Journal of Basics and Applied Sciences Research (JOBASR)
ISSN (print): 3026-9091, ISSN (online): 1597-9962
Volume 3(3) May 2025
DOI: https://dx.doi.org/10.4314/jobasr.v3i3.13

Frequency of *Kdr* Mutation, Permethrin and DDT Resistance in *Anopheles* Mosquito Vectors of Lymphatic Filariasis from Old Katsina Province Nigeria

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ABSTRACT
It has been previously reported that insecticide resistance among the vectors of malaria and lymphatic filariasis (LF) exist in the African continent which could unintentionally affect different species of *Anopheles* mosquitoes found in Northern Nigeria. The aim of this study is to identify the *Anopheles* mosquito species and its profile insecticide resistance. The study was carried out in Batagarawa town. Using a mechanical aspirator, blood-feeding mosquitoes were captured indoors and identified morphologically and molecularly. The WHO procedure was followed for insecticide susceptibility bioassay using F_1 mosquitoes, and PCR was used to check for the existence of the *kdr* mutation in *Anopheles* mosquitoes that had survived exposure to permethrin. *Anopheles* mosquitoes were identified as *Anopheles gambiae s.s* and *Anopheles coluzzi*. Insecticide susceptibility test revealed that *Anopheles gambiae s.l* from Batagarawa town was more susceptible to deltamethrin, bendiocarb, propoxur, and malathion recorded 92.3%, 93.6% and 89% after 24 hours post exposure respectively, but resistant to permethrin and DDT recorded 18%, 42.11% after 24 hours post exposure respectively. The execution of effective vector control strategy can be guided by the presence of permethrin and DDT cross resistance in *Anopheles gambiae s.l* complex.

Keywords:
Permethrin,
propoxur,
bendiocarb,
Anopheles coluzzi.

INTRODUCTION

The parasitic worm infection known as lymphatic filariasis (LF) is caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, and is most commonly seen in tropical and subtropical regions of the world. Over 90% of all cases of LF are caused by *Wuchereria bancrofti* among them (WHO, 2015). *Anopheles* mosquitoes are the main carriers of LF in rural populations of both the north central and southern parts of Nigeria, whereas *Culex quinquefasciatus* is the carrier of LF in urban and semi-urban regions in both the north central and southern parts of Nigeria (WHO, 2019). However, there is a severe lack of knowledge regarding the distribution of the LF vectors and the pattern of insecticide resistance in the Sahel region of Katsina State.

According to reports, the several LF vectors on the African continent exhibit insecticide resistance. The M and S forms of *Anopheles gambiae s.s* had been found to contain the *kdr*mutation's pyrethroid resistance mechanism (Ibrahim*et al*., 2019; Ibrahim *et al*., 2014; Awolola *et al*., 2007; Diabate *et al*., 2003; Weill *et al*., 2000). Moreover, Africa has seen widespread DDT and pyrethroid resistance in *Anopheles gambiae s.s* and *Anopheles arabiensis*, with several resistance mechanisms seen in West Africa (WHO, 2016; Yewhalaw *et al*., 2011). These resistance mechanisms may unintentionally affect the processes that depend on density and the vector competence of different *Anopheles* species.

In addition, the use of indoor residual spraying (IRS) and long-lasting insecticide nets (LLINs) is the two main methods for controlling diseases spread by mosquitoes (Nwoke *et al*., 2010). The widespread use of insecticides, herbicides, and agriculturally related chemicals over many years, however, appears to have contributed to the emergence and quick dissemination of insecticide resistance in Africa (Ibrahim *et al*., 2014; Czeher *et al*., 2008).

Changes in the target locations and faster insecticide metabolism are also key contributors to insecticide resistance in mosquito vectors (Ndiath *et al*., 2012). This research will shed light on the identities, geographic distribution, and insecticide resistance profile of *Anopheles* mosquitoes from Sahel region of Katsina State.

MATERIALS AND METHODS

Materials

Analytical grade laboratory chemicals and reagents were used for this study.

Baby fish food (Tetramin), bioassay tubes, cotton wool, distilled water, droppers, filter paper (12cmx 15cm), impregnated papers (12cmx 15cm), mouth aspirator PCR tubes, paper cups, plastic trays, silica gel, phosphate buffer, potassium acetate, ethanol, proteinase K, Taq polymerase, ddH_2O, agarose gel, 1x TEA buffer, ethidium bromide, 1x HotStar Taq buffer, ATL buffer, buffer AE.

