Insecticide Resistance Profile of Anopheles gambiae Mosquitoes: A Study of a Residential and Industrial Breeding Sites in Kano Metropolis, Nigeria

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ABSTRACT: Monitoring and understanding the trend and dynamics of insecticide resistance is very key to devising efficient control strategies. This study was carried out to characterize the mosquito population, its insecticide resistance profile, and the physicochemical properties of their breeding sites in Sharada and Wailari of Kano State, Nigeria. Six breeding sites from the 2 study areas were sampled and their physicochemical parameters determined. Mosquito larvae were sampled from the sites and reared to adult. The emergent adults were morphologically and molecularly identified to species level. The World Health Organization (WHO) susceptibility assay was carried out on the adult mosquitoes using different classes of insecticides in WHO discriminating concentrations. kdr-mutation was detected by polymerase chain reaction (PCR)-based method using the permethrin (pyrethroid) resistant and susceptible adult mosquitoes. Most of the determined physicochemical parameters were significantly higher in the industrial area, Sharada. Morphologically, the mosquitoes from the 2 sites were identified as *Anopheles gambiae* and 100% of the randomly sampled population were found to be *Anopheles coluzzii* by PCR-based molecular technique. The WHO susceptible assay revealed a graded level of resistance to bendiocarb, dichlorodiphenyltrichloroethane (DDT), and permethrin with mortalities of 78.36%, 75.74%; 43.44%, 56.96%; and 37.50%, 37.50% in both Sharada and Wailari, respectively. Pre-exposure to piperonyl butoxide (PBO) resulted in a significant but minor recovery of susceptibility to permethrin. The kdr mutation frequency was higher in Sharada (45.71%) relative to Wailari (31.43%). Higher kdr mutation frequency was also observed in the resistant population (48.56%) relative to the susceptible (28.54%). The kdr mutation frequency was weakly associated with the resistance status (odds ratio [OR]: 5.9, χ^2:3.58, $P = .058$) and the breeding sites (OR: 3.46, χ^2:2.90, $P = .088$). In conclusion, the study revealed a highly pyrethroid-resistant *A coluzzii* population with low PBO recovery rate. Furthermore, the data suggested the involvement of kdr mutation, detoxification enzyme, and possibly abiotic factors of the breeding sites.

KEYWORDS: Malaria, insecticide resistance, *Anopheles coluzzii*, Nigeria, molecular resistance, metabolic resistance, physicochemical properties

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Introduction

Malaria is a life-threatening disease caused and spread by *Plasmodium* parasite and female mosquito vectors, respectively.[1] It is the world's most important parasitic disease of public health interest.[2] According to the latest world malaria report, African region continues to have an alarmingly high incidence rate of malaria – 92% of malaria cases and 93% of malaria deaths.[1] Nigeria accounts for 25% of the cases in Africa.[3] In Northwest Nigeria, a prevalence range of 60% to 65% has been reported in different studies in Kano State despite the high frequency of use of insecticide-treated nets (ITNs) and indoor residual spraying in the area.[4,5]

In human, malaria is caused by *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale*, and *Plasmodium malariae* – and the infection by *P falciparum* has been reported to pose the greatest threat.[6] *Anopheles gambiae* complex constitutes the main vector that transmits one of the most threatening *Plasmodium* species – *P falciparum* in sub-Saharan Africa.[7] The *A gambiae* complex consists of 7 species (*A gambiae sensu stricto* [s.s.], *Anopheles arabiensis, Anopheles quadriannulatus* species A and B, *Anopheles melas, Anopheles merus, Anopheles bwambae*) which are morphologically indistinguishable. Recently, it has been reported that *A gambiae sensu stricto* exists in 2 molecular forms, denoted M (now *A coluzzii*) and S-form (now *A gambiae* s.s.), which can be distinguished by differences in a 4-Mb region located in the X chromosome.[8] This increases the number of species to 8. *A gambiae sensu stricto* (s.s.), *A coluzzii*, and A *arabiensis* constitute 3 out of the 6 species considered to exhibit the most vectorial capacity.[9] Ecologically, *A melas, A merus*, and *A bwambae* are salt-tolerant and are described as saltwater or mineral water species, while the others are obligate freshwater species.[10] Their public health importance stems from their high anthrophilicity and potential to exploit and adapt to diverse environmental conditions occasioned by human activities – directly or indirectly.[7] This is an indication that they are able to evolve different mechanisms for survival under different environmental conditions defined by various physicochemical parameters.

