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Full Length Research

Investigating the breeding habitats and kdr allele frequency of malaria vectors in Anambra Central Senatorial Zone, Southeast Nigeria

Emmanuel O. Ogbuefi[1]*, Dennis N. Aribodor[1], Tolulope A. Oyeniyi[2] and Emmanuel I. Obiefula[1]

[1]Department of Parasitology and Entomology, Faculty of Biosciences, Nnamdi Azikiwe University Awka 420102, Anambra State, Nigeria.
[2]Molecular Entomology Laboratory, Nigerian Institute of Medical Research, Yaba Lagos 101212, Lagos State, Nigeria.

*Corresponding author. E-mail: oe.ogbuefi@unizik.edu.ng; Tel: +2348032939679.

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ABSTRACT: Malaria, transmitted by infected female *Anopheles* mosquitoes, is a parasitic disease of major public health importance in Nigeria. Various classes of insecticides have played significant role in the control and elimination of malaria by attacking the vectors. Unfortunately, the success of the use of insecticides in the control of malaria vectors is threatened by the emergence of insecticidal resistance. This study was therefore aimed at determining the resistance status of *Anopheles gambiae s. l.* in communities in Anambra State, southeast Nigeria. Larval samples were collected from diverse habitats and reared to adult for susceptibility test. Target site resistance assays were carried out and genotypic differentiation test was performed to assess variability of the allelic frequencies of the kdr (L1014F) mutation. The results showed that *Anopheles gambiae* was the predominant vector (54.2%), followed by *Anopheles coluzzii* (45.8%). The distribution of the frequency of resistant allele of the kdr gene F (L1014F) showed that the number of homozygous resistant genotype (RR) in Awka North LGA for *An. gambiae and An. coluzzii* species was highest (31), followed by Awka South (20), and Njikoka (5) LGAs. Also, the number of Heterozygous resistant genotype (Rr) was highest in Awka South (7), followed by Awka North (2), and none was recorded in Njikoka LGA. Homozygous resistant genotype (RR) was frequently observed in the three study areas of Anambra State and is a threat to the use of pyrethroids for insecticidal control of malaria vectors. There is an urgent need for implementation of insecticide resistance management and monitoring in Anambra State to assess the spread of resistance which may expand malaria vector resistance to the remaining classes of insecticides (organophosphates and carbamates) on the long run.

Keywords: *Anopheles coluzzii*, *Anopheles gambiae*, insecticide, pyrethroids, resistance.

INTRODUCTION

Malaria problem in Africa has caused tremendous health challenges to the populace and they transmit diseases to human especially pregnant women and children which are the most vulnerable group because of their little or no immunity to the disease (Aribodor *et al*., 2011a). In the struggle to both control, eliminate and eradicate malaria vectors, Organochloride, organophosphate, carbamate, and pyrethroid are groups of insecticides approved for Indoor Residual Spray (IRS) but only pyrethroids are presently allowed in Insecticide Treated Nets (ITNs) owing to their low mammalian toxicity and high insecticidal potency (Zufelato *et al*., 2000). Insecticide resistance prompted a series of insecticide changes over time, from DDT to dieldrin, malathion, propoxur, pirimiphosmethyl, lambda-cyhalothrin, and deltamethrin to the present time (Schaefer and Mulligan, 1991). ITNs and IRS are effective in reducing malaria vector-human contact (World Health Organization, 2016), however, extensive use of insecticides has subjected mosquitoes to intensive selection pressure, resulting in the development of physiological resistance and behavioral change (Betson *et al*., 2017). ITNs deliver doses of pyrethroid insecticide

