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Insecticide resistance and target site mutations (G119S *ace-1* and L1014F *kdr*) in *Anopheles gambiae s.l.* in Enugu state, Southeastern Nigeria

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Abstract
A study of insecticide resistant status and detection of G119S and L1014F mutation genes in *Anopheles gambiae sensu lato.* in Enugu State was conducted between May, 2019 and June, 2020. The aim was to investigate the resistance status of *An. gambiae s.l.* to four classes of insecticides used in vector control in the area and to identify the target site resistant genes present in resistant mosquitoes. Three randomly selected communities; Awkunanaw (Enugu East), Obukpa (Enugu North) and Awgu (Enugu West senatorial districts) were used as sampling sites. *Anopheles gambiae s.l.* larvae were collected by dip method and reared to adult for the study. WHO Tube Bioassay was used to determine resistance status. Polymerase Chain Reaction (PCR) was employed for detection of resistance mechanisms. The average mortality of *An. gambiae s.l.* tested with DDT was 28.3%, deltamethrin 63.53%, bendiocarb 88.33%, while malathion had the highest average mortality rate of 100%. The Acetycholinesterase Resistance Gene (GII9S *Ace-1*) mutation was detected at low frequencies in 75(100%) randomly selected *An. gambiae s.l.* from the three senatorial districts while the Knockdown Resistance Gene (LI0I4F *kdr*) mutation was not detected. Implementation of insecticide resistance management strategies in the state to control the spread of resistance is recommended.

Keywords: resistance, insecticides, *Anopheles gambiae*, mutation genes

Introduction

Insecticide resistance is an inherited characterization that allows the development of ability in some insects to tolerate doses of insecticides which would prove lethal to a majority of insects in a normal population [1]. Mechanisms that decrease the insecticide toxicity rely on modifications in one or several genes of the mosquito, and, as a result, resistance is a heritable trait. The individual survivors could then pass the resistance mechanism to the successive generations resulting in pest populations that are more resistant. Insecticide-Treated Nets (ITNs) have played important role in the reduction of malaria parasite transmission in Sub-Saharan Africa [2]. However, concern is growing that insecticide resistance, could reverse this trend and lead to rising incidence of the malaria disease [3] as insecticide resistance is now widespread in a number of mosquito species [4].

Insecticides play a central role in the control of mosquito vectors and will continue to do so for the foreseeable feature. However, the ubiquitous use of a limited number of insecticides for the control of vectors has led to resistance making insecticides used ineffective. Vector control through the use of insecticide plays a key role in the prevention and control of infectious diseases [5]. In West Africa, resistance to pyrethroids due to knockdown resistance (kdr) mutations in *An. gambiae s.l.* populations has been reported in Côte d'Ivoire, Burkina Faso and Benin [6] despite the absence of large-scale mosquito control in these countries.

In recent times, resistance to pyrethroids insecticides by malaria vectors has also been reported in Zambia, Democratic Republic of Congo, Papua New Guinea and Nigeria [7-10]. Also, there have been reports that biting behavior of some mosquitoes changed following pyrethriod-based Long Lasting Insecticide Nets (LLINs) campaigns [11,12]. The problem of insecticide resistance is a challenge, even in Nigeria.

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It has been reported that resistance is becoming a serious threat and may become worse due to the effects of climate change [13]. It has also been noted that there is concern that the mosquitoes are becoming resistant to the other classes of insecticides namely; organochlorines, organophosphates and carbamates [14]. The aim of this study was to investigate the resistance status of the *An. gambiae s.l.* to the four classes of insecticide used in vector control in Enugu State and to identify the target site resistant genes (kdr and Ace-1) present in resistant mosquitoes.

Materials and Methods
Study Area
Enugu State is in the tropical rain forest area of southeastern Nigeria. The state is situated between the coordinates of latitude $6^027'35.87''$N and longitude $7^032'56.22''$E. The state has total area of 7,618 sq. Km and is divided into three senatorial districts politically. The inhabitants of state are predominantly Igbos with a population of at least 4,881,500 people [15]. The occupation of the inhabitants are mainly farming, trading and civil service. The area has an average annual rainfall of about 1300mm and average daily atmospheric temperature of 30^0C. There are two distinct seasons, the wet (March – October) and dry season (November – February). The study was conducted in 3 randomly selected communities in the senatorial districts.