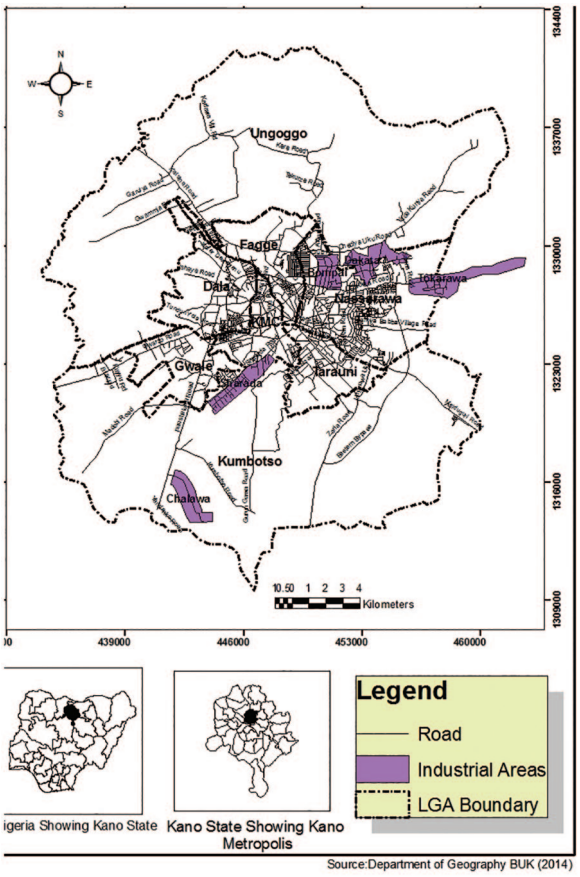
Malaria vector control currently in most part of Africa relies on the use of insecticides through indoor residual spraying and long-lasting insecticides – treated nets (LLINs). This has proven to be effective in the last decade; however, the emergence and spread of insecticide resistance is threatening the sustainability of this approach.[11] In Nigeria, data on the

frequency of use of different insecticides/pesticides in the study locations specifically are lacking; nonetheless, a long list of different pesticide/insecticide products belonging to different classes are available in the local markets and employed both agriculturally and domestically for pest/vector control. They include organophosphate (dimethoate, dichlorvos, primiphos-methyl, chlorpyrifos, malathion), carbamates (isoprocarb, propoxur, bendiocarb), pyrethroids (transfluthrin, cypermethrin, deltamethrin, permethrin), and the least prevalent neonicotinoids (thiamethoxam, thiacloprid).[12] The class organochlorine (dichlorodiphenyltrichloroethane [DDT], endosulfan) is still in use despite the proscription by United Nation Environmental Programme (UNEP).[12,13] It is a common practice in the study area to use all of the mentioned classes of pesticides both domestically and for agricultural purposes with the exception of pyrethroids which is a more preferred option for domestic control of insect vectors. Resistance of *Anopheline* mosquitoes to pyrethroid in West and East Africa has been reported.[14] The major mechanisms related to insecticide resistance to date include an increase in expression of detoxification enzymes (as in permethrin) and the knockdown resistance (kdr) gene (as in pyrethroid and DDT).[15] Knockdown resistance is associated with mutations in the voltage-gated sodium channels of nerve cell membranes (causing decline in the sensitivity), the target of these 2 classes of insecticides.[16] In *A gambiae* s.s., the African malaria vector, 2 point mutations in the voltage-gated sodium-channel gene confer kdr to DDT and pyrethroid insecticides – a leucine-phenylalanine substitution at position 1014 (L1014F) of the gene in the strains from Burkina Faso and Coˆte d'Ivoire and a second mutation, a leucine-serine substitution at the same codon (L1014S) identified in a colony from Kenya.[17] The L1014F mutation has been observed in both M (*A coluzzii*) and S (*A gambiae* s.l) molecular forms of *A gambiae* s.s., whereas the L1014S was observed in only the M-form.[16] Both forms of mutation have been reported to be associated with resistance to pyrethroids; however, high frequency of 1014F kdr allele has been significantly associated with resistance to lambda-cyhalothrin in *A coluzzii*.[16,18]

Effective control of malarial vector amid spreading insecticide resistance presents enormous logistics challenges. Monitoring and understanding the dynamics in relation to some environmental elements such as climate, physicochemical properties are key to addressing the challenges. The impact of environmental elements on mosquito oviposition,[19] larval development/distribution,[20] larval density, and development[7,21] has been reported in some endemic areas of Africa. However, studies that have investigated mosquito vector species, resistance/susceptibility status, and physicochemical properties of the breeding environment are still lacking in Nigeria. While Kabula et al[7] studied insecticide resistance in *A gambiae* s.i. relative to physicochemical properties within Accra metropolis, Awolola et al[14] investigated the kdr mutations in M and S forms of *A gambiae* s.s. in Southwest Nigeria and more recently Ibrahim et al[22] characterized the transmission and resistance profiles of *A coluzzii* population in Nigeria. Both studies from Nigeria did not consider the possible association of abiotic factors of breeding sites to resistance profiles of the sampled mosquitoes. Second, despite these studies, it is important to note that the mosquito insecticide resistance phenomenon is dynamic and thus necessitates continuous study to monitor the trend. It is upon this premise that this study was carried out to characterize the mosquito species, resistance profile, and physicochemical properties of 2 breeding sites – Wailari (residential) and Sharada (industrial) – in Kano state, Northwest Nigeria.