Methods

Study Site:

The study was conducted in Batagarawa town headquarter of Batagarawa local government area just 7km from Katsina city (12°54′17″N, 7°37′11″E) with less agricultural activities.

Indoor Collections of Mosquitoes:

Blood fed female mosquitoes resting indoor in the early morning hours (4:00am-5:00am) were collected using mechanical aspirator in Batagarawa town situated in the Sahel region of Katsina State with the characteristic annual rain fall of 800mm to 1600mm and annual temperature of 25.7ºC to 46.5ºC.The gouged female *Anopheles* mosquitoes were maintained on sugar solution (10%) for six (6) days to reach full gravidity at 25ºC ±2 and percentage humidity of 70-75%. After that, each one was put into a 1.5ml eppendorf tube and left to release eggs as previously described by Hemingway and Ranson, (2000). Paper cups were used in preparation for hatching of eggs to larvae. Larvae were maintained on Tetramin™ baby fish food. Emerged 2- to 4-days old F_1 *Anopheles* females were put randomly into cages for insecticide susceptibility test.

Species Identification

After morphological identification, genomic DNA was extracted using Livak, (1984) method from F_1 *Anopheles* mosquitoes collected from Batagarawa town had tolerated exposure to permethrin. The species of *Anopheles gambiae s.l* complex were molecularly identified using SINE200 PCR (Santolamazza *et al*., 2008).

Bioassay Experiment

In line with the WHO protocols, insecticides bioassay test were conducted with 2-4 day old adult Anopheles mosquitoes (WHO, 2016). About 20-25 *Anopheles* mosquitoes per bioassay tube, in 3-4 replicates were treated for 1 hour to either control paper or insecticide-impregnated papers (0.75% permethrin; 0.05% deltamethrin; 0.01% bendiocarb; 5% malathion and 4% DDT). Alive mosquitoes were moved into fresh holding tube fed on sugar solution (10%), the rate of mortality was calculated 24 hours after treatment.

Frequency of *Kdr* Mutation

Using the methodology outlined by Martinez-Torres et al. (1998), the resistant allele *L1014F* of the *kdr* gene was identified. A DNA fragment encoding the voltage-dependent sodium channel in each tested mosquito is used in this PCR diagnostic test, together with four oligonucleotides or primers (Agd1, Agd2, Agd3, and Agd4), to search by amplification for resistant or susceptible alleles. The *kdr* gene is flanked by the Agd1/Agd2 primer pair, which amplifies a 293 bp product as a checkpoint. Only the resistance region of the kdr gene pairs with the pair of Agd3/Agd1 primers, amplifying a 195pb fragment. By amplifying a 137 bp segment, the Agd4/Agd2 pair binds only the responsive region of the gene. The nucleotide sequences of these primers are as follows: Agd1: 5′-ATA GAT TCCCCG ACC ATG -3′; Agd2: 5′-ACA AGG ATG ATG AACC-3′; Agd3: 5′-AAT TTG CAT TAC TTA CGA CA-3′; Agd4: 5′-CTG TAG TGA TAG GAA ATT TA-3'.

By estimating odds ratios (OR) and calculating statistical significance based on the Fisher exact probability test, it was possible to determine whether there was a correlation between the *L1014F kdr* genotypes and permethrin resistance phenotypes.

Statistical Analysis

The odds ratio (R package) for the *L1014F kdr* genotyping was calculated using R version 3.5.0 (https://cran.r-project.org/bin/windows/base/), and significant differences were considered at $P \leq 0.05$.

RESULTS AND DISCUSSION

Results

Identification of Mosquito Species by Molecular Methods:

Using a mechanical aspirator, a total of 2,800 female mosquitoes that feed on human blood were gathered from fifty (50) randomly chosen homes in the Batagarawa town.

Anopheles gambiae s. l was morphologically identified as the species by 1,300 blood-fed female mosquitoes from Batagarawa town and 50 (50) individuals from this complex were randomly chosen for genetic specie identification. 25 (50%), 3 (6%) and 16 (32%) were found to be *Anopheles coluzzii*, *Anopheles arabiensis*, and *Anopheles gambiae s.s* respectively.

