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THE SUSCEPTIBILITY STATUS OF *ANOPHELES GAMBIAE S.L* POPULATIONS IN IFITE-AWKA, ANAMBRA STATE TO STANDARD INSECTICIDES

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ABSTRACT

This study was carried out to evaluate the susceptibility level of *An. gambiae s.l*, a major malaria vector in Ifite Awka, South East Nigeria to four classes of standard insecticides. *Anopheles* larvae were collected from three communities (Enu-ifite, Ezinato-ifite and Agbana-ifite) and reared to adults. Two to three-day old, non-blood-fed female mosquitoes were exposed to discriminating dosages of 4.0% DDT, 0.05% Deltamethrin, 0.25% Pirimiphos-methyl and 0.1% Propoxur using WHO protocols and susceptibility test kits. Knockdown effect was recorded every 10 minutes for 1hour and mortality recorded after 24 hours of post exposure. The result from this study revealed that the major malaria vector in Ifite Awka, *An. gambiae s.l*, was resistant to DDT, Deltametrin and Pirimiphos-methyl with observed mortality rates of 4.82%, 30.0% and 15.31% respectively while the vector population were susceptible to Propoxur with observed mortality rate of 98.75%. The KDT_{50} (in minutes) of *Anopheles gambiae s.l* exposed to Deltametrin, Pirimiphos-methyl, Propoxur and DDT were 5.794, 8.661, 1.903 and 19.969 respectively while their respective KDT_{95} (in minutes) recorded were 18.022, 14.426, 8.867 and 48.750. From these results obtained, Propoxur is most suitable as alternative to mitigate pyrethroid resistance in this area than DDT and Pirimiphos-methyl. A rationalised use of these insecticides coupled with regular monitoring of resistance status is essential to improve and evaluate the efficacy of the current vector control tools (ITN and IRS).

Keywords: Susceptibility, *Anopheles gambiae s.l*, Ifite-Awka, Standard Insecticides

INTRODUCTION

Malaria remains the most important public health parasitic disease and a major worldwide health problem with the greatest burden in sub-Saharan Africa. Over 90% of all malaria deaths still occur in Africa where it kills over half a million children less than 5 years of age each year. In 2013, malaria killed an estimated 584, 000 people with over 2 million cases. Nigeria is reported to have the unenviable record of contributing about 25% of the world malaria burden. Apart from the health burden, the socio-economic consequences of malaria are enormous such that it was part of the Millennium Development Goals (MDGs) (Aribodor *et al.,* 2016). Malaria constitutes 10% of African's overall disease burden, accounting for 40% of public health expenditure, 30-50% of in-patient hospital admissions and up to 50% of outpatient visits in areas with high transmission (WHO, 2006). Approximately 50% of the Nigerian population experience at least one episode per year. However, official estimate suggests as much as four bouts per person per year on the average (WHO, 2012).

Malaria is caused by a single-celled parasite from the genus *Plasmodium.* Five species of *Plasmodium* commonly infect humans and they include *Plasmodium falciparium, P. vivax, P. ovale, P. malariae* and *P. knowelsi. Plasmodium falciparum* is responsible for most malaria deaths, especially in Africa; it is the most common type of malaria infection and does not have a relapsing phase. The other strains like *Plasmodium vivax, ovale and malariae* can infect the liver and persist in a dormant state for months or even years after exposure to the infection. Humans occasionally become infected with *Plasmodium species* that normally infect animals such as *P. knowelsi.* The malaria parasite is transmitted solely through the bites of infected female *Anopheles* mosquitoes (Aribordor *et al.,* 2013). The mosquito genus, *Anopheles*, includes over 400 species which are widely distributed. The most important malaria transmitting species in Nigeria and other parts of Africa include *Anopheles gambiae, A. funestus*, *A. arabiensis* and *A. mellas* (FOS, 1993; Alaribe *et al.*, 2002).

