Insecticide resistance spectrum and prevalence of L1014F kdr type mutation in Anopheles gambiae s.l. in Abia State, Nigeria


Highlights

• *Anopheles gambiae s.l.* was resistant to permethrin and DDT in all the study sites within Abia State, Nigeria.
• *Anopheles gambiae s.l.* was detected as resistant to bendiocarb for the first time in Agalaba, Abia State, Nigeria.
• *Anopheles gambiae s.l.* was susceptible to malathion in the three geopolitical zones of Abia State.
• *Anopheles gambiae s.s.* was predominant in the three geopolitical zones of Abia State.
• L1014 Kdr mutation is fixed in Abia State with 98-100% homozygote resistant allele (RR) frequency.
Insecticide resistance spectrum and prevalence of L1014F kdr type mutation in *Anopheles gambiae* s.l. in Abia State, Nigeria

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**Abstract:** *Anopheles gambiae* s.l. is the primary vector of malaria, a debilitating disease responsible for substantial mortality and morbidity in Sub-Saharan Africa. This study evaluated the insecticide resistance status and the frequency of L1014F *kdr* mutation in *An. gambiae* [Diptera: Culicidae, Giles 1902] within Abia State, Nigeria. Immature stages of *An. gambiae* (s.l.) were collected from Umudike, Agalaba, and Eben communities and reared to adulthood. Batches of 25 sugar-fed female mosquitoes, aged 3–5 days, were exposed to four types of WHO insecticide-impregnated papers, i.e., 4% DDT, 0.75% Permethrin, 0.1% Bendiocarb, and 5% Malathion, for one hour and the mortalities were recorded after a recovery period of 24 h. Mosquito species were identified using morphological and molecular methods, and *kdr* mutation L1014F was genotyped. *Anopheles gambiae* (s.l.) was highly resistant to permethrin (Umudike-18.8% mortality, Agalaba-17.5%, Eben-49.0%) but showed no resistance to DDT (0.0%) in the three locations. Conversely, all the locations recorded complete susceptibility to malathion (100%). Although complete susceptibility to bendiocarb was reported from Umudike (100%) and Eben-Ohaia (100%), resistance was reported from Agalaba (87.5%). PCR analyses showed that *An. gambiae* (s.l.) were predominantly *An. gambiae* s.s. in Umudike (90.0%), Agalaba (67.5%) and Eben (67.5%), whereas the rest were *An. coluzzii*. Very high frequencies of the *An. gambiae* (s.l.) were observed in all locations [Umudike (1.00), Agalaba (0.98), and Eben-ohafia (0.95)]. The worrisome resistance to bendiocarb in Agalaba suggests the existence of metabolic resistance that needs to be clarified. The high occurrence of L1014F resistance mutation in populations calls for urgent implementation of integrated vector control strategies in Abia State.

**Keywords:** *Anopheles gambiae* s.s.; *Anopheles coluzzii*; insecticide resistance; L1014F *kdr* mutation; Abia State.

**INTRODUCTION**

Malaria accounts for the death of about 400,000 persons yearly, with a vast majority within Sub-Saharan Africa. Nigeria currently reports very high morbidity and mortality, which has become a major health challenge in Africa (Omotayo et al., 2021). The predominant malaria vectors in Nigeria are *Anopheles gambiae* s.s., *An. coluzzii*, *An. arabiensis* and *An. funestus* (Akpan et al., 2018; Awolola et al., 2018; Ibrahim et al., 2020; Chukwuekezie et al., 2020; Awolola et al., 2020). Malaria vector control methods which are largely based on the use of insecticides deployed through Long Lasting Insecticide Treated Nets (LLITNs), Indoor Residual Spraying (IRS) and management of larval sources, have contributed immeasurably in ameliorating the morbidity and mortality associated with malaria in the recent past (WHO, 2017). The pyrethroid class is used for LLITNs, whereas pyrethroids, carbamates, organophosphates and organochlorines are used for IRS (WHO, 2022). However, the huge success recorded through these vector control methods has been continuously threatened by widespread malaria vector resistance to the chemical insecticides used. This has led to the loss of success gained prior to 2015 (Chukwuekezie et al., 2020).

Out of 80 countries endemic to malaria, varying degrees of resistance has been recorded in at least one species against at least one insecticide in 68 countries (WHO, 2018). Within Nigeria, resistance to all the classes of insecticides, which the WHOPES has approved, has been recorded in the majority of the malaria vectors (Awolola et al., 2002; Awolola et al., 2007; Awolola et al., 2009; Okorie et al., 2015; Nwankwo et al., 2017; Chukwuekezie et al., 2020; Muhammad et al., 2021).

