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PYRETHROID RESISTANCE IN THE SUDAN SAVANNAH REGION IN NIGERIA: A STUDY OF THE RESISTANCE PROFILE AND RESISTANCE MECHANISM OF ANOPHELES POPULATIONS FROM HADEJIA TOWN IN JIGAWA STATE

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ABSTRACT

Insecticide-based control measures are key strategies against malaria vectors, and pyrethroid is the only recommended class of insecticide for public health use. The work aimed at determining the pyrethroid resistance in Anopheline mosquitoes and the frequency of the knockdown resistance (*kdr*) gene determinant. Larval samples were collected from two sites in Hadejia Town, Jigawa State in April 2020 and November/December 2020, and reared to adulthood in Bayero University Kano Biochemistry's insectary. Samples were identified by morphological and molecular techniques. Three to five-day-old adult mosquitoes were exposed to standard concentrations of 0.75% permethrin and 0.05% deltamethrin according to WHO criteria. Kdr mutations were investigated using PCR. Results of morphological identification showed an abundance (100%) of the *Anopheles gambiae* complex. However, molecular identification showed varying percentages of *An. gambiae s.s* (15% and 35%), *An. coluzzii* (80% and 45%), and *An. arabiensis* (5% and 20%) each for agricultural and industrial sites, respectively. The result also revealed relatively higher KT_{50} and KT_{90} in the agricultural site and was relatively higher with permethrin based on the KT_{90}. Higher insecticide resistance of *Anopheles* mosquitoes observed in the agricultural site suggests that the practice may affect resistance development. The frequency of negative (homozygous) L1014F kdr mutation genotype was 70% in the resistant (alive) mosquito population and 50% in the susceptible (dead) mosquito population. The frequency of kdr mutation for agricultural and industrial sites was 35% and 15%, respectively. This finding suggests that the *kdr* gene determinant may not be the only mechanism involved in the resistance of the Anopheline mosquito to pyrethroid.

INTRODUCTION

Malaria is a disease that affects global health. About 3.2 billion people worldwide are at risk of malaria infection. The World Health Organization (WHO) reported that 93% of global malaria deaths occur in Africa (WHO, 2019). High mortality rates are recorded in children and pregnant women, and malaria negatively impacts the economy of the African continent (Okorosobo *et al.,* 2011; WHO, 2018). In Nigeria, about 97% of its population is at risk of malaria infection. Nigeria contributes about 27% and 24% of Africa and global malaria deaths, respectively (WHO, 2017; WHO, 2019). The control of malaria primarily involves targeting the disease vectors to stop the transmission of the disease (Mulla, 1994). Of the *Anopheline* species, *Anopheles gambiae complex and Anopheles funestus* are among the primary mosquito species that transmit malaria parasites in Africa. These species are abundant in tropical and subtropical Africa (Coetzee *et al.,* 2013).

The insecticide-based control strategy is part of the key control measures used in targeting disease vectors (Niyang *et al.,* 2018). The most widely effective insecticide-based control measures are indoor residual spraying with insecticides and insecticide-treated materials (bed nets). Pyrethroids are the only WHO-approved class of chemicals for insecticide-treated materials (WHO, 2013). Pyrethroids have relatively reduced toxicity to humans and a fast knockdown effect (Zaim *et al.,* 2000). They perform their insecticidal action by targeting voltage-gated sodium ion channel

