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RESEARCH ARTICLE

The insecticide resistance status of *Anopheles* mosquitoes in the rice agroecosystems of Anambra State, Nigeria

Ukonze, C.B.[1,3], Ezihe, C.K.[4], Egbuche, C.M.[3], Hasan, H.A.[1,2*]

[1]School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia
[2]Vector Control Research Unit, Universiti Sains Malaysia, 11800 Penang, Malaysia
[3]Nnamdi Azikiwe University Awka. PMB 5025, Anambra, Nigeria
[4]Malaria Consortium Maitama, 904101 Abuja, Nigeria
*Corresponding author: hadura@usm.my

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ABSTRACT

Malaria remains one of the leading causes of death worldwide, particularly in Africa, where various control interventions such as case management, insecticide-treated nets (ITNs), intermittent preventive treatment in pregnant women and infants, and indoor residual spraying (IRS) have been implemented. Insecticide resistance in malaria vectors poses a major challenge to vector control efforts. This study assessed the insecticide resistance status of *Anopheles gambiae* s.l. populations in rice agroecosystems of Anambra State, Nigeria. Mosquito larvae were sampled from four rice-farming clusters in Anambra state, reared to adulthood at the National Arbovirus and Vector Research Center (NAVRC) and tested using the WHO susceptibility bioassay against four insecticides namely dichloro-diphenyl-trichloroethane (DDT), pirimiphos-methyl, bendiocarb, and deltamethrin. Knockdown resistance (kdr) mutations were also analyzed. Results revealed confirmed resistance to DDT and deltamethrin, likely due to past agricultural use of DDT and cross-resistance with pyrethroids, which are widely used in ITNs and IRS. However, *An. gambiae* s.l. populations remained susceptible to bendiocarb and pirimiphos-methyl, except in Umunze, where resistance to pirimiphos-methyl was detected. The L1014F kdr mutation was detected at varying frequencies across locations, a key genetic marker for resistance to pyrethroids and DDT, in both *An. gambiae* s.s. and *An. coluzzii*. These findings highlight the importance of continuous insecticide resistance monitoring in rice agroecosystems, improved pesticide regulations and the implementation of integrated vector control management to slow down the spread of resistance in malaria vectors in Anambra.

Keywords: Agroecosystems; knockdown; malaria; mosquito; susceptible.

INTRODUCTION

Malaria remains a significant global public health challenge, particularly in sub-Saharan Africa, where the disease is transmitted by *Anopheles* mosquitoes. The control of malaria vectors has historically relied on insecticide-based interventions such as indoor residual spraying (IRS) and insecticide-treated nets (ITNs) (World Health Organization, 2018). However, the increasing development of insecticide resistance in *Anopheles* mosquitoes threatens the effectiveness of these control measures. Resistance to insecticides among malaria vectors has been documented since the mid-20th century, with reports of resistance to organochlorines such as DDT and dieldrin emerging in the 1950s and 1960s (Gnankiné *et al*., 2013). By 1993, resistance to pyrethroids was detected in African malaria vector populations (Ranson *et al*., 2009), and subsequent studies have confirmed widespread pyrethroid resistance in *Anopheles gambiae* s.l., *Anopheles arabiensis*, and *Anopheles funestus* populations across Africa (Adeleke *et al*., 2018; Kamau *et al*., 2008; Kamau & Vulule, 2006; Okorie *et al*., 2015; Yusuf *et al*., 2021; Zouré *et al*., 2021). More recently, resistance to carbamates and organophosphates has been reported in *An. gambiae* populations in West Africa (Aïkpon *et al*., 2013, 2014; Antonio-Nkondjio *et al*., 2016) following widespread pyrethroid resistance. This pattern is primarily attributed to the subsequent deployment of organophosphates and carbamates for indoor residual spraying (IRS) as alternative insecticides, as well as cross-sectional pressure from agricultural pesticide use, particularly through the spread of target-site mutations such as ace-1.