(standard ITNs) or pyrethroid plus a second active ingredient when the mosquito makes contact with the net surface but widespread insecticide resistance has been observed in *Anopheles gambiae* sensu lato (*s.l.*) and *Anopheles funestus s.l.*, the two African malaria vector groups most effectively targeted by ITNs. Resistance to insecticides which encompasses physiological, biochemical, molecular and behavioral mechanisms (Gatton *et al*., 2013), have developed multiple mechanisms which contribute to the physiological resistance to pyrethroids in mosquitoes, including target-site insensitivity caused by knockdown resistance (kdr) mutations in the *para*-type sodium channel gene and detoxification by mosquito enzymes that metabolize the insecticide before it reaches its target (metabolic resistance) site (Hemingway *et al*., 2004). In Nigeria, Anopheline vector resistance to DDT and pyrethroids have been reported and the emergence of pyrethroid and DDT resistance in the major Afro-tropical malaria vectors would have considerable implications for the success of vector intervention and the monitoring of ongoing control programmes (Manga, 2002). Hence, there is a strong need for the development of appropriate tools to monitor resistance in field populations of Anopheline mosquitoes in order to benefit from the contributions of the appropriate use of chemical insecticides in malaria elimination in Nigeria. But insecticide resistance remains a major obstacle to control of *Anopheles* malaria mosquitoes and requires an improved understanding of the underlying mechanisms (Toure *et al*., 1994). Efforts to discover resistance genes and DNA makers have been dominated by candidate gene and quantitative trait locus studies of laboratory strains, but with greater availability of genome sequences, a shift toward field-based agnostic discovery is anticipated. Resistance can also be mediated by mutations in the target site of the insecticide or its active metabolites (target-site resistance), through enzymatic modification of insecticides to the products of non-toxic metabolites (metabolic detoxification), or by behavioral changes or thickening of the cuticle. DDT and pyrethroids, insecticides commonly used for malaria vector control share a common target site, the *para* voltage-gated sodium channel (Najera and Zaim, 2002) but knockdown resistance (kdr) mutations in this channel can therefore confer cross-resistance to both DDT and pyrethroids (Soderlund and Knipple, 2003). Mechanisms evolve continually to produce elevated resistance yielding multiplicative diagnostic markers, co-screening of which can give high predictive value (Tamarin and Leavitt, 1991). With a shift toward prospective analyses, identification and screening of resistance marker panels will boost monitoring and programmatic decision making in *Anopheles* mosquitoes (Suhrbier, 1991). Despite the various malaria interventions (LLIN distributions, IRS, prompt effective treatment with anti-malarials) rolled-out by WHO in its operational areas, malaria still remains a public health challenge which requires continuous studies in the effort to control and eliminate the disease especially at the unset of both drug resistance to the *Plasmodium* parasite and insecticide resistance to the malaria vectors.

MATERIALS AND METHODS

Study area

The research was carried out in Ezeawulu and Umuanum communities of Nibo in Awka South L.G.A., Amaezike and Akamanator communities of Mgbakwu in Awka North L.G.A., Umu Agidi and Ifite communities of Enugwu Agidi in Njikoka L.G.A. of Anambra Central Senatorial Zone of Anambra State, southeast Nigeria (Figure 1). Anambra State is located on latitude 6˚12’45.68’’N of Equator and longitude 7°04’19.16’’E of Greenwich with an average temperature of 26.8°C/80.2°F. The geographical coordinates of Awka South LGA is located between latitude 6˚10’N of Equator and longitude 7°04’E of Greenwich, Awka North is located between the coordinates of 6°15’N of Equator and 7°10’E of Greenwich while Njikoka LGA is lying between latitude 06°20’58’’N to 06° 21’0’’N and longitude 06°52’55’’E of Greenwich. The 2020 projected population of Anambra State is 6,182,924 (National Bureau of Statistics, 2006). Anambra State has seasonal climatic conditions, the rainy season (which falls between April and October) and the dry season (which falls within November and March) with a short spell of harmattan between November and January which is a period of cold weather when the atmosphere is generally misty (Enete, 2008). The total annual rainfall of the State is above 11,450 mm for the six to seven months of the rainy season (Enete, 2014). The official language of the people of Anambra State is Igbo although English language is widely spoken throughout the state as a secondary language (field observation). Anambra State is an agricultural trade centre where yams, cassava, corn (maize), palm oil and kernels are sold for the people of the surrounding area because the soil in the State sustains forest vegetation but on the low plains further away from the river they maintain good vegetation cover. The research was carried out from September 2021 to August, 2022 covering the two major seasons of the year in Nigeria.