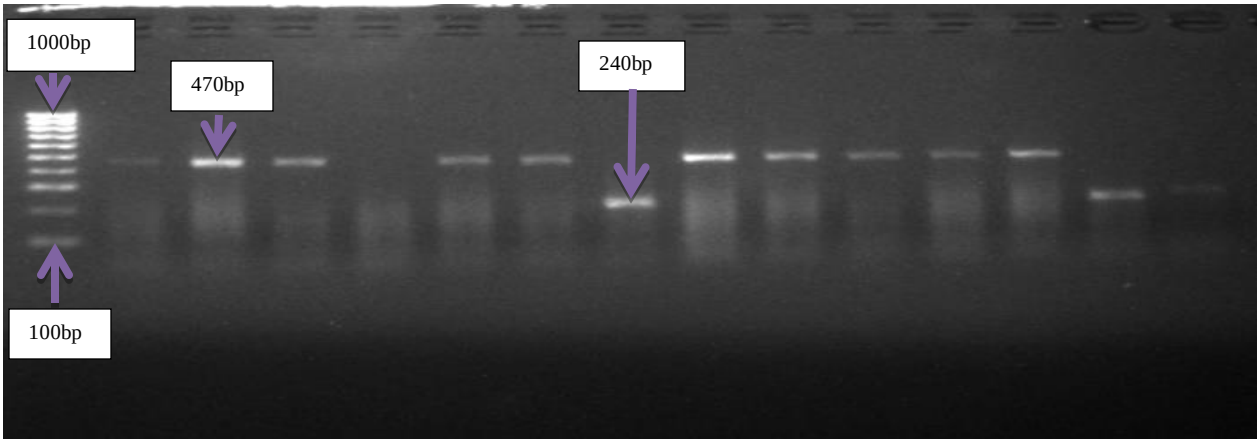

Figure 1: Gel electrophoregram for the identification of members of the *An. gambiae* species complex. Lane M = 100bp molecular weight marker, Lane 1, 2, 3, 5, 6, 8, 9, 10, 11, 12 at 470bps = *Anopheles coluzzii* (formerly M form), Lane 7, 13, 14 at 240bps = *Anopheles gambiae s.s* (formerly S form).

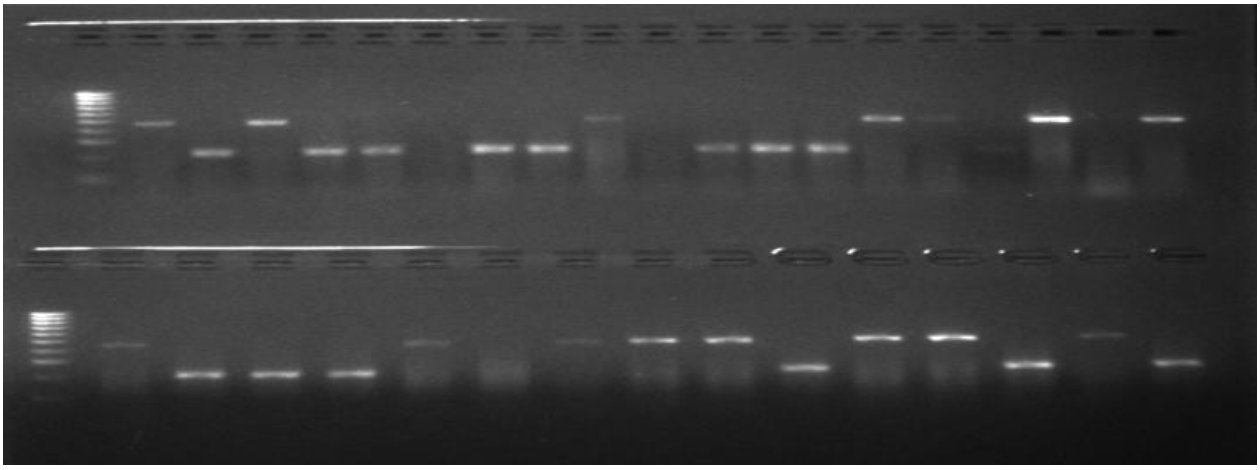

Figure 2: Gel electrophoregram for the identification of members of the *An. gambiae s.l* complex. Lane M = 100bp molecular weight marker, Lane 1, 3, 8, 12, 13, 15, 16, 17, 21, 23, 24, 25, 27, 28, 30 at 470bps = *Anopheles coluzzii* (formerly M form), Lane 2, 4, 5, 6, 7, 9, 10, 11, 14, 23, 26, 28 at 240bps = *Anopheles gambiae s.s* (formerly S form), Lane 18-20 at 220bps = *Anopheles arabiensis.*

