Molecular Characterization of Insecticide Resistance Genes in Mosquito Populations in Port Harcourt Metropolis, Rivers State, Nigeria

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ABSTRACT

Monitoring and understanding the trends and mechanisms of insecticide resistance are critical for developing effective vector control strategies in Nigeria. Malaria and lymphatic filariasis remain major mosquito-borne diseases in Sub-Saharan Africa, primarily transmitted by female *Anopheles* and *Culex* mosquitoes. This study aimed to characterize the mosquito vector population and assess the insecticide resistance profile in three communities within the Obio/Akpor and Port Harcourt Local Government Areas of Rivers State, Nigeria. Mosquito larvae and pupae (*Anopheles* and *Culex* species) were collected from various habitats, reared to adulthood under controlled conditions, and morphologically identified. Insecticide susceptibility was evaluated using the CDC bottle bioassay method. The *kdr* mutation was detected using polymerase chain reaction (PCR) techniques applied to both resistant and susceptible adult mosquitoes exposed to pyrethroids. PCR assays were utilized to identify members of the *Anopheles gambiae* complex, while multiplex PCR confirmed *Culex* species. The CDC bioassays revealed complete susceptibility (100% mortality) to pirimiphos-methyl (organophosphate) and chlorfenapyr (pyrrole) across all sites for both *Anopheles* and *Culex* species. In contrast, high resistance was observed against pyrethroids, with mortality rates ranging from 34-53% in *Anopheles* mosquitoes and 64-91% in *Culex quinquefasciatus*. Morphological identification confirmed the presence of *Anopheles* and *Culex* spp., with an average *kdr* mutation frequency of approximately 90% in the three *Anopheles* species. Molecular analyses identified *An. gambiae* s.s., *An. coluzzii*, and *An. arabiensis* within the *Anopheles gambiae* complex, while *Culex quinquefasciatus* was the sole *Culex* species detected. Furthermore, the data suggested that *kdr* mutations and detoxification enzyme activity may result from environmental factors in contaminated breeding sites. These findings highlight significant public health implications, including increased nuisance biting and a heightened risk of disease transmission in polluted environments. The results underscore the urgent need for improved environmental management and targeted vector control strategies to mitigate the spread of mosquito-borne diseases in Nigeria

Keywords: Insecticide Resistance, mosquitoes, *kdr* mutation, Port Harcourt Metropolis.

INTRODUCTION

Vector control programs in Nigeria have consistently focused on reducing the burden of malaria, often prioritizing baseline studies on vector identification and insecticide susceptibility. Unfortunately, less emphasis has been placed on non-malaria vector species such as *Aedes aegypti* and *Culex quinquefasciatus* (Oduola *et al.*, 2016). These often-neglected species are responsible for the transmission of other life-threatening diseases, including yellow fever, lymphatic filariasis, and dengue fever (Oduola *et al.*, 2016). Environmental changes may influence mosquito physiology and behaviour, potentially enhancing their resistance to insecticides. Malaria control primarily targets the mosquito vectors that transmit the malaria parasite. In Sub-Saharan

Africa, vector control relies heavily on insecticides integrated into bed nets or applied through indoor residual spraying (IRS). This reliance means that vector control—and thus malaria control—can be compromised by resistance to these insecticides in vector populations. Despite the importance of surveillance using standard susceptibility tests, significant gaps exist in monitoring data across Africa (Moyes *et al.*,2020). Resistance to insecticides is defined as a heritable change in the sensitivity of a pest population, reflected in the repeated failure of a product to achieve the expected level of control when used according to label recommendations (Insecticide Resistance Action Committee, 2021). Insecticide resistance allows insects to survive exposure to standard doses of insecticide due to physiological or behavioural adaptations (Immo *et al.,* 2018). Increasing insecticide resistance could lead to a resurgence in disease and mortality rates. The deployment of insecticide-based interventions has been the principal driver of reductions in the global malaria burden since 2000. Unfortunately, resistance to insecticides is widespread in *Anopheles* mosquitoes across Sub-Saharan Africa and India, particularly to pyrethroids—the class of insecticides used in all long-lasting insecticidal nets (LLINs). This resistance diminishes the effectiveness of these interventions, with mathematical models predicting a potential rise in malaria incidence as a result (Immo *et al.*, 2018).

Resistance to four common insecticide classes has emerged in malaria vector populations worldwide, with pyrethroid resistance being particularly concerning. This class is used in all WHO-recommended LLINs and for indoor residual spraying (IRS) in many regions (Immo *et al.,* 2018).

Insecticide-based vector control is crucial in the fight against malaria. Selecting vector-control interventions should consider the resistance status of local mosquito vectors, as well as other factors related to intervention deployment, such as availability, cost-effectiveness, and population acceptance. Therefore, strategic monitoring of insecticide resistance is essential to inform evidence-based vector control strategies. In 2012, the WHO released the Global Plan for Insecticide Resistance Management in Malaria Vectors (GPIRM), which underscored the need for strengthened resistance monitoring and improved management of data, including the establishment of a global database. Understanding insecticide resistance is vital for effective vector control, particularly in regions like Port Harcourt Metropolis, where varying environmental conditions may influence mosquito populations. Mosquitoes have developed diverse mechanisms to resist the effects of insecticides, posing a significant challenge to vector control programs (Liu, 2015). These mechanisms can be broadly classified into four main categories which include target-site resistance which involves genetic mutations in the proteins that insecticides target, reducing the insecticide's ability to bind and disrupt normal function (Namias *et al.,* 2021), metabolic resistance which involves the increased production or enhanced activity of detoxification enzymes that metabolize insecticides, reducing their concentration before they reach their target site (Liu, 2015). cuticular resistance involves changes in the mosquito cuticle that reduce insecticide penetration (Namias *et al.,* 2021). These changes may include thickening of the cuticle or alterations in its composition (Namias *et al.,* 2021) and behavioural resistance which involves changes in mosquito behaviour that reduce their exposure to insecticides (Namias *et al.,* 2021). Target-site reduced sensitivity in *Anopheles* is caused mainly by mutations found in the voltage-gated sodium channel (Nav) (domains II–IV) and they are commonly known as knockdown resistance (*kdr*) mutations. This mechanism has been reported in several studies conducted elsewhere Africa (WHO 2016). *Kdr* point mutations have been characterized and often associated with pyrethroid resistance. (Chouaibou *et al* 2017). One of the *kdr* mutations have been detected in the Nav gene (at position 1014 of the encoded protein) of *Anopheles* populations: the replacement of leucine by phenylalanine (L1014F), found mainly in West Africa (the "*kdr*-west") (Chouaibou *et al* 2017).