(VGSC) by keeping it unusually open for a long time (Hemingway and Ranson, 2000). Malaria vectors in Africa have developed resistance to insecticides such as pyrethroids (Ranson *et al.,* 2011). This resistance increases in major *Anopheles* vectors in Africa and might affect primary malaria vector control programmes (Knox *et al.,* 2014; Stica *et al.,* 2019). The prolonged use of pyrethroids could result in selection pressure in mosquitoes and lead to a mutation known as target site insensitivity. Molecular studies in some vectors have supported the effect of point mutation known as knockdown resistance (*kdr*) mutations in VGSC in pyrethroid-resistant species. *Kdr* mutation was first observed in pyrethroid-resistant house flies and German cockroaches (Narahashi, 1998; Rinkevich *et al.,* 2013). The mutation involves the substitution of leucine by phenylalanine and or substitution of leucine by serine at the sixth segment of domain II. These mutations are referred to as *kdr-w* linked with resistance in West Africa and *kdr-e* linked to East Africa resistance, respectively (Reimer *et al.,* 2008). Other resistance mechanisms include metabolic and penetration resistance (Safiyanu *et al.,* 2019). Multiple resistances were recorded in local *Anopheles* species from North-Eastern Nigeria (Oduola *et al.,* 2019). *Anopheles* mosquitoes in some areas were also reported to resist the effect of deltamethrin (Oduola *et al.,* 2012; Awolola *et al.,* 2014) and permethrin (Awolola *et al.,* 2014).

Human activities like pollution due to urbanization, industrial activities, and the use of agrochemicals can help the disease vectors to develop insecticide resistance (WHO, 2013; Alhassan *et al.,* 2015). Proper knowledge of the current resistance profile and resistance mechanism is significant in the management and control of malaria vectors. This strategy will assist in limiting future selection for insecticide resistance (Nkya *et al.,* 2014; Safiyanu *et al.,* 2019; Stica *et al.,* 2019). Previous studies in the same region (North-Western Nigeria) have shown the escalation of pyrethroid resistance in *Anopheles* mosquitoes (Safiyanu *et al.,* 2016; Safiyanu *et al.,* 2017; Safiyanu *et al.,* 2019; Ibrahim *et al.,* 2019; Ononamadu *et al.,* 2020). The knowledge of mutations leading to resistance phenotype in vectors can give an early insight into the emergence of insecticide resistance, which might not be observed in bioassays. This information is necessary for planning and implementing control strategies. The work aimed at determining the resistance of two populations of Anopheline mosquitoes to permethrin and deltamethrin insecticides and the frequency of an insecticide resistance mechanism, *kdr*, within the population.

MATERIALS AND METHODS

Study Area

The study was carried out in the Sudan savannah region of Jigawa State. Two sites were studied; agricultural and industrial sites from Hadejia town, Hadejia Local Government, Jigawa State (Latitude: 12°44'98"N, Longitude: 10°04'44"E). The State has a total land area of approximately 22,410 km^2 and a density of 251.7 per km^2. It has coordinates of 120 00^{l} N 90 45^{l} E between latitudes 11.00° N to 13.00° N and longitudes 8.00° E to 10.15° E (JGS, 2015). The occupation of the inhabitants includes fishing, rice farming, and the establishment of irrigation-based activities. Farmers use fertilizers and insecticides for agriculture and other purposes in urban areas.

Larval collection

The larvae were collected from their natural breeding sites at different points in the study sites; agricultural (irrigation) and industrial sites as described previously (Service, 1993). Samples were collected from April 2020 and November to December 2020. *Anopheles* mosquito larvae were sampled from randomly selected water bodies on vegetation farms, choked gutters, and waterlogged areas around each study site. These sites were highly polluted with organic materials. *Anopheles* mosquito larvae were identified based on horizontal spatial projections on the water surface. The procedure of Robert *et al.* (2002) was used to obtain larval samples. A dipper was used to obtain *Anopheles* larvae, and they were transferred with their breeding waters to the holding containers. This procedure was done several times until significant numbers of larvae were obtained for each period of the study.

Rearing of *Anopheles* larvae to Adults in the Insectary

The larvae in their breeding site water were transferred into white plastic containers. The containers were covered with an untreated net (Service, 1993). The larvae were transported to the insectary (Bayero University, Kano, Nigeria) under the temperature and humidity conditions of 27± 2°C and 70 ± 20%, respectively, and a 12 h day/night cycle, according to Das *et al.* (2007). The larvae were fed with Tetramin™ baby fish food (about 10 g dissolved in 50 mL of water) once daily. This feeding method continued until the pupae emerged, separated, and placed in mosquito net cages until adulthood. The mosquitoes were fed with a 10% sucrose solution using cotton wool (Service, 1993).

