Original Article

First Report of Target Site Insensitivity in Pyrethroid Resistant *Anopheles gambiae* from Southern Guinea Savanna, Northern-Nigeria

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Abstract

Background: Malaria is a major public health problem and life threatening parasitic vector-borne disease. For the first time, we established and report the molecular mechanism responsible for *Anopheles gambiae* s.l. resistance to pyrethroids and DDT from Yamaltu Deba, Southern Guinea Savanna, Northern-Nigeria.

Methods: The susceptibility profile of *An. gambiae* s.l. to four insecticides (DDT 4%, bendiocarb 0.1%, malathion 5% and deltamethrin 0.05%) using 2–3 days old females from larvae collected from study area between August and November, 2018 was first established. Genomic DNA was then extracted from 318 mosquitoes using Livak DNA extraction protocol for specie identification and kdr genotyping. The mosquitoes were identified to species level and then 96 genotyped for L1014F and L1014S kdr target site mutations.

Results: The mosquitoes were all resistant to DDT, bendiocarb and deltamethrin but fully susceptible to malathion. *An. coluzzii* was found to be the dominant sibling species (97.8%) followed by *An. arabiensis* (1.9%) and *An. gambiae s.s* (0.3%). The frequency of the L1014F kdr mutation was relatively higher (83.3%) than the L1014S (39%) in the three species studied. The L1014F showed a genotypic frequency of 75% resistance (RR), 17% heterozygous (RS) and 8% susceptible (SS) with an allelic frequency of 87% RR and 13% SS while the L1014S showed a genotypic frequency of RR (16%), RS (38%) and SS (46%) with an allelic frequency of 40% RR and 60% SS, respectively.

Conclusion: This study reveals that both kdr mutations present simultaneously in Northern-Nigeria, however contribution of L1014F which is common in West Africa was more than twice of L1014S mutation found in East Africa.

Keywords: *Anopheles gambiae* s.l.; Insecticide resistance; Northern Nigeria; Voltage-gated sodium channels (VGSC)

Introduction

Insecticide resistance is mainly associated with genetic factors that are inherited and can be defined as "the ability in a population to tolerate doses of insecticide which would prove lethal to the majority of individuals in a normal population of the same species, developed as a result of selection pressure to the insecticide" (1, 2). The resistance is mainly acquired through two methods which include the target site insensitivity and metabolic resistance (3). The target site insensitivity is operated through one of the following methods (insensitive acetylcholinesterase (AChE), GABA receptor mutation, or mutations in the voltage-gated sodium channel). The nervous system of mosquitoes is been targeted by specific insecticide through which it acts, though sometimes the sensitivity of the site may reduce as a result of mutations leading to

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resistance (4). The organophosphate and carbamates target the acetylcholinesterase (AChE) through carbamoylating the active serine site thereby stopping it from hydrolyzing the acetylcholine (5-7). Substitution in the GABA receptor of an Alanine to Serine has been reported in *Drosophila melanogaster*, *D. simulans*, *Aedes aegypti*, *Anopheles stephensi* and *An. gambiae* as the cause of resistance (8). Resistance in the Pyrethroids and DDT is mainly due to mutations in the gene that encodes the voltage-gated sodium channel called the knockdown resistance (9). In the *An. gambiae* and *An. arabiensis* mosquitoes, two different knockdown resistance have been reported (10). The leucine-phenylalanine substitution at position 1014 of the sodium channel gene (L1014F), was the first mutation reported from Burkina Faso and Ivory Coast in West Africa (11). While a leucine-serine substitution (L1014S) at the same position was reported as the second mutation from Western Kenya in East Africa (12). A study conducted in Northern Nigeria reported high resistance of *An. gambiae* to permethrin and DDT with less resistant to bendiocarb (13). The resistance profile and kdr mutation of *An. gambiae* s.l. populations was also reported from two locations (Auyo and Bunkure) in northern Nigeria (14). High presence of *An. coluzzii* has been reported from previous studies (13-17). Two different studies conducted in Northern Nigeria both reported lower kdr mutations from both resistant and susceptible mosquitoes (18, 19). Similarly, studies conducted in Kenya reported lower kdr mutations to *An. gambiae* (20, 21).

