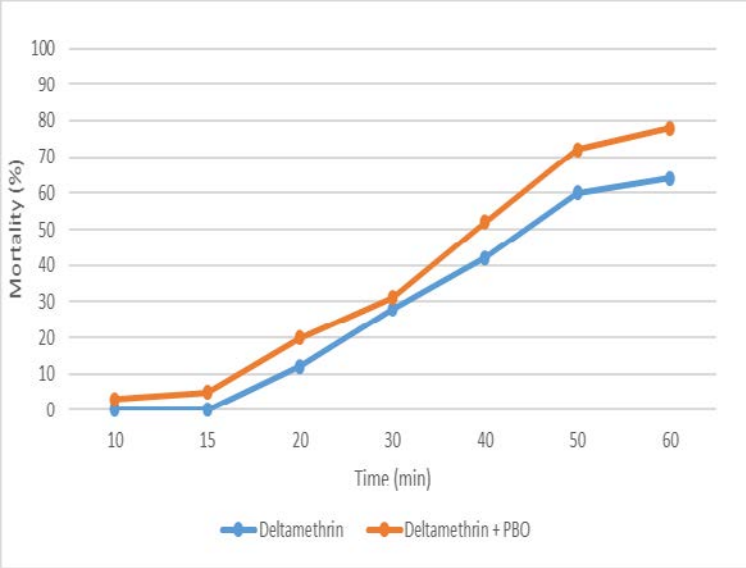

Figure 3. Percentage knockdown of Anopheles gambiae s.l from Badagry, Lagos exposed to Deltamethrin only and Deltamethrin + PBO for 60 mins

Metabolic enzymes activities

Result of metabolic enzymes assay is presented in Table 3. Esterase activities was slightly elevated ($p>0.05$), while that of cytochrome P450 and GST were significantly elevated ($p<0.05$) in DDT exposed samples from Ibadan (1.81, 15.59 and 51.72 mMole/min/mg protein respectively) as compared with unexposed samples (1.30, 5.51 and 12.60 mMole/min/mg protein). The same was observed in values for samples from Badagry (0.24, 13.63 and 48.09 mMole/min/mg protein respectively) when compared with unexposed samples (1.21, 4.73, 15.56 mMole/min/mg protein respectively).

A similar trend was observed in samples exposed to deltamethrin; enzyme activities were generally elevated ($p<0.05$) in the exposed mosquitoes compared with the unexposed except for esterase.

 doi:10.20944/preprints202206.0243.v1

Table 3. Mean values of enzyme activity in Anopheles gambiae s.l mosquitoes exposed to both Deltamethrin and DDT in South-West Nigeria.

Location	Insecticide	Enzyme		
		Esterase	Cytochrome P450	GST
Ibadan, Oyo	DDT	1.81 ± 0.41[a]	15.59 ± 3.87[c]	51.72 ± 7.45[d]
	Deltamethrin	3.00 ± 2.19[a]	12.00 ± 1.94[b]	30.05 ± 4.71[c]
	Unexposed Samples	1.30 ± 0.40[a]	5.51 ± 1.12[a]	12.60 ± 2.05[a]
Badagry, Lagos	DDT	0.24 ± 0.02[a]	13.63 ± 1.89[b]	48.09 ± 3.01[d]
	Deltamethrin	1.27 ± 0.10[a]	15.61 ± 2.41[c]	49.15 ± 5.89[d]
	Unexposed Samples	1.21 ± 0.61[a]	4.73 ± 1.31[a]	15.56 ± 3.35[ab]

Note: Values are presented as mean ± standard error of mean. Values with the same superscript in a column connotes significant difference ($p<0.05$) and vice-versa.

4. Discussion

The development of resistance in Anopheles populations in Nigeria is already alarming and it poses a serious threat to malaria vector control programs. Despite the huge number of LLINs distributed, data on annual malaria incidence still remain unchanged [2, 7]. Studies have shown that local populations of Anopheles are now resistant to all four classes of WHO-approved insecticides used for mosquito control in the country {9-11, 27-28]. This highlights the need for proper resistance monitoring and characterization to inform policy decisions. However, reports on resistance mechanisms such as kdr frequencies are common from different parts of the country but reports on more problematic metabolic resistance are still few and scanty in the country.