Community Visitation and Sensitization
A letter of introduction was obtained from the Department of Parasitology and Entomology, Nnamdi Azikiwe University. The letter was used to make advocacy visits to the leaders of the study communities. They were properly informed about the methods and intent of the study. Their permission was obtained to use their environment for the study.

Study Design
Randomized controlled trail study design was employed with communities as units of study. Three communities (one per senatorial district), were randomly selected. This was done by writing names of all the communities in each of the senatorial districts on sheets of paper. The papers were placed in a bag and one community was blindly picked to represent a senatorial district. The randomly selected communities and their geographical coordinates were; Awkunanaw (Enugu East senatorial district) 6.3907N, 7.4760E; Obukpa (Enugu North senatorial district) 6.890N, 7.4003E; and Awgu (Enugu West senatorial district) 6.0640N, 7.4760E. *Anopheles* mosquito larvae sampling was conducted in the communities between May, 2019 and June, 2020. The WHO bioassay for each class of insecticide tested in each of the three sites was done with one hundred and twenty (120), 2-5days old female *Anopheles* mosquitoes (reared from wild larvae) per class of insecticide. Total susceptibility bioassay for the three senatorial zones was 4 replicates and 2 controls consisting of 20 mosquitoes per replicate/control in all the four classes of insecticide tested per vegetation. These were used to analyze insecticide resistance to the four classes of insecticide used in the three communities for the study.

Larval Sampling
Larval collection was done using 350ml standard dipper [16]. Ladles and pipettes were also used. Collections were made in all forms of breeding sites including puddles, rice fields and other farms, containers, excavations, tire tracks, hoof prints and crab holes amongst others. When *Anopheles* mosquito larvae are present, 10-30 dips were taken depending on the size of larval habitat. Where dipping was not possible, for example, when larvae were seen on polythene, can or leaf, the larvae were transferred from the breeding material into the collection container. Sampling was carried out twice daily, in the morning between 7:00am to 11:00am and in the evening between 4:00pm to 6:00pm (local time) for about 30 minutes in each larval habitat for 5 days. All larvae and pupae collected were transported in containers labeled according to the site of collection to the insectary of the National Arbovirus and Vectors Research Centre, Enugu, Nigeria, were they were reared to adults, for susceptibility tests. The larvae were fed on fish feed (Tropical Flakes, Samyu Pets Corp and Shulin City) in bowls measuring 500ml, while the adult mosquitoes were fed with a 10% sugar solution in cotton wool on emergence. Relative humidity and temperature of $80 \pm 10\%$ and 25 ± 2 °C, respectively were maintained in the insectary throughout the period.

Morphological Identification of *Anopheles gambiae s.l.* Mosquito species
Adult *An. gambiae s.l.* species used in the study were identified at the Entomology Laboratory of the National Arbovirus and Vectors Research Centre, Enugu using morphological keys [17-19].

WHO Tube Bioassay to Determine Resistance Status of *Anopheles gambiae s.l.* Mosquitoes
Non-blood-fed *An. gambiae s.l.* females that were 2 to 5 days post-emergence were used for susceptibility tests [20]. Adults of *An. gambiae s.l.* from all three sites in the study area were tested against 0.1% bendiocarb (carbamate), 0.05% deltamethrin (pyrethroid) and 4% DDT (organochlorine) and 5% malathion (organophoshate) used for the remaining two sites. For each (bendiorcarb, deltamethrin, DDT and malathion) of insecticides, a total of 120 mosquitoes were used for the susceptibility test. 80 mosquitoes were exposed for 60 min to the insecticides using four replicates of 20 mosquitoes each and 40 mosquitoes for the controls using two replicates of 20 mosquitoes each. The test conditions were maintained at a temperature of 26 ± 3 °C and a relative humidity of $74 \pm 4\%$. Mosquitoes that survived were preserved for target site resistance (kdr-w and Ace-1^R) assays, using silica gel in Eppendorf tubes.

