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Susceptibility status of *Anopheles gambiae* complex in NNEWI community, Anambra state

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Abstract
Insecticide resistance in *Anopheles* mosquito is a major concern for vector control interventions in Nigeria. The present base-line study was carried out in Nnewi community, Nnewi North L.G.A., Anambra State, South-east Nigeria to investigate the insecticide susceptibility status of wild *Anopheles gambiae* s.l. to the four classes of insecticide used in vector control interventions. *Anopheles* larvae collected from different breeding sites in the community were reared to adults in the insectary and were morphologically identified following standard protocols. Two to five-day old adult female mosquitoes were exposed to different doses of organochlorine (4% DDT), organophosphate (0.25% pirimiphos-methyl and 5.0% malathaion), carbamate (0.1% propoxur and 0.1% bendiocarb) and pyrethroid (0.05% deltamethrin, 0.75% permethrin) insecticides using WHO susceptibility assay protocol. Data generated were subjected to probit analysis and analysis of variance. The organochlorine insecticide (DDT) had the highest knockdown time KDT_{50} (113.8 minutes) and KDT_{95} (182.1 minutes) while the carbamate insecticide, bendiocarb had the least KDT_{50} (8.97 minutes) and KDT_{95} (28.78 minutes). The mosquitoes were totally susceptible to propoxur and bendiocarb. The two pyrethroid used (deltamethrin and permethrin) and organochlorine (DDT) recorded percentage mortalities of 17.5%, 43.75% and 11.25% respectively. The report of organochlorine and pyrethroid resistance in the study area calls for immediate implementation of management action in the study area. The study has also shown that carbamate insecticides may be a suitable alternative to mitigate pyrethroid resistance in mosquito population in the study area.

Keywords: Insecticide susceptibility, insecticide resistance, *Anopheles gambiae*, Nigeria

Introduction

Insecticides have played a significant role in the control and prevention of vector-borne diseases using Indoor Spraying (IRS) and Long-Lasting Insecticidal Nets (LLINs) as the major control intervention tools (AIRS, 2015) [1]. These two vector control tools have led to a tremendous reduction in malaria mortality in sub-Saharan Africa (Aribodor *et al*., 2015) [3]. However, the problem of insecticide resistance has threatened the efficacy of these control tools in major endemic locations (Aikpon *et al*., 2013) [2]. Previous reports show that *An. gambiae* continued to feed on human hosts despite high LLIN coverage (Chandre *et al*., 2010; Chukwuekezie *et al*., 2020) [4, 5]. This was found to be due to either decrease in the efficacy of LLINs used in the communities or mosquito resistance to pyrethroids. These problems may have severe consequences as they affect the implementation of vector control strategies in major malaria-endemic countries (Corbel and N'Guessan, 2013; Sougoufara *et al*., 2017) [7, 25]. Currently, a restricted number of chemical classes of insecticides are available for mosquito control with all the four classes (organochlorines, organophosphates, carbamates and pyrethroids) used for Indoor Residual Spray (IRS) while LLINS depends exclusively on pyrethroids (Corbel *et al*., 2012) [6]. Pyrethroids are widely used in agriculture and household due to their eco-friendly nature, non-phytotoxicity, bio-degradability, and low toxicity to mammals (Goroubi *et al*., 2018) [8]. However, this sole dependence on pyrethroids raises concerns about the implication of such action especially as regards to the problems of insecticide resistance in malaria vectors (Ranson *et al*., 2011) [23].

Pyrethroid resistance in *An. gambiae* has been reported in many countries in sub-Saharan Africa (Machani *et al*., 2020) [14]. Apart from pyrethroids, resistance in *Anopheles* species has been reported in other insecticide classes. Knockdown resistance (KDR) to pyrethroids and DDT have been reported across several West African countries (Mishra *et al*., 2021; Moyes *et al*., 2021) [15, 16], and this has affected successful and sustainable implementation of insecticide-based malaria control programs in the region (Govella *et al*., 2010; Grau-Bove *et al*., 2021; Henk *et al*., 2021) [9, 10, 11].