DNA extraction

DNA of each mosquito was extracted using the method of Livak (1984). An adult mosquito was homogenized in 100 µL warmed Livak grind buffer (1.6 cm^3 of 5 M NaCl, 5.48 g sucrose, 1.57 g Tris, 10.16 cm^3 of 0.5 M EDTA, 2.5 cm^3 of 20% SDS) and incubated at 65 °C for 30 min. The homogenate was briefly centrifuged, 14 µL of 8 M K-acetate was added, to give a total concentration of 1 M and incubated on ice for 30 min. Again, the solution was centrifuged at 4 °C for 20 min to remove debris, protein, and precipitated SDS. The supernatant was transferred into a sterile 1.5 mL Eppendorf tube. The DNA was obtained from the supernatant by addition of 200 µL of 100% ethanol, then vortexed and spun for 15 min at 4 °C. The supernatant was discarded and the pellet was rinsed in about 100 µL ice-cold 70% ethanol and was left to dry for 1 h in the tube. Thereafter, 100 µL of distilled water was added to the dried pellet and incubated at 65 °C for 10 min.

Species Identification (Morphological Species identification)

The morphological identification was performed as described previously (Coetzee, 2020). Characteristics unique to all *Anopheles* mosquitoes were screened using a Zeiss X10 light microscope (Optika ISO 22196, Microscopes Italy).

Species identification by PCR

Species identification by PCR was performed according to the method of Santolamazza *et al.* (2008) using species-specific primers for *Anopheles gambiae* complexes – the SINE200 X6.1 forward and reverse primers (forward 5'-TCGCCT TAGACCTTGCGTTA-3' and reverse 5'-CGCTTCAAGAATTCGAGATAC-3'). The extracted DNA samples were used for polymerase chain reaction (TaqMan PCR). A 25 µL reaction tube comprised 1 pmol of each primer, 0.2 mM of each dNTP, 2.5 U Taq polymerase, 1.5 mM $MgCl_2$, and 0.5 µL of template DNA extracted from each mosquito. Thermocycler conditions were as follows: denaturation at 94 °C for 10 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 1 min, with final elongation at 72 °C for 10 min. PCR products were analyzed on 2% agarose gel stained with ethidium bromide at 110 V for 35 min, with low and high molecular weight bands corresponding to fragments having or lacking the targeted *SINE200*, respectively.

WHO Insecticide Bioassay Tests

Mosquitoes' insecticides diagnostic kit was used to establish susceptibility and resistant status. Insecticide susceptibility assays were performed using 2-4 day-old adults. They were randomly selected from pooled families and used for insecticide bioassays as described previously (Cuamba *et al.,* 2010; Wondji *et al.,* 2012) using the WHO protocol (WHO, 1998). The following insecticides were tested: 0.75% permethrin (type I pyrethroid), and 0.05% deltamethrin (type II pyrethroid). These insecticides were procured from the Universiti Sains Malaysia (USM), Malaysia.

For each insecticide, adult mosquitoes were divided into batches of 20-25 per test (four replicates) and exposed to insecticides treated papers for 1 h, and paper with only carrier oils as control. The knockdown rate was recorded every 15 min for 1 h. Thereafter, they were transferred back to the resting tubes for 24 h and fed using 10% sucrose solution soaked on a pad of cotton wool placed on the mesh-screen end of the holding tubes. Mosquitoes were considered dead

or knocked down when immobile or not able to take off. Mosquitoes were transferred to appropriately labeled 1.5 mL Eppendorf tubes after the bioassay. The resistant and susceptible mosquitoes were separately placed in labeled 1.5 mL Eppendorf tubes and stored at -80°C for subsequent assays.