Sampling methods

The study was carried out using experimental study design alongside laboratory based molecular characterization.

Collection of mosquito larvae

The larval collections were performed monthly during three consecutive days from 9:00 am to 12:00 pm. Tyres,

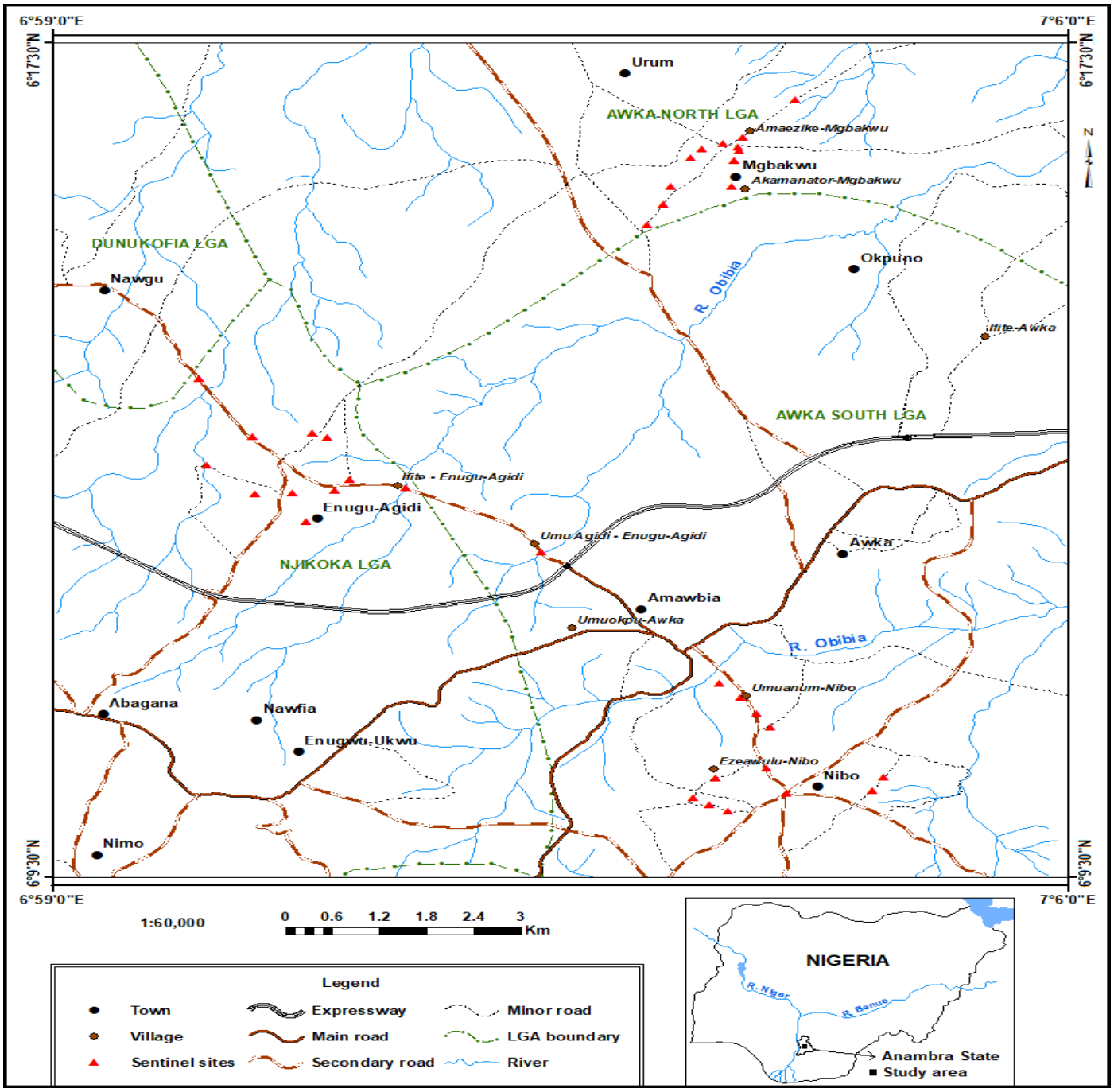

Figure 1. GIS map of the study areas showing Nibo in Awka South L.G.A., Mgbakwu in Awka North L.G.A. and Enugwu Agidi in Njikoka L.G.A. of Anambra State. **Source:** GIS and Cartography Laboratory of Department of Geography and Metereology, Nnamdi Azikiwe University Awka, Anambra State on 20/12/2022.