Methods

Study area



Source: Department of Geography, Bayero University, Kano State, Nigeria.

The study was conducted in 2 areas of Kano Metropolis. First, Wailari area of Kumbotso local government, which is located on longitude 8.503 E, latitude 11.888 N (residential site). This area is predominantly characterized by residential houses and activities. Second, Sharada industrial area of Kano Municipal (KMC) local government, which is located on longitude 8.4897 E and latitude 11.9490 N (industrial site). This area is majorly characterized by industries and pockets of farms and living settlements. The 2 sites were chosen because of the relatively divergent human activities in them (residential vs industrial).

Sampling sites and laboratory rearing of larva

Anopheles mosquito larvae were collected from 3 ponds (earthen turbid ponds) randomly selected from each site around industrial and residential areas within Kano metropolis within the months of June to September, 2018. The larvae were identified on the basis of their spatial projections on the surface of the waters (horizontally inclined). A 35-mL dipper attached to the end of an approximately 1.2-m pole was used to scoop larvae from the waters. The cup was then inspected for the presence of *Anopheles* larvae. If no larvae were present, the cup was emptied and tried on another spot nearby. If larvae were present, they were removed using a small pipette and transferred to another holding cup prior to taking another dip. This process was continued until a large number of larvae were collected.[23]

Larvae collected were taken to the insectary and allowed to develop into pupae. The pupae were then separated and kept inside the net cage. The pupae developed into fully grown adult mosquitoes within 24 hours. The insectary condition was at temperature 25°C to 33°C and humidity 70% to 80% with a 12-hour day/night cycles.[24] The mosquitoes were fed with 10% sucrose solution and allowed to grow into adulthood in 72 hours before selecting and subjecting the females to bioassay.

Morphological identification

Morphological identification was conducted using the Gilles and Coetzee[25] characteristic under a Zeiss ×10 light microscope. The upper margins of the fore wings contained some dark spot (wing spots), a feature common to all *Anopheles* mosquitoes. The palpis are elongated and segmented into 3 parts with a pale spot on the second dark area.

DNA extraction

DNA was extracted from individual mosquitoes using the method of Livak.[26] Each *A gambiae* female mosquitoes was homogenized in 100 µL warmed Livak grind buffer (1.6 cm^3 5 M Nacl, 5.48 g sucrose, 1.57 g Tris, 10.16 cm^3 0.5 M EDTA, 2.5 cm^3 20% SDS) and incubated at 65°C for 30 minutes. The homogenate was briefly microfuged and 14 µL 8 M K-acetate added to make a final concentration of 1 M. The homogenate was then incubated on ice for 30 minutes. Debris and precipitated SDS and protein were removed by 20 minutes centrifugation at 4°C in a refrigerated centrifuge. The resulting supernatant was transferred into a new 1.5 µL eppendorf tubes. The nucleic acid was obtained from the supernatant by adding 200 µL of 100% ethanol and then vortexed and spun for 15 minutes at 4°C. The supernatant was removed and discarded and the pellet was rinsed in approximately 100 µL ice cold 70% ethanol and allowed to dry for 1 hour in the tube. The dried pellet was suspended in 100 µL distilled water and incubated at 65°C for 10 minutes.

Species identification by PCR method

The specimens were identified to species and molecular forms by the method of Santolamazza et al[27] using species-specific primers for *A gambiae* complexes – the *SINE200 forward and reverse primers* (forward 5′-CGCTTCAAGAATTCGAG ATAC-3′ and reversed 5′-CGCTTCAAGAATTCGAGA TAC-3′). Fifty-six *A gambiae* mosquitoes (30 from Sharada and 26 from Wailari) were used. The extracted DNA pellets were subjected to polymerase chain reaction (PCR) (Taqman). The reactions were carried out in a 25 µL reaction tube which contained 1 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 2.5 U Taq polymerase, and 0.5 µL of template DNA extracted from a single mosquito. Thermocycler conditions were 94°C for 10 minutes followed by 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute, with a final elongation at 72°C for 10 minutes, and a 4°C hold. The resulting products were analysed on 1.5% agarose gels stained with ethidium bromide at 85 V, with low- and high-molecular-weight bands corresponding to fragments containing or lacking the targeted *SINE200*, respectively.