Insecticide Susceptibility Bioassays

To undertake an insecticide susceptibility bioassay, 620 F_1 adult female *Anopheles gambiae s. l.* complexes from Batagarawa town were used. In accordance with the findings, susceptible to deltamethrin, bendiocarb, propoxur, and malathion recorded 78.3%, 68%, 88.8%, and 98.11% knockdowns respectively after one hour post exposure and resistant to permethrin and DDT recorded 5.77% and 23.21% knockdown after one hour exposure (Figure 3). Mortality was also found to be 18%, 42.11%, 92.3%, 93.6% and 89%. In control tubes, there was no evidence of death.

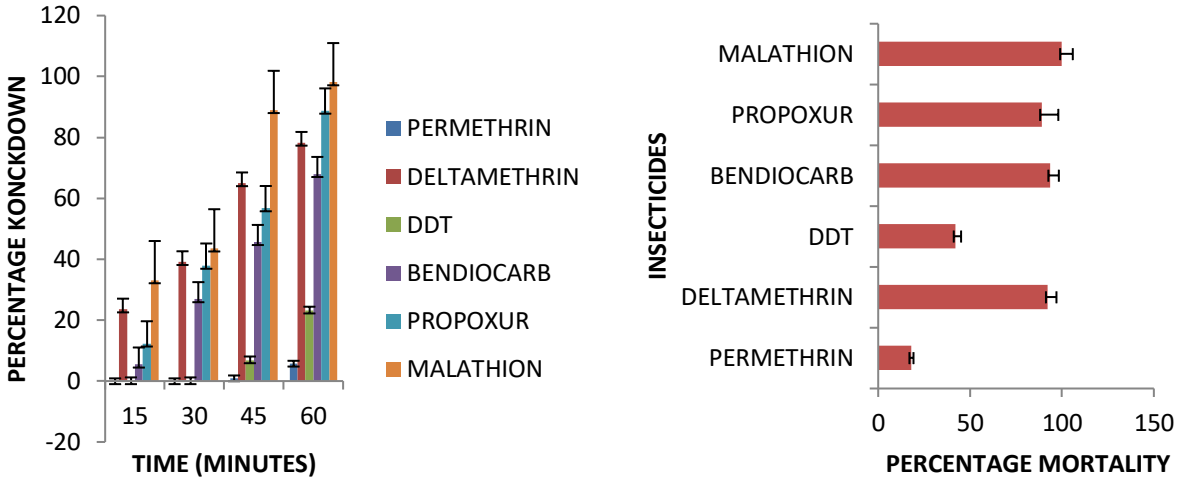

Figure 3: *Anopheles gambiae s. l.* complex from Batagarawa town: knockdown profile and insecticide susceptibility/resistance status. Error bars show how the data can vary.

***Kdr* mutation frequency**

The existence of both the *West-kdr* (*L1014F*) and *East-kdr* (*L1014S*) mutations in 114 mosquitoes from Batagarawa town that were subjected to Permethrin (type 1 pyrethroids) was genotyped. Figures 5 and 6 indicated that forty-six (46) mosquitoes were still alive after exposure (16 *Anopheles coluzzii* and 32 *Anopheles gambiae s.s*, 8 *Anopheles gambiae s.s*, and 3 hybrids). The homozygote resistant (RR), heterozygote resistant (RS), and homozygote susceptible (SS) genotypes for the *L1014F-kdr* as well as the *L1014S-kdr* genotypes were shown in Table 1 along with their respective resistance phenotypes to permethrin.

In light of this, of the 22 genotyped *Anopheles coluzzii* individuals who survived exposure, 23.7% (5) were homozygote resistant (RR) for *L1014F-kdr*, 31.7% (7) were heterozygote resistant (RS), and 50% (11) were homozygote susceptible (SS) for *L1014S-kdr*.

Anopheles gambiae s.s., in contrast to *Anopheles coluzzii*, had only 8 survivors following exposure, of which 25% (2) were homozygote resistant (RR) for *L1014F-kdr*, 37.5% (3) were heterozygote resistant (RS), and 37.5% (3) were homozygote susceptible (SS) for *L1014S-kdr*. The genotyping of three hybrid (*Anopheles coluzzii/Anopheles gambiae s.s*) individuals revealed that they were homozygote susceptible (SS) for *L1014S-kdr*.