The overuse and repeated application of insecticides in both agriculture and public health programs have been identified as major drivers of resistance evolution in malaria vectors (Riveron *et al*., 2018; Sonhafouo-Chiana *et al*., 2022). This occurs through selective pressure, where resistant individuals survive exposure to insecticides and pass on resistance genes to subsequent generations (Sumarnrote *et al*., 2017). The frequent use of insecticides with a limited range of activity can also contribute to the emergence of resistance by allowing resistant individuals to persist and proliferate (Meier *et al*., 2022). Resistance in *Anopheles* mosquitoes develops

through several mechanisms, including metabolic detoxification, reduced penetration or knockdown resistance, and behavioural avoidance (Gan *et al*., 2021; Siddiqui *et al*., 2023).

One of the primary mechanisms responsible for pyrethroid and DDT resistance in *An. gambiae* in West Africa is a mutation in the voltage-gated sodium channel gene, commonly referred to as the L1014F kdr mutation (Gnankiné *et al*., 2013; Martinez-Torres *et al*., 1998; Oduola *et al*., 2012; Santolamazza *et al*., 2008). A related mutation, L1014S, has also been detected in some populations (Dabiré *et al*., 2014; Namountougou *et al*., 2012). Additionally, resistance to carbamates and organophosphates has been linked to the ace-1R mutation, which results in an altered acetylcholinesterase enzyme that is less susceptible to inhibition by these insecticides (Weill *et al*., 2004). In southeastern Nigeria, *An. gambiae* populations harbour both kdr and ace-1R mutations, which are attributed to extensive insecticide usage in agriculture and vector control (Chukwuekezie *et al*., 2020).

Despite progress in malaria control, Nigeria continues to bear a significant burden of the disease. Between 2010 and 2018, malaria parasite prevalence in the country declined from 42% to 23%, with Anambra State recording a 9% prevalence (World Health Organization, 2019). The primary malaria control strategy in Nigeria involves vector control through IRS and ITNs, with pyrethroids being the most commonly used insecticides (Federal Ministry of Health, 2015). However, the continuous use of pyrethroids for both agricultural and public health purposes has exacerbated resistance in local mosquito populations.

Rice farming ecosystems play a crucial role in this context, as rice fields serve as ideal breeding habitats for *Anopheles* mosquitoes (Amarasinghe & Weerakkodi, 2014; Yasuoka & Levins, 2007). Given the increasing reports of insecticide resistance, it is essential to assess the resistance status of *Anopheles* mosquitoes in rice agroecosystems to inform the development of effective vector control strategies. Implementing integrated pest management approaches that incorporate diverse control measures rather than relying solely on insecticides is crucial to mitigating resistance development. Additionally, routine monitoring of insecticide resistance patterns in mosquito populations enables early detection of emerging resistance and facilitates timely interventions.

Therefore, this study aims to evaluate the insecticide-resistant status of *Anopheles* mosquitoes in the rice agroecosystems of Anambra State, Nigeria. The findings can provide critical insights into the current resistance trends and inform evidence-based strategies for sustainable malaria vector control in agricultural settings.

MATERIALS AND METHODS

Study Area

This study was conducted in Anambra State, located in southeastern Nigeria at latitude 6.2758° N and longitude 7.0068° E, covering an area of 4,844 km². Anambra shares boundaries with Enugu State to the east, Delta State to the west, Imo and Rivers States to the south, and Kogi State to the north. The predominant ethnic group in Anambra is Igbo (98%), with a minor Igala population (2%) residing in the northwestern part of the state.

Anambra has a tropical climate with two distinct seasons: the wet season (April to October) and the dry season (November to March). The average annual temperature is 25.9°C, with March being the warmest month (28.1°C) and August the coldest (24.2°C). The state receives an average annual rainfall of 1,386 mm, with peak precipitation in September (279 mm) and the driest month being January (8 mm of rain).

The study was conducted in four rice-producing Local Government Areas (LGAs) in Anambra State namely Awka North, Anyamelum, Orumba North, and Orumba South (Figure 1). These LGAs are known for extensive rice farming activities and serve as ideal sites for studying mosquito populations in rice agroecosystems. Sampling was carried out at specific rice farm clusters within these LGAs.