puddles, streams and river bodies, gutters, catchment pits, containers, excavations, tyre tracks, hoof prints, crab holes and rice fields were sampled among others. Ladles and pipettes were used to ensure that each site in the study areas were combed in the course of sourcing for mosquito larvae. Coarse debris like sticks and plant leaves that were collected alongside with the larvae were handpicked and thrown away and a sieve of 0.55 mm mesh size (kitchen sieve) was used to separate the larvae from other debris. The larvae collection was conducted covering both rainy and dry seasons of the year. The reason was to ensure that mosquito populations of all possible vectors breeding in the study areas were collected through embarking on comprehensive larval sampling.

Rearing of mosquito larvae

Mosquito larvae collected were placed in cock plastic containers and labeled according to the site of collection, time and date collected, and then transported to the Insectary Department at the National Arbovirus and Vectors Research Centre (NAVRC), Enugu, Nigeria where they were reared to adult stages. The larvae were fed with yeast in 500 ml larval bowls covered with a transparent net. On emergence to adult stage, the mosquitoes were transferred to a mosquito cage with the aid of an aspirator and fed with a 10% sugar solution soaked in cotton wool.

World Health Organization (WHO) bioassay tests

Insecticide susceptibility/resistance bioassay tests were carried out using World Health Organization susceptibility test-kits (impregnated/control papers) and standard procedures that were provided from Universiti Sains Malaysia (USM), Penang, Malaysia to determine the resistance status of the malaria vectors in the study areas. As described by World Health Organization (2018), four (4) replicates of 25 non-blood-fed 3-5 day old adult female mosquitoes was used in the study. On completion of the susceptibility test, the mosquitoes were transferred individually to clearly labelled Eppendorf tubes with silica gel in airtight lids (separating dead and life mosquitoes into separate tubes) for preservation until further analysis was carried out for target site resistance (kdr) assays. The detection of the mutations of the knock down resistance (*kdr*) gene for pyrethroid resistance were the east (leucine to leucine substitution; L1014L) and west (leucine to phenylalanine substitution; L1014F).

NB: Abbott's formula was used to correct the observed mortality when the mortality in the control is between 5–20% as described by Abbott (1925).

Data analysis

Data obtained from the study were summarized and analyzed using descriptive and comparative statistics. The resistance status of mosquito samples was determined according to the WHO protocol for insecticide resistance monitoring using pyretroids (0.05% deltamethrin and 0.75% permethrin), Organophosphates (5% malathion) and carbamates (0.1% bendiocarb) group of insecticides as follows: mortality rate > 98%, the population was considered fully susceptible; mortality rates of 90–98%, resistance suspected in the population; mortality rates < 90%, the population was considered resistant to the tested insecticides (World Health Organization, 2016). The genotypic differentiation test was performed using the method described by Goudet *et al*. (1996) to assess variability of the allelic frequencies of the kdr (L1014F) mutation across populations.

RESULTS AND DISCUSSION

Breeding habitat of malaria vectors in Awka South, Awka North and Njikoka L.G.A.s in Anambra State

A total of 641 *Anopheles* mosquitoes collected during the study were found to breed in the following habitats: Broken buckets/tins, clay pots, domestic containers (cups, bottles and cans), ground pools, reservoir tanks, used tyres, crab holes, catchment pits, rivers and streams, gutters, tire tracks and excavations. Result shows that in all the LGAs, the highest number of malaria vector species collected in the study was from domestic containers 17.0% (109/641), followed by catchment pits 16.4% (105/641) and the least number was from rivers and streams 3.3% (21/641) as shown in Table 1.

