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Malaria Vector Resistance to Some Selected Insecticides in Karu and Kokona Local Government Areas of Nasarawa State, Nigeria

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Abstract

The fight against malaria is increasingly threatened by failures in vector control due to increasing insecticide resistance. Monitoring and understanding the trend and dynamics of insecticide resistance by the Anopheles mosquito are very essential to devising efficient control strategies. The greatest burden of the disease is felt in Africa, particularly, Nigeria. More information on insecticide resistance is needed in many parts of the country; hence, this study will also contribute to the supply of information in the North-central zone. The study was carried out in 2021, to characterize the mosquito population and its insecticide resistance profile, in their agricultural breeding sites of Karu and Kokona local government areas of Nasarawa state, North-central Nigeria. Mosquito larvae from the breeding sites were sampled and reared to adulthood. The emergent adults were morphologically and molecularly identified to species level. These were *An. gambiae, An. coluzzi* and *An. arabiensis*. Susceptibility tests were carried out on the adult mosquitoes using CDC bottle bioassay insecticide discriminating times (in minutes); 12.5 µg for deltamethrin and alpha-cypermethrin, 20 µg for pirimiphos-methyl and 12.5 µg for bendiocarb. The CDC bottle bioassay revealed a graded level of resistance. Knockdown resistant (Kdr)-mutation was detected by PCR, the observation of which implied that selection pressure on the Anopheles population in Karu and Kokona LGAs has occurred. This result is critical for the planning and implementation of malaria vector control interventions based on IRS and ITNs, as currently ongoing in Nigeria.

Keywords: Cdc bottle bioassay • Deltamethrin • Alpha-Cypermethrin • Pirimiphos-Methyl and Bendiocarb

Introduction

Insecticide resistance is defined as an inherited ability of a population to survive an insecticide dose that would normally have proven lethal to individuals of a susceptible population of the same species administrated under the same conditions [1].

Insecticides play a pivotal role in the control of mosquito vectors and will continue to do so for the foreseeable future. However, the pervasive use of some insecticides for both agricultural pests and vector of human and livestock diseases has led to resistance making insecticides used ineffective and limiting the available option for disease control. Vector borne diseases are among the major causes of illness and death, particularly in tropical and subtropical countries; vector control through the use of insecticide plays a key role in the prevention and control of infectious diseases. In Nigeria, mosquitoes are regarded as public health nuisance. Mosquito-borne diseases are among the leading causes of human deaths worldwide, with the estimated 200 million cases of malaria in 2012 leading to about 27,000 deaths, mostly of African children [2].

All these diseases cause high morbidity and mortality in human and animal population, leading to huge economic losses. One method for the control of these deadly mosquito borne diseases is the interruption of disease transmission by killing or preventing mosquitoes from biting man and animals. Mosquito control remains an important component of human and animal diseases [3].

Six classes of insecticides namely organochlorines Organophosphates (OP), carbamates, pyrethroids, pyrroles and phenyl pyrazoles are recommended for use against adult mosquitoes. Indoor residual spraying and long-lasting insecticidal nets treated with pyrethroids are the two most important measures for human protection from malaria-carrying mosquitoes. Anopheles vector control relies heavily on a single class of insecticides, the pyrethroids. These insecticides are the only class approved for use on insecticide treated nettings and are being increasingly deployed in Indoor Residual Spray (IRS) programmes in Africa and Long-Lasting

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Insecticide Treated Nets (LLINs). A rise of pyrethroids resistance by mosquitoes has become the latest threat to combating malaria in Nigeria, where roughly up to 300,000 people die each year from the killer disease [4]. The problem of insecticide resistance is very real and growing in Nigeria, there are signs that it might worsen due to the effects of climate change and there is concern that the mosquitoes are becoming resistant to the entire classes of insecticide in use. Reported studies, on the distribution of Anopheles in Nigeria between 1900 to 2010 as follows; *An. gambiae s.1* (181), 65.2%, *An. gambiae s.s* (156), 6.5%, *An. arabiensis* (122), 5.0%, *An. funestus* complex (95) 17.3%, *An. funestus s.s* (21), 2.5% while other species (57) constitute 4.5%. In a related investigation carried out in the Southern part of Nigeria *An. gambiae s.1* constitutes 77.7% of the total number of mosquitoes caught followed by *An. funestus* 22.3% which confirms it as the most common mosquito in the country. Knowledge on insecticide resistance in target species is a basic requirement to guide insecticide use in malaria control programs [5]. The purpose of this study is to add some knowledge gaps on the role of agrochemicals in the development of insecticide resistance in malaria vectors and this is of utmost importance for vector control. This will assist in future decisions on insecticide usage made by authorities responsible for malaria vector control [6].