The aim of this study was to investigate species composition, the insecticide susceptibility status, and to explore type of kdr mutations conferring pyrethroids and DDT resistance in members of the *An. gambiae* complex from Northern-Nigeria.

Materials and Methods

Study Location

Yamaltu Deba (10° 13′ 0″ N, 11° 23′ 0″ E) is one of the eleven Local Government Areas in Gombe State, Nigeria (Fig. 1). It has a population of 255,248, an area of 1,981km² and is located in the north-eastern part of Nigeria, stretching through the Sudan savannah, northern and southern guinea savannah (22, 23).

Study Sample

Dipping method was used to collect larvae samples from different breeding places in the study site as described by (13) in order to provide laboratory stock of mosquitoes. The samples were transported to the insectary at Bayero University Kano with a rearing condition of 28±2 °C temperature, 65±5% relative humidity (RH) and 12:12 hrs D: L. Two to three days old female sugar fed mosquitoes were used for susceptibility tests (24).

WHO susceptibility tests

Adult susceptibility test was conducted according to the recent WHO bioassay guideline (25). Twenty five female mosquitoes of 2–3 days old fed on 10% sugar solution, were exposed to malathion 5%, bendiocarb 0.1%, DDT 4.0% and deltamethrin 0.05% impregnated papers for 60 minutes in the standard WHO test kit. Oil-impregnated papers were used for the control group. There were four replicates for the treated and two replicates for the control group. At the end of the exposure time, both the treated and control mosquito groups were allowed to recover in holding tubes with cotton pads containing 10% sucrose solution on the top for 24 hours and then the number of dead and alive mosquitoes were recorded. A mosquito is considered alive if it is able to fly, regardless of the number of legs remaining.

DNA Extraction

Genomic DNA was extracted from 318 individual mosquitoes using Livak DNA extraction protocol template preparation kit (26, 27).

Specie Identification

The mosquitoes were first identified morphologically using morphological identification

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keys (28, 29). Molecular species identification was performed by PCR-SINE200 technique as previously described (14). Sine PCR reagents were carried out in 15ul master mix containing amplification reaction of 0.51mol of each primer sine 200F and sine 200R, 0.12mM of each dNTP, 0.75mM of MgCl2, 1.5U *Taq* DNA polymerase, PCR Buffer 10x [200mM Tris HCl (pH 8.4), 500mM KCl], 1.0µl of template DNA extracted from each mosquito. The primer sequence and thermal cycling conditions are shown in (Table 1).

PCR for kdr west (L1014F) and kdr East (L1014S)

The amplification protocol used for the detection of 1014F and 1014S mutations was performed using allele specific PCR in a 12.5µl reaction containing 1µl of template DNA, 1x Qiagen PCR buffer, 0.5mM MgCl2, 0.5nM of each primer, 0.5µM of dNTPs, and 1U of *Taq* DNA polymerase revised from the protocols previously established by Martinez-Torres et al. (11) and Ranson et al. (30). The primer sequences and thermal cycling conditions are shown in (Table 2). The primers Agd1, Agd2, Agd4 and Agd5 were used to detect the 1014S mutation whereas primers Agd1, Agd2, Agd3 and Agd4 were used to detect the 1014F mutation.

Data analysis

The 24hrs mortality was accessed manually, while the susceptibility was defined as; 98–100% mortality indicates susceptibility, 90–97 % mortality requires confirmation of resistance and between 0–89% suggests resistance (25). The Hardy-weinberg equilibrium equation was used to calculate the genotypic and allelic frequencies. Microsoft office excel, version 2003 was used to create charts, calculate the standard deviation, sort and clean the data. Abbott's formula (30) was used to correct for natural mortality, if the control mortality was between 5 and 20%. The results of the tests with >20% mortality in controls, were discarded and the test repeated (25).

Results

Female mosquitoes exposed to deltamethrin, DDT and bendiocarb showed 74% (95%, CI: 68–79); 53% (CI: 49–56) and 44% (CI: 38–49) mortalities after 24 hours, respectively (Fig. 2). Whereas, malathion was found to be susceptible (Fig. 2).