This study aims to provide crucial insights that can inform local malaria control strategies and improve public health outcomes. It also aims to identify and characterize insecticide resistance genes present in mosquito populations in Port Harcourt Metropolis and assess the prevalence of resistance genes among different mosquito species, including both malaria and non-malaria vectors as well as evaluate the potential impact of environmental factors on the expression of resistance genes in these populations.

METHODS

Study Area

The study was conducted in two Local Government Areas (LGAs) of Port Harcourt Metropolis: Port Harcourt Local Government and Obio/Akpor Local Government (Figure 1). Port Harcourt (4.8156° N, 7.0498° E), the

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capital of Rivers State, Nigeria, lies along the Bonny estuary in the south-south region of the country, also referred to as the Niger Delta. The area is characterized by a tropical mangrove ecosystem, with rainfall extending from February to December (Mohammad *et al.*, 2021). As a major economic centre in Nigeria, Port Harcourt has experienced rapid urbanization and significant industrial and commercial growth (Obianuju *et al.*, 2017). The heaviest rainfall occurs in September, averaging 367 mm, while December is the driest month, with only 20 mm of rainfall (Mohammad *et al.*, 2021).

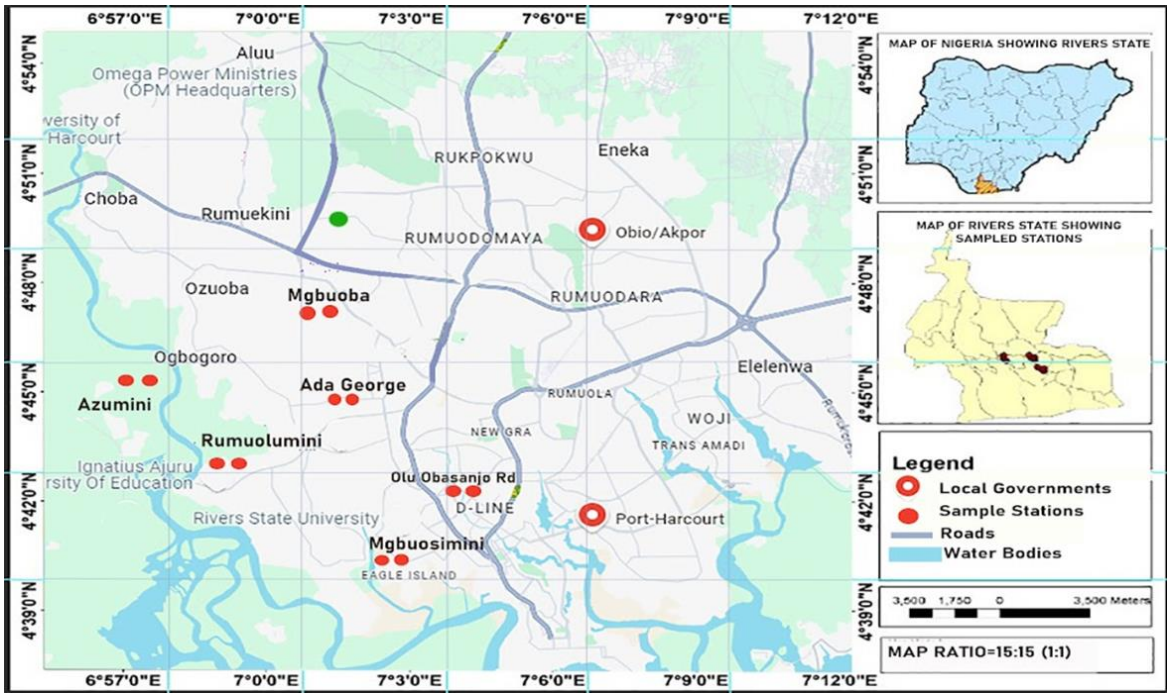

Figure 1: Map Showing the Study Area

Selection of Study Sites

The two LGAs were chosen due to their divergent human activities and to compare insecticide resistance profiles. Obio/Akpor and Port Harcourt are two of the three cities that make up the Port Harcourt metropolitan area. Obio/Akpor is located between latitudes 4°49'53.51" N and longitudes 6°59'20.62" E, with a subtropical climate and an average temperature of 20.6ºC. Mosquito larvae were collected from six communities within these LGAs and transported to the insectary at Rivers State University for sorting and rearing into adult stages for susceptibility bioassay tests.

Data Collection

Sampling of Mosquito Larvae

Anopheles mosquito larvae were randomly collected from various breeding sites, including drainage systems, ground cisterns, roadside ditches, and low-lying pools. A 35-ml dipper attached to a 1.2-m pole was used to scoop larvae from the water. The larvae were identified based on their spatial projections on the water surface (Gillies & Coetzee, 2020).

Field Sampling Procedures

Larval collection for susceptibility tests was conducted intensively around the communities in Obio/Akpor and Port Harcourt. Specific coordinates for the sampled locations include:

- **Obio/Akpor LGA**: Azumini (4.804116 N, 6.94332 E), Mgbuoba (4.81452 N, 6.94855 E), Rumuolumeni (4.84210 N, 6.97211 E).
- **Port Harcourt LGA**: Ada George (4.805406 N, 6.993755 E), Olu Obasanjo Road (4.79176 N, 7.002305 E), Mgbuosimini (4.80804 N, 6.98249 E), Control site (4.79734 N, 6.97935).

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Rearing of Larvae in the Laboratory

Collected larvae were kept in individual containers and labelled accordingly. They were transported alive and undamaged to the insectary at the Department of Animal and Environmental Biology, Rivers State University, maintained at 25–28°C and 70–80% relative humidity, allowing them to develop into pupae and subsequently into adult mosquitoes within 24 hours (Das *et al*., 2007). The insectary conditions included a 12-hour day/night cycle, with mosquitoes fed a 10% sucrose solution prior to sorting for susceptibility bioassay tests. Larvae were transported to the insectary, sorted, and transferred to larval rearing pans filled with water from the same breeding habitat. They were fed twice daily with ground biscuits carefully dissolved in water to ensure proper nourishment.

Emerged adult mosquitoes were collected from the rearing pans with an aspirator and placed into adult cages. They were fed with a 10% sugar solution in preparation for insecticide susceptibility tests.