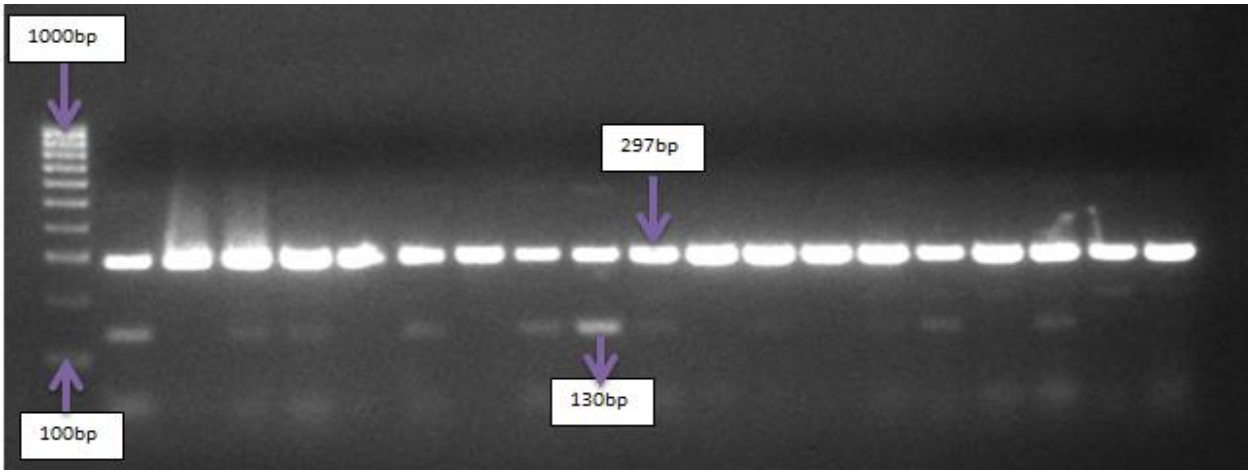

Figure 4: *Anopheles gambiae s.l* complex gel electrophoregram for the presence of both *L1014F* mutations. Lane M is a molecular weight marker with a 100 bp length, Upper Lane 1–19 is a kdr amplified gene at 297 bp, Lane 1, 6, 8, 9, 12, 18, 19 is a susceptible allele (SS) with a 130 bp length, and Lane 3, 4, 10, 14, 15, and 17 is a resistant allele (RS) with a 100 bp length.

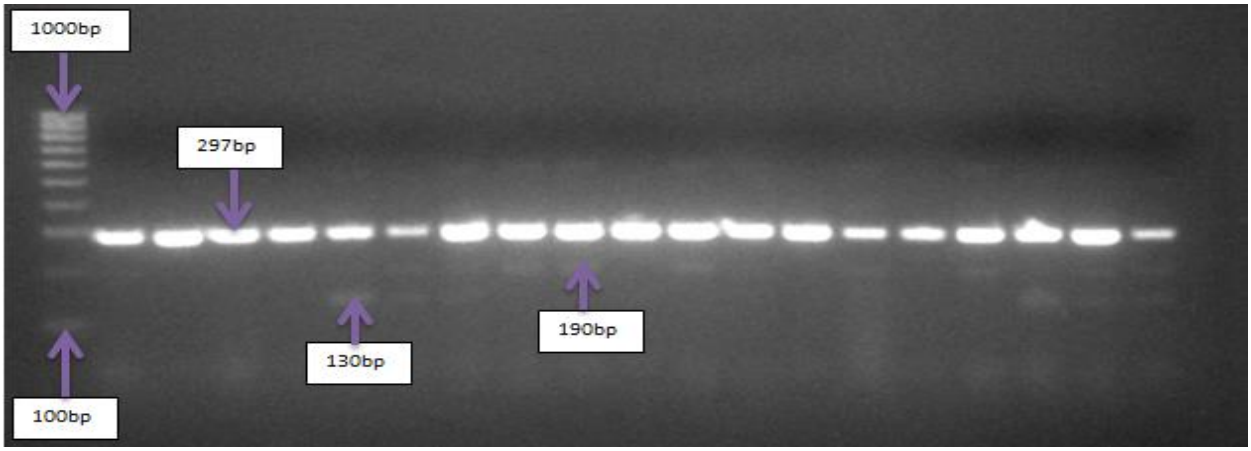

Figure 5: Anopheles gambiae s.l complex gel electrophoregram for the presence of both *L1014F* mutations. Lane M is a 100 bp molecular weight marker. Upper Lane 20–38 is an amplified version of the kdr gene at 297 bp. Lane 24, 25, and 26 are susceptible alleles (SS) at 130 bp, Lane 27, 28, 30, 31, 34, and 36 are resistant alleles (RR) at 190 bp, and Lane 37 and 38 are resistant alleles (RS), which indicate the existence of both susceptible and resistant alleles.