Resistance of malaria mosquitoes to insecticides could be a result of a mutation in the target site of insecticide action, giving rise to decreased target site sensitivity (target site resistance mechanism) or it can be an amplification, increased up-regulation or mutation of the coding sequence in metabolic enzyme genes leading to over-expression and subsequent increase in the insecticide detoxification activity of enzymes before the insecticide reaches its site of action (Hemingway and Ranson, 2000; Karunaratne et al., 2018). Three major groups of enzymes responsible for this detoxification of insecticide in mosquitoes are glutathione S-transferases, cytochrome P450s and esterases (WHO, 1998). Many other studies have proved that these mechanisms, together with other minor mechanisms such as reduced penetration and behavioural resistance, act...
as major contributing factors to insecticide resistance in malaria vectors (Awolola et al., 2009; Ojuka et al., 2015).

The two target sites of insecticide action are the acetylcholinesterases and Na channel proteins, and mutations at these sites lead to decreased sensitivity. When there is a single amino acid substitution of glycine to serine at position 119 in the ace-1 gene the GS119 target site mutation arises, conferring resistance to bendiocarb and organophosphates (Weill et al., 2004). However, two amino acid changes in the sodium channel gene give rise to the more predominant knockdown resistant mutation (kdr) responsible for resistance to pyrethroids and cross-resistance to dichlorodiphenyltrichloroethane (DDT). Martinez-Torres et al. (1998) explained that the L1014F (West African kdr) mutation occurs when there is a replacement of the leucine residue located at codon 104 with phenylalanine, but when it is replaced with serine, it gives rise to the L1014S mutation (East African kdr) (Ranson et al., 2000).

The L1014F kdr type mutation and other resistance mechanisms have been reported in Nigeria (Awolola et al., 2009; Oduola et al., 2012; Awolola et al., 2018; Chukwuekzie et al., 2020; Fabohun et al., 2020). However, unlike the Northern and South Western parts of Nigeria, the South-Eastern part of Nigeria suffers a vast dearth of information on the insecticide resistance mechanisms of malaria vectors. This study, therefore, sought to investigate the prevalence of West African knock down resistance (kdr) and its relationship with insecticide resistance in the three geopolitical zones of Abia State, South Eastern, Nigeria, and hence fulfills the recommendation of Chuwuekezie et al., (2020), which considered just one community from each state of South Eastern Nigeria.

MATERIALS AND METHODS

Study Area

Abia State is located within the South Eastern part of Nigeria with 17 Local Government Areas (L.G.A.), and covers an area of 5,234.7 km² (Ogbuewu et al., 2016). Abia lies between 5°25 - 5°42 N latitude and 7°30 - 7°50 E longitude. It is positioned 223 m above sea level and the climate is classified as tropical, with an average temperature of 26.7°C, humidity 75.5% and an annual rainfall range of 2,500 mm/year from April to October (Ogbuewu et al., 2016). Within the state, the study was carried out across three geopolitical zones of Abia State: Ebem, Ohafia L.G.A. (5.61861° S, 7.81305° E); Umudike, Ikwuano, L.G.A (5.48079° N, 7.54208° E); and Agalaba, Osisioma L.G.A. (5.14796° N, 7.32988° E) (Figure 1).

Larval Survey

Larval collections were made from different points within the study area across the dry and wet seasons (August, 2021 to July, 2022). Larvae and pupae were collected from various natural breeding sites including ground pools, tire tracks, rice paddies, puddles, containers, foot and hoof prints, and other points. Water was scooped using a plastic scooper pipette and poured into small transparent plastic bowls. A strainer was used to sieve and pool together the third and fourth instars larvae to have sufficient adult emergence of the same physiological age. The mosquito larvae collected were transported in labelled plastic bottles to the insectaria in the Entomology unit of Michael Okpara University of Agriculture, Umudike, where they were maintained and reared at 26±3°C and 74±4% relative humidity to the adult stage. Larvae were fed on ground biscuits in 400 ml bowls, while adults were provided with 10% sugar solution in cotton wool. The resulting adults were identified according to the morphological keys of Gillies and D eMeillon (1968), Gillet (1972) and Gillies & Coetzee (1987). All bioassays were performed on adult females aged 3–5 days (WHO, 2022).