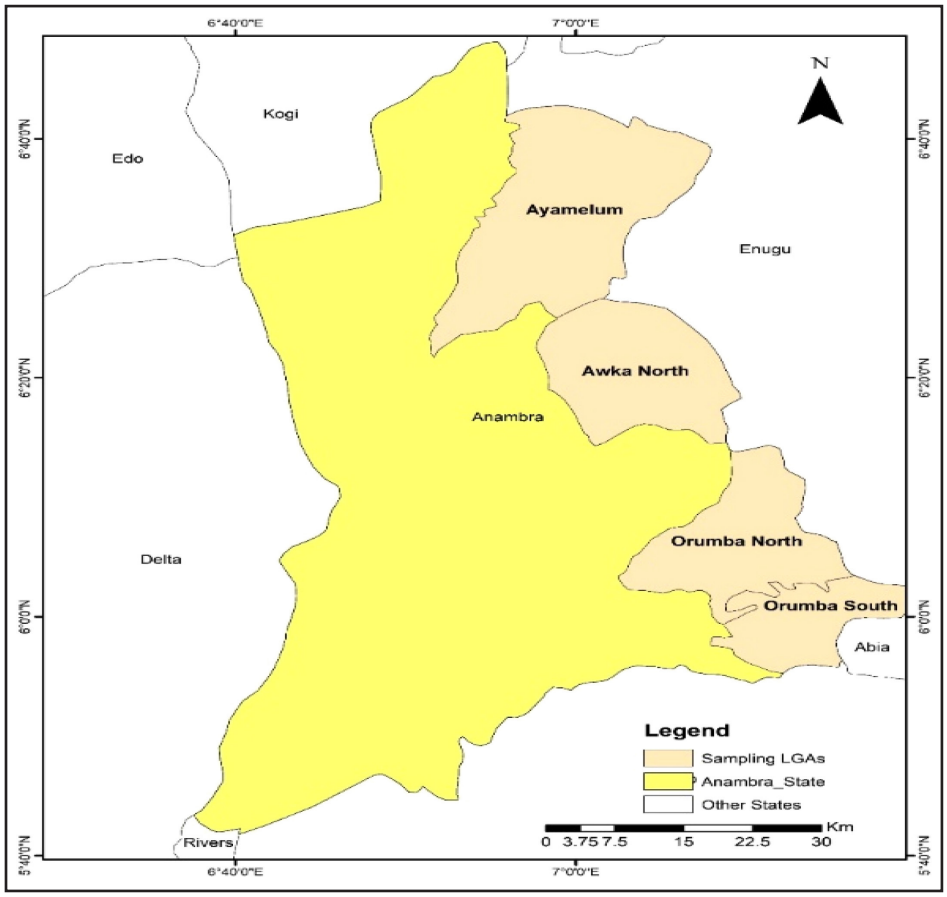

Figure 1. Map of Anambra state showing the four local government area study sites.

***Anopheles* mosquito sampling and rearing**

Anopheles mosquito larvae were randomly collected from breeding habitats across the four selected LGAs using the standard larval dipping method. The larvae were sampled from potential breeding habitats of the rice farms such as pools, paddy, marshes and canals using a WHO 350ml standard dipper. The larvae were pooled together irrespective of their breeding habitat and transported in sampling containers containing source water to the insectary at the National Arbovirus and Vector Research Centre (NAVRC) in Enugu for rearing into adults under controlled conditions.

In the insectary, all specimens were maintained at a controlled temperature of 27±2°C and 70–80% relative humidity. The pupae were collected daily and placed in plastic cups inside cages. Emerged adult mosquitoes were provided with a 10% sucrose solution absorbed on cotton wool for feeding.

WHO susceptibility bioassay test

The susceptibility of *Anopheles* mosquitoes to insecticides was assessed using the World Health Organization (WHO) standard adult bioassay test. The WHO insecticide monitoring test kit was obtained from the Vector Control Research Unit (VCRU), Universiti Sains Malaysia, Penang. The test kit consisted of WHO test tubes (holding tubes and exposure tubes) and WHO insecticide-impregnated papers.

Mosquitoes were tested for resistance against the following four insecticides including 4% dichloro-diphenyl-trichloroethane (DDT), 0.25% pirimiphos-methyl, 0.1% bendiocarb, 0.05% deltamethrin. The bioassay followed WHO guidelines (World Health Organization, 2018). A total of 25 non-blood-fed, 2 to 5-day-old female mosquitoes were aspirated from the cages into holding tubes (marked with a green dot) and allowed to acclimatize for one hour. Mosquitoes that could not fly or were dead after acclimatization were discarded.