Table 1: Relationship between resistance phenotypes to permethrin and the frequency of the *L1014 F* allele

Species	Phenotype	N	*L1014F* alleles		Odds ratio	*P*- value
			RR	RS		
Anopheles coluzzi	Alive	22	5		0.89	0.56
	Dead	16	7			
	Total	38	4			
			5			
			23.7%			
			31.7%			
Anopheles gambiae s.s	Alive	8	2		1.20	0.04
	Dead	32	3			
	Total	40	8			
			12			
			25%			
			37.5%			

According to the Fisher's exact probability test, the terms RR—Homozygote Resistant—and RS—Heterozygote Resistant—test significantly different at P<0.05.

Discussion

One of the main factors preventing effective vector control is resistance to the main insecticides used in public health. Because of their low human toxicity and great potency at low dosages quickly knock down and eventually kill the mosquitoes, synthetic pyrethroids and organochlorines have demonstrated tremendous success for mosquito control approach for more than decades (Prasittisuk, 1994). Arthropod species, such as *Anopheles gambiae s.l*. and *Anopheles funestus s.l*. complex, have been found to be resistant to these synthetic insecticides, though.

*Anopheles gambiae s.l*complex strains had demonstrated relatively high levels of resistance to permethrin and DDT, as was clearly seen in this study. Cross-resistance between insecticides may be to blame for this.

The sodium volte-gate channels of the nerve sheath are the same target location shared by permethrin and DDT (Feyereisen, 2015). Additionally, agricultural related chemicals and pesticides that have been employed for pest management may have contaminated the mosquito breeding grounds. *Anopheles gambiae s.l*. from sub-Saharan Africa has been found to have broad resistance to DDT and permethrin in previous investigations (Rousseau *et al*., 2016; Yewhalaw *et al*., 2011). The findings of Ibrahim *et al*, (2014), who reported a first-of-its-kind DDT and permethrin resistance in the *Anopheles gambiae s.l* complex, are supported by the current investigation. *Anopheles gambiae* from Batagarawa town was similarly reported by Ibrahim *et al*, (2019) to be resistant to DDT and permethrin in a similar circumstance.

In addition, agricultural related chemicals and pesticides that have been employed for pest

management may have contaminated the mosquito breeding grounds. Despite the possibility that repeated indoor residual spraying and the use of insecticide-treated nets for many years contributed to the evolution of resistant genes that support physiological resistance in a particular mosquito population.

However, the F_1 adult mosquitoes collected in Batagarawa town has shown full susceptibility to deltamethrin, bendiocarb, propoxur, and malathion. Previous research on deltamethrin susceptibility has demonstrated that the same mosquito populations can be both susceptible and resistant to several insecticides from the same family. This might be related to the fact that this population's up-regulated cytochrome P450 (CYP6P4) chose to metabolize permethrin but not deltamethrin.

Furthermore, the susceptibility to deltamethrin suggests that kdr alone may not be sufficient to confer resistance to pyrethroids; metabolic resistance is likely also involved, in contrast to the findings of Okorie *et al*, (2015) from Ibadan, Nigeria, who claimed that high resistance to pyrethroids was present in both Type I (permethrin) and Type II (deltamethrin) pyrethroids.

In a similar manner, the F_1 adult mosquitoes obtained in the study locations were shown to be susceptible to bendiocarb, propoxur, and malathion. This vulnerability may be explained by an excess of the acetylcholinesterase enzyme in the synapses of the nerves and/or a down regulation of the detoxifying enzymes.

Ibrahim *et al*, (2014) indicated that mosquitoes collected from Auyo were entirely sensitive to malathion, and the present investigation agreed with them. The susceptibility pattern was corroborated by the lack of the *ace-1R* mutation in all the mosquitoes analyzed.