In the absence of a vaccine, malaria control programmes rely on the combination of effective vector control, and effective treatment of clinical cases (WHO, 2008). Indoor Residual Spray (IRS) of insecticides and Long Lasting Insecticide treated Nets (LLINs) have shown to be very effective not only in reduction of adult Anopheline population densities but also preventing malaria parasite transmission (Kamau *et al.*, 2007). Progress in malaria control in African countries in the past decade can be attributed largely to the massive scale up of insecticide-based interventions targeting malaria carrying mosquitoes (Ibrahim *et al.,* 2013).

Out of the four classes of insecticide (organochlorine, pyrethroids, carbamates and organophosphates) approved for malaria vector control pyrethroids are the most compounds used for IRS and the only insecticide class recommended for use in ITNs (WHO, 2010). However, the emerging and rapid spread of insecticide resistance in major malaria mosquito vectors across the

African countries threatens the efficacy and sustainability of these control tools (Ibrahim *et al.,* 2013). The resistance describes the situation in which the vectors are no longer killed by the standard dose of insecticides or manage to avoid coming into contact with the insecticide (WHO, 2012). The vectors accomplish resistance through any or combination of increased metabolism of toxic compounds, decreased target site sensitivity, decreased rate of insecticides penetration and increased rate of insecticides excretion but the two most important mechanisms are metabolic or enzymes based detoxification and target site insensitivity (David *et al.,* 2013; Nkya *et al.,* 2013). In Nigeria resistance of *Anopheles gambiae* to common classes of insecticides is well documented (Awolola *et al.,* 2007; Oduola *et al.,* 2012).

Currently most resistance monitoring is dependent on bioassays, using fixed insecticide concentrations and exposure times, and the data is reported as percentage mortality and/or Knockdown (KD) effect. The existence of resistance could render ineffective the mass use of insecticide treated bed nets for malaria control. It is obligatory that tests on mosquito susceptibility to insecticide be performed before the introduction of insecticide-treated bed nets. Each country may be divided into sections according to similar geographical and demographic parameters and baseline susceptibility data collected at representative sites. Based on such baseline data, susceptibility levels can be extrapolated for areas with similar socio-economic status, demographic and ecological characteristics.

Implementation of vector control measure before careful evaluation of target mosquito population risks success of control programme hence a good knowledge of the species, its abundance and distribution, levels and dynamics of insecticide resistance in local mosquito population followed by continuous monitoring of resistance are required for a successful malaria control programmes. Although there have been carbamate, DDT and pyrethroid resistance by *Anopheles gambiae s.s* in Southern part of the country with reported 96% and 80% mortality due to bendiocarb exposure in residential and irrigation areas respectively in DDT resistant *A. gambiae* of Auyo town northwest Nigeria (Oduola *et al.,* 2012), it is not known whether the strength and scale of resistance have increased overtime or being extended beyond the locations investigated in previous studies.

This study therefore, seeks to monitor the susceptibility status of *A. gambiae complex* populations in Ifite Awka North, Anambra State to WHO approved standard insecticides. It is hoped that the findings from this study will promote and improve effective vector control decision making. The aim of this study is to determine the susceptibility status of test female *Anopheles* mosquitoes to four insecticides for Indoor Residual Spray baseline data collection in Anambra State, Nigeria.

MATERIALS AND METHODS

Study Area

The study was based in Ifite-Awka, a village located in Awka-south Local Government Area, Anambra, Nigeria. Awka

lies within coordinates 6°12′ N and 7°04′ E in the tropical zone of Nigeria (Onyido *et al*., 2011). Ifite is an urban community that shares boundary with the Government house and comprise three villages namely: Enu-ifite, Ezinato-ifite and Agbana-ifite. Ifite-Awka is about 150 meters above the sea-level when compared with the Ridges of the Mamu River. The town accommodates most students and staff of Nnamdi Azikiwe University. Despite development in the area, Ifite-Awka is also an agrarian community.