In Anambra State, Nigeria, knockdown resistance in *An. gambiae* mosquitoes to DDT, pyrethroid, and organophosphate were reported in Amansea community (Nwankwo *et al*.,) [19]. Household and agricultural use of these insecticides have been implicated in the occurrence of resistance in the *Anopheles* vector (Muhammed *et al*., 2021; Nkya *et al*., 2013; Nwankwo *et al*., 2019; 2017; Corbel and N'Guessan, 2013) [17, 18, 20, 7]. In addition, there have been two major rounds of LLINs mass distribution and IRS campaigns conducted between 2009 and 2014 in Anambra State. Nnewi community where the study was conducted benefited from the interventions deployed in the community in 2013 and 2014, respectively. Nevertheless, there is a dearth of information on the susceptibility status of *Anopheles gambiae* to insecticides used in these interventions in this community, where large-scale use of LLINS and IRS are the major components of Roll Back Malaria Programme (Nwankwo *et al*., 2017; Corbel and N'Guessan, 2013) [19, 7].

Therefore, it is imperative that mosquito susceptibility to the major classes of insecticides used in these control interventions be conducted before further introduction of the control tools. This would involve routine testing with a standardized protocol for resistance monitoring. Against this backdrop, a global database on insecticide resistance was established in 2014 by World Health Organization (WHO) to provide timely tracking of insecticide-resistance patterns in endemic communities and to identify where data are not sufficient. It is necessary therefore that states in Nigeria should be conducting sectional studies based on geographical and demographic parameters and baseline data collected at representative sites. It is hoped that based on such baseline data, susceptibility levels could be extrapolated for areas with similar socio-economic status, demographic and ecological characteristic. In this regard, the Federal Ministry of Health has boosted prevention and control efforts for malaria in Nigeria.

The present investigation was conducted to evaluate the susceptibility status of *Anopheles gambiae* complex from Nnewi community in Anambra State, Nigeria. It is hoped that base-line information obtained from this study would help in tackling the threat of insecticide resistance in the State.

Materials and methods

Study area

The study was conducted in Nnewi community, the second largest commercial city in Anambra State. Geographically, it falls within the lowland tropical rain forest region of Nigeria located between latitude 5°5941°.64 "N and 6°03'28. 44"N and longitude 6°03'2844"E and 6°52'41.64". It has an estimated population of 391,227 inhabitants based on 1991 population census. Majority of the inhabitants are traders, farmers, civil servants, students and professionals.

Mosquito sampling

Breeding sites of *Anopheles* mosquitoes were surveyed in some villages in Nnewi using standard dipping method. Wild larvae of *Anopheles* identified by their parallel positions on the water surfaces were collected with a ladle and kept in 1000ml loosely capped plastic containers. They were taken to the insectary of the National Arbovirus and Vector Research Center (NAVRC) Enugu. The larvae were fed with a mixture of yeast and cabin biscuit and reared to adult at temperature of 26±3 ºC and relative humidity of 74±4%. They were closely monitored and those that pupated were transferred into plastic cups using Pasteur pipettes and placed in labeled cages for adult emergence. All adults that emerged were fed on 10% sugar solution, and 2-5 day old non-blood fed female adults were used for the bioassay.

Insecticide susceptibility assay

Test papers impregnated with four classes of insecticides namely; organochlorine (4% DDT), organophosphate (0.25% pirimiphos-methyl and 5.0% malathaion), carbamate (0.1% propoxur and 0.1% bendiocarb) and pyrethroid (0.05% deltamethrin, 0.75% permethrin) were used for the susceptibility assays. Susceptibility tests were performed in the insectary according to WHO (2013) protocols. Twenty five *An. gambiae* s.l mosquitoes per tube were used for each replicate. The same number of mosquitoes were exposed to untreated papers that served as control. Knockdown effect after exposure to each insecticide was recorded in 5, 10, 15, 20, 30, 40, 50 and 60 minutes. Mortality observed in adult mosquitoes was assessed after 24 hours post insecticide exposure and each mosquito was scored as either dead (susceptible) or alive (resistant).

Data analysis

Percentage mortality and knockdown values were subjected to analysis of variance (ANOVA) and means separated using LSD. Probit analysis was used to determine knockdown times for 50% and 95% (KDT_{50} and KDT_{95}). The resistance status of mosquito samples was determined based on the WHO criteria (WHO, 2013) [29]. In line with this criteria, 98% - 100% mortality indicate that the population is susceptibility. Mortality between 90% - 97% means resistance is suspected and requires further investigation. Mortality of less than 90% confirms the presence of resistance genes, and additional bioassay may not be necessary. However, the mechanism of resistance must be investigated.