Materials and Methods

Study area

The study was conducted between March and June 2021 in the localities of Karu and Kokona LGAs in Nasarawa state. Both areas vary by agricultural profile (in terms of crops grown) and the subsequent use of agrochemicals. Maps of the study areas are shown in Figure 1. Kokona LGA sits on a total area of 1,844 square kilometers and has an average temperature of 29°C. The area witnesses two major seasons which are the dry and the rainy seasons. The average wind speed in Kokona LGA is put at 10 km/h while estimated total precipitation in the LGA is put at 1450 mm of rainfall per annum [7].

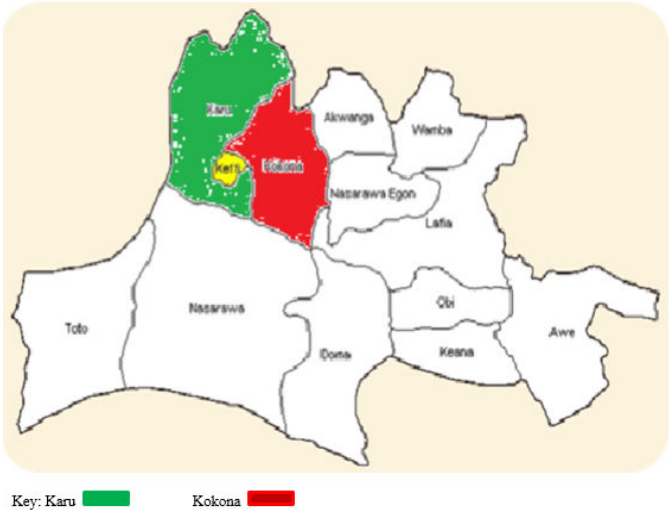

Figure 1. Map of Nasarawa state, Nigeria, showing study areas of Karu and Kokona.

Karu LGA in Nasarawa state covers an area of 2640 km and has an annual temperature range between 21°C to 32°C. Karu has two distinct seasons, wet (rainy) and dry seasons. The average wind speed in Karu LGA is put at between 6-15 km/h with a mean annual rainfall between 1100 mm to about 2000 mm. Both sites (Karu and Kokona) have similar vegetation composed of woody Savannah and gallery forests; the most common agricultural products being cereals, tubers and cashew. Both sites are extensive mosquito breeding areas and malaria persists throughout the year. Malaria is the leading cause of morbidity among the population [8].

Larval density/larval sampling methods

In all breeding sites dipping method was used. Dipping method was used to sample from relatively large water bodies such as swamps, ditches, streams and rice fields. The dipper was lowered gently at an angle of about 45 to minimize disruption. The larvae collected were transferred into white plastic buckets and taken to the insectary. Using pipettes, the larvae were later separated and transferred into rearing bowels/containers. The number of dips and time were recorded. Pupae that emerged were separated and kept in separate plastic containers for adult emergence [9].

Susceptibility test using CDC bottle bioassay

Four replicates of CDC bottles were coated with 1 ml of insecticide each and this was repeated for the insecticides provided (Deltamethrin, Alphacypermethrin, Pirimiphos-methyl and Bendiocarb). The coated bottles were left for 24 hours. A total of 25 Anopheles mosquitoes were introduced into each of the coated and uncoated (control) using 2 different aspirators. One for control which remains separate at all times to avoid insecticide contamination. Susceptibility was determined from number of mosquitoes knocked down first after 5 minutes and later after every 10 for 30 minutes depending on the insecticides [10].

According to the updated WHO guidelines (2016) test procedures for insecticide resistance monitoring in malaria vector mosquitoes. 2nd ed. Geneva: World Health Organization.

Susceptibility ntensity assays must be interpreted as follows:

- <90% mortality (confirmed resistance)
- >90%-97% mortality (possible resistance)
- >98% mortality (susceptible)

Molecular analysis

All survivors (pyrethroid resistant) *Anopheles gambiae s.l* mosquito samples from all pyrethroid susceptibility were sent to the Nigerian institute of medical research for molecular analysis in Lagos for PCR analysis and determination of Knockdown resistance (Kdr). The aim is to identify and determine the proportion of *An. coluzzii* and A*nopheles gambiae* from survivors (resistant samples) from pyrethroid exposed *An. gambiae s.l* from Deltamethrin, Alphacypermethrin and Primiphos methyl to estimate the frequency of the *kdr* gene in the pyrethroid resistant population. The specimens analyzed were selected from (i) mosquito that survived insecticide exposure during routine insecticide susceptibility tests. The identification of *An. coluzzii* and *An. gambiae*

and *Kdr* PCR assays was preceded by a priori Polymerase Chain Reaction (PCR) assay for identification of members of the Anopheles gambiae complex. This includes DNA extraction using essential extraction kits followed by PCR analysis [11].