WHO susceptibility tests

Insecticide susceptibility bioassays were carried out using 3 to 5 days old, sugar-fed female mosquitoes to determine their insecticide resistance status. The mosquitoes from all the locations were tested against 4% DDT (an organochlorine), 0.75% permethrin (a pyrethroid), 0.1% bendiocarb (a carbamate) and 5% Malathion (an organophosphate) impregnated papers (procured from Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia) according to the WHO (2022) test procedures. A maximum of 100 female mosquitoes in four replicates were exposed to each insecticide for 60 min, with 50 mosquitoes in two replicates for the control. They were then carefully transferred to the holding (recovery) tubes and kept for 24 h during which they were fed with 10% sucrose solution. Records of final mortality were taken after 24 h recovery period and the susceptibility status of the population was graded according to WHO recommended protocol (WHO, 2022). Dead and survived mosquitoes from this bioassay were separately preserved in clearly labelled 1.5 ml Eppendorf tubes containing silica gel for target site resistance (kdr-w) assays. All susceptibility tests were carried out at 26±3°C temperature and 74±4% relative humidity.

Molecular identification of Anopheles species

DNA extraction

About 120 pyrethroid-resistant and susceptible An. gambiae (s.l.) (40 from each location) were selected randomly and subjected to DNA extraction. Genomic DNA from whole female mosquitoes was extracted according to the standard manufacturer’s protocol of genomic DNA extraction kit (Jena Bioscience Cat Number PP-213L), and then stored at -20°C.

PCR for molecular species identification

Species identification was carried out at the Nigerian Institute for Medical Research (NIMR). Combined An. gambiae complex and ribosomal DNA type assay for M/S discrimination by Wilkins et al., (2006) was used to identify members of the An simultaneously. gambiae complex. The Wilkins et al. (2006) method of An. gambiae complex discrimination is based on species-specific single nucleotide polymorphisms (SNPS) in the intergenic spacer region (IGS). Primers were added to this method to elucidate the ribosomal DNA type simultaneously. To constitute the PCR master mix (12.5 µl), 5 µl of distilled
Figure 1: Location Map of Abia State, Nigeria, showing the study locations.

Water (ddH₂O), 2.5 μl of pre-mix, and 0.5 μl of each primer [universal primer (IMP-UN), arabiensis primer (AR-3T), gambiæ primer (GA-3T), merus primer (ME-3T), primer for gambiæ S form (IMP-S1), primer for gambiæ M form (IMP-M1)], 1.0 μl quadrannulatus primer (QD-3T) (Table 1), and 1 μl of DNA template were added to a 1.5 ml Eppendorf tube. Each sample tube containing 12.5 μl of PCR master was subsequently loaded in the PCR machine (BIO- RAD T100 Thermal Cycler, California, USA), and the PCR conditions were: 95°C for 5 minutes 1 cycle; followed by 30 cycles of 95°C for 30 seconds, 59.2°C for 30 seconds, 72°C for 30 seconds) 30 cycles; and the final extension step of 72°C for 5 minutes 1 cycle, and then 4°C hold.

PCR products were detected on 2% agarose gel and images were interpreted using a Syngene bio-imaging system (Syngene, Cambridge, UK) based on the fragment sizes; 636 for An. quadrannulatus, 528 An. merus, 463 An. gambiæ s.s, 387 An. arabiensis, 333 An. coluzzii M and 221 An. gambiæ S. Primers used for the PCR identification of sibling species of An. gambiæ s.l. includes:

IMP-UN 5’ GCTGCGAGTTGTAGAGATGCG 3’
AR-3T 5’ GTGTAAAGTGTCCTTCTCCGT 3’
GA-3T 5’ GCTTACTGTTTTGGTCGACGT 3’
ME-3T 5’ CAACCCACTCCCTTTGACGT 3’
QD-3T 5’ GCATGTCCACCAACGTAAATCC 3’
IMP-S1 5’ CCAGACCAAGATGGTCGCTG 3’
IMP-M1 5’ TAGCCAGCTCTTGTCCACTAGTTT 3’

Source: Wilkins et al., 2006
PCR for kdr-type resistance mechanism detection

Knockdown resistance-west (kdr-w) genotyping to identify the presence of the L1014F mutation was carried out as described by Huynh et al., (2007). To constitute the PCR master mix (10.5 μl), 5 μl of distilled water (ddH₂O), 2.5 μl of Pre-mix, and 0.5 μl of each primer [outer forward (IPCF-F), outer reverse (AltRev-R), inner reverse (WT-R)] and 1.5 μl of inner forward (West-F) were added to an Eppendorf tube (Table 2). A 2 μl DNA template of each specimen was added separately to 10.5 μl master mix prepared. The PCR conditions were: 95°C for 5 min followed by 35 cycles of 95 °C for 30 s, 59°C for 30 s, 72°C for 30 s and the final extension step of 72 °C for 5 min.