Six sheets of clean white paper (12 x 15 cm), rolled into a cylinder shape, were inserted into six holding tubes (one per tube) and fastened into position with a steel spring-wire clip. The tubes were attached to slides. A total of 120 active female mosquitoes were aspirated (in batches) from a mosquito cage into the six holding tubes through the filling hole in the slide to give six replicate samples of 20 mosquitoes per tube. Once the mosquitoes have been transferred, the slide unit was closed and the holding tubes set in an upright position for one hour. At the end of this time, damaged insects were removed. Six exposure tubes were prepared in much the same way. Each of the 4 red-dotted exposure tubes were lined with a sheet of insecticide-impregnated paper, while the 2 yellow-dotted control exposure tubes were lined with oil-impregnated papers. Each was fastened into position with a copper spring-wire clip. The empty exposure tubes were attached to the vacant position on the slides and with the slide unit open, the mosquitoes were blown gently into the exposure tubes. Once all the mosquitoes were in the exposure tubes, the slide unit

was closed and the holding tubes were detached and set to one side. Mosquitoes were kept in the exposure tubes, which were set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour (60 minutes). At the end of the 1-hour exposure period, the mosquitoes were transferred back to the holding tubes by reversing the procedure outlined. The exposure tubes were detached from the slide units. A pad of a cotton-wool soaked in sugar water was placed on the mesh-screen end of the holding tubes. Mosquitoes were maintained in the holding tubes for 24 hours (the recovery period). During this time, the holding tubes were kept in a shady, sheltered place free from extremes of temperature (an insectary is ideal). Temperature and humidity were recorded during the recovery period. At the end of recovery period (24 hours post-exposure), the number of dead mosquitoes were counted and recorded. An adult mosquito was considered to be alive if it was able to fly, regardless of the number of legs remaining. Any knocked-down mosquitoes, whether or not they have lost legs or wings, were considered dead. On completion of the susceptibility test, mosquitoes were transferred to individual, clearly labeled Eppendorf tubes (separating dead and live mosquitoes into separate tubes) for storage until such time that they can be transferred to suitable facilities for supplementary testing [20].

Polymerase Chain Reaction (PCR) for Detection of Resistance Mechanisms

Sub-samples from survivor and dead mosquitoes used for insecticide exposure were subjected to molecular diagnostic test for the presence of the *kdr* and *ace-1* mutation genes.

Detection of Knockdown Resistance Gene (*kdr*)

The PCR-restriction fragment length polymorphism diagnostic test was performed to identify the presence of the L1014F mutation (*kdr)* using a described method [21]. Primers used; Agd1 5' ATA GAT TCC CCG ACC ATG 3', Agd2 5' AGA CAA GGA TGA TGA ACC 3', Agd3 5' AAT TTG CAT TAC TTA CGA CA 3', Agd4 5' CTG TAG TGA TAG GAA ATT TA 3'. The lyophilized primers were rehydrated by adding nuclease-free water to 300um stock solution, from this stock aliquots, 30um were collected and used for the primers. Master mixing reaction set up used includes 5x Green Go Taq Buffer 10ul, 25 mM MgCl2 2.5ul, 25mM each dNTP'S 2.ul, Agd1-30um (1ul), Agd2-30um-(1ul), Agd3-30um-(1.5ul), Agd4-30um (1.5ul), PCR water 25ul, GoTaq flex DNA polymerase 5u/ul 0.5ul, volume/tube 45ul, DNA template 5ul, total reaction volume 50ul. Extra reactions were made to compensate for pipetting errors. The cycling parameters were 35 cycles at 95 ºC for 5 minutes, followed by 94 ºC for 30 seconds, then 46 ºC for 30 seconds, 72 ºC for 30 seconds, 72 ºC for 7 minutes. Agarose gel electrophoresis was used for size separation of the PCR product by comparison with a DNA ladder (a molecular weight marker). PCR product was verified on 2% agarose gel at 120v for at least 1 hour 30 minutes. The expected product size include; control 293bp, 1014F (RR) 195 bp, and 1014L (SS) 137bp [21].