WHO Insecticide susceptibility/resistance bioassay tests

Insecticide susceptibility/resistance bioassay tests were carried out using World Health Organization (WHO) susceptibility test-kits and standard procedures[28,29] with 4 replicates of 20 to 25 non-blood-fed adult female mosquitoes. The assays were carried out in batches by exposing the mosquitoes to papers impregnated with a recommended concentration of a given insecticide (purchased from Universiti Sains, Penang, Malaysia) in the assay tubes. The following insecticides (classes) were used for the assay: (1) 0.75% permethrin (a pyrethroid: inhibitor of closure of voltage-gated sodium channels), (2) 4% DDT (organochlorine: inhibitor of closure of voltage-gated sodium channels), (3) 4% bendiocarb (carbamate: reversible inhibitor of acetylcholinesterase), and (4) 0.1% malathion (organophosphate: cholinesterase inhibitor) and 0.75% permethrin + 5% piperonyl butoxide (PBO) (a synergist that inhibits cytochrome P450 oxidase system and carboxyesterase). Each replicate was exposed to the insecticide-impregnated filter paper for 60 minutes. During the exposure period, the number of mosquitoes knocked down was recorded after 15, 30, 45, and 60 minutes. The mosquitoes were then transferred into the holding tube and fed on glucose solution via a pad of a cotton wool soaked in 10% glucose solution and placed on the mesh-screen end of the holding tubes. The time taken to achieve 50% of population knockdown (KDT_{50}) was assessed using log-probit analysis. Mortality was determined 24-hour post-exposure by counting the number of dead and alive mosquitoes. An adult mosquito is considered to be alive if it was able to fly regardless of the number of legs remaining. Any knocked down mosquito, whether or not it has lost a leg or wing, was considered moribund and counted as dead. Mosquitoes were classified as dead or knocked down if they were immobile or unable to

stand or take off. On completion of the susceptibility test, the mosquitoes were transferred individually to clearly labelled tubes with a lid for airtight locking (separating dead and alive mosquitoes into separate tube) for preservation until further analysis.

For the synergist bioassay to establish the potential involvement of metabolic enzyme system using PBO and permethrin, replicates of about 20 to 25 female mosquitoes were pre-exposed to 5% PBO for 30 minutes in a tube. They were subsequently transferred to a tube containing permethrin for 1 hour. The mosquitoes were treated as in conventional bioassay described above and mortalities scored after 24 hours.

Detection of kdr mutation (pcr kdr)

Genomic DNA was extracted from 35 *A coluzzii* (18 randomly sampled from Sharada and 17 from Wailari) mosquitoes as described earlier above. Knock down resistance (L1014F) was detected by the methods of Martinez-Torez et al.[17] Amplification was performed using the following primers – Agd1 (5′-atagattccccgaccatg-3′), Agd2 (5′-agacaaggatgaatgaacc-3′), Agd3 (5′-aatttgcattacgaca-3′), and Agd4 (5′-ctgtagtgatgatag-gaaattta-3′) all in a single set and kappa Taq DNA polymerase. The cycling conditions were initial 95°C denaturation for 3 minutes followed by 10 cycles of 1-minute denaturation at 94°C, 30 seconds of annealing at 54°C, and 30 seconds of extension at 72°C followed by 30 cycles of a 1-minute denaturation at 94°C, 30 seconds of annealing at 47°C and 30-minute extension at 72°C and a final extension at 72°C for 10 minutes. Amplification products were checked on a 2% agarose gel and visualized after ethidium bromide stain in syngene bio-imaging system. The genotype frequency was calculated by dividing the number of individual mosquitoes with a given genotype, by the total number of analysed mosquitoes.

Physicochemical parameter determination

A total of 18 parameters were measured from water samples collected from 2 sites which were chosen to reflect the type of human activities taking place in the areas. Standard methods as described by American Public Health Association (APHA)[30] were used: temperature, pH, electrical conductivity, total dissolved solutes (TDS), dissolved oxygen (DO), sulphate, phosphate, carbonate, sodium, potassium, calcium, magnesium, zinc, lead, nickel, iron, cadmium, and manganese. Metals were analysed using atomic absorption spectrophotometer. Conductivity, TDS was measured using conductivity-TDS meter, while pH and temperature were measured using a pH meter and thermometer, respectively (Hanna Instrument, United States).