Results

Exposure time effect on knockdown

The exposure time effect on knockdown of *An. gambiae* after one hour is presented in Table 1. The results show that within 5, 10, and 15 minutes of exposure to insecticides, the mean knockdown rates were 1.36±1.0, 2.14±1.48, and 3.36±1.90, respectively. However, as the time of exposure increased to 40 and 60 minutes, the knockdown rates were 9.71±3.24 and 12.82±3.19 respectively. *Anopheles* mosquitoes exposed to DDT had the least mean knockdown rate of 0.56±0.19, while bendiocarb caused the highest mean knockdown rate of 15.94±1.77. Analysis of variance showed significant differences ($p<0.05$) in the knockdown time effect at different exposure times.

Knockdown Time Effect (KDT_{50} and KDT_{95}) of the Insecticides on *An. gambiae* Complex from Nnewi

The knockdown times recorded for the different insecticides are presented in Table 2. The results showed that the carbamate and organophosphate had the lowest KDT_{50} and KDT_{95} with values of 8.97 min and 28.78 min recorded for bendiocarb while 22.38 and 35.74 min were recorded for Malathion respectively. The highest KDT_{50} and KDT_{95} recorded for DDT were 113.8min and 182.1 min respectively.

Mortality rate and Susceptibility Status

Percentage Mortality and Susceptibility Status of *An. gambiae* complex from Nnewi, Exposed to the Four Classes of Insecticides. The mortality rates and susceptibility status of *An. gambiae* complex against the insecticides are shown in Table 3. The F_1 population of *An. gambiae* were resistant to discriminating doses of 4% DDT (17.50% mortality), 0.05% deltamethrin (43.75% mortality) and 0.75% permethrin (11.25% mortality) 24 hours post- exposure mortalities respectively. However, they were fully susceptible to 5% Malathion (98.75% mortality), 0.1% propoxur and 0.1% bendiocarb (100% mortalities) respectively. However, resistance was suspected in pirimiphos-methyl with 93.75% mortality on mosquitoes and requires further studies.

Table 1: Effect of 60 minutes exposure to insecticide on the knockdown (KD) of *Anopheles gambiae* s.l from Nnewi community.

Insecticide (conc)	Time of exposure (minutes)								Mean (±s.e)	% KD
	5	10	15	20	30	40	50	60		
DDT (4%)	0.00	0.00	0.25	0.25	0.50	1.00	1.50	1.50	0.56±0.2	7.50
Pirimiphosmethyl (0.2%)	0.00	0.00	0.25	1.25	3.50	5.00	8.00	16.75	4.34±2.03	83.75
Malathion (5.0%)	0.00	1.00	3.50	8.50	17.50	19.50	19.75	20.00	11.22±3.2	100.0
Propoxur (0.1%)	2.50	3.50	6.00	7.00	10.00	17.00	18.50	20.00	10.56±2.5	100.0
Deltamethrin (0.05%)	0.00	0.00	0.00	0.75	3.25	5.00	7.00	9.50	3.19±1.3	47.50
Permethrin (0.75%)	0.00	0.00	0.00	0.00	1.00	1.00	2.00	2.00	0.75±0.3	10.00
Bendiocarb (0.1%)	7.00	10.50	13.50	17.50	19.50	19.50	20.00	20.00	15.94±1.8	100.0
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00±00	0.00
Mean±SE	1.3±1.0	2.14±1.5	3.36±1.9	5.04±2.45	7.89±2.9	9.71±3.2	10.96±3.1	12.82±3.2		

Insecticide: $p<0.001$, LSD=5.022; **Time**: P = 0.015, LSD = 7.258

Table 2: Knock-down Time (KDT) of *An. gambiae* s.l mosquitoes from Nnewi community exposed to four classes of insecticides

Insecticide (conc)	Insecticide class	Number exposed	KDT_{50} (mins)	95%CI	KDT_{95}(mins)	95%CI
DDT (4%)	Organochlorine	80	113.8	75.85-12334	182.1	111.09-23973
Pirimiphosmethyl (0.25%)	Organophosphate	80	48.99	44.44-55.06	75.57	66.72-91.36
Malathion (5.0%)	Organophosphate	80	22.38	19.87-25.31	35.74	31.57-42.79
Propoxur (0.1%)	Carbamate	80	25.50	21.55-29.60	52.48	45.68-63.48
Bendiocarb (0.1%)	Carbamate	80	8.97	3.55-12.35	28.78	23.57-40.21
Deltamethrin (0.05%)	Pyrethroid	80	58.16	51.18-70.55	93.84	78.71-125.88
Permethrin (0.75%)	Pyrethroid	80	95.60	70.61-321.6	148.7	100.88-606.00

Table 3: Percentage mortality and susceptibility status of *An. gambiae* complex from Nnewi community exposed to the four classes of insecticides.