Study Design

The research involved both field and laboratory assessment which included the mosquito breeding sites identification, collection of mosquito larvae from those sites, rearing of the collected *Anopheles* mosquito larvae to adult in the insectary, morphological identification of adult mosquitoes to determine species and carrying out of susceptibility tests with Primiphos methyl (Organophosphate), DDT (Organochlorine), Propuxur (Carbamate), Deltametrin (Pyrethroids) on adult female *Anopheles* mosquitoes using standard WHO procedure and susceptibility test kits.

Ethical Considerations

An Introduction letter was obtained from the Head, Department of Zoology of Nnamdi Azikiwe University Awka. This letter was submitted to office of the Chief Medical Officer, National Arbovirus and Vectors Research Centre Enugu.

Larval Identification

Identification of *Anopheles* larvae in their breeding sites was done using their position in the water. Larval collections were done in larval breeding sites in Ifite-Awka community using a scooping method whereby ladles were used to scoop the larvae into a holding bowl. Collected samples were transferred into a big transparent jar with perforated lid. The jar was properly labeled stating the date of collection, time of collection and site. Most larvae were either at their fourth instar stage or pupal stage.

Sample collection and processing

Anopheles larval sample were collected from different larval breeding sites in Ifite-Awka. The collection lasted from June to August usually from 10:00am to 1:30pm. All larvae collected were transported to the insectary attached to Entomology section of National Arbovirus and Vectors Research Centre (NAVRC), Enugu.

Study sites were made up of pot holes, swamps, and gutters from which the larvae were collected. All larvae were scooped with laddle into plastic containers while making sure that little water from breeding sites goes in with the larvae were turned into a well labelled plastic container with perforated lid and transported down to the insectary. At the insectary, the larvae were emptied into a plastic bowl 26cm by 26cm in length and with and 12.5cm deep with each of these white plastic bowls covered with a nylon mesh. The larvae were fed with yeast and cabin biscuit blend together at ratio 1:2 and reared to adult.

They were maintained at temperature of 26 ± 3℃ and 74 ± 4% relative humidity. The emerging pupae were sucked out of the

larval containers using pipette and kept in white cups containing water inside a metallic mosquito cage which is properly labeled to show age of the mosquitoes and collection site. Adults which emerged overnight in the larval bowls were collected with the aid of entomological aspirator and transferred to adult mosquito cages. The adults are maintained on 10% sucrose solution in the insectary before being assayed.

Morphological Identification

The reared adults were identified using morphological criteria. The mosquitoes were identified under a dissecting microscope at X10 magnification. The resulting adults were identified as *An. gambiae s.l* according to the morphological keys (Gillies and Coetzee, 1987). The morphological character include; absence of lateral abdominal tufts of hair, completely colored tarsal segments, speckled legs, 3-banded palps and pale interruptions on the 3rd chain dark area of the first wing vein

Mosquito Sex Determination

Despite the large difference in susceptibility between the sexes, females were exclusively used because they survive better and show lower mortalities than the males (WHO, 1998). An entomological aspirator guided the separation of females from males. Males were distinguished from females by their bushy (plumose) antennae and club-shaped palps whereas females have scanty antennae (pliose), with the palps pointed sideways usually same length with the proboscis.

Insecticide Susceptibility Bioassay

This was carried out on all the samples that were 2-3days old, glucose-fed females. Mosquitoes' insecticides diagnostic kit consists of holding tubes, exposure tubes and post-exposure tubes with green, red and yellow markings respectively, rings, lids and impregnated papers. A pre-test was carried out by carefully introducing 20-25 mosquitoes into each holding tube with an aspirator and was allowed to stand for 10minutes after which they were transferred to the exposure tubes through a hole on the lid that separated the holding tube and the exposure tube. It was with the aid of this diagnostic kit that the susceptibility and resistant status using 4% DDT and 0.1% propoxur, 0.05% deltametrin and 0.25% Primiphos-methyl impregnated paper was established according to WHO procedure (WHO, 1998).