Detection of Insensitive Acetycholinesterase Resistance Gene (*Ace-1*)

The presence of the G119S mutation was screened with PCR thermal cycler following the protocol established by Bass and Colleagues. Deoxyribonucleic Acid (DNA) from the mosquitoes were extracted using Quick-DNA™ Tissue/Insect Miniprep Kit manual Catalog No. D6016 produced by Zymo

Research, USA 2019.

Procedure for Deoxyribonucleic Acid Extraction

Five mosquitoes from same senatorial districts were placed in a 1.5ml Eppendorf tube and 50ul BashingBead™ Buffer added to the Eppendorf tube and grounded slowly and properly with a grinding stick. Three hundred and fifty (350ul) of BashingBead™ Buffer was added to the tube to bring the volume to 400ul and the tube was vortex to permit total mixing of the solution. ZR BashingBead™ Lysis Tube (2.0mm) was centrifuged in a microcentrifuge at 10,000 rpm for 1 minute. The supernatant was transferred to a Zymo-Spin™ III-F Filter placed in a Collection Tube and then centrifuged at 8,000 rpm for 1 minute. The collection tube was collected with the filtrate and Zymo-Spin™ III-F Filter was discarded. A measure of 1,200ul of Genomic Lysis Buffer was added to the filtrate in the Collection Tube and the mixture vortex for 10 seconds. The Zymo-Spin™ IICR Column 1 was placed in a Collection Tube and 800ul (the remaining mixture was not discarded as it was used at a later stage) of the mixture from the previous step was transferred to it and the mixture was centrifuged at 10,000 rpm for I minute. The flow was discarded through filtrate from the Collection Tube and the Zymo-Spin™ IICR Column 1 placed in the Collection Tube. The remaining mixture from the previous stage was transferred to Zymo-Spin™ Column 1 and centrifuge at 10,000rmp for 1 minute. The flow was discarded from the Collection Tube through the filtrate and the Zymo-Spin™ IICR Column 1 placed in a new Collection Tube. Two hundred (200ul) DNA Pre-Wash Buffer was added to the Zymo-Spin™ IICR Column in the new Collection Tube and centrifuged at 10,000rmp for 1 minute. A measure of 500ul g-DNA Wash Buffer was added to the Zymo-Spin™ IICR Columnin the Collection Tube and centrifuged at 10,000 rpm for 1 minute. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml Eppendorf tube and 100ul DNA Elution Buffer added to it. The Zymo-Spin™ IICR in the Eppendorf tube was centrifuged at 10,000 rpm for 30 seconds to elute the DNA. The eluted solution in the Eppendorf tube containing the mosquito DNA was stored at-20ºC for subsequent PCR analysis (Quick-DNA™ Tissue/Insect Miniprep Kit Manual Catalog No. D6016).

Master Mixing (reaction setup) One Taq 2x Master Mix (14µl)**,** EX2AGDIR 1 (Foward primer) 2µl, EX4AGREV 2 (Reverse primer) 2µl**,** Nuclease free water 9µl**,** and DNA template 3µl**.** Total PCR reaction volume 30µl. Primer sequence used are: EX2AGDIR 1 AGG TCA CGG TGA GTC CGT ACG A**,** EX4AGREV 2 AGG GCG GAC AGC AGA TGC AGC GA. DNA Amplification: Amplification Conditions used were as follows: hold (95 ºC for 90 seconds), 35 cycles (94 ºC for 30 seconds, 68 ºC for 30 seconds and then 72 ºC for 60 seconds) and hold for final extension (72 ºC for 5 minutes). The amplification protocol was adopted and modified from already described methods [22, 23]. Expected Band size was 924bp.

Data Analysis

Linear regression, Correlation and Analysis of Variance (ANOVA) were used to analyze the result using SPSS software. 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 [20]. Abbott's formula was used to correct the

observed mortality when the mortality in the control is between 5–20% [24].