Insecticides (conc.)	Insecticide class	Number exposed	Mean (s.e) mortality	% mortality	Susceptibility status
DDT (4%)	Organochlorine	80	3.50±1.3	17.5	Resistant
Pirimiphos-methyl	Organophosphate	80	18.75±0.8	93.75	Resistance suspected
Malathion (5.0%)	Organophosphate	80	19.75±0.3	98.75	Susceptible
Propoxur (0.1%)	Carbamate	80	20.00±0.0	100.0	Susceptible
Bendiocarb (0.1%)	Carbamate	80	20.00±0.0	100.0	Susceptible
Permethrin (0.75%)	Pyrethroid	80	2.25±0.3	11.25	Resistant
Deltamethrin (0.05%)	Pyrethroid	80	8.75±1.9	43.75	Resistant

Discussion

The present study has provided base-line data on the susceptibility status of *Anopheles gambiae* s.l mosquito to DDT (4%), pirimiphos-methyl (0.25%), 5.0% malathaion (5.0%), (0.1% propoxur (0.1%), bendiocarb (0.1%), deltamethrin (0.05%), and permethrin (0.75%) in Nnewi community, Anambra State. The *An. gambiae* population tested recorded high level of resistance to DDT (17.5%), permethrin (11.25%) and deltamethrin (43.75%) while resistance was suspected in primiphos-methyl (93.75). However, the mosquitoes were completely susceptible to malathion (98.75%), propoxur (100%) and bendiocarb (100%). The knockdown time showed that propoxur, bendiocarb and malathion took 25.5, 22.38 and 8.97 minutes respectively to knockdown 50% of the mosquito population while pirimiphos-methyl, deltametrin, permethrin and DDT took a longer time (48.99, 58.16, 95.60 and 113.80 minutes) respectively to knockdown 50% of the mosquito population.

Studies have shown that knockdown time is a measure of early detection of reduced susceptibility and has long been accepted (Wei *et al*., 2021) [27]. Thus, the measurement of knockdown time could be incorporated in monitoring programs for insecticide resistance in mosquitoes since it provides initial information on the plausible involvement of the knockdown resistant (kdr) gene in these mosquitoes. Hence, in the present study, small numbers of mosquitoes were knocked down by DDT and pyrethroid insecticides after 60 minutes of exposure. The observed KDT_{50} and KDT_{95} of these insecticides suggest decreased susceptibility of *Anopheles* mosquitoes to the discriminating doses of the insecticides. This also may be indication that a knockdown resistance mechanism could be operating in this mosquito population. This supports the study carried out by Nwankwo *et al.* (2017) [19]; Chukwuekezie *et al.* (2020) [5] and Wei *et al.* (2021) [27]. Similarly, Chandre *et al*. (2010) reported that *An. gambiae* s.l. populations from southern Benin were resistant to DDT and pyrethroid insecticide owing to the Leu-Phe kdr mutation mechanism operating in the population. On the contrary, large numbers of mosquitoes knocked down by malathion (an organophosphorous insecticide) and the cabamate insecticide as well as reduced KDT_{50} and KDT_{95} of these insecticides depict complete susceptibility of the mosquito population to these insecticides.

Studies have shown that insecticide resistance in malaria vector population is a global phenomenon, which is highly predominant in the sub-Saharan Africa (Kleinschmidt *et al*., 2018) [13]. Resistance has been reported in all the classes of insecticides recommended by WHO for public use namely; pyrethroids, organochlorines, carbamates and organophosphates (Susanna and Pratiwi, 2022; WHO, 2018) [26, 28]. Significant amount of resistance was found in both DDT and pyrethroid in the present study, and this is consistent with the study by Nwankwo *et al.* (2017) [19] in Amansea, Anambra State. This has serious implication as it may likely affect sole dependence on LLINs and IRS for vector control, particularly the recent campaigns for LLINs distribution in Nigeria. Cases of cross resistance between DDT and pyrethroid have been reported in *An. gambiae* s.l (Corbel and N'Guessan, 2013; AIRS, 2015) [7, 1]. This was also confirmed by Idowu *et al*. (2020) [12] in southwest, Nigeria and Okorie *et al*. (2015) [22] in Ibadan southwest Nigeria. This type of resistance between DDT and pyrethroid may be possible because they share similar mode of action.