Moreover, vulnerability to malathion has already been noted in populations of *Anopheles gambiae* in Africa, including those in Burkina Faso (Kwiatkowska *et al*., 2013) and Cameroon (Antonio-Nkondjio *et al*., 2011).

In controlling mosquitoes from the Sahel region of Katsina State, type II pyrethroids (deltamethrin), malathion, bendiocarb, and propoxur may still be useful. In populations of *Anopheles gambiae s.l* in Batagarawa town, this study indicates the presence of permethrin and DDT insecticide resistance.

According to earlier research (Himeidan *et al*., 2011; Rogan and Chen, 2005), *Anopheles gambiae* from sub-Saharan Africa exhibits extensive resistance to DDT and pyrethroids. The presence of DDT and permethrin resistance in Batagarawa town is a blatant sign of the spatial spread of DDT and permethrin resistance in Northern Nigeria's Sahel Savannah region.

The presence of hybrids (*Anopheles gambiae/Anopheles coluzzii*) found in Batagarawa town are homozygotes susceptible (SS- allele), which is consistent with earlier findings made by several authors (Djogbenou *et al*., 2010; Della Torre *et al*., 2001; Péka, 2001) and supports the theory of gene flow between the two species despite the well-developed speciation. According to Taylor *et al*, (2010), the existence of these hybrids may be related to *Anopheles coluzzii* and *Anopheles gambiae's* insufficient reproductive differentiation. *Anopheles coluzzii* and *Anopheles gambiae s.s* in Batagarawa town, are updated in this study with current levels of resistance and frequencies of the *kdr* mutation. Only two *Anopheles* species were found in the town of Batagarawa: *Anopheles coluzzii* (M form) and *Anopheles gambiae s.*s (Ibrahim *et al*., 2019).

Ibrahim *et al*, (2019) reported that, the most prevalent species in Batagarawa town was *Anopheles gambiae s.s*. *Anopheles coluzzii* (M form) predominated in urban or semi-urban settings, whereas Anopheles gambiae s.s (S form) was more prevalent in rural settings, according to earlier research in Nigeria (Kristan *et al*., 2003) and Cameroon (Wondji *et al*., 2005).

Anopheles coluzzii and *Anopheles gambiae s.s* both had frequencies of the *L1014F kdr* mutations of 62.5 and 54.5%, respectively. In the populations of *Anopheles gambiae* and *Anopheles coluzzii* in Batagarawa town, there was no discernible change in the frequencies of the resistant allele *L1014F* of the *kdr* gene (P>0.05). Since more than ten years ago (Dabire *et al*., 2012; Dabire *et al*., 2009), Burkina Faso has been well-versed in the spread of the *L1014F kdr* mutation in the *Anopheles gambiae s.l* complex, which includes *Anopheles gambiae*, *Anopheles coluzzii*, and *Anopheles arabiensis*. Numerous investigations found that *Anopheles gambiae s.s* and *Anopheles coluzzii* populations, particularly those from the Sudan Savannah region where mutation frequency was becoming fixated, had a high frequency of this mutation (Dabire *et al*., 2012; Dabire *et al*., 2009).

The frequency of the *kdr* mutation and permethrin resistance were shown to be positively correlated in this study in *Anopheles gambiae s.s* (odd ratio= 1.20 and P= 0.04). Both the strongest permethrin resistance and the highest frequency of the *L1014F kdr* mutation were discovered in Batagarawa town, indicating that a stronger physiological resistance that is more permethrin-selective has been present in this town for a long time. As a result, increased use of insecticides for pest control and/or extensive LLIN use in the town may be to blame for the high frequency of kdr mutations that have previously been noted in another population of *Anopheles gambiae* in the "M" form living in a setting in Burkina Faso (Kwiatkowska *et al*., 2013). Permethrin and DDT, which are utilized in the battle against malaria and LF vectors, are inexorably trending toward inefficiency.

CONCLUSION

The success of the present insecticide-based control strategy in the Sahel region of northern Nigeria may be

hampered by the cross resistance of permethrin and DDT in the main malaria and LF vectors described in this study. In these areas where such knowledge was previously insufficient, it is crucial to keep track of this resistance and its underlying mechanisms. This could serve as guidance for putting in place effective vector control strategies in Sahel region of northern Nigeria.

Competing Interests

No competing interests are disclosed by the authors.

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