Percentage mortality, phenotypic characteristics and knockdown times of malaria vectors to different insecticides in Awka South, Awka North and Njikoka Local Government Areas, Anambra State, Nigeria

The percentage mortality of malaria vectors to different insecticides in Awka South, Awka North and Njikoka Local Government Areas, Anambra State, Nigeria was outlined in Table 2 whereas the Phenotypic characteristics and knockdown times of malaria vector in Awka South, Awka North and Njikoka Local Government Areas, Anambra State, Nigeria was outlined in Table 3.

Knock down resistance (kdr) for *Anopheles gambiae s. l.* complex in Awka South, Awka North and Njikoka Local Government Areas, Anambra State

(A): Lane 1 is DNA ladder. Lanes 2, 3, 6, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18, 19 and 20 were the products of RR while Lanes 4 and 7 were the products of Rr, Lane 5 was the product of rr whereas Lane 12 was unamplified and thus unidentified (Figure 2).

(B): Lane 1 is DNA ladder. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 18, 19 and 20 were the products of RR whereas Lane 14 and 16 were unamplified and thus unidentified (Figure 2).

(C): Lane 1 is DNA ladder. Lanes 2, 3, 7, 8, 9, 10, 11, 12, 15, 16, 17 and 18 were the products of RR while Lanes 4,6 and 14 were the products of Rr, Lane 5 was the product of rr whereas Lane 13,19 and 20 were unamplified and thus unidentified (Figure 2).

On the breeding habitat of malaria vectors in Awka South, Awka North and Njikoka Local Government Areas, Anambra State, a total number of 641 mosquito larvae were collected in all the study areas ranging from broken buckets/tins, clay pots, domestic containers, ground pools,

Table 1. Malaria vector larvae collected from different breeding habitats in Awka South, Awka North and Njikoka LGA, Anambra State.

Breeding habitats	Awka South LGA	Awka North LGA	Njikoka LGA	Grand Total (%)
Domestic containers	45 (41.3)	23 (21.1)	41 (37.6)	109 (17.0)
Catchment pits	53 (50.5)	45 (42.9)	7 (6.7)	105 (16.4)
Broken buckets/tins	50 (53.2)	33 (35.1)	11 (11.7)	94 (14.7)
Reservoir tanks	19 (38.0)	8 (16.0)	23 (46.0)	50 (7.8)
Used tyres	17 (34.0)	21 (42.0)	12 (24.0)	50 (7.8)
Gutters	18 (36.0)	9 (18.0)	23 (46.0)	50 (7.8)
Clay pots	6 (15.0)	18 (45.0)	16 (40.0)	40 (6.2)
Excavations	3 (8.3)	0 (0)	33 (91.7)	36 (5.6)
Ground pools	7 (20.0)	10 (28.6)	18 (51.4)	35 (5.5)
Tyre tracks	22 (62.9)	7 (20.0)	6 (17.1)	35 (5.5)
Crab holes	11 (47.8)	3 (13.0)	9 (39.1)	23 (3.6)
Banks of rivers & streams	12 (57.1)	3 (14.3)	6 (28.6)	21 (3.3)
Total (%)	263 (41.0)	180 (28.1)	198 (30.9)	641 (100)

χ^2=160.858, df=22, Pv=0.000.