Insecticide-impregnated papers were placed in the exposure tubes (marked with a red dot), and mosquitoes were transferred from the holding tubes to the exposure tubes. Exposure was conducted for one hour under standardized conditions (27±2°C and 70–80% RH). The knockdown was recorded at 5-minute intervals for 60 minutes. After exposure, mosquitoes were transferred back to holding tubes and provided with 10% sucrose solution. Mortality was assessed 24 hours post-exposure.

Live mosquitoes were identified as those capable of flying, while knocked-down or dead mosquitoes were immobile or unable to stand or fly. All mosquito samples were properly labelled and preserved on silica gel in Eppendorf tubes for further molecular analysis and species identification.

Detection of resistance mechanisms

Knockdown resistance (kdr) was assessed using polymerase chain reaction (PCR) to detect the presence of the L1014F mutation in the voltage-gated sodium channel gene, which confers target-site resistance to pyrethroids. Genomic DNA was extracted from individual adult *Anopheles* mosquitoes using a standard extraction procedure. The PCR amplification was carried out following the protocol described by Huynh *et al*. (2007) using a 5X Hot FIREPol® Blend Master Mix with 7.5 mM $MgCl_2$ (Solis BioDyne Estonia). Each reaction was prepared in a total volume of 12.5µl, comprising 2.5µL of the master mix, 0.5µL of the primers, 1µL of template DNA and nuclease-free water. Amplification was carried out under the following thermal cycling conditions: initial-denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, elongation at 72°C for 30 seconds with a final elongation at 72°C for 5 minutes.

DNA extraction, amplification and electrophoretic analysis were conducted at the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos. PCR products (10 µl) were resolved on a 2% ethidium bromide-stained agarose gel and visualized under ultraviolet illumination. A 314 bp band confirmed successful amplification, while a 214 bp band indicated the wild-type allele and a 156 bp band indicated the resistant allele (Figure 3).

Data Analysis

Data was recorded using a standardized WHO bioassay data collection form. Mortality was calculated as the proportion of dead mosquitoes out of the total exposed mosquitoes. Insecticide resistance was classified based on WHO criteria:

98–100% mortality: Susceptible population
90–97% mortality: Possible resistance (requires further investigation)
< 90% mortality: Confirmed resistance

Abbott's formula (Abbott, 1925) was applied to correct mortality rates when control mortality ranged between 5 to 20%. However, in this study, control mortality was consistently below 5%, so corrections were unnecessary. Percentage mortality data were analyzed using two-way analysis of variance (ANOVA) to evaluate the effect of the insecticides and study location on mosquito mortality. Statistical significance was set at $P < 0.05$.

Kaplan-Meier survival analysis was used to estimate knockdown times (KDT_{50}) of *Anopheles* mosquitoes exposed to different insecticides across four study sites. Significant spatial variation in knockdown response was observed.

The genotype frequencies of the L1014F kdr mutation were compared between resistant and susceptible mosquito populations using Fisher's exact test.

RESULTS

Kaplan-Meier survival analysis revealed significant spatial and insecticide-specific variation in knockdown responses of *An. gambiae* s.l. varied across the four study locations (Table 1). Overall, DDT consistently exhibited the slowest knockdown responses, with prolonged media knockdown times (KDT_{50} = 6-7 min) and low proportions of mosquitoes knocked down within the 60-minute exposure, particularly in Anaku (KDT_{50} = 7.0 min) and Umunze (KDT_{50} = 7.0 min). This indicates a widespread resistance to organochlorines. In contrast, bendiocarb generally produced the most rapid knockdown across all locations with uniformly shorter

Table 1. Kaplan-Meier estimates of knockdown time KDT_{50} of Anopheles mosquitoes exposed to different insecticides across study sites