PCR products were checked on 2% agarose gel and all the PCR positive products were expected to contain a band at 314 bp (outer primers). In addition, a band of 214 bp indicates the homozygous susceptible (wild type) allele (SS), 156 bp the homozygous resistant allele (RR), and 156 bp, 214 bp the heterozygote resistant allele (RS) indicates co-dominance.

Primers used for the screening of target-site modification (L1014F) in resistant An. gambiae s.l. include:
- IPCF: 5’GATAATGTGGATAGATTCCCCAGCATG3’
- AltRev: 5’TGCCGTTGGTGCAGACAAGGATG 3’
- WT: 5’GGTCCATGTTAATTTGCATTACTTAATAGAAATGTT3’
- West: 5’CTTGGCCACTGTAGTGATAGGAAAATGTT3’

Source: Huynh et al., 2007

Data interpretation and analysis

Percentage mortality for each insecticide was calculated as the proportion of mosquitoes found dead after the 24 h recovery period out of the total number of mosquitoes exposed. The mortality rate in the control tubes were always less than 5%, and hence was not corrected using Abott formula (Abott, 1987). The resistance status of various mosquito populations for each insecticide was determined according to WHO criteria (WHO, 2022). The mortality rates of less than 90% indicated confirmed resistance, whereas those between 90 to 98% mortality indicated possible resistance and those greater than or equal to 98% were susceptible. Analysis of Variance (ANOVA) was also used to compare the mortalities between the insecticides, and the Least Significant Difference (LSD) was used to separate the means. The genotypic differentiation test was performed to evaluate the variability of allelic frequencies of the kdr (L1014F) mutation across An. gambiae populations from different locations (Goudet et al., 1996; Chukwuekezie et al., 2020).

RESULTS

Susceptibility of Anopheles gambiae (s.l.) from Abia State to Bendiocarb, Permethrin, Malathion and DDT

Percentage mortalities of An. gambiae (s.l.) for bendiocarb (Carbamate) in the three locations from Abia state were: 87.6% in Agalaba (Osisioma L.G.A.), 100% in Umudike (Ikwuano L.G.A.) and 100% in Ebem (Ohafia L.G.A.; Fig. 2, 3). For permethrin, the observed mortalities were: 17.5% in Agalaba, 18.8% in Umudike and 49.0% in Ebem. A mortality of 100% was observed in all the locations for malathion, whereas 0% mortality was observed in all the locations for DDT (Figures 2 and 3).

Distribution of sibling species of Anopheles gambiae (s.l.) across Abia State

All the Anopheles larval samples collected from the three senatorial zones of Abia State were morphologically identified to be An.gambiae (s.l.). Further, molecular identification of them by PCR showed that 90% of the tested mosquitoes from Umudike (Abia Central geopolitical zone) were An. gambiae s.s., while 10% were An. coluzzii. A similar trend was seen at Agalaba (Abia south geopolitical zone) and Eben-Ohafia (Abia north geopolitical zone) with 67.5% An. gambiae s.s. and 32.5% An. coluzzii, respectively in both zones (Table 1).

Figure 2: Percentage mortalities of Anopheles gambiae (s.l.) for Bendiocarb, Permethrin, Malathion and DDT across the three geopolitical zones of Abia State, Nigeria.
Distribution of insecticide resistance in *Anopheles gambiae* in Abia State, Nigeria

**Figure 3**: Distribution of insecticide resistance in *Anopheles gambiae* in Abia State, Nigeria

**Table 1**: Distribution of *Anopheles gambiae* (s.s.) and *An. coluzzii* across the three geopolitical zones of Abia State.

<table>
<thead>
<tr>
<th>Species</th>
<th>Distribution (%)</th>
<th>Umudike (Ikwuano L.G.A)</th>
<th>Agalaba (Osisioma LGA)</th>
<th>Ebem-Ohafia (Ohafia L.G.A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n=40</td>
<td>n=40</td>
<td>n=40</td>
</tr>
<tr>
<td><em>An. gambiae</em>s.s.</td>
<td>90.0</td>
<td>67.5</td>
<td>67.5</td>
<td></td>
</tr>
<tr>
<td><em>An. coluzzii</em></td>
<td>10.0</td>
<td>32.5</td>
<td>32.5</td>
<td></td>
</tr>
</tbody>
</table>

**Distribution of L1014F mutation of voltage gated sodium channel (VGSC) gene across Abia State**

Target site resistance due to L1014F mutation in voltage-gated sodium channel gene was assayed in *An. gambiae* (s.l.) mosquitoes collected from the three geopolitical zones of Abia State. The West African knockdown resistance mutation L1014F was detected in very high frequencies (Table 2). The genotypic differentiation test showed that there was no significant difference in the L1014F (*kdr*) resistance mutation of mosquitoes that died and those that survived pyrethroid exposure across the different locations sampled (Odds ratio [OR]: 1.375) (P > 0.05) (Table 2).