Species identification by PCR

All *Anopheles* mosquito from agricultural and non-agricultural sites were separately analysed (Table 1). The DNA extracted from each specimen using standard method was amplified with the *Anopheles gambiae* species specific multiplex PCR. PCR products were separated in agarose gel, stained with ethidium bromide and visualised under UV Trans illuminator. The PCR diagnosis bands for this assay include: A 464-base pair (bp) band for *An. melas*, 390 bp for *An. gambiae s.s.* and 315 bp for *An. arabiensis*.

Based on the outcome of the species specific-PCR assay, aliquot of DNA from each sample was processed for subsequent test to identify *An. coluzzii* and *An. gambiae* using established protocols [12].

Table 1. PCR identification of resistant *An. gambiae s.l.* and proportion of *An. arabiensis*, *An. coluzzii* and *An. gambiae* from Kokona and Karu LGA.

	Species identified	Number identified (%)	P-Value
Kokona and Karu	*An. arabiensis*	3 (7.7)	(7.7 *vs.* 6.3)
		2 (6.3)	$\times^2$=0.29, df=1, p=0.592
		5 (7.04)	
	An. coluzzii	7 (18)	(18 *vs.* 25)
		8 (25)	$\times^2$=1.14, df=1, p=0.285
		15 (21.13)	
	An. gambiae	29 (74.4)	(74 *vs.* 68.8)
		22 (68.8)	$\times^2$=0.17, df=1, p=0.675
		51 (71.83)	
Grand total		71 (100%)	

Knock Down Resistance (KDR) genotyping

The presence of the *kdr* alleles was tested as earlier described.

The presence of both the west (*kdr-w*) and east (*Kdr-e*) African *kdr* mutations were determine using specific primers and protocols designed for these assays. Details procedures of laboratory protocols are contained in the references provided. Specifically, the West African *kdr* genotype is characterized by three different PCR bands: 293 bp common to both susceptible and resistant specimens; 137 bp susceptible band and 195 bp kdr band [13].

The presence of the three bands in a single specimen indicates heterozygote.

The frequency of the *kdr* gene was calculated using established genotype formula:

f (R)=(2RR+Rr)/2n

Where, f=frequency, n=number of samples analyzed, RR=number of homozygote resistant, Rr=number of heterozygote resistant.

Results

PCR analysis of (71) resistance *An. gambiae s.l.* samples drawn from Alphacy-permethrin, deltamethrin and pirimiphos-methyl exposure indicated the samples were composed of predominantly *An. gambiae* 51 (71.83%), *An. coluzzii* 15 (21.13%) and *An. arabiensis* 5 (7.04%) of this number, the composition of resistant samples from Kokona comprised of *An. arabiensis* 3 (7.7%), *An. coluzzii* 7 (18%) and *An. gambiae* 29 (74.4%). While in Karu resistants were composed of *An. arabiensis* 2 (6.3%), *An. coluzzii* 8 (25%) and *An. gambiae* 22 (68.8%) [14].

No significant difference in the proportion of all three malaria vectors between Kokona and Karu *An. arabiensis* (7.7 *vs.* 6.3) $\times^2$=0.29 P=0.29, *An. coluzzii* (18 *vs.* 25) $\times^2$=1.14, P=0.285 and A*n. gambiae* (74 *vs.* 68.8) $\times^2$=0.17 P=0.675 (Table 2).

Table 2. *An. coluzzii*.

Insecticides	LGA	Species identified	Alpha-cypermethrin resistant								
			Number tested for *Kdr*	*Kdr*				*Kdr-e*			
				RR	Rr	rr	*Kdr* frequency	RR	Rr	rr	*Kdr* frequency
Alpha-cypermethrin	Karu	*An. coluzzii*	1	0	0	1	0	-	-	-	0
	Kokona	*An. coluzzii*	6	1	3	2	0.42	-	-	-	0

Polymerase chain reaction analysis indicated *Kdr-w* gene frequencies in resistant *An. coluzzii* varied with the type of pyrethroid. The *Kdr-w* gene frequency varied from 00 in alphacy-permethrin exposed resistant samples in Karu to 0.50 in deltamethrin exposed resistant in both Kokona and Karu. Significant difference was recorded in *Kdr-w* gene frequency of Alphacy-permethrin exposed *An. coluzzii* was recorded between Kokona and Karu (0.0 *vs.* 42%) $\times^2$=80.05 P=0.0001 in Table 3. While no significant difference *Kdr-w* gene frequency of Deltamethrin exposed mosquitoes from Karu and Kokona was recorded (50 *vs.* 50) $\times^2$=0.000 P=1 (Table 4).