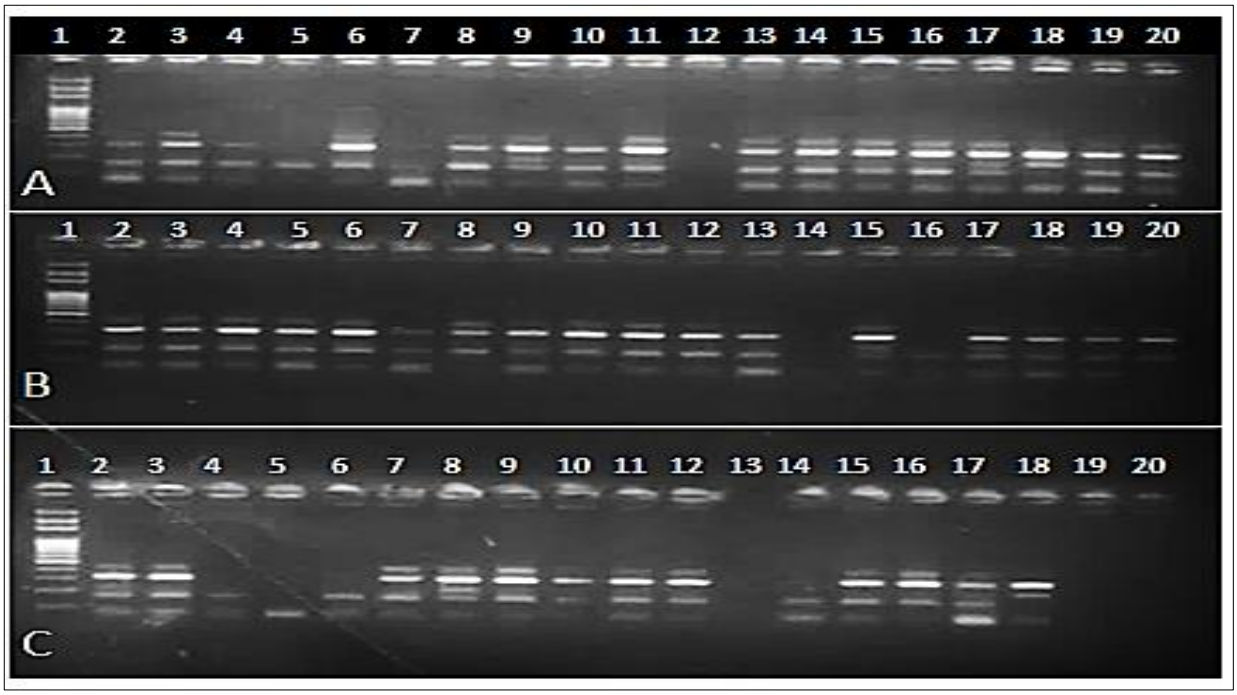

Figure 2. The characteristic PCR plate of Agarose gel electrophoresis knock down resistance (kdr) for *Anopheles gambiae s. l.* complex.

reservoir tanks, used tyres, crab holes, catchment pits, rivers & streams, gutters, tire tracks, to excavations with a significance difference. This indicates that the malaria vector larva were evenly distributed across all the communities and the major factor that would influence the uneven distribution could be the availability of breeding sites and the nature of the settlements of the inhabitants in the different communities. The number of larvae collected in the present study was lower than that of Eze *et al*. (2018) who collected 1,180 larvae of malaria vectors in Obi-Akpor, Rivers State. Egwu *et al*. (2018) collected 2,641 larvae in Ohafia, Abia State whereas Okonkwo *et al*. (2014) collected 2,319 larvae in Oba, Anambra State. A significantly higher number of 4,256 larvae were collected by Afolabi *et al*. (2013) in Akure, Ondo State and 4,871 larvae by Dalhatu *et al*. (2016) in Azare Bauchi State. High

Table 2. Percentage mortality of malaria vectors to different insecticides in Awka South, Awka North and Njikoka Local Government Areas, Anambra State, Nigeria.

Insecticides discriminating concentration (%)	Classification	No. of *An. gambiae s.l.* tested	(%) Mortality rate after 24 h	Resistance? (< 98%)	No. of control used (%)	No. of control after test (%)
Awka South LGA						
Deltamethrin (0.05%)	Pyrethroid	100	62	38	50	45
Permethrin (0.75%)	Pyrethroid	100	65	35	50	45
Malathion (5%)	Organophosphates	100	100	0	50	50
Bendiocarb (0.1%)	Carbamates	100	100	0	50	45
Awka North LGA						
Deltamethrin (0.05%)	Pyrethroid	100	54	46	50	41
Permethrin (0.75%)	Pyrethroid	100	22	78	50	47
Malathion (5%)	Organophosphates	100	100	0	50	49
Bendiocarb (0.1%)	Carbamates	100	100	0	50	50
Njikoka LGA						
Deltamethrin (0.05%)	Pyrethroid	100	63	37	50	42
Permethrin (0.75%)	Pyrethroid	100	42	58	50	50
Malathion (5%)	Organophosphates	100	100	0	50	46
Bendiocarb (0.1%)	Carbamates	100	100	0	50	48

Table 3. Phenotypic characteristics and knockdown times of malaria vector in Awka South, Awka North and Njikoka Local Government Areas, Anambra State, Nigeria.