The *kdr* mutation was high in *An. gambiae* s.s. which recorded a frequency of 1, and *An. coluzzii* which had a frequency of 0.9 across all the locations from which they were collected (Table 3). Genotypic differentiation test showed that the frequency of the L1014F (*kdr*) mutation did not differ significantly between the dead and alive mosquitoes after permethrin exposure across the sibling species [Odds ratio (OR): 0.85, p > 0.05;Table 3].
Table 2: Frequency of knock-down resistance mutation \((kdr)\) in \textit{Anopheles gambiae} s.l. across the Local Government Areas.

<table>
<thead>
<tr>
<th>Location</th>
<th>N</th>
<th>RR</th>
<th>RS</th>
<th>SS</th>
<th>F(kdr)</th>
<th>Odd ratio</th>
<th>95 % CI</th>
<th>Z Test</th>
<th>P &gt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umudike (Ikwuano)</td>
<td>40</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Agalaba (Osisioma)</td>
<td>40</td>
<td>39</td>
<td>1</td>
<td>0</td>
<td>0.98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ebem-Ohafia</td>
<td>40</td>
<td>38</td>
<td>2</td>
<td>0</td>
<td>0.95</td>
<td>1.375</td>
<td>0.08 - 23.67</td>
<td>0.219</td>
<td>0.8264*NS</td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td>117</td>
<td>3</td>
<td>0</td>
<td>0.98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Abbreviations: n, number tested; SS, homozygous susceptible; RS, heterozygous for resistance; RR, homozygous resistant; F, frequency*

Table 3: Frequency of knock-down resistance mutation \((kdr)\) across mosquito species.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>RR</th>
<th>RS</th>
<th>SS</th>
<th>F(kdr)</th>
<th>Odd ratio</th>
<th>95 % CI</th>
<th>Z Test</th>
<th>P &gt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Anopheles gambiae} (s.s.)</td>
<td>90</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.85</td>
<td>0.07 - 10.61</td>
<td>0.126</td>
<td>0.8996NS</td>
</tr>
<tr>
<td>\textit{Anopheles coluzzii}</td>
<td>30</td>
<td>27</td>
<td>3</td>
<td>0</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td>117</td>
<td>3</td>
<td>0</td>
<td>0.98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Abbreviations: n, number tested; SS, homozygous susceptible; RS, heterozygous for resistance; RR, homozygous resistant; F, frequency*

Figure 4: Gel electrophoresis of PCR products of \textit{An. gambiae} s.s and \textit{Anopheles coluzzii} mosquito samples. Lane 1: DNA ladder (100 bp); Lane 1: \textit{Anopheles gambiae} positive control (463 and 221 bp); Lane 2: Negative control; Lane 19: \textit{Anopheles coluzzii} positive control (463 and 333 bp); Lanes 6, 9, 11-12, 16 and 18: \textit{Anopheles gambiae}; Lanes 14-15, 17: \textit{Anopheles coluzzii}

Figure 5: Gel electrophoresis of PCR products to detect \(kdr\) L1014F mutation. Lane 1: DNA ladder (100 base pair); Lanes 1-4, 6-13, 15-16, 18: susceptible (wildtype) allele (314 and 156 bp); Lane 17: homozygous resistant (314 and 214 bp)
DISCUSSION

This is the first comprehensive study on the susceptibility status and L1014F mutation distribution across the three geopolitical zones of Abia State, Nigeria. It presents a high level of L1014F (kdr) resistance mutation occurrence in two geopolitical zones of Abia State that has not been previously reported by Chukwukezie et al. (2020). The resistance to Bendiocarb (Figs. 2, 3) observed in Agalaba within Aba, the industrial nerve of Abia State is the first report of Bendiocarb resistance in Abia State, Nigeria. This Bendiocarb resistance agrees with a study which reported An. gambiae (s.l.) bendiocarb resistance (44 to 45% mortality) in North East and North-Western Nigeria (Yusuf et al., 2020).