Detection of kdr mutation (kdr PCR)

The knockdown resistance (*kdr*) mutation (L1014F) of mosquitoes was determined by the method of Martinez-Torres *et al.* (1998). Genomic DNA was extracted from mosquito samples collected from the two study sites namely: agricultural and industrial sites. Thirty samples were analyzed, i.e., 15 dead and 15 alive mosquitoes. DNA amplification was performed by use of the following primers – Agd1 (5'-ATAGATTCCCCGACCATG-3'), Agd2 (5'-AGACAAGGATGAATGAACC-3'), Agd3 (5'-AATTTGCATTACGACA-3'), and Agd4 (5'-CTGTAGTGATGATAGGAAATTTA- 3') all in a single set and kappa Taq DNA polymerase. Thermocycler conditions were as follows: initial denaturation at 95 °C for 3 min, then 10 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 30 sec, and extension at 72 °C for 30 sec, 30 cycles of denaturation at 94 °C for 1 min, annealing at 47 °C for 30 sec, extension at 72 °C for 30 min and a final extension at 72 °C for 10 min. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide and visualized in Syngene bio-imaging system (Syngene UGenius Gel Imaging System, ID #: 21791, LabX, United States).

Statistical analysis

The mortality rate between 98-100% indicated full susceptibility, 90-97% as possible resistance, and less than 90% was considered resistant to the tested insecticides. The frequency of genotype was obtained by dividing the number of individual mosquitoes with a given genotype by the total number of analyzed mosquitoes. KT_{50} and KT_{90} were analyzed using regression analysis (probit).

RESULTS

The results obtained showed the species composition based on the morphological identification of adult Anopheline mosquitoes from agricultural and industrial sites in Hadejia town, Jigawa State, Nigeria. All the samples morphologically identified belonged to the members of the *An. gambiae s.l.* complex. The species composition of adult *Anopheles* mosquitoes from the two study sites based on molecular identification showed that *An. gambiae*, *An. coluzzii* was predominant in the agricultural and industrial study sites (Table 1).

Table 1: Species composition of adult *Anopheles* mosquitoes from the two study sites in Hadejia town, Jigawa State, Nigeria based on molecular species identification.

		Anopheles gambiae complex	
Sites	*An. gambiae s.s*	*An. coluzzii*	*An. arabiensis*
Agricultural	3 (15%)	16 (80%)	1 (5%)
Industrial	7 (35%)	9 (45%)	4 (20%)

The average knockdown time KT_{50} and KT_{90} for the studied insecticides are presented in Tables 2 and 3. Deltamethrin showed a significantly lower KT_{50} (50.58 and 43.48 min in agricultural and industrial sites, respectively) and KT_{90} (126.00 and 98.05 min, agricultural and industrial, respectively) relative to permethrin with KT_{50} (113.57 and 91.36 mins) and KT_{90} (291.33 and 180.50 min) in the agricultural and industrial sites, respectively. The result also revealed higher KT_{50} and KT_{90} for the agricultural site compared with the industrial site. This observation was statistically significant with permethrin in terms of recorded KT_{90} (291.33 min) according to results obtained from regression analysis (probit). The lower KT_{50} and KT_{90} observed with deltamethrin suggest a superior knockdown potential relative to permethrin. It also indicated a lower resistance

Table 2: KT_{50} for the two studied sites.

	Agricultural		Industrial	
	KT_{50} (min)	95% C.I	KT_{50} (min)	95% C.I
Deltamethrin	50.58	(42.08 - 59.08)	43.48	(22.19 - 64.77)
Permethrin	113.57	(74.84 - 152.30)	91.36	(84.41- 98.31)

Table 3: KT_{90} for the two studied sites.

	Agricultural		Industrial	
	KT_{90} (min)	95% C.I	KT_{90} (min)	95% C.I
Deltamethrin	126.00	(98.90 - 153.09)	98.05	(84.16 - 111.93)
Permethrin	291.33	(159.63 - 423.03)	180.50	(150.53 - 210.47)

Kdr mutation genotype frequencies of Anopheles species from the two study sites were presented. Figure 1 describes the gel-electropherogram of the *kdr* genotype for the L1014F mutations in *Anopheles* mosquitoes from the study sites. The frequency of *kdr* mutation genotype of the *Anopheles* species from the two study sites was 35% and 15% in the agricultural and industrial sites, respectively.