The number of mosquitoes knocked down were recorded every ten minutes of exposure for I hour after which the specimen were maintained for the next 24 hour on 10% sucrose solution and the percentage mortality recorded at the end of the 24th hour. Mortality rate between 98 – 100% indicate full susceptibility, 90 -97% require further investigation, less than 90% is considered resistant to the tested insecticides. The resistant and susceptible mosquitoes were separately placed in labeled silica gel contained ependorf tubes and at - 80˚C for subsequent enzyme assay.

Data Collection and Statistical Analysis

The susceptibility status of the population tested was determined using WHO criteria. A resistant population is defined by mortality rates less than 90%

after the 24hour observation period while mortality rates greater than 98% are indicative of susceptible populations. Mortality rates between 90-98% are suspected resistance that requires confirmation.

$$\% \text{ Knockdown} = \frac{\text{Total Knockdown}}{\text{Total No.of exposed mosquitoes}} \times 100$$

$$\% \text{ Mortality} = \frac{\text{Total No.of dead mosquitoes after 24 hours}}{\text{Total No.of exposed mosquitoes}} \times 100$$

Percentage mortality and knockdown of treatments and control groups were compared using Analysis of Variance. (ANOVA). For Probit analysis, knockdown times for 50% (KDT50) and 95% (KDT95) of the test population were estimated by the log time probit model using the SPSS (version 20.0) statistical software.

RESULTS

Mean Percentage Knockdown of Anophelines Population from Ifite Awka during exposure to Four Insecticides

Table 1 shows the mean percentage of adult female *An. gambiae s.l* knocked down at various time (in min) interval during one hour exposure to four classes of insecticides. The Table revealed that the highest knockdown at 1 hour (60 mins) of exposed mosquitoes was recorded by Propoxur (94.04±5.995) followed by Deltamethrin (60.15±18.102) while least in DDT (2.38±4.760). However, the Analysis of Variance result revealed that there was significant difference between the mean knockdown of mosquitoes at one hour after exposure ($P<0.05$).

KDT_{50} and KDT_{95} of the Anophelines Population from Ifite Awka during exposure to Four classes of Insecticides

Table 2 shows the KDT_{50} and KDT_{95} of the insecticidal classes to exposed mosquitoes. The result revealed that Propoxur (1.903) had the lowest KDT_{50} followed by Deltamethrin (5.974) while highest in control (56.206). However, Propoxur (18.02) had the lowest value for KDT_{95} followed by Pirimiphos-methyl (14.43) while highest in control (1102.996). The T-test of significance revealed that there was no significant difference in the KDT_{50} and KDT_{95} of the four classes of insecticides ($P>0.05$).

Figure 1 revealed a strong, positive relationship ($R^2= 0.658$), Figure 2 a weak relationship ($R^2= 0.345$), Figure 3 a strong relationship ($R^2= 0.794$), while Figure 4 revealed a linear relationship between knockdown and time of exposure of mosquitoes to Deltamethrin, Pirimiphos-methyl, Propoxur and DDT respectively.

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Table 1: Progressive mean number of knockdown rates of anophel population from Ifite Awka during exposure to insecticides