Molecular specie identification

A total of 318 mosquitoes composing of 138 alive (45 exposed to deltamethrin, DDT, bendiocarb and 3 exposed to malathion) and 180 dead (45 exposed to deltamethrin, DDT, bendiocarb and malathion) were identified to specie level. All the dead mosquitoes identified were *An. culluzzi*, the alive mosquitoes exposed to DDT, malathion and bendiocarb also were *An. culluzzi* while for deltamethrin exposed, 53.3% were *An. culluzzi*, 40% *An. arabiensis* and 6.7% *An. gambiae* s.s (Table 3).

Genotyping kdr west (L1014F)

A total of 96 mosquitoes: 76 alive (69 *An. coluzzii*, 6 *An. arabiensis*, 1 *An. gambiae* s.s) and 20 dead (13 *An. coluzzii*, 6 *An. arabiensis*, 1 *An. gambiae* s.s) were used for kdr genotyping. The following result was recorded: alive mosquitoes, the only *An. gambiae* s.s 1/1 (100 %) was homozygote resistant (RR); *An. arabiensis* 4/6 (67%) RR, 2/6 (33%) heterozygote resistant (RS); *An. coluzzii* 55/69 (80%) RR, 9/69 (13%) RS, 5/69 (7%) homozygote susceptible (SS). Dead mosquitoes, the only *An. gambiae* s.s 1/1 (100%) RR; *An. arabiensis* 3/6 (50%) RR, 2/6 (33%) RS, 1/6 (17%) SS; *An. coluzzii* 8/13 (62%) RR, 3/13 (23%) RS, 2/13 (15%) SS (Table 4).

Genotypic and allelic frequencies of L1014F

The Hardy-weinberg equilibrium equation was used to calculate the genotypic and allelic frequencies. The result was found to be 72 (75%) RR, 16 (17%) RS and 8 (8%) SS; 87% RR and 13% SS (Table 5).

Genotyping kdr west (L1014S)

A total of 96 mosquitoes were used, L1014S mutation was only found in 37 (39%) mosquitoes distributed as follows: 24 alive (15 *An. coluzzii*, 8 *An. arabiensis*, 1 *An. gambiae* s.s) and 13 dead (8 *An. coluzzii*, 4 *An. arabiensis*, 1 *An. gambiae* s.s) were used for kdr genotyping. The following result was rec orded: alive mosquitoes, the only *An. gambiae* s.s 1/1 (100 %) RS; *An. arabiensis* 2/8 (33%) RR, 5/8 (56%) RS, 1/8 (11%) SS; *An. coluzzii* 4/15 (27%) RR, 3/15 (20%) RS, 8/15 (53%) SS. Dead mosquitoes: the only *An. gambiae* s.s 1/1 (100%) RS; *An. arabiensis* 3/4 (75%) SS, 1/4 (25%) RS; *An. coluzzii* 3/8 (38%) RS, 5/8 (62%) SS (Table 4).

Genotypic and allelic frequencies (L1014S)

The Hardy-weinberg equilibrium equation was used to calculate the genotypic and allelic frequencies. The result was found to be 6 (16 %) RR, 14 (38%) RS and 17 (46%) SS: 40% RR and 60% SS (Table 5).

Table 1. (A) PCR primer sequences and (B) thermal cycling conditions used for specie identification of the *Anopheles gambiae* complex from Yamaltu Deba (Gombe state), Northern Nigeria, 2018

A

Primer name	sequence (5′ to 3′)	Identified species	Size of the PCR product (bp)
sine200F	TCG-CCT TAG ACC TTG CGT TA	*An. gambiae s.s.*	240
sine200R	CGC TTC AAG AAT TCG AGA TAC	*An. coluzzii*	470
		An. arabiensis	220

B

Step	Temperature °C	Time	Cycle
Initial Denaturation	95	5Mins	1
Denaturation	94	30Sec	35
Annealing	54	1Min	
Extension	72	1Min	
Final Extension	72	10Mins	1

Table 2. (A) PCR primer sequences and (B) thermal cycling conditions used for detection knockdown resistance mutations (L1014F and L1014S) in the *Anopheles gambiae* complex from Yamaltu Deba (Gombe state), Northern Nigeria, 2018