The differential variation in the Bendiocarb susceptibility status of An. gambiae (s.l.) across the three localities sampled in Abia State is in tandem with a study in four communities of Adamawa State, North-Eastern Nigeria, with suspected bendiocarb resistance in Vtimint and Imburu communities, and full resistance in Muchala and Bachure communities (Wahedi et al., 2021). Bendiocarb resistance in Agalaba An. gambiae (s.l.) suggests the existence of other target site resistance mechanisms, like the insensitive acetylcholinesterase-1 mutation, that also contributes to carbamate resistance. Chukwukezie et al., (2020), reported low frequencies of the Ace-1 mutation, with Anopheles gambiae s.l. Bendiocarb susceptibility in their study locations. Bendiocarb resistance could also be linked to metabolic detoxification genes, as reported in Cameroon where most of the sampled mosquitoes were resistant to both bendiocarb and malathion (Ngangue-Siewe et al., 2022).

This resistance has the potential to jeopardize any IRS using bendiocarb within Agalaba with known resistance to pyrethroids and DDT. Anopheles gambiae (s.l.) larvae from Agalaba were found breeding in sympathy with Culex quinquefasciatus in much-polluted ground pools and could have possibly developed resistance to bendiocarb because of selection pressure. This can only be confirmed by further studies to confirm the bendiocarb susceptibility status of Cu. quinquefasciatus in Agalaba, because resistance to bendiocarb in Cu. quinquefasciatus from Umudike has been reported by Ukpai and Ekedo (2019). Conversely, the susceptibility to bendiocarb observed in Umudike and Ebem agrees with earlier studies by Ekedo and Ukpai (2019) and Chukwukezie et al. (2020) respectively, within the same geopolitical locations. Unlike Umudike and Ebem, the Bendiocarb resistance in Agalaba could be associated with the very high discharge of industrial effluents, possibly inducing resistance to the insecticides within that area.

The overwhelming resistance to permethrin across all the locations in this study is consistent with reports on the widespread of pyrethroid resistance in major malaria vectors across the country (Chukwukezie et al., 2020; Oyeniyi et al., 2020; Awolola et al., 2018; Olayemi et al., 2011) (Figures 2 and 3). This is also in agreement with reports on resistance to pyrethroid insecticides across Africa. Awolola et al. (2014) reported the presence of deltamethrin, permethrin, and lambdacyhalothrin resistance amongst populations of An. gambiae s.l. at Ilara, Irolu, and Ijesa villages in Remo North L.G.A of Ogun State. The study of Oyewole et al. (2018) on the susceptibility pattern of Anopheles mosquitoes in Ila-Orunang, Southwest Nigeria showed that the sampled population was resistant to permethrin. Many other studies have also reported varying degrees of permethrin resistance within and beyond Nigeria (Awolola et al., 2005; Djouaka et al., 2007, 2008; Awolola et al., 2007; Okorie et al., 2015; Oduola et al., 2019; Ononamadu et al., 2020; Idowu et al., 2020). This overwhelming resistance to pyrethroid insecticides is thought to have been triggered by the over-dependence on pyrethroids for use in LLINs for around 12 years and IRS (WHO, 2012; WHOPES, 2011; Kabula et al., 2011) in Abia and other South Eastern states.

Malathion, an organophosphate insecticide, was 100% effective against An. gambiae (s.l.) mosquitoes from all three locations of this study showed to be the most effective insecticide for its control in the state (Figures 2 and 3). This agrees with a study where the population of An. gambiae (s.l.) from Misau in Bauchi State, North-east Nigeria was resistant to permethrin, DDT and bendiocarb was fully susceptible to malathion (Umar et al., 2014). This malathion susceptibility is in further agreement with another study which showed that An. coluzzi populations from Port Harcourt, Rivers State were resistant to DDT (6.3% mortality) and permethrin (6.7% mortality), but fully susceptible to malathion and bendiocarb (Muhammad et al., 2021). Similarly, Yusuf et al. (2020) showed a high level of Malathion susceptibility (99 to 100%) in An. gambiae (s.l.) populations resistant to DDT (37 to53%), and bendiocarb (44 to 55%). Furthermore, Wanjala and Kekwa (2018) reported An. gambiae (s.l.) susceptibility to malathion at all the seven sites sampled in Western Kenya, with DDT and deltamethrin resistance occurring in most sites.

The high resistance to DDT evenly distributed in the three geopolitical zones of Abia State (Figures 2 and 3) could be attributed to the extensive use of DDT for agricultural purposes within the study locations, as suggested in the study of Corbel et al. (2007) and Czeher et al. (2008). Umudike houses the National Root Crops Research Institute and the Michael Okpara University of Agriculture Umudike, Ebem has large farm areas, while Agalaba a major industrial hub, and all contribute to insecticide/chemical availability in their environments that could be aiding resistance development. An. gambiae s.s. dominance across the three geopolitical zones of Abia is in agreement with the reports of Chukwukezie et al., (2020) in Southeastern Nigeria and Oyeniyi et al. (2020) in the six geopolitical zones of Nigeria.