Mosquito populations in the present study were highly resistant to Deltamethrin and DDT which indicate cross-resistance within the populations [13]. This resistance data is consistent with previous reports from South-West Nigeria [9, 10, 28]. In many populations, cross-resistance has been largely attributed to the presence of kdr resistance gene [29-31]. Albeit, the frequency of kdr in the studied populations was not investigated, previous data suggest that kdr is one of the resistance mechanisms in populations of Anopheles gambiae s.l. from South-West Nigeria [27]. Also, the high KDT50 values for both Deltamethrin and DDT in the study suggests high resistance intensity [32], which has been attributed to presence and high frequencies of kdr gene [29].

Results from synergist assay where mortality was increased after pre-exposure to PBO suggest metabolic enzymes are also involved in resistance development to Deltamethrin in the populations. This result is also consistent with a recent report in South-West Nigeria that showed the involvement of cytochrome P450 in resistance process against pyrethroid based insecticides in malaria vectors [11]. Also, evidence for this has been partly provided in a population of Anopheles in Nigeria where Permanet 3.0 (PBO + Deltamethrin) performed better than Permanet 2.0 in a phase III trial [33].

Levels of cytochrome P450 was significantly higher in the Deltamethrin exposed samples from the two populations thereby suggesting its involvement in resistance develop-

ment. Cytochrome P450 elevation had earlier been reported in different pyrethroid resistant populations in South-West Nigeria [11]. A study by [34] also reported cytochrome P450 elevation in Anopheles mosquitoes resistant to pyrehtroids and DDT from some localities in Kenya. The impact of significant high level of cytochrome P450 in Anopheles population from South West Nigeria will be a strong disadvantage to the use of pyrethroid only net and this provide evidence for introduction of LLIN impregnated PBO and Pyrethroid as the introduction of PBO will increase the potency of pyrethroids as seen in the present study. Also, GST elevation was observed in DDT-resistant samples from both Ibadan and Badagry populations. This is similar to the work of [35] who reported elevated level of GST in DDT-resistant Anopheles population. In Nigeria, AlHassan et al. [36] also reported significant GST elevation in DDT-resistant Anopheles population from North-West, Nigeria and this is consistent with data from other parts of Africa [13, 37-39].

It is essential to monitor the development of insecticide resistance and properly characterize resistance mechanisms in populations of Anophelines in Nigeria. This is highly necessary due to geometric spread of insecticide resistance in all common vectors of malaria within the country. Such data will inform policy decisions especially on usage of LLINs in several parts of the country. This is because LLIN is a major vector control intervention currently in use [40] and data providing enough details to make choice of LLINs will be helpful to achieving the overall goals of malaria control program. More essentially for policy direction are data from studies implicating metabolic enzymes in populations of Anopheles gambiae s.l. resistant to pyrethroid and organochlorides in South-West, Nigeria, as this will serve as guide in making sound judgement about allocation of LLINs impregnated with PBO.

5. Conclusions

Cytochrome P450 and Glutathione-S-Transferases are responsible in part for resistance of Anopheles gambiae and Anopheles coluzzii to Deltamethrin and DDT insecticides in South-West, Nigeria.

Author Contributions: Conceptualization, AA. and LO.; methodology, AA. BA. OA. and LO. ; software, AB.; validation, AO., OT and OA..; formal analysis, AB. JT. And LO; investigation, JR. OT. and OA.; resources, AS. LO. AA.; data curation, OA, AA and JT.; writing—original draft preparation, OA. and JT.; writing—review and editing, AA. and BA..; visualization, BA. AA. And OA .; supervision, AA. and LO.; project administration, JT., OA. and OT. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Data Availability: All data used in this study have been provided in forms of Tables and Figures

Acknowledgments: The authors appreciate the support of project staff at the Molecular Entomology and Vector Control Research Laboratory, Department of Public Health and Epidemiology, Nigeria Institute of Medical Research, Yaba, Lagos, Nigeria..