Susceptibility Tests Using CDC Bottle Bioassays

CDC bottle bioassays were conducted following standard operating procedures. Mosquitoes from each of the two LGAs were exposed to five insecticides: alpha-cypermethrin, deltamethrin, permethrin, pirimiphos-methyl, and chlorfenapyr. A group of 25 female Anopheles mosquitoes (2-5 days old) was introduced into each insecticide-coated bottle, with mortality recorded at 15-minute intervals for 30 minutes for pyrethroids and one hour for organophosphates

The diagnostic dosages for each insecticide include Alphacypermethrin and deltamethrin (12.5 µg/bottle), Permethrin (21.5 µg/bottle) Pirimiphos-methyl (20 µg/bottle) and chlorfenapyr (100 µg/bottle).

Morphological Identification

Morphological identification of *Anopheles* species was conducted using identification keys (Gillies & Coetzee, 2020). The larvae were reared to adulthood and identified as part of the *An. gambiae* complex. All female adults were identified morphologically (Mohammad *et al.*, 2021).

DNA Extraction

To determine species identity, adult female *Anopheles* mosquitoes were randomly selected for DNA extraction using the methods outlined by Livak (1984). The buffer was prepared by dissolving specific reagents and stored at -20ºC. Mosquitoes were homogenized in preheated buffer, and DNA was extracted through a series of centrifugation and precipitation steps.

Molecular Species Identification

***Anopheles* Species Identification Using SINE200 PCR**

The SINE200 PCR protocol was employed using primers SINE200F and SINE200R to amplify fragments for species identification within the *Anopheles gambiae* complex. The amplification conditions followed standard protocols (Santolamazza *et al*., 2008).. For the species identifications for both *Anopheles* and *Culex*, 2% gel was used.

Genotyping for L1014F Mutation in Voltage-Gated Sodium Channel (VGSC) in *Anopheles gambiae*

The genotyping reaction mix was prepared as per Martinez-Torres *et al.*, (1998), with specific primers targeting the L1014F mutation. PCR conditions included initial denaturation and subsequent cycles of denaturation, annealing, and extension, with products visualized on agarose gels. *Kdr* genotyping was conducted using methods described by Martinez-Torres *et al.*, (1998). for *kdr* genotyping, 2.5 % gels were used to separate the fragments and were all run at 110V for 40min.

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Data analysis

Mosquito mortality was calculated by dividing the number of dead mosquitoes following exposure by total number exposed for each insecticide. *Anopheles* mosquito mortalities were corrected using Abbott's formula if the mortality recorded in control bottles was ≥ 5% and < 20%. Tests assays were discarded and repeated if control mortalities were ≥ 20% (Abbott 1925) . Susceptibility levels of *An. gambiae* s.l. were evaluated using Guidelines for evaluating insecticide resistance in vectors using the CDC bottle bioassay method (Brogdon and Chan 2010). Mean and standard deviatins were calculated using standard methods.

Genotype frequencies for the various species was calculated as the relative frequency of the homozygote resistant and heterozygote resistant individuals. The allelic frequencies of L1014F were calculated as follows: F(R) = [2RR + RS]/[2(RR + RS + SS)]. The Hardy–Weinberg equation was used to calculate the expected genotype frequency of L1014F in *An. gambiae s.s.* and *An. coluzzii*. The expected and observed genotype frequencies were compared using Pearson's Chi-squared tests to determine statistical significance of differences.

RESULTS

CDC Bottle Bioassay Test

The results from the insecticide susceptibility tests using the CDC Bottle bioassay provided insights into the resistance profiles of *Anopheles* mosquitoes in Obio/Akpor and Port Harcourt LGAs. The data include various classes of insecticides, their effectiveness, and the mortality rates observed.

For *Anopheles gambiae* s.l., the Susceptibility test results shown in Figure 2. and Table 1 reveal, that the control site generally showed higher susceptibility to the insecticides compared to the study sites. Again, for Resistance, the Obio Akpor study site exhibits the highest resistance across all the insecticides, with chlorfenapyr and pirimiphos-methyl showing the highest levels of Susceptibility.

For Deltamethrin, Port Harcourt showed 20.50% mortality (mean ± SD: 20.50±1.67) with 82 alive; classified as resistant (R). Obio Akpor showed 13.25% mortality (mean ± SD: 13.25±1.58) with 53 alive; also classified as resistant (R). Reference area: 23.25% mortality (mean ± SD: 23.25±0.25) with 93 alive; classified as partially resistant (PR). Both study sites showed resistance to deltamethrin, with significantly higher mortality observed in the reference group.

Concerning Alphacypermethrin, Port Harcourt had 24.00% mortality (mean ± SD: 24.00±1.33) with 96 alive; classified as partially resistant (PR). Obio Akpor had 21.25% mortality (mean ± SD: 21.25±1.58) with 85 alive; classified as resistant (R). The reference is recorded 24.25% mortality (mean ± SD: 24.25±0.25) with 97 alive; classified as partially resistant (PR). Similar trends of resistance are observed, with higher efficacy in the reference group compared to the study sites.

For Permethrin, Port Harcourt had 15.00% mortality (mean ± SD: 15.00±8.67) with 60 alive; classified as resistant (R). Obio Akpor, 8.50% mortality (mean ± SD: 8.50±7.00) with 34 alive; also resistant (R). Control: 22.50% mortality (mean ± SD: 22.50±0.33) with 90 alive; classified as partially resistant (PR). Resistance is evident in both study areas, with the reference area showing significantly higher effectiveness.

For Organophosphate (Pirimiphos-Methyl), both Port Harcourt and Obio Akpor showed 100% mortality (mean ± SD: 25.00±0.00) with no mosquitoes alive; classified as susceptible (S). Primiphos-Methyl was highly effective in both areas, indicating no resistance.

For Pyrroles (chlorfenapyr), both Port Harcourt and Obio Akpor showed 100% mortality (mean ± SD: 20.00±0.00) with no mosquitoes alive; classified as susceptible (S). Chlorfenapyr is also highly effective across both study sites, demonstrating no resistance.