Sites	Insecticide	Diagnostic time (min)	KDT_{50} (min)	95%CI (min)	Log-rank p-value
Anaku	DDT	60	7.0	6.42-7.58	0.001
	Deltamethrin	60	4.0	2.72-5.28	
	Bendiocarb	60	4.0	2.72-5.28	
	Pirimiphos-methyl	60	5.0	3.88-6.12	
Achalla	DDT	60	6.0	5.22-6.79	0.136
	Deltamethrin	60	4.0	2.72-5.28	
	Bendiocarb	60	5.0	3.91-6.09	
	Pirimiphos-methyl	60	5.0	3.93-6.07	
Ufuma	DDT	60	4.0	2.77-5.23	0.497
	Deltamethrin	60	4.0	2.78-5.22	
	Bendiocarb	60	4.0	2.72-5.28	
	Pirimiphos-methyl	60	5.0	3.91-6.09	
Umunze	DDT	60	7.0	–	0.008
	Deltamethrin	60	5.0	4.02-5.98	
	Bendiocarb	60	4.0	2.76-5.24	
	Pirimiphos-methyl	60	5.0	4.00-6.00	

The p-values greater than 0.005 indicates no significant difference ($P < 0.005$).

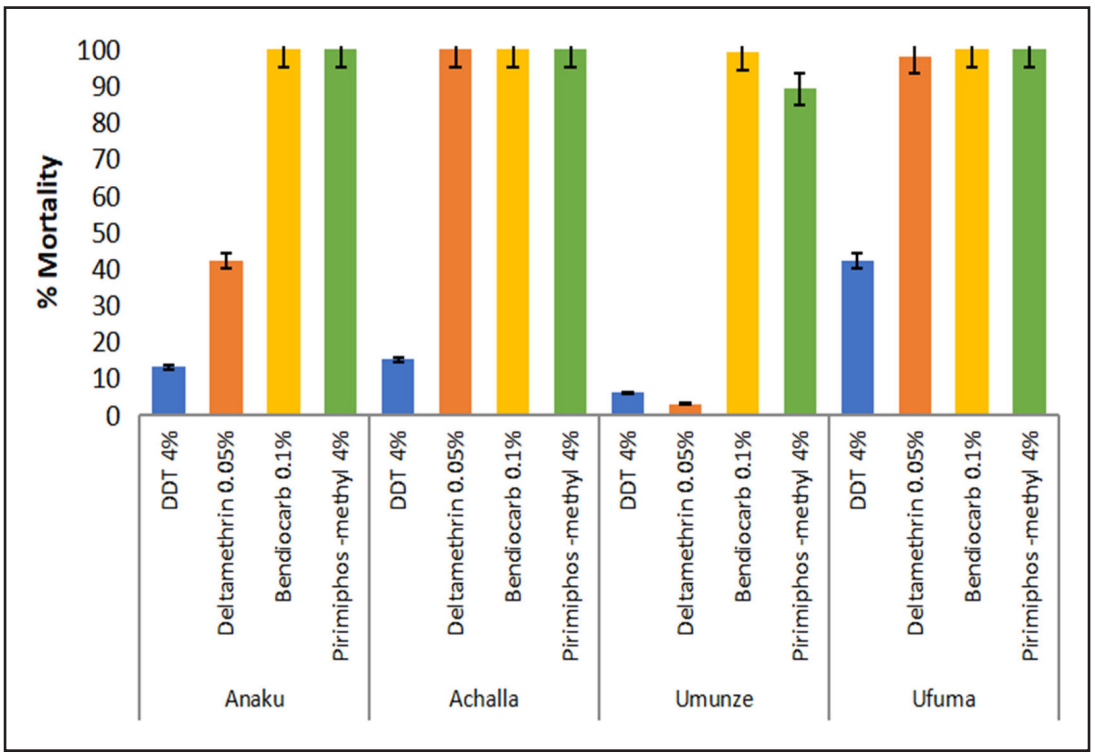

Figure 2. Percentage mortality (%) of mosquitoes in response to insecticide bioassay test. The p-value among locations in mortality response = 0.000 (P < 0.005). The p-value among insecticides = 0.000 (P < 0.005).

KDT_{50} values of 4.0 min in Anaku, Achalla, Ufuma and Umunze. This suggests retained efficacy of carbamates. Responses to pyrethroid (deltamethrin) and organophosphate (pirimiphos-methyl) were intermediate and varied across sites, with KDT_{50} values ranging from 4.0 to 5.0 min, reflecting heterogeneity in susceptibility patterns among mosquito populations.