Other studies have reported the occurrence of DDT resistance across Africa (Ranson et al., 2011; Santolamazza et al., 2008; Ranson et al., 2009; Okorie et al., 2015; Chukwukezie et al., 2020). However, the susceptibility level of An. gambiae (s.l.) mosquitoes to DDT has degenerated over time. In 2005, Awolola et al. reported that An. gambiae was susceptible to DDT in seven localities (across the mangrove, forest, and Sudan-savanna), and the L1014F kdr mutation was absent (0%) in the mosquito
population in these localities. Similarly, the study of Yadouleton et al. (2010) in Aglangandan, Southern Benin Republic, showed that An. gambiae mosquitoes were fully susceptible to DDT (100% mortality), but were resistant in Southern Benin Republic. Oduola et al. (2010) also reported resistance to DDT in 12 communities sampled across Lagos, Oyo and Niger States, with percentage mortalities ranging from 9.8% - 80%. Umar et al. (2014) in their study reported that An. gambiae (s.l.) had 78.33% mortality after exposure to DDT, indicating resistance. By 2015, a review of insecticide resistance in Nigeria showed that DDT resistance had been recorded across all the geographical zones of Nigeria (Mohammed et al., 2015). The highest record of DDT and, subsequently pyrethroid resistance was between 2014 and 2015 (Chukwuuekezie et al., 2020; Okorie et al., 2015; AIRS, 2014).

In agreement with previous studies, (Awolola et al., 2007; Djouaka et al., 2008; Okorie et al., 2014 Okorie et al., 2015; Akpon et al., 2018; Thabet et al., 2022) findings of this study also reported sympatric breeding of An. gambiae s.s. and An. coluzzii (Table 3). Conversely, Chukwuuekezie et al. (2020), reported allopatric breeding pattern of a single member of the species complex, An. gambiae s.s. from eastern Nigeria including Abia State. The higher occurrence of An. coluzzii (32.5%) in Agala and Eben could be linked to cross-border migration from the Niger Delta region that has been confirmed to be dominated by An. coluzzii by the reports of Muhammed et al. (2021). This abundance of An. gambiae s.s. within Abia State invariably incriminates it as the primary malaria vector, and therefore calls for species-specific efforts aimed at its control.

The only mechanism of insecticide resistance analysed in this study was the kdr type target site resistance, primarily due to the availability of funds. Across the three locations sampled, the West African knockdown resistance (L1014F) mutation occurred in very high frequencies (0.95 – 1.00) (Table 2). These high frequencies observed agree with the report of Chukwuuekezie et al., 2020 which recorded frequencies within 0.60-0.90 across the five states of southeastern Nigeria. Many other researchers have reported high frequencies of kdr mutation as contributing to pyrethroid resistance across Nigeria and Africa (Awolola et al., 2009; Oduola et al., 2012; Gnanguenon et al., 2015; Fodjo et al., 2018; Ononamadu et al., 2020; Oyeniyi et al., 2020; Kpanou et al., 2021). This is probably the first study that is recording a fixation of L1014F kdr resistance mutation within the southeastern part of Nigeria. These very high kdr frequencies could have possibly contributed to the overwhelming resistance to permethrin insecticide and cross-resistance to DDT in all the locations surveyed. This assertion is supported by the study of Djouka et al. (2008), which reported high kdr frequencies (0.84 -0.86) in two locations within Benin Republic and related it to kdr mutation resistance and other resistance mechanisms. A report of Oduola et al., 2012 also attributed the pyrethroid/ DDT resistance observed to kdr -w point mutation at allelic frequencies between 45%-77%.

However, pyrethroid bioassays tests showed that there was no significant difference in the frequency of the L1014 kdr mutation observed between the live and dead mosquitoes (Odds ratio [OR]: 1.375) (P > 0.05). This observation supports a study where no correlation was found between the resistance phenotype and the kdr genotype of Anopheles mosquitoes at Bodija and Ojoo, Ibadan (Okorie et al., 2015). Ononamadu et al. (2020) in Sharada and Wailari metropolis of Kano showed that the kdr mutation frequency was weakly associated with the pyrethroid resistance status of An. coluzzi. This situation suggests the possible existence of other resistance mechanisms in the Anopheles population of Abia State. Djouaka et al. (2008) reported high frequencies of the West African kdr genes, alongside upregulation of two cytochrome P450 genes (i.e CYP6F3 and CYP6M2). Over-expression of the P450’s and glutathione S-transferase (GST) has been reported as being responsible for the high DDT and permethrin resistance witnessed in An. gambiae from Kpome, Benin Republic, with no evidence of kdr mutations (Tchigossou et al., 2018). Fagbohun et al. (2019) also indicated high expressions of cytochrome P450 mono-oxygenase in DDT and pyrethroid-resistant An. gambiae from Kosofe, Lagos State, Nigeria.