Knock down time (min)	LGA Insecticide / concentration											
	Awka South LGA				Awka North LGA				Njikoka LGA			
	D (0.05%)	P (0.75%)	M (5%)	B (0.1%)	D (0.05%)	P (0.75%)	M (5%)	B (0.1%)	D (0.05%)	P (0.75%)	M (5%)	B (0.1%)
10	0	1	0	0	0	0	0	0	0	0	0	0
15	0	0	1	0	0	2	0	1	0	5	0	4
20	0	0	12	36	1	1	12	34	0	1	69	41
30	3	0	27	63	5	1	57	43	2	1	24	18
40	12	1	49	1	2	0	30	22	1	0	7	31
50	19	0	11	0	2	0	1	0	0	0	0	6
60	8	1	0	0	1	0	0	0	1	3	0	0
24 hr	20	62	0	0	43	18	0	0	59	32	0	0
Total observed mortality	62	65	100	100	54	22	100	100	63	42	100	100

Keys: D = Deltamethrin, P = Permethrin, M = Malathion, B = Bendiocarb.

abundance of *Anopheles gambiae* larvae have also been reported by Aribodor *et al*. (2011b) and Onyido *et al*. (2011) in different parts of Anambra State, Nigeria. The differences in the number of larval collections made in different areas may depend on the period of the year and length of the period of the studies. Also, other factors responsible for abundance of larval mosquitoes such as poor sanitation, poor environmental management and availability of breeding sites may abound in these areas. As was observed in the present study, similar types of breeding sites were also observed in different studies in Nigeria (Egwu *et al*., 2018; Goselle *et al*., 2017; Dalhatu *et al*., 2016). This observation agrees with the work of Mbanugo and Okpalaononuju (2003) who noted that the preponderance of malaria vector species in Awka metropolis was due to prevailing mosquito breeding habitats in the area. These various sites identified collect and hold waters that form breeding sites for malaria vectors especially during the wet season. In a study done by Onyido *et al*. (2014) of the 329 mosquitoes collected, 162(49.24%) were *A. gambiae*. This indicates that virtually a half of the mosquito population collected in the study area were malaria vectors, especially *A. gambiae s. l.*, which were collected from the temporary ground pools sustained by constant availability of water in fresh water swamps due to overflow of the streams and flooding during the rains. Also, Irikannu and Chukwuekezie (2015) observed that in Nigeria, the urban landscapes were often littered with garbage, plastic and tin cans, bottles, disposable cups, discarded vehicle tyres and earthen wares which form breeding grounds for mosquitoes especially during the wet season. World Health Organization (1995) noted that in places like Nigeria which Anambra State is part of it, there are higher breeding rates of malaria vectors due to rainfall patterns of the area and the amount of rainfall determines the abundance of mosquito breeding sites. As was observed in the present study, *Anopheles* species was found to breed in all the habitats as against former notion that they only breed in clean water. Simon-Oke *et al*. (2012) noted that mosquito distribution and abundance are related to population, land use and human activities. Also, Afolabi *et al*. (2013) suggested that anthropogenic related factors such as open drainage systems contribute to the increasing abundance of mosquitoes in the breeding sites in highly developed and populated area such as was observed in Awka South and Awka North LGAs. Therefore, rural community such as Njikoka LGA that has no such open drainage system and has fewer inhabitants had less number of malaria vector breeding in their area. World Health Organization (2010) noted that in places like Nigeria, there are higher breeding rates of malaria vectors due to rainfall patterns of the area and that the amount of rainfall determines the abundance of mosquito breeding sites. The study revealed that *Anopheles gambiae* was not only abundant but breed in all the villages and this calls for mass education of the people on malaria infection, prevention, and control through environmental management.