Data analysis

Percent mortality was calculated as a percentage of the total number of mosquitoes used for the screening in the 4 replicates. The KDT_{50} values were estimated using Probit Analysis. Sample size was determined by *Sampsi* (sample size and power for proportions) command. A 1-way analysis of variance (ANOVA) and *T* test were used to compare the mortalities observed with each insecticide and each site, respectively. Chi-square test and classical test of hypothesis by *csi* and p*rtesti* (2-sample proportion comparison calculator) command in Stata were used to test for association and difference between molecular kdr allele frequency and the resistance status as well as the studied breeding sites. The relationship between physicochemical properties of the breeding sites (water), the kdr allele frequency, and insecticide resistance profiles were determined using Pearson bivariate correlation analysis. *P*-values $<.05$ were considered significant. All statistical analyses were carried out using Stata version 12.1 for Windows (Stata Corp LP, United States).

Results

Identification of the A gambiae complex species

Following morphological identification, out of 607 *A gambiae* s.l. mosquitoes, 56 were selected for PCR-based molecular identification and were all identified as *A coluzzii* (with band at 479 bp) using the SINE PCR species identification method.

WHO insecticide susceptibility test

The result of the WHO insecticidal bioassay is presented in Figures 1A and B and 2 and Table 1. The result generally showed that DDT, permethrin, and permethrin + PBO recorded a significantly ($P<.05$) poor knock down (low percentage knockdown and longer median knockdown time). The pre-exposure to synergist, PBO, recorded a significant but low improvement in the knock down potential of permethrin. The 24-hour mortality assay result (Figure 2) revealed a very high mortality (no resistance) for malathion and bendiocarb (with low level of resistance) and low mortality and high resistance to DDT, permethrin, and permethrin + PBO. The pre-exposure with PBO did not increase the mortality observed with permethrin beyond 60%. Comparatively, lower mortality was observed in Walari relative to Sharada; however, a significant ($P<.05$) difference in mortality between the 2 sites was only observed with DDT.

Physicochemical properties of breeding sites

The physical and chemical parameters of the breeding sites are presented in Tables 2 and 3. The result showed a significantly higher total dissolved solids (TDS) and electrical conductivity (EC) in Sharada relative to Wailari for the physical parameters. There was no significant difference in the mean pH and temperature of the 2 sites. All the chemical parameters (Tables 2 and 3) for Sharada were significantly higher ($P<.05$) than that of Wailari.

Kdr mutant alleles distribution and correlation with physicochemical parameters

The distribution the kdr alleles is presented in Table 4. A total of 37 mosquitoes (dead and alive from permethrin susceptibility