Results

The WHO bioassay of four insecticides belonging to different classes was conducted (Fig 1). The average mortality of *An. gambiae s.l.* tested with bendiocarb was 88.33%. Observed mortality in the zones was as follows; Awgu 100% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.943) and a very high R^2 (0.889). Correlation and ANOVA were both significant at P = 0.001) and Awkunanaw 87.5% (A linear regression test yielded a very strong positive R (0.955) and a very high R^2 (0.913) Correlation was significant at *P*<0.001; ANOVA was significant P = 0.001). The lowest mortality with bendiocarb was observed in Obukpa Nsukka 77.5% (A linear regression test yielded a very strong positive R (0.943) and a very high R^2 (0.889). Correlation was significant at P = 0.014 and ANOVA was also significant at P = 0.028).

The efficacy of deltamethrin to *Anopheles gambiae s.l.* collected from three sites (Obukpa Nsukka in Enugu North senatorial zone, Awgu in Enugu West senatorial zone and Awkunanaw Enugu East senatorial zone) in Enugu state show an average mortality of 63.53% Observed mortality in the zones was as follows; Obukpa Nsukka 98.75% (A linear regression test of knockdown time and number of knockdown yielded a strong positive R (0.703) and a low R^2 (0.494). Correlation was significant at P = 0.039 and ANOVA was not significant at P = 0.078) and Awkunanaw 64% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.988) and a very high R^2 (0.976). Correlation and ANOVA were both significant at *P*<0.001). The lowest mortality with delthamethrin was observed in Awgu 28.75% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.937) and a very high R^2 (0.877). Correlation was significant at P = 0.001and ANOVA was significant at P = 0.002).

With malathion, the average mortality of *An. gambiae s.l.* is 100%. At the senatorial zone level, observed mortality was 100% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.960) and a very high R^2 (0.921). Correlation and ANOVA were significant at *P*<0.001) in Obukpa Nsukka and 100% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.961) and a very high R^2 (0.923). Correlation was significant at *P*<0.001; ANOVA was significant at P = 0.001) in Awkunanaw. Also, the mortality with malathion observed in Awgu was 100% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.960) and a very high R^2 (0.922). Correlation was significant at *P*<0.001and ANOVA was significant at P = 0.001).

The DDT test for Enugu recorded average mortality of 28. 3% for *An. gambiae s.l.* At the senatorial zone level, observed mortality was 28.75% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.953) and a very high R^2 (0.908). Correlation was significant at *P*<0.001 ANOVA was significant at P = 0.001) in Awkunanaw. The lowest mortality recorded at Awgu was 26.25% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.961) and a very high R^2 (0.923). Correlation was significant at P < 0.001 and ANOVA was significant at P =

0.001) in Awkunanaw. The highest mortality with DDT was observed in Obukpa Nsukka 30% (A linear regression test of knockdown time and number of knockdown yielded a very strong positive R (0.955) and a very high R^2 (0.913). Correlation was significant at *P*<0.001 and ANOVA was significant at P = 0.001). The Knockdown rate of bendiocarb, deltamethrin, malathion and DDT to the mosquitoes in the three study communities are shown in figures 2-4.
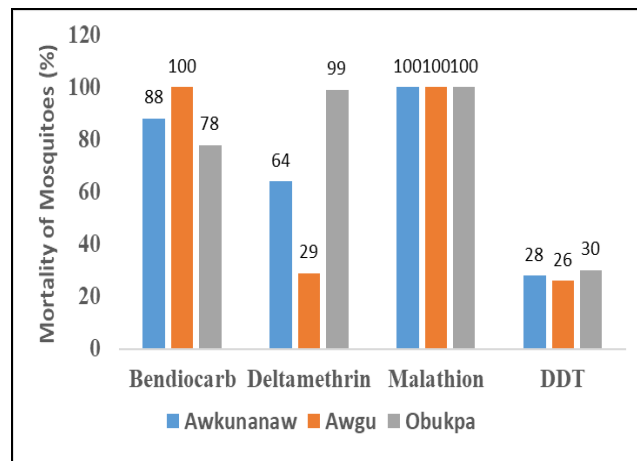