A

Primer name	sequence (5′ to 3′)	Primer type	Combination and Size of PCR product (bp)
Agd1	ATAGATTCCCCGACCATG	Common forward	Agd1+Agd2=293
Agd2	AGACAAGGATGATGAACC	Common reverse	
Agd3	AATTTGCATTACTTACGACA	Specific reverse for L1014F	Agd1+Agd3=195
Agd4	CTGTAGTGATAGGAAATTTA	Specific forward for susceptible L1014L	Agd2+Agd4=137
Agd5	ATTTGCATTACTTACGACTG	Specific reverse for L1014S	Agd1+Agd5=195

B

Step	Temperature °C	Time	Cycle
Initial Denaturation	95	3Mins	1
Denaturation	94	30Sec	35
Annealing	60	1Min	
Extension	72	1Min	
Final Extension	72	5Mins	1

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Table 3. Molecular species identification *Anopheles gambiae* s.l. specimens (A: alive, B: dead) following exposure to the insecticides from Yamaltu Deba (Gombe state), in Northern Nigeria, 2018

A

Insecticide	No. Exposed	*An. coluzzii* (%)	*An. arabiensis* (%)	*An. gambiae s.s* (%)
Deltamethrin 0.05%	45	53.3	40	6.7
DDT 4%	45	100	0	0
Malathion 5%	3	100	0	0
Bendiocarb 0.1%	45	100	0	0

B

Insecticide	No Exposed	*An. coluzzii* (%)	*An. arabiensis* (%)	*An. gambiae s.s* (%)
Deltamethrin 0.05%	45	100	0	0
DDT 4%	45	100	0	0
Malathion 5%	45	100	0	0
Bendiocarb 0.1%	45	100	0	0

Table 4. Genotyping of kdr west (L1014F) and east (L1014S) mutations of *Anopheles gambiae* s.l. from Northern Nigeria, 2018

kdr Mutation (L1014F)						
	Alive			**Dead**		
Species	***An. gambiae s.s.***	***An. arabiensis***	***An. coluzzii***	***An. gambiae s.s.***	***An. arabiensis***	***An. coluzzii***
Homozygote resistance	100%	67%	80%	100%	50%	62%
Heterozygote resistance	0	33%	13%	0	33%	23%
Homozygote susceptible	0	0	7%	0	17%	15%
	n=1	n=6	n=69	n=1	n=6	n=13

kdr Mutation (L1014S)						
	Alive			**Dead**		
Species	***An. gambiae s.s***	***An. arabiensis***	***An. coluzzii***	***An. gambiae s.s***	***An. arabiensis***	***An. coluzzii***
Homozygote resistance	0	33%	27%	0%	0%	0%
Heterozygote resistance	100%	56%	20%	100%	25%	38%
Homozygote susceptible	0	11%	53%	0	75%	62%
	n=1	n=8	n=15	n=1	n=4	n=8

Table 5. The allelic and genotypic frequencies of the L1014F and L1014S, mutations of *Anopheles gambiae* s.l. from Northern Nigeria, 2018

Genotypic frequencies (L1014F)			
Homozygote resistance	**Heterozygote resistance**	**Homozygote susceptible**	
75%	17%	8.00%	
			n=96
Allelic frequencies (L1014F)			
Homozygote resistance		**Homozygote susceptible**	
87%		13%	
Genotypic frequencies (L1014S)			
Homozygote resistance	**Heterozygote resistance**	**Homozygote susceptible**	
16%	38%	46%	
			n=96
Allelic frequencies (L1014S)			
Homozygote resistance		**Homozygote susceptible**	
40%		60%	