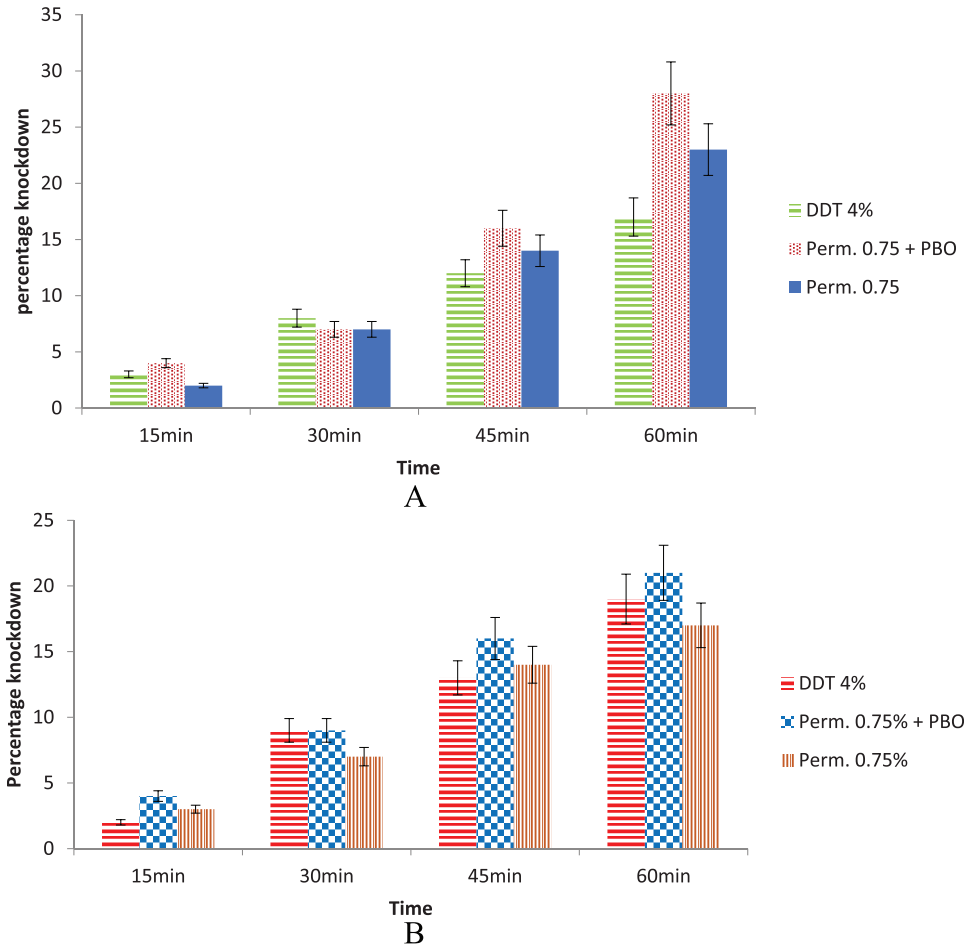


Figure 1. Knockdown profile for *Anopheles coluzzii* mosquitoes from (A) Sharada industrial area and (B) Walari residential area.
DDT indicates dichlorodiphenyltrichloroethane; PBO, piperoxyl butoxide; Perm, permethrin.

assay) were screened for kdr mutation. An overall frequency of 77.14% kdr mutation (homozygous + heterozygous) was observed. A frequency of 33.43% kdr (+) (11.43% homozygous and 20.00% heterozygous) was observed in Walari and 45.71% (17.14% homozygous and 28.57% heterozygous) in Sharada. Comparing the kdr mutant frequency by resistance/susceptible status, a frequency of 48.56% (17.4% homozygous and 31.42% heterozygous) kdr mutation was observed in the resistant population, while the susceptible gave a frequency of 28.54% (11.40% homozygous and 17.14% heterozygous). Statistically, a weak association was observed between the kdr mutation and breeding site (odds ratio [OR]: 3.46, χ^2: 2.90, P=.088) and also between the kdr mutation and resistance/susceptibility status of the mosquito population (OR: 5.9, χ^2: 3.58, P=.058).

The bivariate correlation of the frequency of kdr mutant alleles (homozygous and heterozygous) with the physicochemical parameters of the studied sites (Table 5) revealed that there was a significant correlation (P< .05) between the frequency kdr mutant and the following physicochemical properties: total dissolved solids, phosphate, sulphate, potassium, manganese, and iron.

Discussion

The study was carried out in 2 environmentally diverse areas of Kano State, Wailari and Sharada, categorized residential and

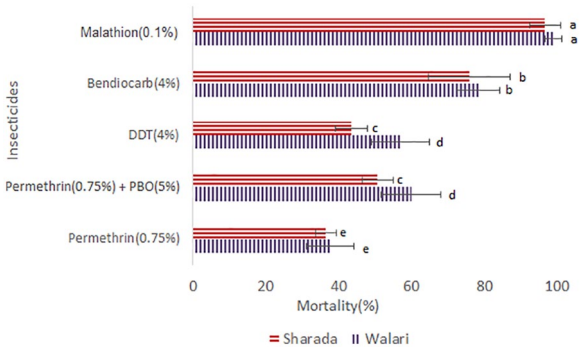


Figure 2. Resistance profiles of an *Anopheles coluzzii* mosquitoes from Sharada and Walari using different classes of insecticides.
Different alphabets across the bars column are statistically different (P < .05).
DDT indicates dichlorodiphenyltrichloroethane; PBO, piperoxyl butoxide.

industrial area, respectively, by their predominant human and land use. In this study, a preponderance of chemical parameters was observed in Sharada breeding sites. This may be a result of pollution from the industrial waste water and effluents.[7,31]

The morphological and molecular identification of the studied mosquito population showed that *A coluzzii* was the only member of the *A gambiae* complex in the 2 study sites. There was no variation in the *Anopheline* species found in the 2 sites despite divergent environmental factors. Similar findings

Table 1. Knockdown time of the *Anopheles gambiae* exposed to organochlorine and pyrethroid.

PESTICIDES	WALARI		SHARADA	
	KT50 (MIN)	95% CONFIDENCE INTERVAL	KDT_{50} (MIN)	95% CONFIDENCE INTERVAL
Permethrin	169.28[a]	156.83-191.72	143.60[a]	113.37-173.63
Permethrin + PBO	141.35[b]	107.09-145.61	123.64[a]	110.26-137.00
DDT	166.06[b]	127.64-204.47	191.74[b]	156.37-227.11

Abbreviations: DDT, dichlorodiphenyltrichloroethane; KDT_{50}, time taken to knockdown 50% of the mosquito sample population; PBO, piperoxyl butoxide.
Different superscripts along a column or across a row are statistically different ($P < .05$).

Table 2. Mean distribution of physical parameters across Sharada (industrial area) and Wailari (residential area) breeding sites in Kano metropolis.