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Table 1. Mean distribution and Resistance profiles of Insecticide Susceptibility Test for *Anopheles gambiae* s.l. using CDC Bottle bioassay for Obio/Akpor and Port-Harcourt LGAs

CLASS OF INSECTICIDES	INSECTICIDES USED	Study Area	No Mosquitoes EXPOSED	No of Mosquitoes dead	No of mosquitoes alive	% Mortality	Status
				Mean±SD	Mean±SD		
Pyrethroids	Deltamethrin	Port Harcourt study site	100	20.50±1.67[a]	4.50±1.67[a]	82	R
		Obio Akpor study site		13.25±1.58[b]	11.75±1.58[b]	53	R
		Reference Area		23.25±0.25[c]	1.75±0.25[c]	93	PR
	Alphacypermethrin	Port Harcourt study site	100	24.00±1.33[a]	1.00±1.33[a]	96	PR
		Obio Akpor study site		21.25±1.58[b]	3.75±1.58[b]	85	R
		Reference Area		24.25±0.25[ac]	0.75±0.25[ac]	97	PR
		Harcourt study site					
	Permethrin	Port	100	15.00±8.67[a]	10.00±8.67[a]	60	R
		Obio Akpor study site		8.50±7.00[b]	15.00±14.00[b]	34	R
		Reference Area		22.50±0.33[c]	2.50±0.33[c]	90	PR
Organophosphate	Primiphos-Methyl	Port Harcourt study site	100	25.00±0.00[a]	0.00±0.00[a]	100	S
		Obio Akpor study site		25.00±0.00[a]	0.00±0.00[a]	100	S
Pyrroles	Chlorfenapyr	Port Harcourt study site	100	20.00±0.00[a]	0.00±0.00[a]	100	S
		Obio Akpor study site		20.00±0.00[a]	0.00±0.00[a]	100	S

Means that do not share a letter are significantly different. *Susceptibility: Mortality 98-100%*

Possible resistance (to be confirmed): Mortality 90-97%

Resistance: Mortality < 90%

The results from the insecticide susceptibility tests using the CDC Bottle assay provided an assessment of the resistance profiles of *Culex* mosquitoes in Obio/Akpor and Port Harcourt LGAs. The data in table 1 encompass various classes of insecticides, their mortality rates, and resistance statuses.

With regards to the Pyrethroids (Deltamethrin), mortality was 22.50% (mean ± SD: 22.50±13.67) with 90 alive; classified as partially resistant (PR) in Port Harcourt. In Obio Akpor mortality was 21.75% (mean ± SD: 21.75±0.92) with 87 alive; classified as resistant (R) according to WHO standard recommendations. Reference area showed 22.75% mortality (mean ± SD: 22.75±0.92) with 91 alive; classified as partially resistant (PR). Resistance is observed in Obio Akpor, while Port Harcourt and the reference site show a partial resistance.

For Alphacypermethrin, mortality was 23.75% (mean ± SD: 23.75±2.25) with 95 alive; classified as partially resistant (PR) in Port Harcourt. In Obio Akpor, mortality was 22.75% (mean ± SD: 22.75±0.92) with 91 alive; classified as partially resistant (PR). Reference area showed 23.50% mortality (mean ± SD: 23.50±0.33) with 94 alive; classified as partially resistant (PR). All sites show partial resistance to alphacypermethrin, suggesting a consistent level of effectiveness across locations but indicating potential resistance.

For Permethrin, the mortality recorded was 21.00% (mean ± SD: 21.00±0.67) with 84 alive; classified as resistant (R) in Port Harcourt. Mortality was 16.00% in Obio Akpor (mean ± SD: 16.00±16.67) with 64 alive;

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classified as resistant (R). Reference area showed 4.75% mortality (mean ± SD: 4.75±8.25) with 19 alive; classified as resistant (R). Resistance is evident across all sites, with significantly lower mortality rates in the control site.

For Organophosphate (Primiphos-Methyl), the mortality was 24,75% (mean ± SD: 24.75±0.25) with 99 alive; classified as susceptible (S). For Obio Akpor, mortality was 25.00% (mean ± SD: 25.00±0.00) with 100 alive; classified as susceptible (S). Both areas show high efficacy of Primiphos-Methyl, with no resistance detected.

Tests conducted in Port Harcourt with mosquitoes exposed to chlorfenapyr (pyrroles) indicated that mortality was 20.00% (mean ± SD: 20.00±0.00) with 100 dead classified as susceptible (S). In Obio Akpor mortality was 20.00% (mean ± SD: 20.00±0.00) with 100 dead; classified as susceptible (S). Chlorfenapyr shows complete effectiveness in both locations, demonstrating no resistance and providing a strong option for vector control.

Table 2 Mean distribution for insecticide resistance profile of *Culex* mosquitoes using CDC Bottle Assay for Obio/Akpor and Port-Harcourt LGAs

CLASS OF INSECTICIDES	**INSECTICIDES USED**	**Study Area**	**No Mosquitoes EXPOSED**	**No of Mosquitoes dead**	**No of mosquitoes alive**	**% Mortality**	**Status**
				Mean±SD	**Mean±SD**		
Pyrethroids	Deltamethrin	Port Harcourt study site	100	22.50±13.67^{a}	2.50±13.67^{a}	90	PR
		Obio Akpor study site		21.75±0.92^{a}	3.25±0.92^{a}	87	R
		Reference Area		22.75±0.92^{a}	2.25±0.92^{a}	91	PR
	Alphacypermethrin	Port Harcourt study site	100	23.75±2.25^{a}	1.25±2.25^{a}	95	PR
		Obio Akpor study site		22.75±0.92^{a}	2.25±0.92^{a}	91	PR
		Reference Area		23.50±0.33^{a}	1.50±0.33^{a}	94	PR
	Permethrin	Port Harcourt study site	100	21.00±0.67^{a}	8.25±72.92^{a}	84	R
		Obio Akpor study site		16.00±16.67^{ab}	9.00±16.67^{ab}	64	R
		Reference Area		4.75±8.25^{c}	20.25±8.25^{c}	19	R
Organophosphate	Primiphos-Methyl	Port Harcourt study site	100	24.75±0.25^{a}	0.25±0.25^{a}	99	S
		Obio Akpor study site		25.00±0.00^{a}	0.00±0.00^{a}	100	S
Pyrroles	Chlorfenapyr	Port Harcourt study site	100	20.00±0.00^{a}	0.00±0.00^{a}	100	S
		Obio Akpor study site		20.00±0.00^{a}	0.00±0.00^{a}	100	S

Means that do not share a letter are significantly different. Susceptibility: Mortality 98-100%

Possible resistance (to be confirmed): Mortality 90-97%

Resistance: Mortality < 90%

Molecular Species Identification of *Anopheles* species using SINE200 PCR

A fragment of 479 bp confirmed *An. coluzzii*, 240 *Anopheles gambiae* s.s while 220 bp confirmed *Anopheles arabiensis* species identification.