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Theoharides, T.C. Mast cells promote malaria infection? Clinical Therapeutics., **2015,** 37(5):1374-1377. doi.org/10.1016/j.clinthera.2015.03.014
2. World Health Organization. World Malaria Report. WHO, **2019.,** Geneva, Switzerland.
3. Dawaki, S. Is Nigeria winning the battle against malaria? Prevalence, risk factors and KAP assessment among Hausa communities in Kano State. *Malar J*., **2016**;15:351.
4. Oyewole, I.O.; Awolola, T.S. Impact of Urbanisation on Bionomics and Distribution of Malaria Vectors in Lagos, Southwestern Nigeria. J Vector Borne Dis., **2006,** 43:173–8.

5. Oduola, A.O.; Ezra, A.; Olukayode, A.; Adeolu, T.; Kennedy, P.; Awolola, T. Widespread Report of Multiple Insecticide Resistance in Anopheles gambiae s.l. Mosquitoes in Eight Communities in Southern Gombe, North-Eastern Nigeria. J Arthropod-Borne Dis., **2019,** 13(1): 50–61.
6. Omotayo, A.I.; Ande, A.T.; Oduola, A.O.; Olakiigbe, A.K.; Ghazali, A.K.; Adeneye, A.; Awolola, S.T. Community Knowledge, Attitude and Practices on Malaria Vector Control Strategies in Lagos State, South-West Nigeria. Journal of Medical Entomology, **2021** p.tjaa278.
7. World Health Organization. World Malaria Report. WHO, **2021,** Geneva, Switzerland.
8. Djouaka, R J.; Seun. M.A; Genevieve, M.T.; Jacob, M.; Riveron, H. Evidence of a multiple insecticide resistance in the malaria vector Anopheles funestus in South West Nigeria. Malar J. **2016,** 15:565 DOI:10.1186/s12936-016-1615-9.
9. Adeogun, A.O.; Popoola, K.O.; Oduola, A.O.; Olakiigbe, A.K.; Awolola, T.S.. High Level of DDT Resistance and Reduced Susceptibility to Deltamethrin in Anopheles gambiae, Anopheles coluzzi, and Anopheles arabiensis from Urban Communities in Oyo State, South-West Nigeria. *J Mosq Res*. **2017,** 7(16): 125–133.
10. Oduola, A.O.; Idowu, E.T.; Oyebola, M.K.; Adeogun, A.O.; Olojede, J.B.; Otubanjo, O.A.; Awolola, T.S. Evidence of Carbamate Resistance in Urban Populations of Anopheles gambiae s.s. Mosquitoes Resistant to DDT and Deltamethrin Insecticides in Lagos, South-Western Nigeria. Parasites & Vectors. **2012**, 5:116 https://doi.org/10.1186/1756-3305-5-116 PMid:22686575 PMCid:PMC3409038
11. Fagbohun, I.K.; Tolulope, A.O.; Idowu E.T.; Otubanjo, O.A.; Awolola, S.T. Cytochrome P450 Mono-Oxygenase and Resistance Phenotype in DDT and Deltamethrin-Resistant Anopheles gambiae (Diptera: Culicidae) and Culex quinquefasciatus in Kosofe, Lagos, Nigeria. Journal of Medical Entomology, **2019,**. 56(3):817–821. doi: 10.1093/jme/tjz006
12. Nkya, T.E.; Akhouayri, I.; Kisinza, W.; David, J.P. Impact of Environment On Mosquito Response to Pyrethroid Insecticides: Facts, Evidences and Prospects. *Insect Biochem. Mol. Biol*., **2013,** 43: 407–416.
13. Riveron, M.J.; Cristina, Y.; Sulaiman, S.I.; , Djouaka, R.; Helen, I. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. Genome Biology, **2014,** 15:R27.
14. Hemingway, J.; Hawkes, N.J.; McCarroll, L.; Ranson, H. The Molecular Basis of Insecticide Resistance in Mosquitoes. Insect Biochem. Mol. Biol., **2004,** 34: 653–665.