Awolola et al. (2018) also reported higher resistance in An. gambiae populations with both the kdr and up-regulation of detoxification enzymes than with kdr alone, concluding that increased P450s and GSTs were responsible for the high pyrethroid resistance seen in their study across Anambra, Edo, Lagos, Ogun, Kwara and Niger. Overexpression of GSTs, cytochrome P450s, esterases, trypsins and cuticle proteins in permethrin, dieldrin and DDT-resistant mosquitoes compared to susceptible ones have been reported too (Atoyebi et al., 2020). Their study identified GSTe2 as the most upregulated detoxification gene in permethrin, dieldrin and DDT resistant mosquito populations.

Although Umudike had the highest kdr mutation frequency (1.00), Agalaba An. gambiae population with a lower frequency (0.98) was the most resistant population to permethrin and bendiocarb indicating that other resistance mechanisms support the L1014F kdr resistance in Agalaba. They were resistant to bendiocarb, a carbamate insecticide that Anopheles populations from Umudikde and Eben-Ohafia could not resist. The possible existence of multiple resistance mechanism in Agalaba, Osisioma L.G.A. is further explained by the report of Ngangue-Siewe et al. (2022), where bendiocarb (carbamate) and malathion (organophosphate) resistance was discovered to be as a result of the overexpression of P450s gene Cyp6p3 associated with carbamate resistance and the glutathione S-transferase gene Gste2 associated with organophosphate resistance.

The homozygous resistant state (RR) of the kdr mutation dominated the allelic frequency in all the study locations (Table 2). This is particularly worrisome, since this can resist pyrethroid insecticides, which are primarily used for LLINs. Selection pressure from agricultural insecticides, as well as insecticides used for vector control over the period could have exacerbated these high kdr mutations.
frequencies. The effect of the previous use of agricultural insecticides can never be overlooked. Matiya et al. (2019) reported over transcription of several P450s genes associated with metabolic resistance in An. gambiae s.l. mosquitoes collected from agricultural areas.

Presence of high kdr frequencies in both An. gambiae s.s. and An. Coluzzii populations (Table 3) agrees with earlier reports (Gnanguenon et al., 2015; Okorie et al., 2015; Chukwuekezie et al., 2020). However, it contradicts the AIRS Nigeria report (2014), where all An.coluzzii populations were not positive for the African knockdown resistance (L1014F) mutation. Reports from this study show that the homozygous susceptible state and the homozygous resistant state occurred in An. coluzzii, while An. gambiae had only the homozygous resistant state. This may suggest that the An. gambiae s.s. population is adapting faster to the insecticide pressure than An. coluzzii. The higher mutation frequency in An. gambiae s.s. in comparison to An. coluzzii also agrees with the study of Chukwuekezie et al. (2020).

The limitations of the study were that the intensity of the resistance was not considered and the presence of other kdr-type target site mutations like the G119S, 1575Y and L014S, which are paramout target site mutations in An. gambiae (s.l.) was not assessed. In addition, the transcriptional analyses for the detection of metabolic resistance mechanisms were not carried out in this study because of limited funds to perform the analysis and this was a significant limitation. All these aspects together would produce a clearer picture of the complete resistance mechanism of An. gambiae (s.l.) population in Abia State.

CONCLUSION

The study reports An. gambiae (s.l.) differential resistance to bendiocarb, widespread resistance to pyrethroids and DDT, and homogenous susceptibility to malathion in Abia State, Nigeria for the first time. An. gambiae s.s., the dominant species incriminated within the State displayed a similar trend of resistance with the less dominant An. coluzzii, with the homozygote resistance allele (RR) prevalent in the analyzed mosquitoes. The West African knockdown resistance (L1014F) mutation was detected in very high frequencies, and had become fixed in Umudike, Abia South geopolitical zone of the state. This widespread resistance to three of the four classes of insecticides used in public health could have grave consequences in the control of malaria and its vectors within the State, since most of the interventions depend solely on insecticides within these classes. Therefore there is an urgent need for insecticide management strategies in the state to assess and curtail the spread of resistance to carbamates and organophosphates in areas where An. gambiae (s.l.) are still susceptible to these insecticides.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest to the manuscript.

REFERENCES