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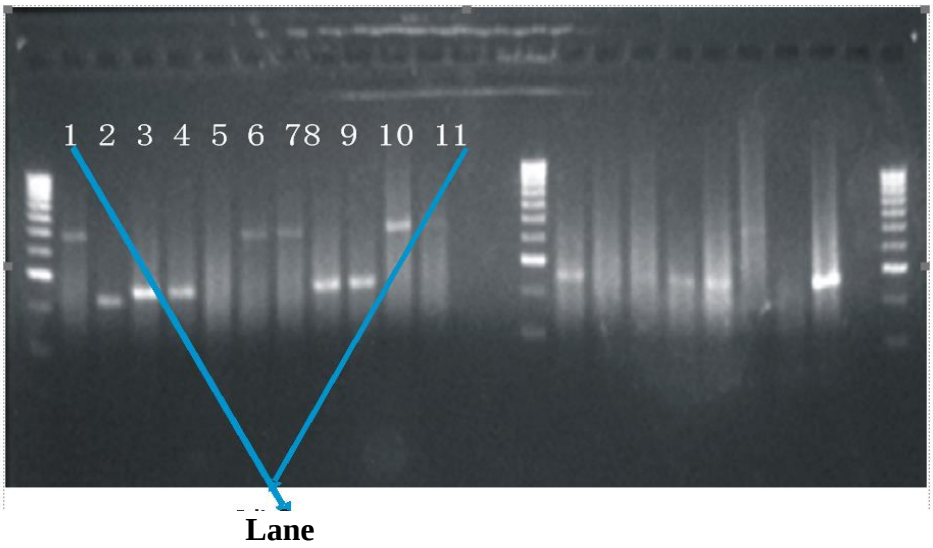

Plate 1. Agarose gel electrophoresis picture showing the bands observed after amplification of the Short interspersed Element 200 (SINE200) for species identification *An. gambiae* sibling species from Polluted sites in Obio/Akpor and Port Harcourt LGAs. L: hyper ladder 100 Bioline Biosysterm, (100-1013bp) bp. Bands for *An. coluzzii* seen at 479bp (lane 1,6,7,10 and 11), *An. gambiae ss* at 240bp (lane 3,4,8 and 9) and *An. arabiensis* at 220bp lane 2.

Molecular Species Identification of *Culex* species using Multiplex PCR

The *Culex pipiens* have a band at 610 bp, while *Cx. quinquefasciatus* have a 274 bp band, and the *Cx. torrentium* 416 bp. From the experiment all the *Culex* species identified were *Cx. quinquefasciatus* with characteristic pattern of bands at 274 bp (Plate 2).

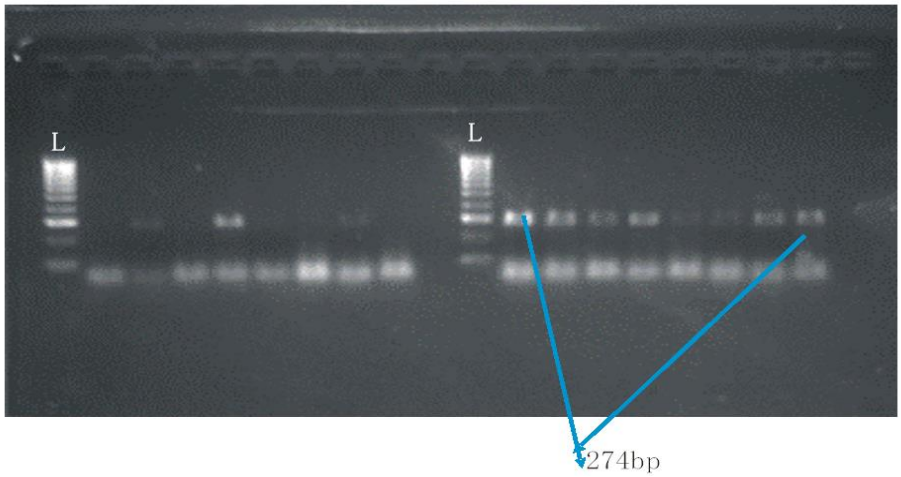

Plate 2. Agarose gel electrophoresis picture showing the bands observed after amplification of the Short-interspersed Element 200 (SINE200) for species identification of *Cx. quinquefasciatus* with characteristic pattern of bands at 274 bp.

Genotyping for L1014F mutation in voltage gated sodium channel (VGSC) in *Anopheles gambiae*

The results shown in Plate 3 illustrate the successful amplification of the *kdr* gene using specific primer pairs designed for the L1014F mutation. The amplification process was conducted through Polymerase Chain Reaction (PCR), which allows for the selective replication of the target gene segment. The plate shows distinct bands representing the amplified products of the *kdr* gene. Each band corresponds to the presence of the target gene, indicating successful amplification. Control lanes are included to validate the PCR process. Individual lanes represent different samples tested for the L1014F mutation. The intensity and position of the bands can indicate the presence and quantity of the gene variant. The presence of bands in specific lanes signifies

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samples that carry the L1014F mutation, which is crucial for understanding resistance patterns in this target insect populations.

The significance of the *kdr* Gene identification (knockdown resistance gene) is crucial in this study, for it helps in the understanding of the resistance mechanisms in insect populations, particularly in relation to pesticides. The L1014F mutation within this gene is associated with resistance to pyrethroid insecticides. Regarding the Allele-specific PCR genotyping of the L1014F mutation, the Agd1/Agd2 primers pair flanks the *kdr* gene by amplifying a 293 bp product as a control. The pair of Agd3/Agd1 primer pairs only with the resistance portion of the *kdr* gene to amplify a 195pb fragment. The Agd4 /Agd2 pair associates only with the portion of the susceptible gene by amplifying a 137 bp fragment.