15. Corbel, V.; N'Guessan, R.; Brengues, C.; Chandre, F.; Djogbenou, L.; Martin, T.; Akogbéto, M. Multiple Insecticide Resistance Mechanisms in Anopheles gambiae and Culex quinquefasciatus from Benin, West Africa. Acta Trop., **2007,** 101: 207–216.
16. Liu, N. Insecticide Resistance in Mosquitoes: Impact, Mechanisms, and Research Directions. Annu. Rev. Entomol., **2015,** 60: 537–559.
17. Hien, S.A.; Soma, D.D.; Hema, O.; Bayili, B.; Namountougou, M.; Gnankiné, O.; Baldet, T.; Diabaté, A.; Dabiré, K.R.. Evidence That Agricultural Use of Pesticides Selects Pyrethroid Resistance Within Anopheles gambiae s.l. Populations from Cotton Growing Areas in Burkina Faso, West Africa. PLoS One, **2017,** 3:1–15. https://doi.org/10.1371/journal.pone.0173098
18. Namountougou, M.; Diloma, S.D.; Kientega, M.; Balbon´e, M.A.; Kabor´e, D.P.; Drabo, S.; Coulibaly, A.Y.; Fournet, F.; Baldet, T.; Diabate, A.; Dabire, R.K.; Gnankine, O. Insecticide resistance mechanisms in Anopheles gambiae complex populations from Burkina Faso, West Africa, Acta Tropica, **2019**, https://doi.org/10.1016/j.actatropica.2019.105054
19. Awolola, T.S.; Oyewole, I.O.; Amajoh, C.N.; Idowu, E.T.; Ajayi, M.B.; Oduola, A.O.; Manafa, O.U.; Ibrahim, K.; Koekemoer, L.L.; Coetzee, M. Distribution of the molecular M and S forms of Anopheles gambiae and pyrethroid knockdown resistance gene in Nigeria. Acta Tropica., **2005**, 95: 204-209
20. Muhammad, A.; Ibrahim, S.S.; Mukhtar, M.M.; Irving, H.; Abajue, M.C.; Edith, N.M.A. High pyrethroid/DDT resistance in major malaria vector Anopheles coluzzii from Niger-Delta of Nigeria is probably driven by metabolic resistance mechanisms. PLoS ONE, **2021,** 16(3): e0247944. https:// doi.org/10.1371/journal.pone.0247944
21. Service, M.W. Studies on sampling larval population of Anopheles gambiae complex. Bull WHO, **1971,** 45: 169–80.
22. WHO. World Health Organization. Test Procedures for Insecticide Resistance Monitoring in Malaria Vector Mosquitoes. **2013,** Geneva, Switzerland. Available at: (www.africairs.net/.../Test-procedures-for-insecticide-resistance-monitoring-WHO.pdf).
23. Gillies, M.T.; Coetzee, M. A supplement to the Anopheline Africa south of the Sahara (Afrotropical region), Johannesburg, South Africa. S Afr Inst Med Res., **1987,** 55: 1–143.
24. Scott, J.A., Brogdon, W.G.; Collins, F.H. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. Am J Trop Med Hyg., **1993,** 49: 520–529.
25. Favia, G.; Della, T.A.; Bagayoko, M.; Lanfrancotti, T.; Sagnon, N.F.; Toure, Y.T.; Coluzzi M. Molecular identification of sympatric chromosomal forms of Anopheles gambiae and further evidence of their reproductive isolation. Insect Mol Biol., **1997,** 6: 377–383.
26. WHO. World Health Organization. Techniques to detect Insecticide Resistance Mechanisms (Field and Laboratory manual). WHO/CDS/CPC/MAL/98.6., **1998**
27. Awolola, T.A.; Oduola, I.O.; Oyewole, J.B.; Obansa, C.; Amajoh, L.; Koekemoer, L.L.; Coetzee, M. Dynamics of Knockdown Pyrethroid Insecticide Resistance Alleles in A Field Population of Anopheles gambiae s.s. In Southwestern Nigeria. Journal of Vector Borne Diseases, **2007**, 44(3): 181-188.