Class of insecticides (conc)	**N**	**Mean number of Knockdown (KD) mosquitoes**							
		10 min	**15 min**	**20 min**	**30 min**	**40 min**	**50 min**	**60 min**	**> 60 min (%)**
Deltamethrin (0.05%)	80	0.00± 0.00 [a]	1.50± 1.00 [a]	2.00± 1.63[b]	6.00± 2.82[b]	9.00± 3.83[b]	9.00± 2.82[b]	12.00 ±3.83[c]	60.15± 18.10[c]
Pirimiphosmethyl (0.25%)	98	1.00± 2.00 [a]	0.50± 1.00 [a]	1.00± 1.15[ab]	1.50± 1.91[a]	3.00± 1.15[a]	6.50± 1.00[b]	8.00± 0.00[b]	32.67± 0.76[b]
Propoxur (0.1%)	83	6.75± 1.89[b]	9.00± 1.41[b]	13.00 ±1.4[c]	15.75 ±2.98[c]	16.75 ±1.70[c]	19.25 ±0.95[c]	19.50 ±1.29[d]	94.04± 5.99[d]
DDT (4.0%)	83	0.00± 0.00 [a]	0.00± 0.00[a]	0.00± 0.00[a]	0.00± 0.00[a]	0.00± 0.00[a]	0.50± 0.00[a]	0.50± 1.00[a]	2.38±4. 76[a]
Control	87	0.50± 0.57[a]	0.50± 0.57 [a]	1.00± 0.81[ab]	1.25± 0.95[a]	2.00± 2.16[a]	2.75± 2.97[a]	2.75± 2.99[a]	13.25± 15.13[a]

Columns sharing similar superscripts (a,b,c,d) are not significantly different at P>0.05

Table 2: KDT_{50} and KDT_{95} of the Anophelines Population from Ifite Awka during exposure to four classes of Insecticides

Class of insecticides	**N**	**KDT_{50}**	**KDT_{95}**
Deltamethrin (0.05%)	80	5.974	18.022
Pirimiphosmethyl (0.25%)	98	8.661	14.426
Propoxur (0.1%)	83	1.903	8.867
DDT (4.0%)	83	19.969	48.750
Control	87	56.206	1102.996