Fig I: Efficacy of Insecticides to *Anopheles gambiae s.l.* in the three senatorial districts in Enugu State
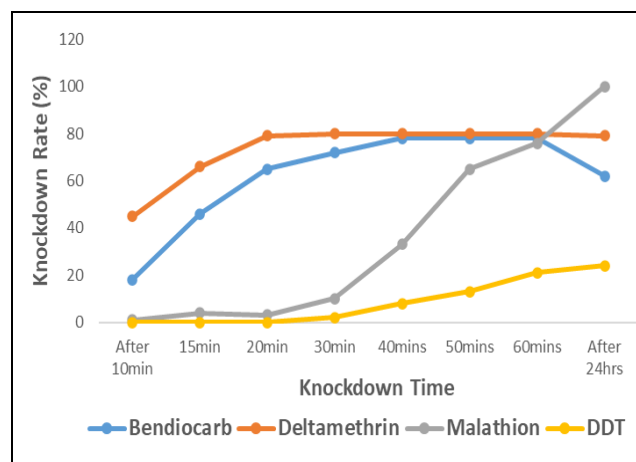

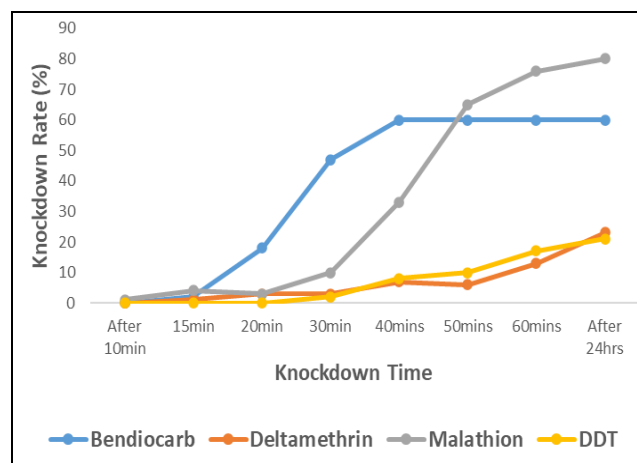**Fig 2:** Knockdown rate of four classes of insecticides to *Anopheles gambiae s.l.* in Obukpa Nsukka

Fig 3: Knockdown rate of four classes of insecticides to *Anopheles gambiae s.l.* in Awgu
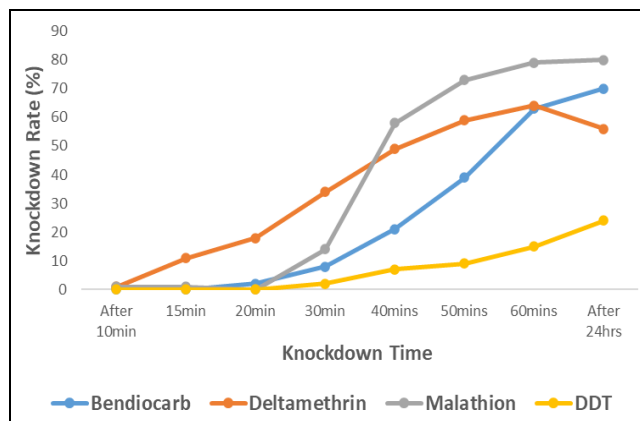
Fig 4: Knockdown rate of four classes of insecticides to *Anopheles gambiae s.l.* in Awkunanaw

Target site resistance was assayed in the *An. gambiae s.l.* collected from the three study communities in the senatorial districts. The GII9S *Ace-1* mutation was detected at low frequencies in mosquitoes collected from all three sites. On the other hand, mutations in the LI0I4F *kdr* gene were not detected in *An. gambiae s.l.* collected from all sites in the senatorial districts (Table 1).