Location-specific comparisons further highlighted differences in knockdown dynamics. In Anaku, knockdown times differed significantly among insecticides (log-rank $X^2 = 10.18$, $p = 0.001$), with deltamethrin and bendiocarb achieving rapid knockdown (KDT_{50} = 4.0 min). In contrast, pirimiphos-methyl showed a slightly delayed response (KDT_{50} = 5.0 min), and DDT performed poorly (KDT_{50} = 7.0 min). In Achalla, no significant difference in knockdown was observed among the insecticides (log-rank $X^2 = 2.22$, $p = 0.136$), although deltamethrin produced the fastest knockdown (KDT_{50} = 4.0 min), followed by bendiocarb and pirimiphos-methyl (KDT_{50} = 5.0 min), whereas DDT showed delayed knockdown (KDT_{50} = 6.0 min). Similarly, knockdown response in Ufuma did not differ significantly among the insecticides (log-rank $X^2 = 0.46$, $p = 0.497$), with relatively uniform KDT_{50} for DDT, deltamethrin, and bendiocarb each at 4.0 min, whereas pirimiphos-methyl showed a slightly longer KDT_{50} (5.0 min). In contrast, significant differences were observed in Umunze (log-rank $X^2 = 0.46$, $p = 0.497$), where bendiocarb remained the most effective insecticide (KDT_{50} = 4.0 min), followed by deltamethrin and pirimiphos-methyl (KDT_{50} = 5.0 min), and DDT again exhibited the slowest knockdown response (KDT_{50} = 7.0 min).

Mortality rates of *An. gambiae* s.l. varied significantly across locations with DDT showing the lowest mortality across all sites, ranging from 6% to 42%, confirming high resistance. Conversely, bendiocarb and pirimiphos-methyl demonstrated the highest mortality rates, ranging from 89% to 100%, indicating susceptibility. Deltamethrin showed variable responses, with high mortality in Achalla (100%) and Ufuma (98%), but significantly lower mortality in Anaku (25%) and Umunze (3%), suggesting emerging resistance.

The susceptibility bioassay further confirmed these trends (Figure 2). In Anaku, mosquitoes were resistant to DDT (13% mortality) and deltamethrin (42% mortality) but susceptible to bendiocarb and pirimiphos-methyl (100% mortality). Similarly, Achalla exhibited resistance to DDT (15%) but susceptibility to all other insecticides (100% mortality). Umunze mosquitoes were resistant to DDT (6%), deltamethrin (3%), and pirimiphos-methyl (89%), whereas bendiocarb remained highly effective (99% mortality). In Ufuma, only DDT resistance was detected (42%), while all other insecticides were effective (98%-100% mortality). A statistically significant difference was observed among locations in mortality response ($p = 0.000$) and among insecticides ($p = 0.000$).

In Table 2, the distribution of kdr genotypes varied significantly among locations. Anaku recorded the highest frequency of the homozygous susceptible genotype (59.3%), whereas Ufuma showed a predominance of the homozygous resistant genotype (66.7%). Umunze had a relatively higher proportion of heterozygous individuals than at the other locations (33.3%). Fisher's exact test showed a significant association between location and genotype

Table 2. Frequency of knockdown-resistant mutation (kdr) genotypes across the study sites

Location	n	rr (%)	Rr (%)	RR (%)	F (r)	F (R)
Anaku	54	32(59.3)	7(13.0)	15(27.8)	0.65	0.343
Achalla	59	28 (47.5)	10(16.9)	21(35.6)	0.559	0.441
Ufuma	45	12(26.7)	3(6.7)	30(66.7)	0.300	0.700
Umunze	42	18(42.9)	14(33.3)	10(23.8)	0.595	0.40

rr – Homozygous susceptibility; Rr – heterozygous resistance; RR – homozygous resistance; f (r) = frequency of susceptible allele; f (R) = frequency of resistant allele; Fisher's exact Test, $p < 0.001$.