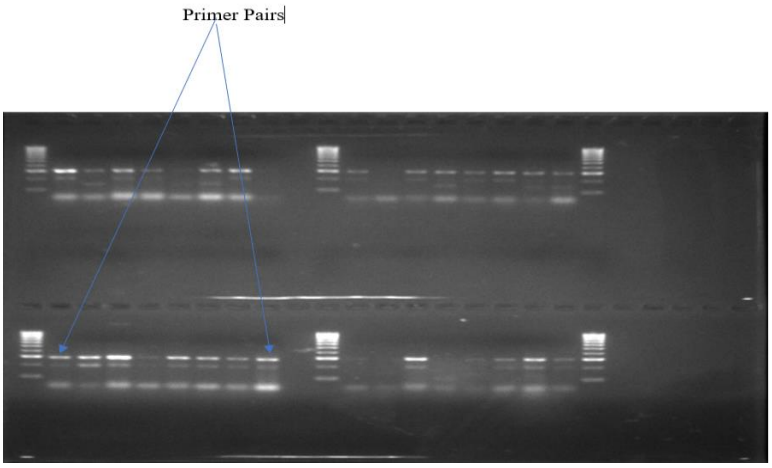

Plate 3. Picture showing Identification of *kdr* gene after Amplification by PCR Genotyping of the L1014F Mutation-Based Diagnostic Test.

Species Composition

The Species Composition of different *Anopheles* mosquito species after exposure to two different insecticides - Deltamethrin and Permethrin are presented on Figure 3. For *Anopheles* mosquitoes exposed to Deltamethrin, *An. gambiae* s.s. predominated (43.8%), followed by *An. coluzzii* (21.9%). *An. arabiensis* made up 6.3% of the population while unamplified species accounted for 28.1%. For mosquitoes exposed to Permethrin, *An. coluzzii* predominated (34.4%) followed by *An. gambiae* s.s. (31.3%). *An. arabiensis* made up 9.4% of the population while unamplified species were 25.0% of the population. The chart allowed for a comparison of how the insecticides differentially impacted the relative abundance of the *Anopheles* species (Figure 2).

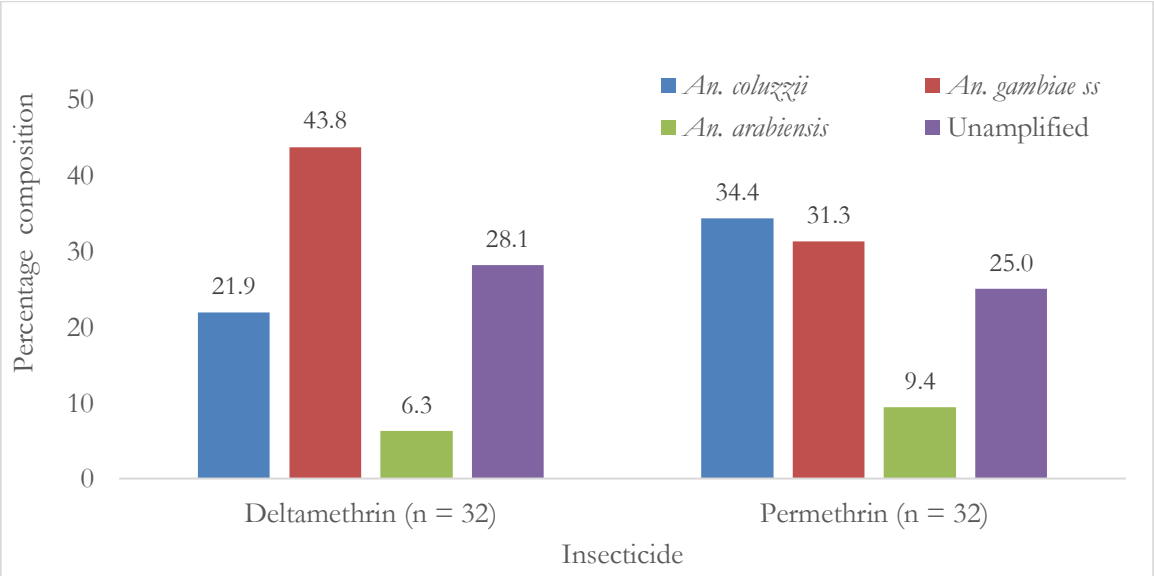

Figure 2. The Species Composition of Mosquitoes Exposed to Deltamethrin and Permethrin in the study area.

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Figure 3 highlights the distribution of different *Anopheles species* in relation to their resistance status indicating that the significant majority of the resistant samples were *An. coluzzii* and *An. gambiae* s.s. the chart also showed that these species are more prevalent among resistant populations. *An. arabiensis* was found to be absent in resistant samples while in the susceptible samples, *An. gambiae* s.s. and *An. arabiensis* are present, which also showed their potential susceptibility to insecticides. The percentage of *An. coluzzii* is lower in susceptible populations compared to resistant ones.

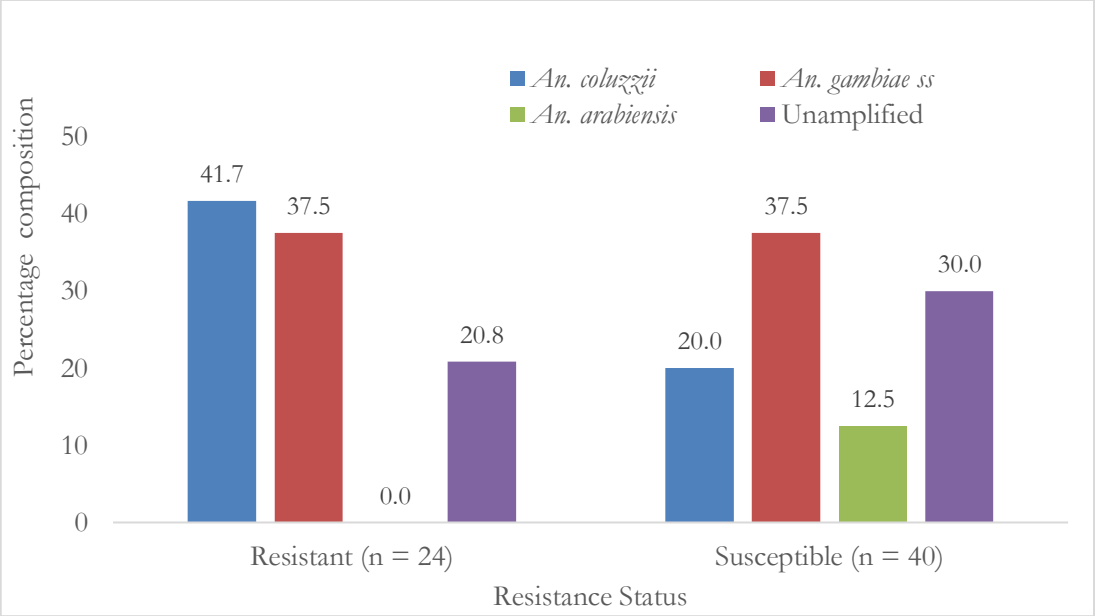

Figure 3 Species Composition showing the Resistance Status of Local mosquitoes exposed to insecticides

Figure 4 shows the species composition of different *Anopheles* mosquito species found at study and study areas. In the reference (control site), *An. coluzzii* made up 25.0% of the mosquito population. *An. gambiae* ss was the dominant species at 39.6%. *An. arabiensis* accounted for 8.3%. The Unamplified category represented 27.1% of the population.