PARAMETER	WALAIRI	SHARADA	SIG (2-TAILED)
Temperature (°C)	33.47 ± 3.17	32.18 ± 2.46	.561
pH	8.63 ± 1.82	9.24 ± 0.89	.015
DO (mg/L)	2.24 ± 0.12	1.86 ± 0.08	.014
TDS (mg/L)	240 ± 11.79	351 ± 10.54	<.001
EC (dS/m)	0.42 ± 0.013	0.58 ± 0.04	.002

Abbreviations: DO, dissolved oxygen; EC, electrical conductivity; TDS, total dissolved solid.
Values are presented as mean values ± standard deviation.

Table 3. Mean distribution of chemical parameters across Sharada (industrial area) and Wailari (residential area) breeding sites in Kano metropolis.

PARAMETER	WAILARI	SHARADA	SIG (2-TAILED)
PO_3^{2-} (mg/L)	6.967 ± 0.316	13.023 ± 0.166	<.001
Cl^- (mg/L)	536.433 ± 28.335	732.227 ± 32.287	.001
HCO^3 (mg/L)	58.033 ± 2.902	81.134 ± 2.510	<.001
SO_4^{2-} (mg/L)	117.177 ± 6.503	51.122 ± 2.20	<.001
Mg^{2+} (mg/L)	6.046 ± 0.064	6.819 ± 0.745	<.001
K^+ (mg/L)	9.739 ± 0.257	17.791 ± 0.184	<.001
Ca^{2+} (mg/L)	19.76 ± 0.316	45.17 ± 1.460	<.001
Na^+ (mg/L)	7.907 ± 0.138	13.038 ± 0.518	<.001
Fe^{2+} (mg/L)	0.174 ± 0.005	0.704 ± 0.006	<.001
Mn^{2+} (mg/L)	0.081 ± 0.003	0.159 ± 0.080	.002

Values are presented as mean values ± standard deviation.

have been reported in previous studies carried out in Northwestern and Northeastern part of Nigeria.[21,32,33] All 3 independent studies, spanning over 3 consecutive years, reported a preponderance of *A coluzzii* (formerly M-form) of *A gambiae* in different parts of Northern Nigeria. In contrast, studies from distal Southern Nigeria have reported both forms with a higher frequency of the *A gambiae* s.s. (S-form).[14] Interestingly, previous studies have opined that the *A coluzzii* is an obligate freshwater with low tolerance for salinity and pollutants.[34] But the findings of this study showed that this species is beginning to thrive in polluted sites possibly due to selective pressure from ecological changes or human activities. Similar observations have been made by Awolola et al[31] and Nwaefuna et al[35] in different studies.

Table 4. Frequency distribution of kdr mutant alleles by breeding sites and resistance profile.

	GENOTYPE	SUSCEPTIBLE (%)	RESISTANT (%)	OR	χ^2	*P*-VALUE
kdr (+)	L1014F/L1014F	11.40	17.14	5.9	3.58	.058
	L1014L/L1014F	17.14	31.42			
kdr (–)	L1014L/L1014L	17.14	5.70			
	GENOTYPE	SHARADA (%)	WALARI (%)	OR	χ^2	*P*-VALUE
kdr (+)	L1014F/L1014F	17.14	11.43	3.46	2.90	.088
	L1014L/L1014F	28.57	20.00			
kdr (–)	L1014L/L1014L	5.80	17.14			

Abbreviations: L1014L/L1014L, homozygous normal mosquitoes; L1014L/L1014F, heterozygous mutant mosquitoes; L1014F/L1014F, homozygous mutant mosquitoes; OR, odd ratio.

Table 5. Correlation of physicochemical properties with frequency of kdr mutant alleles.

PHYSICOCHEMICAL PARAMETERS	P	CL	HCO^3	SO_4^{2-}	MG	K	CA	NA
r (*P*-value)	0.812* (.047)	0.685 (.133)	0.747 (.088)	−0.804* (.043)	0.789 (.062)	0.867* (.048)	0.794 (.059)	0.808 (.052)
PHYSICOCHEMICAL PARAMETERS	FE	MN	EC	TDS	TEMPERATURE	DO	PH	
r (*P*-value)	0.815* (.048)	0.817* (.049)	0.744 (.091)	0.838** (.037)	0.693 (.127)	−0.670 (.140)	0.861 (.162)	

Abbreviations: DO, dissolved oxygen; EC, electrical conductivity; TDS, total dissolved solid.
The Pearson correlation coefficients are expressed as *r* (*P*-value).
r values bearing * are statistically significant.