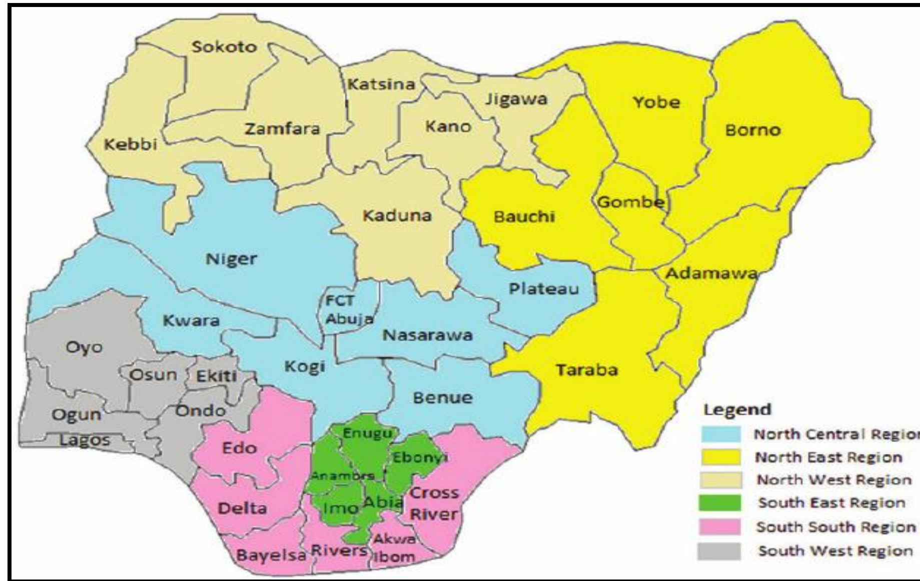

Fig. 1. Map (adapted from google earth) showing the geographical location of the study site, Gombe state (red circle) in Northern Nigeria, 2018

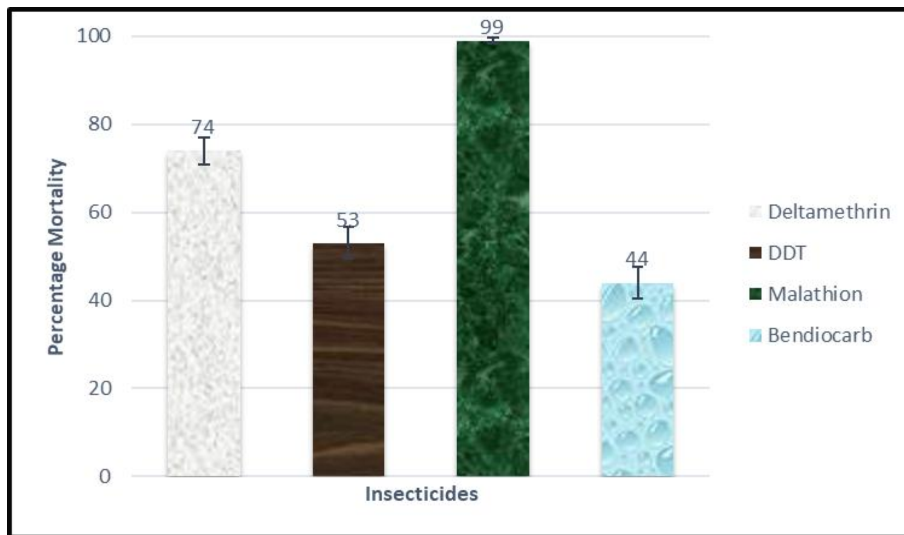

Fig. 2. Susceptibility (24hrs) profile of *Anopheles gambiae* s.l. to four insecticides from Yamaltu Deba (Gombe state), Northern Nigeria, 2018

Discussion

Susceptibility test

Bendiocarb showed very high level of resistance. This finding agrees with previous study (32), where they reported a percentage mortality range of 2.3–100%. Similarly, a study from Kumasi in Ghana, reported 38–56% mortality to bendiocarb (33). This study reports moderate level of resistance to deltamethrin from the study site. This finding is in agreement with study conducted in the northern guinea savanna of Nigeria (34) where they reported percentage mortality of 83%, and from north western part of Nigeria where they reported 78% mortality to

deltamethrin (14). However, a study conducted around the study location disagrees with our finding where they reported a very high resistance of 38% mortality to deltamethrin (35). DDT was found to be resistant and this finding is in agreement with previous studies from Sudan, Guinea and Sahel savanna of Nigeria (13, 14, 19, 30, 36, 37). Malathion was found to be susceptible and agrees with studies from different regions within and outside Nigeria (14, 19, 33, 34, 37).