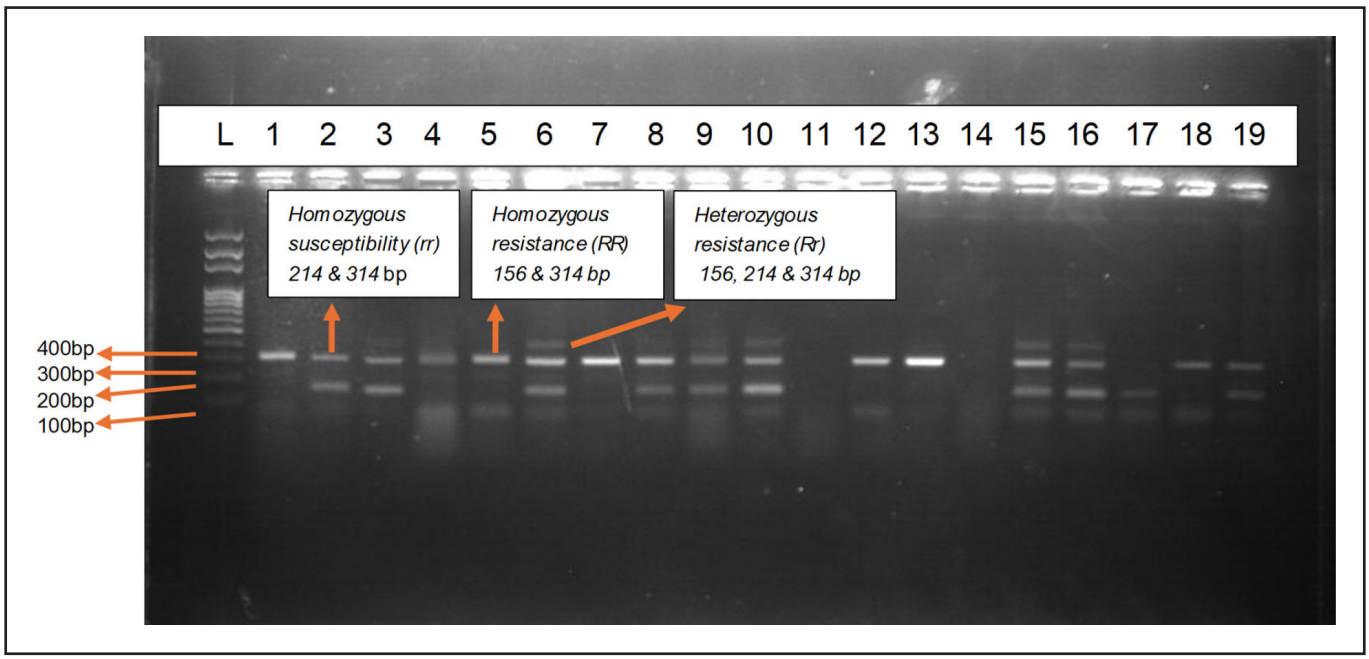

Figure 3. Electrophoresis gel image of the kdr.

Table 3. Frequency of kdr across mosquito species

Species	n	rr (%)	Rr (%)	RR (%)	F (r)	F (R)
An. gambiae	100	43(43.0)	15(15.0)	42(42.0)	0.505	0.495
An. colluzi	100	47(47.0)	23(23.0)	30(30.0)	0.585	0.415

rr – Homozygous susceptibility; Rr – heterozygous resistance; RR – homozygous resistance; f (r) = frequency of susceptible allele; f (R) = frequency of resistant allele; Fisher's exact Test, p = 0.150.

distribution ($p < 0.001$), indicating that genotype frequencies differed across the study sites. In contrast, Fisher's exact test found no significant association between mosquito species and kdr genotype distribution ($p = 0.150$). The frequencies of kdr genotypes were similar between *An. gambiae* and *An. coluzzi*, suggesting that the distribution of kdr mutations is not species-dependent in this population (Table 3).

DISCUSSION

The findings of this study highlight the growing concern of insecticide resistance among *An. gambiae* s.l. populations in the rice agroecosystems of Anambra State, Nigeria. Resistance to DDT and deltamethrin was widespread, while bendiocarb and pirimiphos-methyl remained effective in most study sites. The detection of knockdown resistance (kdr) mutations in the mosquito populations further supports the evidence of increasing resistance selection due to prolonged insecticide exposure. These results are consistent with previous studies across Nigeria and other parts of Africa, which have reported high levels of pyrethroid and organochlorine resistance in malaria vectors (Adeleke *et al*., 2018; Kamau *et al*., 2008; Kamau & Vulule, 2006; Okorie *et al*., 2015; Yusuf *et al*., 2021; Zouré *et al*., 2021).