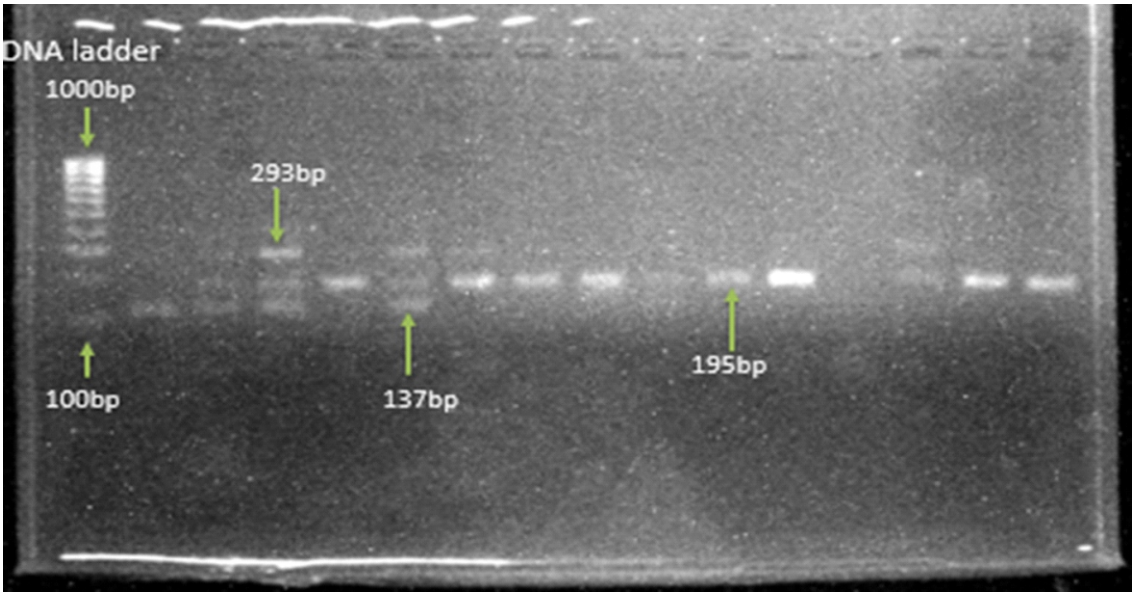

Figure 1: Gel electropherogram of *kdr* genotype for the L1014F mutations in *Anopheles* mosquitoes from the two study sites.

Legend:
Control band: 293bp
Susceptible: 137 bp
Kdr: 195 bp

DISCUSSION

Adult mosquitoes were identified morphologically, and the results showed an abundance of the *Anopheles gambiae* complex in the agricultural and industrial sites. *Anopheles gambiae s.s.* and *Anopheles funestus* complex are the main mosquito species that transmit malaria parasites in Africa and are widely spread over subtropical and tropical Africa. Also, *Anopheles gambiae* Giles is the main African malaria vector in humans (CDC, 2010; Coetzee *et al.,* 2013). However, molecular species identification showed that in each study site, the adult *Anopheles* mosquitoes comprised of the *An. gambiae s.l* complex consisting of *An. gambiae s.s*, *An. coluzzii*, and *An. arabiensis*, respectively. Also, *An. arabiensis* were observed in this study during the dry season. This observation is similar to the report of Coetzee *et al.* (2013), that *An. arabiensis* is located in drier environments, while *An. coluzzii* is distributed in West and Central Africa. Proper identification of mosquitoes is significant to the control of the diseases that the vectors transmit (Ajamma *et al.,* 2016). Morphologically indistinguishable *Anopheles* often consists of genetically distinct sibling species in which one member of the species complex might be a significant malaria vector, even though others are not. Entomological assessment of the risk of malaria transmission can therefore involve the exact identification of the *Anopheles* species.