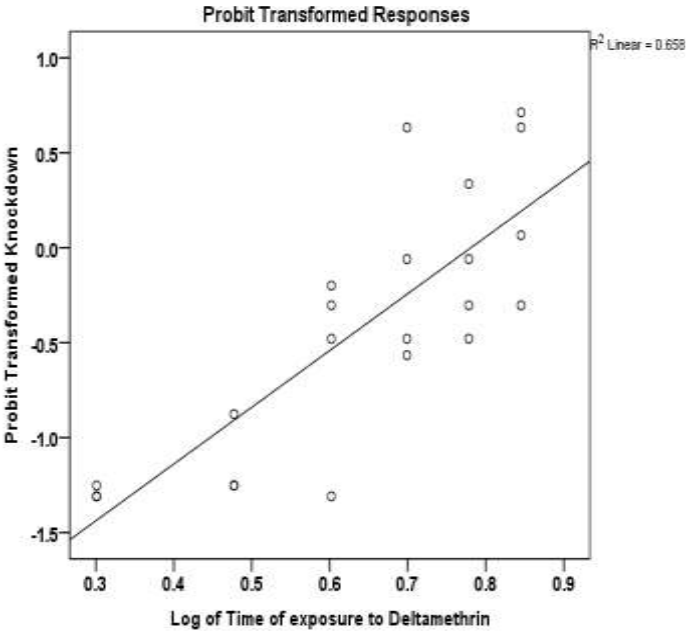


Figure 1: Log of Time of exposure to Deltamethrin

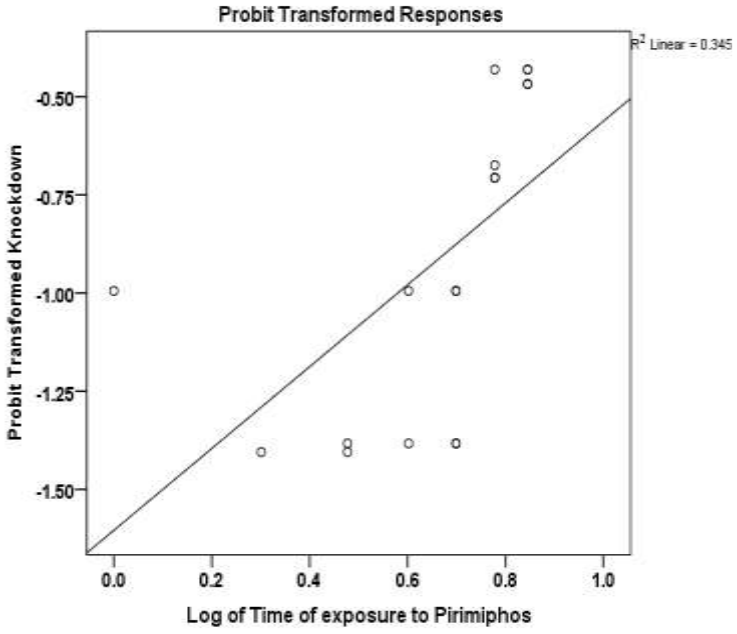


Figure 2: Log of Time of exposure to Pirimiphos-methyl

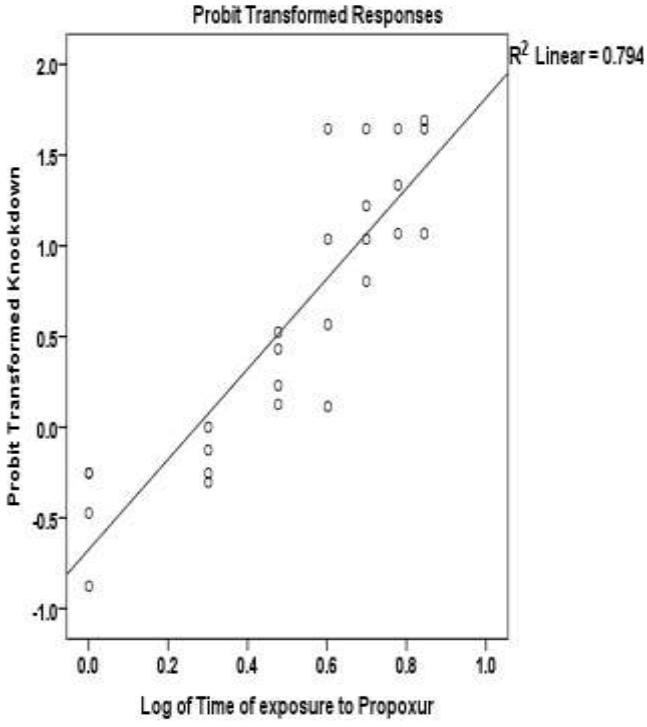


Figure 3: Log of time of exposure to Propoxur

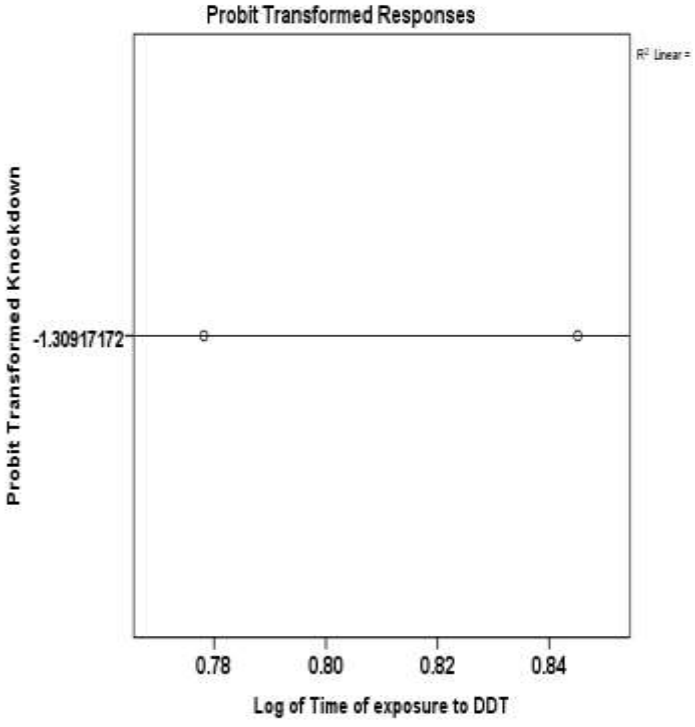


Figure 4: Log of time of exposure DDT

Mean Mortality of the Anopheline Population from Ifite Awka at 24 hours of exposure to four classes of Insecticides