28. Oduola, O.A.; Olojede, J.B.; Ashiegbu, C.O.; Adeogun, A.O.; Olojede, J.B.; Oduola, A.O.; Awolola T.S. Efficacy of a combination long lasting insecticidal net Permanet 3.0: An Enhanced Efficacy Combination Long-Lasting Insecticidal Net Against Resistant Populations of Anopheles gambiae s.s. Malaria Chemotherapy, Control and Elimination, **2012,** 1 https://doi.org/10.4303/mcce/235543
29. Chandre, F.; Darriet, F.; Manguin, S.; Brengues, C.; Carnevale, P.; Guillet, P. Pyrethrid cross resistance spectrum among populations of Anopheles gambiae s.s from Cote d'Ivoire. J.Am. Mosq. Cont. Ass., **1999**; 15(1):53-59
30. Dai, Y.; huang, X.; Cheng, P.; Liu, L.; Wang, H.; Wang, H.; Kou , J. Development of insecticide resistance in malaria vector Anopheles sinensis populations from Shandong province in China. Malaria Journal, **2015**, 14:62
31. Hancock, P.A.; Wiebe, A.; Gleave, K.A.; Bhatt, S.; Cameron, E.;, Trett, A.; Weetman, D.; Smith, D.L.; Hemingway, J.; Coleman, M.; Gething, P.W.; Moyes, C.L.. Associated patterns of insecticide resistance in field populations of malaria vectors across Africa. Proceedings of the National Academy of Sciences of the United States of America, **2018**,115(23):5938-5943.
32. The PMI VectorLink Project. January 2019. The PMI VectorLink Nigeria Annual Entomology Report, November 2018-September 2019. Rockville, MD. VectorLink, Abt Associates Inc.
33. Adeogun, A.O.; Olojede, J.B.; Oduola, A.O.; Awolola T.S. Efficacy of a combination long lasting insecticidal net Permanet 3.0: An Enhanced Efficacy Combination Long-Lasting Insecticidal Net Against Resistant Populations of Anopheles gambiae s.s. Malaria Chemotherapy, Control and Eliminatio, **2012**, 1 https://doi.org/10.4303/mcce/235543
34. Wanjala, C.L.; Kweka, E.J. Malaria vectors insecticides resistance in different agroecosystems in Western Kenya. Frontiers in Public Health., 2018; 6:55
35. Perera, M.B.; Devika, H.J.; Parakram, S.H. Multiple Insecticide Resistance Mechanisms Involving Metabolic Changes and Insensitive Target Sites Selected in Anopheline Vectors of Malaria in Sri Lanka. Malaria Journal, 2008, 7(1)168.
36. AlHassan, A.J.; Sule, M.S.; Dangambo, M.A.; Yayo, A.M.; Safiyanu, M.; Sulaiman, D. Detoxification Enzymes activity in DDT and Bendiocarb Resistant and Susceptible Malarial Vector (Anopheles gambiae) Breed in Auyo Residential and Irrigation Sites North-West, Nigeria. European Scientific Journal. 2015; 11(9): 315-326
37. Ibrahim, S.S.; Riveron, J.M.; Scott, R.; Irving, H.; Wondji, C.S. The Cytochrome P450 CYP6P4 is Responsible for the High Pyrethroid Resistance in Knockdown Resistance-free Anopheles arabiensis. Insect Biochemistry and Molecular Biology, 2016; 68:23-32.
38. Marcombe, S.; Bobichon, J.; Somphong, B.; Phommavan, N.; Maithaviphet, S.; Nambnya, S.; Corbel, V.; Brey, P.T. Insecticide Resistance Status of Malaria Vector in Lao PDR. PLoS one., **2017**, 12(4):e0175984.
39. Simma, E.A.; Dermauw, W.; Balabanidou, V.; Snoeck, S.; Bryon, A.; Clark, R.M.; Yewhalaw, D.; Vontas, J.; Duchateau, L.; Van Leeuwen, T. Genome-wide Gene Expression Profiling Reveals That Cuticle Alterations and P450 Detoxification are Associated with Deltamethrin and DDT Resistance in Anopheles arabiensis Populations from Ethiopia. Pest Management Science, 2019; 75(7). doi.org/10.1002/ps.5374
40. National Malaria Strategic Plan (NMSP) Nigeria (2014-2020), Federal Ministry of Health, Abuja, Nigeria.