The level of resistance in mosquito population in the present study was higher for permethrin compared to deltamethrin, which is also in line with the study by Nwankwo *et al*. (2017) [19]; Chukwuekezie *et al*., (2020) [5]; Moyes *et al*., (2021) [16]. This could also be as result of wide use of permethrin than deltamethrin for both agricultural and public health purposes (Gorouhi et *al*., 2018; Chukwuekezie *et al*., (2020) [8, 5]. In addition, deltamethrin being a type II pyrethroid, which contains an alpha-cyano group in their chemical structure, may have resulted in better kill against the mosquito than permenthrin.

Furthermore, the present study have revealed that the mosquito population were not fully resistant to pirimiphos-methyl, an organophosphate reported to be effective in IRS field trials (WHO, 2011; Yakob *et al*., 2011) [30, 31] but were susceptible to bendiocarb, propoxur and malathion. Complete susceptibility to bendiocarb has been reported in Nigeria (Okorie *et al*., 2015) [22] and in other African countries (Aikpon *et al.*, 2013) [2]. On the contrary, resistance to propoxur has been reported in Lagos (Oduola *et al*., 2012) [21]. Increased level of carbamate resistance in *An. gambiae* s.l mosquito populations is a major concern for malaria control because these insecticides are increasingly used alone or in synergy with pyrethroids for IRS. However, studies have shown that combination of organophosphates and pyrethroid for IRS can increase and enhance protection against malaria (Corbel *et al*., 2012) [6] owing to differences in their mode of action.

Multiple resistance *An. gambiae* s.l to different classes of insecticides in the present study was noted and this has been reported elsewhere in Nigeria (Riveron *et al*., 2015; Nwankwo *et al*., 2017) [24, 19]. This has serious consequences for the Nigeria malaria control programme as it has the tendency of reducing the gain already made using LLINs and IRS as major vector control tools. However, the carbamate insecticides (bendiocarb, and propoxur) particularly bendiocarb as observed in the present study may be a useful alternative chemical class to organochlorine (DDT) and the pyrethroids (deltermathrin and permethrin) for vector control. This has also been suggested by Muhammad *et al*. (2021) [17] with continuous monitoring through bioassay of the carbamate insecticides.

This investigation did not involve studies on mechanisms conferring resistance in *An. gambiae* s.l and therefore could not associate the susceptibility tests with the knockdown resistant (kdr) mutation or the detoxifying enzymes (cytochrome P450s, carboxylesterases and glutathione S-transfereases), which are the two ways insects become resistant to insecticides. Hence, further characterization to unveil the mechanism of resistance in the mosquito vector is necessary in future studies to assist the implementation of recommended vector control tools.

Conclusion

The study has clearly demonstrated that *Anopheles gambiae* complex population in Nnewi Nigeria is highly resistant to deltamethrin, primiphos-methyl and DDT. Comparatively, bendiocarb, propoxur, and malathion were found to be the most effective (*i.e* least resistant) among the seven insecticides tested. This study also shows that exposure time has effect on the knockdown time of all the insecticides but at different levels. The study has confirmed and equally disputed several studies on insecticide susceptibility in *An. gambiae* mosquitoes carried out elsewhere in Africa and Asia. The differences observed in their findings may be attributed to the environmental and geographical differences within the study areas.

The reasons for the high level of resistance in DDT and pyrethroid in the study area may be attributed to the routine use of these pesticides by farmers for pest control operations and insecticidal aerosols and mosquito repellents for household use. The level of resistance reported in this study may have implication in the country on the current reliance on LLIN and IRS as vector control tools. There is therefore urgent need to adopt insecticide resistance management practices in line with global best practices.

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Declaration of competing interest

The authors declare that they have no known competing financial or personal interests that could influence the findings reported in this paper.

Author contribution

Ikeh, Roseline E.: contributed in developing the concept, methodology, performing laboratory work.

Nwankwo Edith N.: contributed in writing the original draft of the manuscript, writing of reviews and provided significant editorial assistance and supervision.

Okorie Patricia N.: contributed in writing of reviews and

provided significant editorial assistance.
Ikeh Franklin: contributed in the field collection of mosquitoes and laboratory work.
Ogbonna Confidence U.: contributed in data analysis and interpretation.
Ezihe, Ebuka K.: contributed in mosquito rearing, assays and data analysis.

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