One of the most striking findings was the variation in resistance levels across the four study locations. The highest resistance was observed in Anaku and Umunze, where mortality rates for deltamethrin were as low as 42% and 3%, respectively. This suggests a significant decline in the efficacy of pyrethroids in these areas, which is concerning given that pyrethroids remain the primary class of insecticides used in long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (World Health Organization, 2018). The high resistance to DDT across all locations further reinforces the limited effectiveness of this insecticide, which aligns with previous studies that have documented widespread resistance to DDT due to its historical use in malaria control and agriculture (Gnankiné *et al*., 2013; Ranson *et al*., 2009).

The presence of the kdr L1014F mutation in the *An. gambiae* populations across all study sites are a key factor driving pyrethroid resistance. The highest mutation frequency was found in Ufuma (66.7%), with a significant proportion of mosquitoes being homozygous resistant (*RR*). This is consistent with findings from other parts of Nigeria, where the kdr mutation has been widely reported (Chukwuekezie *et al*., 2020; Oduola *et al*., 2012; Santolamazza *et al*., 2008). The mutation is known to confer resistance to pyrethroids and DDT by reducing the sensitivity of the mosquito's voltage-gated sodium channels to these insecticides (Martinez-Torres *et al*., 1998; Weill *et al*., 2004).

The role of agricultural insecticide use in driving resistance cannot be overlooked. Rice farming ecosystems provide ideal breeding habitats for *Anopheles* mosquitoes, and the extensive use of insecticides in these environments likely contributes to the selection of resistant mosquito populations (Amarasinghe & Weerakkodi, 2014; Yasuoka & Levins, 2007). Similar trends have been observed in other malaria-endemic regions, where agricultural pesticide use has been implicated in the evolution of resistance in malaria vectors (Riveron *et al*., 2018; Sonhafouo-Chiana *et al*., 2022). The frequent exposure of mosquito populations to the same classes of insecticides in both agricultural and public health sectors amplifies selection pressure, thereby accelerating the spread of resistance genes (Meier *et al*., 2022; Sumarnrote *et al*., 2017).

Despite high resistance to pyrethroids and DDT, the study identified bendiocarb and pirimiphos-methyl as effective alternatives, with mortality rates ranging from 89% to 100%. Bendiocarb, a carbamate, and pirimiphos-methyl, an organophosphate, target different biochemical pathways than pyrethroids and organochlorines, which likely explains their continued effectiveness (Aɪkpon *et al*., 2013, 2014; Antonio-Nkondjio *et al*., 2016). However, the observed resistance pattern also suggests the involvement of additional resistance mechanisms, including metabolic detoxification mediated by cytochrome P450 monooxygenases and esterases,

which are known to contribute to pyrethroid resistance and may influence cross-resistance dynamics. These findings suggest that IRS programs in Anambra State should consider incorporating carbamates and organophosphates into vector control strategies to mitigate resistance development. However, the long-term sustainability of this approach requires careful monitoring, as resistance to these insecticides has already been detected in *An. gambiae* populations in West Africa (Dabiré *et al*., 2014; Namountougou *et al*., 2012; Weill *et al*., 2004).

Given the rapid spread of resistance, an integrated vector management (IVM) approach is essential for effective malaria control. Strategies such as insecticide rotation, the use of insecticide mixtures, and the deployment of next-generation bed nets treated with novel active ingredients should be prioritized (World Health Organization, 2019). Additionally, biological control measures, such as larval source management and the introduction of natural predators, could help reduce mosquito populations in rice-growing areas (Chandra *et al*., 2008; Gan *et al*., 2021; Siddiqui *et al*., 2023). The importance of regular insecticide resistance surveillance cannot be overstated, as continuous monitoring will enable timely interventions before resistance becomes widespread and unmanageable.

Overall, this study provides critical insights into the resistance patterns of *Anopheles* mosquitoes in the rice agroecosystems of Anambra State. The findings underscore the urgent need for adaptive vector control strategies that incorporate multiple approaches to delay resistance emergence and sustain malaria control efforts. Future research should assess the impact of agricultural pesticide use on resistance trends and evaluate the effectiveness of novel insecticide formulations in these rice-farm areas.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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