Resistance (mortality of less than 90%) was recorded from insecticide bioassay for both insecticides, although percentage mortality was higher in both sites using deltamethrin than permethrin. KT_{50} and KT_{90} are of great value in determining insecticidal knockdown potential or capacity of insecticides in understudied conditions and can serve as an indicator of the emerging resistance intensity to insecticides. The observation with regards to deltamethrin with lower KT_{50} and KT_{90} values is an indication of its superior knockdown potential relative to permethrin. It also showed a lower resistance selection pressure against deltamethrin. The relatively higher KT_{50} and KT_{90} in the agricultural site suggest resistance due to selection pressure relative to the industrial site and more prominent with permethrin. This finding is in line with the report by Reid and McKenzie (2016). They observed in a descriptive review that in 23 out of 25 relevant publications across Africa, higher insecticide resistance was associated with agricultural insecticide use. Permethrin was reported to be cheaper, readily available, and more used by local farmers compared to deltamethrin, suggesting a selection of pressure against permethrin. Also, this result might be attributed to the fact that deltamethrin is a type II pyrethroid insecticide compared to permethrin as a type I pyrethroid. The two types of pyrethroids differ from each other in terms of the presence or absence of the alpha-cyano group. Presently, there are four classes of chemical insecticides: pyrethroids, carbamates, organophosphates, and organochlorines (DDT exclusively). However, only pyrethroids are approved by WHO for treating bed nets because of their fast knock-down effect and relatively low toxicity to humans (Zaim *et al.,* 2000). Pyrethroid resistance observed in this study is similar to permethrin and deltamethrin resistance reported by Adeogun *et al.* (2012) and Awolola *et al.* (2007) in North-Central and South-Western Nigeria, respectively. The rate of pyrethroid resistance in *An. coluzzii* from Northern Nigeria has increased in less than a decade, threatening the efforts of WHO projection (by the year 2030) to reduce the malaria burden by 90%. Therefore, it is important to continue monitoring the resistance and its underlying mechanisms in the region. Surveillance will assist in malaria control- programmes and aid in the implementation of evidence-based control measures (Ibrahim *et al.,* 2019).

Mutations in the target site proteins are the most popular pyrethroid resistance mechanism observed in insects (Nkya *et al.,* 2014). The L1014F *kdr* mutation occurs in the central nervous system of insects involving non-synonymous mutations of the gene that encodes the VGSC (Nkya *et al.,* 2013). Pyrethroids affect the gating ability of VGSC and keep it unusually open for a long period. This can lead to paralysis and the death of insects. Long-term use of pyrethroids can exert selection of pressure on *Anopheles* vectors and could lead to substitution or point mutation leading to the insensitivity of the target site. Molecular studies of some vectors support the effect of *kdr* mutation (point

mutation) in VGSC in pyrethroid-resistant vectors (Narahashi, 1998; Rinkevich *et al.,* 2013). Increased rate of insecticide resistance genes in *Anopheles gambiae* giles *sensu stricto* poses threats to vector control measures (Reimer *et al.,* 2008).

Knowing vectors that possess a trait known to cause resistance is of significance because resistance can spread fast in such a population until selection pressure is eased or when genetic cost relative to the resistant allele is high. Resistance genes range from dominant to semi-dominant to recessive. Dominant and semi-dominant are both significant in terms of the expression of resistance genes (Corbel and N'Guessan, 2013). *Kdr* mutation genotype frequency of *Anopheles species* from the agricultural site (35%) was higher relative to that of the industrial site (15%). This finding can be attributed to the resistance recorded in this study. Also, other mechanisms different from *kdr* mutation alone might have contributed to the *Anopheles* resistance observed in this study. Corbel and N'Guessan (2013) reported that failure to detect kdr may not indicate the absence of resistance in a population as it induced by other mechanisms. This observation is in line with a similar study reported by Ononamadu *et al.* (2020). Hence, molecular assays should complement bioassays. Also, resistance might be attributed to the kind of activities occurring in the study sites and/or the time of sample collection which is the dry season (April 2020 and November/December 2020) because mosquito larval samples for similar work have been usually collected during rainy season due to their abundance.

CONCLUSION

This study showed that *Anopheles gambiae s.l* is the primary malaria vector in both agricultural and industrial sites in the study area. *Anopheles coluzzii* was found to be dominant in both study sites. The high level of pyrethroid resistance observed in this study could be linked to other forms of resistance including the rapid spread of *kdr* L1014F mutations. Understanding the composition and distribution of major vectors of malaria including their resistance profile will assist in the proper management and monitoring of insecticide resistance. This will help in vector control programmes.

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