For the study site, *An. coluzzii* accounted for 37.5% of the mosquito population. *An. gambiae* ss made up 31.3% of the population. *An. arabiensis* had the lowest proportion at 6.3%. The unamplified category represented 25.0% of the population.

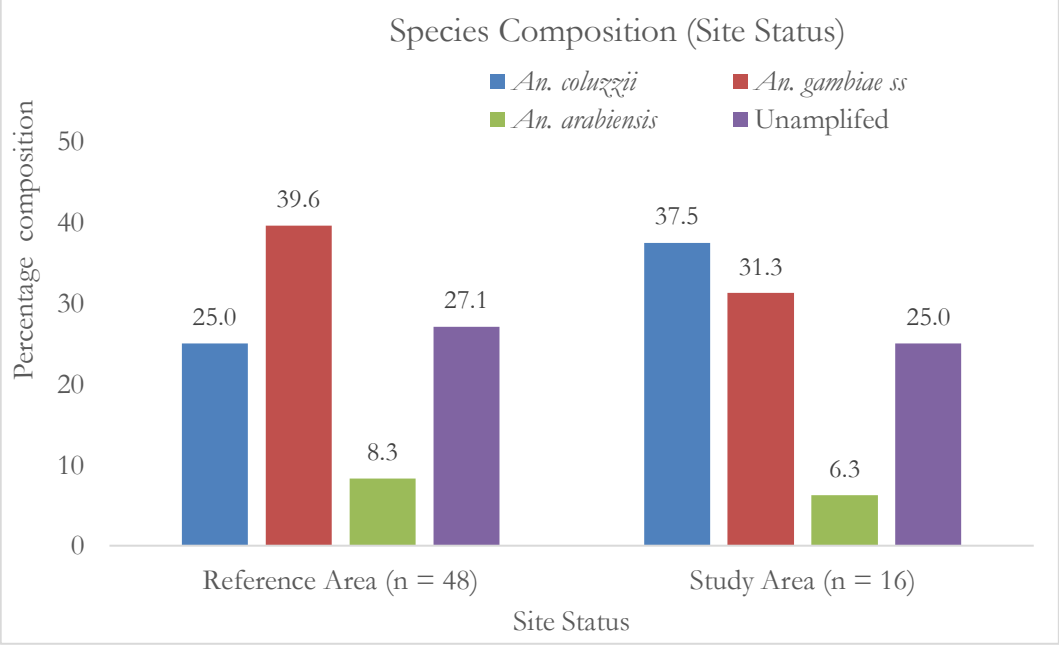

Figure 4 Species Composition of Mosquitoes exposed to insecticides at the Study Site

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The comprehensive chart depicts the species composition of different *Anopheles* mosquito species across various combinations of site status (study vs. control), Insecticide (permethrin and deltamethrin), and resistance status (resistant and susceptible). At the control site, *An. coluzzii* was the dominant species (50.0%) among the permethrin resistant mosquitoes while at the study site, *An. gambiae* s.s. was the dominant species (50.0%) among the permethrin resistant mosquitoes. Percentage occurrence of *An. coluzzii* and *An. gambiae* s.s. was 37.5% respectively among mosquitoes resistant to deltamethrin at the study site while *An. coluzzii* was dominant at the control site. *An. gambiae* s.s. predominated (37.5%) among permethrin and deltamethrin susceptible mosquitoes at the control site while *An. coluzzii* was dominant (37.5%) at the study site.

Investigation of The L1014F *kdr* Mutation (*kdr* -west*)*

Assessment of *kdr-w* (L1014F) mutations in permethrin and deltamethrin -resistant *Anopheles* mosquitoes indicated the presence of *kdr-w* point mutations across the study sites. *Kdr-w* gene frequencies in *An. coluzzii* exposed to permethrin at the study site was 0.50 while those of the reference (control) sites was 1.00 but these did not vary significantly (p=1.0000). *Kdr-w* gene frequencies in *An. gambiae* s.s. exposed to permethrin were 1.00 respectively in both study and reference sites. Additionally, *kdr-w* gene frequencies in *An. arabiensis* exposed to permethrin was observed to be 1.00 in the reference site while none was detected in the study site.

For deltamethrin, *kdr-w* gene frequencies in *An. coluzzii* was 1.00 at the reference site while none was detected at the study site. For *An. gambiae* s.s., whereas no gene frequency was detected in the reference site, *kdr-w* gene frequencies were 1.00 in the study site while the same was recorded in *An. arabiensis* (1.00) in the same site (Table 3).

To detect the L1014F *kdr* mutation, 8 susceptible (alive) and 8 resistant (dead) female *Anopheles* s.l. mosquitoes exposed to deltamethrin and permethrin respectively were used for genotyping using PCR. In total 18 alive females and 31 dead were successfully genotyped. From the alive cohort 15 (83.33%) were homozygote resistant, RR (Table 3), 2 (11.11%) were heterozygotes (RS) and only one female (5.56%) was homozygote susceptible (SS). From the dead mosquitoes, 23 (74.19%) were RR, 6 were RS (19.35%) and 2 were ss (6.45%). For both alive and the dead, 38 females were RR (93.88%) and 8 were RS (16.33%). The *kdr* genotypes were 94.4% for alive mosquitoes, 93.6% for dead and 93.9% for both alive and dead.