Table 1: Distribution of L1014F and G119S mutations genes across the state

Location	No. Tested (%)	*Kdr* LI0I4F (%)	*Ace-1* G119S (%)
Awgu	25 (33.33)	0 (0.00)	25 (33.33)
Obupa Nsukka	25 (33.33)	0 (0.00)	25 (33.33)
Awkunanaw	25 (33.33)	0 (0.00)	25(33.33)
Total	75 (100)	0 (0.00)	100 (100)

Plate 1: Electrophoresis gel image for Ace-1 gene confirmed at 924bp

Discussion

This study revealed that *An. gambiae s.l.* in the study area were resistant to carbamates, pyrethroids and DDT, but were only susceptible to the organophosphate (malathion). Previous studies in different parts of Nigeria have reported similar resistance level to different pyrethriods and DDT [25-27]. Another study also noted that malaria vector populations showed the greatest resistance against DDT and pyrethroids [28]. This may be due to excessive use of DDT in the past, which may also have conferred resistance on the pyrethroids through cross-resistance. Additionally, LLINs have been widely distributed in the South-East Zone for about a decade. The fact that the pyrethroids are the only class of insecticide used in LLINs is likely to have aided the situation. But the observation in this study contrasted with another in Anambra

State [7], where malaria vectors were found to be susceptible to only the carbamate, bendiocarb. The high pyrethroid resistance in *An. gambiae s.l.* from Enugu state may be due to the prevailing insecticide selection pressure on vector populations following the rapid scale up and use of pyrethroid-based vector control interventions and agricultural usage of pyrethroid insecticides in these areas [25].

Resistance of *An. gambiae s.l.* to DDT and pyrethroids has been reported in various studies in Nigeria [25-27, 29-34] and in other parts of Africa [35]. This may be due to cross-resistance, use of agricultural insecticides and/or use of pyrethroids in LLINs and IRS. On the contrary, resistance to organophosphate is not as widespread as those of DDT and the pyrethroids as shown by this study. Also, *An. gambiae s.l.* showed susceptibility to organophosphate (Malathion) in all sites studied. This agrees with other studies where *An. gambiae s.l.* susceptibility to organophosphate (pirimiphos-methyl) was reported in some parts of South-West Nigeria [7, 28]. Susceptibility to organophosphate in 21 out of 24 sentinel sites in Akwa Ibom, Bauchi, Ebonyi, Nassarawa, Oyo, and Sokoto states in Nigeria has been reported [29].

Target site resistance was the only mechanism of resistance analyzed in this study. Nevertheless, target site resistance is a major mechanism driving vector insecticide resistance in Nigeria [36]. The West African knockdown resistance (L1014F) was not detected in all *An. gambiae s.s.* tested. This was contrary previous findings in Nigeria [26, 28, 29, 31, 33, 37] where the L1014F mutation was found in mosquito populations. The result of the study also contrast others from several parts of West and Central Africa where L1014F mutation were observed [29, 38-40]. L1014F mutation has been associated with DDT and pyrethriods cross resistance in *An. gambiae s.l.* [21,39] though it has been argued that this mutation alone may not solely be responsible for this phenotypic response [41]. Despite the absence of the L1014F mutation, DDT, pyrethriod and carbamate resistance were observed in all the sites. This suggests that other resistance mechanisms such as metabolic resistance which confers cross resistance to organophosphates and carbamates could be involved in the vector populations.

In contrast to L1014F, acetylcholinesterase resistance (G119S mutation gene) was observed in all the sites. The dominant *An. gambiae s.s.* tested from all the site were positive for G119S mutation. This is in agreement with recent results in Nigeria [33, 42] where G119S mutation genes were detected. The *Ace-1R* mutation has been linked to carbamates and organophosphate cross resistance in malaria vectors. The detection of *Ace-1R* mutation in *An. gambiae s.s.* in Enugu state could be detrimental to the utilization and the efficacy of IRS, a major strategy in the control of malaria vectors in sub Saharan Africa. Previous studies have associated the extensive use of agricultural pesticides [43-45] and spread of resistance genes from neighbouring countries [23] with development of carbamate and organophosphate cross resistance, but this may not be applicable to this study as all sites studied were completely susceptible to organophosphate (malathion).

Conclusion

This study has demonstrated a widespread resistance of *An. gambiae s.l.* to three of the four classes of insecticides used in vector management in the study area. This could have huge negative impact in the control of malaria and its vectors in the state as most of the vector control interventions rely heavily

on insecticides from these classes. Also, the detection of G119S resistance gene is a danger sign. There is an urgent need for implementation of insecticide resistance management strategies in the state to assess and control the spread of resistance.

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