Table 3 shows the mean mortality of the anopheline population from Ifite Awka at 24 hours of exposure to four classes of insecticides. The result revealed that Propoxur (20.50±1.29) caused the highest mortality at 24 hours followed by Deltamethrin (6.00±2.828) while least in the control (0.25±0.500). There was significant difference in the mean mortality of mosquitoes exposed to the four classes of insecticides at 24 hours ($P<0.05$).

Result of the Susceptibility of the Anopheline Population from Ifite Awka at 1 hour of exposure to four classes of Insecticides

Figure 6 revealed that the anopheline population from Ifite Awka was more susceptible to Propoxur followed by Deltamethrin while least in DDT. The test of significance revealed that there was no significant difference in the susceptibility of the anophline mosquitoes exposed to the four classes of insecticides ($P>0.05$).

Table 3: Mean Mortality of the Anopheline Population from Ifite Awka at 24 hours of exposure to four classes of Insecticide

Class of insecticides	N	Mean mortality at 24 hrs
Deltamethrin (0.05%)	80	6.00 ± 2.828^{b}
Pirimiphosmethyl (0.25%)	98	3.75 ± 1.708^{b}
Propoxur (0.1%)	83	20.50 ± 1.291^{c}
DDT (4.0%)	83	1.00 ± 1.155^{a}
Control	87	0.25 ± 0.500^{a}

Columns sharing similar superscripts are not significantly different at $P<0.05$

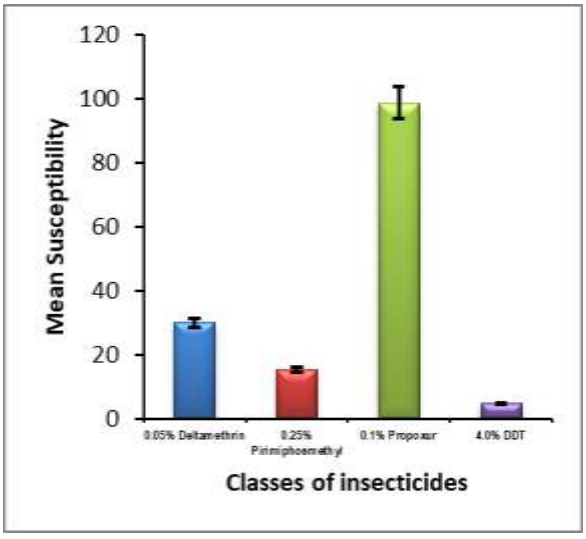


Figure 5: Susceptibility of the Anopheline Population from Ifite Awka at 1 hour of exposure to four classes of Insecticides

DISCUSSION

The study has provided an update on the current resistance status of *Anopheles* mosquitoes in Ifite-Awka after two rounds of IRS conducted in the LGA in 2013 and 2014. Larval sampling revealed that *Anopheles* larvae are seen in open, sunlit fresh water collections, ditches made by animal foot or car tyres as reported by Awolola *et al*. (2007), Okorie *et al.* (2015) and many other Nigerian researchers with similar findings. These diverse breeding sites according to Service 1993 as cited by Aribordor *et al.* (2013) are part of the challenges militating against effective and sustained control of mosquitoes.

Anopheles gambiae were seen to breed in the same environment with *Culex quinquefasciatus* or with aedes spp. Breeding sites where this malaria vectors were seen with *Culex quinquefasciatus* looked polluted which is unusual for *Anophelines* but the curiosity was erased when found to be in line with the findings of Awolola *et al.* (2007) which reported that *Anopheline* larvae were collected in polluted water bodies which contained some heavy metals and the larvae shared the breeding site with *Culex species*. Breeding sites where *Anopheles gambiae* were seen with *Aedes* spp looked unpolluted.