Table 3. *An. gambiae*.

	LGA	Species identified	Alpha-cypermethrin resistant								
			Number tested for *Kdr*	*Kdr-w*				*Kdr-e*			
				RR	Rr	rr	*Kdr* frequency	RR	Rr	rr	*Kdr* frequency
Alpha-cypermethrin	Karu	*An. gambiae*	4	2	2	0	0.75	-	-	-	0
	Kokona	*An. gambiae*	15	1	9	5	0.37	-	-	-	0

Table 4. *An. coluzzii*.

	LGA	Species identified	Deltamethrin resistant								
			Number tested for					*e*			
				RR	Rr	rr	frequency	RR	Rr	rr	frequency
Deltamethrin	Karu	*An. coluzzii*	1	0	1	0	0.5	-	-	-	0
	Kokona	*An. coluzzii*	1	0	1	0	0.5	-	-	-	0

Also, *kdr-w* gene frequencies in resistant *An. coluzzii* from organophosphate exposed samples varied according to LGA with significantly higher *Kdr-w* gene frequency recorded in *An. coluzzii* from Karu (33 *vs.* 00) ($\times^2$=62.06 P=0.0001) (Table 5).

Table 5. *An. gambiae*.

	LGA	Species identified	Deltamethrin resistant								
			Number tested for *Kdr*	*Kdr-w*				*Kdr-e*			
				RR	Rr	rr	*Kdr* frequency	RR	Rr	rr	*Kdr* frequency
Deltamethrin	Karu	*An. gambiae*	2	0	1	1	0.25	-	-	-	0
	Kokona	*An. gambiae*	3	0	2	1	0.33	-	-	-	0

The PCR analysis also indicated that *kdr-w* gene frequencies in resistant *An. gambiae* varied with the insecticide exposed. *Kdr-w* gene frequency from 0.33 in deltamethrin exposed resistant *An. gambiae* from Kokona to 0.75 in alphacy-permethrin exposed A*n. gambiae* from Karu. Significantly higher *kdr-w* gene frequencies were recorded in Alphacy-permethrin resistant *An. gambiae* from Karu than Kokona 0.75 *vs.* 0.37 ($\times^2$=1.10 P=0.293) (Table 6).

Table 6. *An. coluzzii*.

	LGA	Species identified	Pirimiphos-methyl resistant								
			Number tested for *Kdr*	*Kdr*				*Kdr-e*			
				RR	Rr	rr	*Kdr* frequency	RR	Rr	rr	*Kdr* frequency
Pirimiphos-methyl	Karu	*An. coluzzii*	6	0	4	2	0.33	-	-	-	0
	Kokona	*An. coluzzii*	0	0	0	0	0	-	-	-	0

Although higher *kdr-w* gene frequency was recorded in Pirimiphos-Methyl exposed *An. gambiae* from Karu 0.44 than Kokona 0.36. There was no significant difference in *kdr-w* gene frequency between the two sites $\times^2$=0.80 P=0.371 (Table 7).

Table 7. *An. gambiae*.

	LGA	Species identified	Pirimiphos-methyl resistant								
			Number tested for *Kdr*	*Kdr-w*				*Kdr-e*			
				RR	Rr	rr	*Kdr* frequency	RR	Rr	rr	*Kdr* frequency
Pirimiphos-methyl	Karu	*An. gambiae*	16	2	10	4	0.44	-	-	-	0
	Kokona	*An. gambiae*	11	0	8	3	0.36	-	-	-	0

Discussion

In Nigeria, merging operational wide scale vector control decisions for LLIN mass campaign with specific malaria vector surveillance and insecticide resistance monitoring data is crucial for the success of targeted control efforts.

Globally resistance to pyrethroids-the only insecticide class used in Insecticide Treated Nets (ITNs)-continues to be widespread. It was detected in at least one malaria vector in 69.9% of the sites for which data were available [15].

This study was conducted to provide an understanding of the resistance status of *An. gambaie* and *An. coluzzii* and the proportion of each member of *An. gambiae s.l* in resistant samples from two LGAs of Nasarawa state, Nigeria. This is part of a general effort to link species distribution to information on insecticide resistance for the purpose of management of insecticide use.