The WHO insecticides susceptibility test revealed a high level of resistance to DDT and permethrin. A relatively low level was also observed with bendiocarb. This is an indication of local selective pressure from intensive use of these classes of insecticides for agricultural purposes, as well as for indoor residual spraying.[6] It has become a common practice in these areas to use pesticides meant for outdoor agricultural purposes, indoor as a preferred method for controlling mosquitoes and other insects with little or no consideration of the consequences. Comparatively, Sharada recorded higher resistance to all the insecticides relative to Wailari. Although this was not significant, it is a likely indication that some factors peculiar to Sharada such as the significantly high physicochemical parameter may have contributed (directly or indirectly) to the resistance/susceptibility status of the mosquitoes in some little measures. The poor, but significant, recovery of susceptibility to permethrin with pre-exposure to PBO (cytochrome P450 inhibitor) prior to permethrin susceptibility assay implicated the involvement of detoxification enzyme, cytochrome P450 oxidase system, and carboxyesterase (metabolic resistance) to the resistance of permethrin. This is in line with previous studies,[36-38] but the low recovery of susceptibility also suggests that other mechanisms aside the expression of cytochrome P450 oxidase system and carboxyesterase may be largely involved as posited by Riveron et al,[39] in a study in Mozambique. This is of grave consequence to malaria vector control via long-lasting insecticide-treated nets (LLIN), considering the fact that the vast majority of the nets presently deployed control are treated with pyrethroids only.[6]

The data from the study revealed a higher frequency of the kdr mutant alleles in the resistant mosquito population of *A coluzzii* and also in the Sharada breeding site; however, the association between the frequency of 1014F kdr mutation and permethrin resistance profile (OR: 5.9, χ^2: 3.58, $P = .058$) as well as the breeding sites (OR: 3.46, χ^2: 2.90, $P = .088$) was statistically poor. This corroborates our earlier position that 1014F kdr mutation may not be the major factor responsible for the observed level of resistance to permethrin in this study, detoxification by cyt P450, and a cluster of other mechanisms may be hugely implicated.

The observed higher frequency of kdr mutation (although not significant), preponderance of some physicochemical parameters, and the significant correlation with kdr mutation frequency is an indication that some of the physicochemical parameters may contribute in different measures to the observed insecticide resistance and adaptation of *A coluzzii*. Studies that investigate the direct impact of some physicochemical parameters on development of resistance or resistance profile of *A*

coluzzii are still lacking. However, pockets of literature have reported the possible impact of some physical and chemical parameters of breeding sites on mosquito breeding. Fossog et al[40] reported that DO and ammonia significantly correlated with larval tolerance in a study in Cameroun. In Tanzania, Emidi et al[41] reported the association of high salinity and conductivity to *Anopheles* and *Culex* mosquito larvae abundance while Kabula et al[7] posited that nitrite and fluoride were the best predictors of pyrethroid resistance in deltamethrin and permethrin. This study revealed a significant correlation between kdr mutation frequency and the following physicochemical parameters – total dissolved solids, phosphate, sulphate, potassium, manganese, and iron. It also recorded a high kdr mutation frequency in the breeding site (Sharada) that showed a preponderance of most of the physicochemical parameters. This result generally indicates that these physicochemical parameters may be implicated in the mechanism of resistance of *A coluzzii*. However, the true nature of their involvement is still uncertain. It is very unlikely that they are directly involved. Oliver and Brooke[42] demonstrated under controlled condition that metal pollution increases insecticide tolerance to malathion and deltamethrin in *A gambiae* larva, but this was linked to increase in the detoxification enzyme rather than molecular mechanism of mutation. Thus, further studies are required in this area.

In conclusion, the ability of *A gambiae* to adapt and tolerate increasingly abiotic diverse breeding waters and the dramatic wide spreading resistance to pyrethriod insecticides in Northern Nigeria will portend problems for control in the near future in this region. Monitoring and understanding the dynamics is very key to devising efficient control strategies. This study revealed a preponderance of *A coluzzii* mosquitoes (a member of the *A gambiae* complex) with high level of resistance to DDT and permethrin and a potentially emerging resistance to bendiocarb in the study sites. The study further revealed a high frequency of kdr mutation in *A coluzzii* in both sites contrary to previous findings in Southwest, Nigeria.[14,43] Overall, the pattern of resistance and kdr mutant allele distribution suggests that molecular mutation in association with other mechanisms and some physicochemical factors may account for the observed resistance to permethrin in the study. However, the study is considered preliminary and provides baseline data for a more robust and large-scale study. The study was limited by scale, size, as well as the failure to determine some chemical parameters such as residual hydrocarbons and pesticides which may have also contributed to the resistance profile.

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Author Contributions

AAI conceived, designed, and supervised the study; JTD carried out the field and benchwork while CJO analysed the data and drafted the manuscript.

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