Species identification

Anopheles coluzzii was found to be the dominant sibling species followed by the *An. arabiensis* and *An. gambiae s.s* (Table 3). This is in agreement with a study conducted in northern Nigeria, where they reported *An. coluzzii* as the dominant specie 86.8% followed by *An. arabiensis* 77% (14). Also, the high presence of *An. coluzzii* reported is supported by previous studies (13, 15, 17). However, a study conducted on molecular identification of *An. gambiae* s.l mosquitoes in Kamuli District of Uganda, disagrees with our finding where they reported 98% of the mosquitoes to be *An. gambiae* s.s (38). Another study conducted in Nigeria by Oyewole and colleagues (2011), reported *An. gambiae* s.s as the dominant species (74.6%) followed by *An. arabiensis* 26.4% in contrast to our finding (39).

Knockdown resistance (kdr) West (L1014F) and East (L1014S)

The kdr mutations were observed in the study location with high frequency of the L1014F in the *An. coluzzii* species (Table 4). This agrees with study conducted by Ibrahim et al. (2014) where they found the kdr mutations in 80.1% of the *An. coluzzii* and 13.5% in *An. arabiensis* mosquitoes (12). Oyewole et al. (2011), in their study from south-western Nigeria reported that 87% of the mosquitoes resistant to deltamethrin carried the kdr mutations and 80% of the DDT resistant mosquitoes as well (36). Furthermore, increase L1014F was reported from Ghana by Lynd et al. (2010) Niger, by Czeher et al. (2008) and sharp et al. (2007) from Equatorial Guinea (40-42). A study by Awolola et al. (2003) contradicts our finding, where they reported high frequency of L1014F in *An. gambiae* s.s compared to *An. coluzzii* (43). Derrick et al. (2011) from Kenyan, also reported increased presence of kdr in *An. gambiae* s.s compared to the *An. coluzzii*. Also, increased presence of L1014S was reported by Protopopoff et al. (2008), from Burundi and Verhaeghen et al. (2010), from Uganda (44, 45).

Genotypic and allelic frequencies

This study reports high genotypic frequency particularly in the L1014F kdr compared to the L1014S gene from the study location (Table 5). This is in agreement with previous study where they reported homozygous resistant of 74.1%, heterozygous resistant of 19.7% and homozygous susceptible of 6.2% for the L1014F in the *An. coluzzii*. While from the *An. arabiensis*; 69.2% were homozygous susceptible, 23.1% heterozygous and 7.7% homozygous resistance (14). A study by Habibu and colleagues (2017), contradicts our finding, where they reported 65.6% homozygous susceptible, 10% homozygous resistance and 24.4% as heterozygous resistance in the L1014S (36). While the L1014F showed 54.4% as homozygous susceptible, 21.6% as homozygous resistance and 24% as heterozygous resistance. The L1014S was seen both in the *An. coluzzii* and *An. arabiensis* (36). Our study also reports a very high allelic frequency in the L1014F compared to the L1014S (Table 5). This agrees with the results of Habibu et al. (2017) where they reported an allelic frequency of 48.9% and 65.9% in *An. gambiae* s.s and 20% and 61.8% in *An. arabiensis* in the L1014F (13). While the L1014S mutation recorded an allelic frequency of 40% and 55.3% in *An. coluzzii*; 20% and 30.8% in *An. arabiensis*. The L1014F has higher association with *An. coluzzii* (36). Studies by Derrick (2011) and Stump (2004) from Kenyan re-

ported lower allelic frequency compared to our findings (20, 21). The high level of insecticide resistance observed may be associated with increased use of pyrethroids treated bed nets and carbamate for indoor residual spraying (IRS) in public health and agricultural applications (35, 46). Farmers in the study location use a wide range of pesticides and herbicides to protect their crops and these pesticides marketed under different trade names belong to all the chemical classes including organophosphates, organochlorine, pyrethroids and carbamates (36). The high presence of kdr gene seen in this population of mosquitoes could be explained by the increase usage and abuse of insecticides by farmers and the increase coverage of LLIN distribution.

Conclusion

This study reveals the co-occurrence of L1014F and L1014S mutations with dominance of *An. coluzzii* and high genotypic and allelic frequencies in the L1014F over L1014S-kdr. Very high level of resistance to DDT, deltamethrin and bendiocarb was also observed.

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