Pyrethroid resistance affecting both *An. gambiae* and *An. coluzzii* is widespread and has been recorded in at least 20 out of 36 states of Nigeria but the actual number could be higher because there is no in-country platform for insecticide resistance monitoring to present accurate data [16].

In this study, insecticide resistance tests and PCR assays indicated that pyrethroid resistance had increased significantly to both Deltamethrin and Alphacypermethrin in the Guinea savannah of North central Nigeria in both *An. coluzzii* and *An. gambiae*. Also, recorded complete absence of resistance in *An. coluzzii* as at 2005. This finding is consistent with who recently reported an escalation of pyrethroid resistance in *An. coluzzii* from 3 sites in the Sahelo-sudan ecozone of Nigeria [17].

Polymerase chain reaction analysis for vector composition of resistant *An. gambiae s.l.* samples drawn from alphacy-permethrin, deltamethrin and pirimiphos-methyl indicated that the samples were composed of predominantly *An. gambiae* followed by *An. coluzzii* and An. arabiensis with no significant difference in the proportion of each of the three malaria vectors in exposed samples between Kokona and Karu [18]. The predominance of *An. gambiae* corroborates who recorded an almost pure collection of the S form (*An. gambiae*) in this ecozone in 2005.

Molecular analysis for knockdown resistance indicated that the highest *kdr* L1014F gene frequencies of 0.75 was recorded in Alphacypermethrin exposed *An. gambiae* from Karu while the highest *kdr* L1014F was recorded in *An. coluzzii* from the Guinea Savannah in the central part of Nigeria. This may be explained by the hypothesis that the source of the sharp increase in *kdr* mutation in *An. coluzzii* presumed to be introgression from the highly resistant *An. gambiae*. Recent studies in Burkina Faso showed that up to 89.3% of *An. coluzzii* with L1014F *kdr* mutation was from introgression from *An. gambiae*; accompanied by enhanced susceptibility to *Plasmodium falciparum* infection. Recent findings from Burkina Faso indicate that introgressed A*n. coluzzii* were significantly more susceptible to *P. falciparum* than non-intogressed *An. coluzzii*. In neighboring Niger republic, resistance in *An. coluzzii* was accompanied by a dramatic rise in sporozoite rate in *An. coluzzii* from 3% to 13%. Increasing

resistance in *An. coluzzii* could lead to a significant increase in malaria transmission [19]. The current trend of complexities in resistance accompanied by an escalation in pyrethroid resistance in *An. coluzzii* indicates that the level of efficacy intended for LLINs might not be met if we continue with the distribution of pyrethroids only LLINs especially in areas with metabolic based resistance [20]. Pyrethroid-PBO nets and neonicotinoid insecticides for IRS with proven efficacy have been recommended by the WHO for use. Hence the need for continual monitoring and update on the dynamics of the spatial distribution of *An. coluzzii, An. gambiae* and *kdr* mutations across Nigeria.

Conclusion

This study provides strong evidence that indiscriminate use of insecticides can cause insecticide resistance in malaria vector populations. Insecticide resistance driven by agrochemical usage should be considered when vector control strategies are developed. The collective goal is to mitigate the impacts of insecticide resistance, hence protecting public health from mosquito-borne disease by improving the efficacy of mosquito control. The CDC bottle bioassay was selected for this study because it can detect resistance to insecticides in mosquitoes and other insects. The technique is simple, rapid and economical, compared with alternatives. From the study, it was adduced that insecticide resistance emerged from multi-generational selection from exposure to sub lethal doses of insecticides. In each population of the mosquitoes, some individuals have alleles for resistance to insecticides, possibly from interactions with plant allelochemicals. Alleles for insecticide resistance are selected when the population of insects is exposed to insecticides, ultimately fixing the alleles in the insect population and resulting in the failure of chemical-based control, though There may also be non-lethal (biological, behavioral) effects of insecticides on mosquitoes, such as changes in blood feeding habits, fecundity, fertility and/or other effects.

Recommendation

A regular monitoring of the status of resistance in a population is encouraged to

Provide a baseline data for program planning and pesticide selection before the start of control operation.

Detect resistance at an early stage, so that timely management can be implemented. Vector Control Programs (VCPs) should be informed about the variables influencing insecticide resistance and ultimately, mosquito mortality.

Continuously monitor the effect of control strategies on insecticide resistance. More should be done to investigate the occurrence and potential causes of insecticide resistance to improve policies aimed at mitigating resistance.

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Conflicts of Interest

The authors declare no conflicts of interest.

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