Knockdown during the susceptibility test signaled what resistance would look like. There was no significant difference in time taken for each insecticidal class to knock down 50% and 95% of the test population. While DDT took 5.974 minutes to knockdown 50% of its tested population and 18.022 minutes for 95% of the tested population, Propoxur knocked down 50% of its tested population within 1.903 and 95% of tested population before expiration of 8.867 minutes.

The mosquitoes showed very low susceptibility to three of the four insecticides with least susceptibility to DDT (4.82% observed mortality) followed by Primiphos methyl (15.31% Observed mortality) and then Deltametrin (30.0% 0bserved mortality). As recorded by Djouaka *et al.* (2011), level of DDT susceptibility observed in this population (with 4.82% mortality recorded in females after 1h exposure) is not only the least among the insecticides tested in Ifite-Awka, but is among the least level of susceptibility reported for an *An.*spp. population across Africa until now. The population was highly susceptible to Propoxur which recorded 98.79% observed mortality. The efficacy of propoxur has also been reported by various researchers within and outside the shores of Nigeria (Oduola *et al.,* 2012; Pennetier *et al.,* 2006).

In contrast, Ibrahim *et al.* (2013) recorded reduced susceptibility and susceptibility to 0.75% permethrin and 0.05% deltametrin with 24 h post exposure mortalities ranging from 83.5 to 87.7% and 98.5% respectively in Ibadan. Okorie *et al.* (2015) has previously reported that the vectors from their different study areas were significantly less susceptible to DDT and the Pyrethriods used.

Results recorded by Ibrahim *et al.* (2013) revealed malaria vectors to be resistant to 0.1% bendiocarb (same class with propoxur) with observed mortalities of

70.0% and 52.5% in the two study areas used. Oduola *et al.* (2012) also reported the growing carbamate resistance now spreading in malaria vectors especially in West Africa (Benin, Cote d'ivoire and Nigeria) but present work was in accordance with reports from Okorie *et al.* (2015) which recorded susceptibility of the malaria vector to carbamate.

The increased level of resistance to carbamates as recorded by Oduola *et al.* (2012) and high level of susceptibility to carbamate as recorded by the present study though within the same country maybe due to difference in geographical zones because south east has few information on resistance status of the malaria vector peculiar to them while the southwest has more information on resistance status of malaria vector peculiar to them. With more information, the pyrethroids were being replaced with carbamates hence the increased resistance as recorded by Oduola *et al.* (2012).

A report by United Nations Environmental Programme (UNEP) in 2012 showed it is obvious that insecticide resistance in malaria vectors is increasing worldwide due to the increasing selection pressure on mosquito populations caused by presence of urban, domestic and/or agricultural pollutants in the environment. This may be the case in Ifite, as most of the inhabitants revealed they make use of one form of insecticide or pesticide. ESA (2016) also recorded that certain insects are more likely to develop resistance due to their biology, genetic predisposition, and their level of insecticide exposure.

Ibrahim *et al.* (2013) did not observe carbamates as suitable replacement to pyrethroid for IRS in south west Nigeria but this study reveals carbamate to be a suitable replacement to pyrethroid for IRS. As part of an insecticide management strategy cabamates may be the right candidate for malaria control in South east Nigeria.

Based on the findings of this study, it is concluded that procurement of IRS insecticides for deployment in the state should be guarded until the susceptibility status of malaria vectors in the area is confirmed to have changed. It is recommended that there should be continuous monitoring of resistance in Ifite, Anambra State and indeed the country at large. This would ensure that policy makers are abreast with information on respect to the dynamics of resistance. Further studies should be geared towards determining the mechanisms of resistance in the vector population. This will enhance decision making as well as detecting presence of resistant genes early before they lead to control failure.

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