The (L1014F)-kdr resistance mutation frequencies were high among the mosquito populations tested and also significantly associated with mosquito survival following deltamethrin and permethrin exposure which implies that L1014F-*kdr* mutations conferred significant protection against the pyrethroids used in the survey. In the current study, *Anopheles gambiae* and *An. coluzzii* showed similar L1014F-*kdr* frequencies where the West African knockdown resistance gene (L1014F) was found in high frequencies (between 0.13–0.94) across the three LGAs. The homozygous resistance genotype (RR) (between 0.40-0.94) dominated the allelic frequency in all the LGAs followed by the heterozygous resistance genotype (Rr) (between 0.14–0.8) and then the homozygous susceptible genotype (rr) (between 0.03–0.20). The frequency of the L1014F-*kdr* resistant allele varied significantly among the different LGAs using the Fisher's Exact test; (p = 0.0005). The L1014F-kdr allele was identified with the majority of malaria vectors samples presenting homozygous and heterozygous *kdr* profiles 80.4% and 13.6% respectively which showed that there was an association between L1014F-kdr allele frequency and ability of mosquitoes to survive insecticide exposure for 60 minutes. This agrees with the findings of Gnanguenon *et al*. (2015) and Okorie *et al*. (2015) where the L1014F mutation was found in both populations of study. However, it contrasts a report by AIRS Nigeria (2015) where populations of *An. coluzzii* collected from the study were all negative for the L1014F mutation. The result also contrasts with reports from Burkina Faso where unregulated detoxification enzymes were responsible for extreme pyrethroid resistance in *An. coluzzii*, with N1575Y associated with more limited tolerance to deltamethrin (Toé, *et al*., 2015). The presence of the L1014F-*kdr* mutation has also been reported in a single *An. arabiensis* from the Sudan savannah of northern Nigeria by Ibrahim *et al*. (2014). However, Habibu *et al.* (2017) reported a higher frequency of L1014F-*kdr* mutation in *An. coluzzii* and *An. arabiensis* mosquitoes than previously reported by Ibrahim *et al*. (2014). The number of large samples (n = 240) used for PCR in the present study shows a more accurate estimation of the presence of resistance, therefore these findings suggests an increasing L1014F-kdr mutation rate in Anambra State, Nigeria. This is a very important consideration in the fight against insecticide resistance of malaria vectors as immediate action is crucially needed to avert this menace.

Conclusion

Insecticide resistance monitoring is essential for evidence-based control of mosquito-borne diseases. There is an urgent need for implementation of insecticide resistance management strategies in the State to assess the spread of resistance since the detection of kdr (L1014F) resistance may likely expand malaria vector resistance to the remaining classes of insecticide (organophosphate

and carbamate). Since the World Health Organization already recommends immediate and pre-emptive action to delay and possibly avert insecticide resistance prompt interventions with integrated mosquito control measures and massive health education will equally help to curtail malaria vectors and this requires the use of tools with proven optimal efficacy to be deployed rationally as part of an evidence based insecticide resistance management strategy in Anambra State particularly and Nigeria at large.

Recommendations

The global commitment to eliminate malaria by 2030 needs immediate efforts, unfortunately, the presence of L1014F-*kdr* allele mutation which was reported in the study area was associated to pyrethroid resistance, suggesting the reinforcement and spread of this resistance. Hence, there is an urgent need for continuous and expanded insecticide resistance monitoring in the State at large to obtain a broader and clearer idea of the situation. Based on the results of this study and the previous ones, the alarm is being sounded again, to draw the attention of decision-makers, especially the Nigeria Malaria Control Programme (NMCP), to the urgent need of resistance management programme intervention and implementation. This is to avoid the introduction of insecticides that have similar insecticide resistance mechanism/s that could confer cross resistance to the replaced insecticide. Also, monitoring insecticide resistance can help designing strategies to delay or prevent its onset and spread in vector populations.

COMPETING INTEREST

The authors declare that they do not have any conflicts of interest.

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