Table 3. Allele frequencies of *kdr* mutation L1014F of *An. gambiae ss, An. coluzzii and An. arabiensis* in the study area

Sites	Species Identified	Resistant				
		Number Tested for *Kdr*	*Kdr*			
			RR	RS	SS	*Kdr* frequency
Reference site Permethrin Resistant	*An. coluzzii*	2	2	0	0	1.00
	An. gambiae ss	5	5	0	0	1.00
	An. arabiensis	1	1	0	0	1.00
Study site Permethrin Resistant	*An. coluzzii*	4	1	2	1	0.50
	An. gambiae ss	3	3	0	0	1.00
	Unamplified	0	0	0	0	0.00
Reference Site Deltamethrin Resistant	*An. coluzzii*	3	3	0	0	1.00
	An. gambiae ss	0	0	0	0	0.00
	Unamplified	0	0	0	0	0.00
Study site deltamethrin Resistant	*An. coluzzii*	0	0	0	0	0.00
	An, gambiae ss	7	7	0	0	1.00
	An. arabiensis	1	0	1	0	1.00
Total		26	22	3	1	0.90

DISCUSSION

This study investigated the resistance profiles and molecular mechanisms underlying insecticide resistance in major malaria vectors, *Anopheles gambiae* s.l. and *Culex* spp., using CDC bottle bioassays across three

locations in Rivers State, Nigeria. Understanding the breeding sites and the physicochemical factors influencing mosquito abundance is crucial for effective malaria control (Amawulu *et al.,* 2020).

The findings demonstrated that field-collected larvae from study sites exhibited tolerance to pyrethroids but remained susceptible to Primiphos-methyl (the organophosphate) and chlorfenapyr (pyrrole). This result aligns with previous research by Billy *et al.,*. (2012), which reported high tolerance to permethrin and deltamethrin, suggesting low selective pressure in the breeding sites of the study areas. *Anopheles gambiae* was susceptible to pirimiphosmethyl (organophosphate) and chlorfenapyr (pyrrole). The findings from this assessment could provide critical data that could guide the selection of an appropriate insecticide for indoor residual spraying program in Nigeria. There was no phenotypic resistance to pirimiphos-methyl and chlorfenapyr reported in this study.

Resistance to pyrethroids was confirmed across all sites, with mortality rates for *An. gambiae* s.l. ranging from 34% to 53%. These rates suggest that environmental factors may contribute significantly to the resistance status of local mosquitoes exposed to different insecticides. . Notably, full susceptibility to primiphos-methyl and chlorfenapyr was observed, indicating potential alternatives for vector control.

For *Culex* spp., resistance to pyrethroids was also noted, with mortality rates between 64% and 91%. Similar to *Anopheles* mosquitoes, *Culex* populations exhibited full susceptibility to both Primiphos-methyl and chlorfenapyr. These findings are consistent with earlier studies highlighting high resistance levels in *Anopheles* mosquitoes across various states in Nigeria (Oduola *et al.,* 2012; Aïkpon *et al.,* 2014; Kabula *et al.,* 2011).

Resistance mechanisms often involve physiological changes, including mutations in target proteins (Nkya *et al.,* 2013). Molecular identification confirmed the presence of *An. coluzzii*, *An. gambiae* s.s., and *An. arabiensis*, with specific bands corresponding to each species (Amawulu *et al.,* 2020). Urbanization in Nigeria has led to an increase in malaria cases, with *An. gambiae* s.s. and *An. arabiensis* adapting to contaminated water sources, as reported by Awolola *et al.,* (2007) who worked in Lagos.

Research indicates that *An. coluzzii* can thrive in polluted environments, challenging earlier assumptions of its preference for low salinity (Nwaefuna *et al.,* 2019). The adaptability of *An. coluzzii* to urban conditions, coupled with its resistance to pyrethroids, poses significant challenges for malaria control, particularly in rapidly urbanizing areas (Muhammad *et al.,* 2021; Longo-Pendy *et al.,* 2021).

The growing prevalence of *An. coluzzii* in urban breeding sites underscores the potential for increased malaria transmission, especially in coastal regions where ecological changes are prevalent. This species thrives in environments previously unsuitable for malaria vectors, highlighting the urgent need for adaptive vector control strategies to address the evolving dynamics of malaria transmission (Kudom *et al.,* 2015; Longo-Pendy *et al.,* 2021). This assessment also demonstrated the *kdr* L1014F resistance mutation to be present at high frequency in the study area. This did not come as a surprise putting into consideration the high level of phenotypic resistance to pyrethroids. The L1014F mutation has been previously reported from Burkina Faso, Benin, Cameroon and Cote d'Ivoire (Jones *et al.,* 2012; Djedge *et al.,* 2014)

CONCLUSION

The findings of this study highlight significant resistance in both *Anopheles* and *Culex* mosquitoes to commonly used pyrethroid insecticides, presenting substantial challenges for malaria control efforts. However, alternative insecticides such as primiphos-methyl and chlorfenapyr remain effective, offering viable options for vector management in the studied regions.

Genetic mutations in *An. gambiae*, particularly the knockdown resistance (*kdr*) mutations associated with pyrethroid resistance, complicate vector control strategies. The rising dominance of *An. coluzzii* in urban settings suggests that traditional control measures may no longer suffice to effectively manage malaria transmission.

This study underscores the urgent need for integrated vector management strategies that incorporate ecological insights and targeted interventions. Public health policies should prioritize improvements in sanitation and

waste management to reduce potential breeding sites while adapting control measures to address evolving resistance patterns. As *An. coluzzii* continues to expand its habitat range and develop resistance, it is essential to reassess and innovate vector control strategies to counter the rising threat of malaria in urbanized environments.

The emergence of *Anopheles* mosquitoes in contaminated breeding sites poses a significant risk to malaria control efforts. Effective urban planning, enhanced waste management, and targeted vector control strategies are vital for mitigating malaria transmission in rapidly urbanizing regions. Without appropriate interventions, urban malaria could escalate into a major public health challenge.

This study also highlights critical resistance mechanisms in *An. gambiae* and *A. coluzzii*, particularly the role of genetic mutations, including *kdr* mutations, in conferring resistance to pyrethroids. These mutations induce structural changes in voltage-gated sodium channels, diminishing the effectiveness of insecticides. Enhanced metabolic pathways further contribute to the resilience of these mosquito populations against insecticides. The ecological adaptability of *A. coluzzii*, enabling it to thrive in contaminated urban settings, emphasizes the complexity of resistance mechanisms in evolving habitats and the necessity for integrated vector management strategies that account for these resistance traits. The resistance profile of *An. gambiae* in Port Harcourt is relatively similar to that found elsewhere in West Africa. It is characterized by a high level of pyrethroid resistance and an almost stable L1014F mutation. Additionally, given the high frequency of the L1014F mutation, it is recommended that NMEP should plan to deploy nets treated with non-pyrethroids for future mass distribution cycles and routine distribution channels. These results have formed the basis for further entomological studies and supported the state ministry of Health (SMEP) and NMEP in their development of insecticide resistance monitoring and management strategies.

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