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Widespread Multi-Insecticide Resistance Associated with Metabolic Mechanisms in Wild *Anopheles funestus* s.s. Populations from Central Senegal

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Abstract

Background: The insecticide resistance profile of the malaria vector *Anopheles funestus* and its underlying mechanisms remain poorly characterized in several regions of Senegal. To address this gap in knowledge, we investigated insecticide resistance profiles and assessed the contribution of metabolic resistance in *Anopheles funestus* populations from central Senegal. **Methods**: Indoor-resting blood-fed and gravid female mosquitoes were collected from randomly selected human dwellings in each study site using mouth aspirators. WHO susceptibility tests, Piperonyl butoxide (PBO) synergist assays, and resistance intensity assays were conducted on unfed 3-5-day-old F1 female progeny derived from wild-caught blood-fed *Anopheles funestus* females. Four classes of public health insecticides were tested. **Results:** *Anopheles funestus* populations from Camara, Keur Mamadou, and Tawa Mboudaye showed moderate to high resistance intensity to pyrethroids, whereas populations from Dielmo exhibited low intensity to lambda-cyhalothrin and moderate intensity to alpha-cypermethrin. Moderate resistance to bendiocarb was detected in Nioro, but neither in Sokone nor Ndoffane. Overall, populations remained susceptible to largely susceptible to the organochlorine (DDT) and organo-

phosphates across the study areas. However, in the localities of Tawa Mboudaye and Keur Mamadou, resistance to fenitrothion (86.4% and 76.1% mortality, respectively) and suspected resistance to DDT (91.4% and 88.3% mortality, respectively) were observed. PBO synergist assays suggest the involvement of cytochrome P450 monooxygenases mediating resistance to pyrethroids and bendiocarb. **Conclusions:** This study demonstrates that *Anopheles funestus* populations in central Senegal exhibit moderate to high levels of pyrethroid resistance, together with localized resistance to bendiocarb and fenitrothion in some locations. The results suggest that metabolic resistance mechanisms, particularly those involving cytochrome P450 monooxygenases, may contribute substantially to the observed resistance phenotypes; however, these findings provide only indirect evidence, and additional mechanisms beyond P450s cannot be excluded, especially for compounds such as DDT and bendiocarb. These results support the consideration of PBO-based LLIN deployment across the study areas.

Keywords

Anopheles funestus, Insecticide Resistance, Metabolic Resistance, Piperonyl Butoxide, Central Senegal

1. Introduction

Malaria remains a major public health challenge in endemic regions of sub-Saharan Africa, including Senegal. In 2024, an estimated 282 million cases and 610,000 deaths were reported globally, with approximately 95% of malaria-related deaths occurring in the WHO African Region. Children under five years of age remain the most vulnerable group, accounting for nearly 75% of malaria deaths in the region [1]. In Senegal, the malaria burden has progressively declined over recent years, with malaria-related mortality decreasing from 2.7% in 2020 to 1.62% in 2022 [2]. To sustain this progress and advance towards malaria elimination, the country has intensified malaria control strategies. Vector control remains the cornerstone of these efforts and relies mainly on indoor residual spraying (IRS) and the mass distribution of long-lasting insecticidal nets (LLINs) every three years [3]. These targeted interventions have substantially improved household access to and use of LLINs, contributing significantly to the reduction of malaria transmission and overall disease burden [4].

Among malaria vectors, *Anopheles funestus* is one of the principal species in sub-Saharan Africa, including Senegal. In Senegal, *Anopheles funestus* sensu stricto (s.s.) has been identified as an important contributor to malaria transmission [3] [4]. Effective vector control strategies depend on a thorough understanding of vector biology and behaviour, and insecticide susceptibility. However, despite its epidemiological importance, *An. funestus* s.s. remains relatively understudied in Senegal compared with the members of the *An. gambiae* complex, with most existing studies focusing mainly on bionomic aspects. This highlights the need for

more comprehensive data on insecticide resistance profiles in natural populations of *An. funestus,* and mechanisms to better guide vector control interventions. In particular, significant knowledge gaps persist regarding the monitoring of insecticide resistance and the characterization of the underlying resistance mechanisms.

In Senegal, malaria vector control strategies have not specifically targeted *An. funestus* populations. As a result, the control of this vector remains challenging for current interventions, due to factors such as changes in biting behaviour, the ecological adaptability of its breeding habitats, and the emergence of insecticide resistance. Notably, *An. funestus* has shown a strong ability to develop resistance to commonly used public health insecticides, particularly pyrethroids [5]-[7]. Across Africa, resistance to pyrethroids, carbamates, and organochlorines in *An. funestus* populations has been widely documented [8]-[13].

In Senegal, studies conducted in the Senegal River basin have reported resistance of *An. funestus* populations to pyrethroids commonly used in LLINs and IRS, as well as moderate resistance to dichlorodiphenyltrichloroethane (DDT) and the carbamate bendiocarb [7]. Molecular analyses from this region have highlighted the involvement of metabolic resistance mechanisms, particularly cytochrome P450 monooxygenases and glutathione S-transferases, including the overexpression of genes such as CYP6M7, CYP4H17, CYP304B1, CYP4C27, and CYP4H25 [7]. The increasing prevalence of these resistance mechanisms may reduce the effectiveness of LLINs and IRS interventions. Addressing these challenges requires strengthened vector control strategies alongside continuous monitoring of insecticide resistance. A comprehensive understanding of resistance profiles, underlying mechanisms, and their operational implications for vector control effectiveness is therefore essential for the development of evidence-based resistance management strategies. However, in several regions of Senegal, particularly in central areas, the insecticide resistance profile of *An. funestus* populations remains poorly documented. To support malaria vector control efforts and guide resistance management strategies, this study aimed to assess insecticide resistance patterns in *An. funestus* populations and investigate the contribution of metabolic resistance mechanisms in central Senegal.

2. Materials and Methods

2.1. Study Area and Mosquito Collection

Blood-fed and gravid *Anopheles* females resting indoors were collected in human dwellings between 8:00 and 12:00 during two sampling campaigns conducted in January 2023 and April 2024 in four villages located in central Senegal. The study was carried out in the villages of Camara (13˚38'N, 15˚37'W) and Keur Mamadou (13˚41'N, 16˚02'W) in the Nioro health district, Tawa Mboudaye (13˚58'N, 16˚12'W) in the Ndoffane health district, and Dielmo village (13˚43'N, 16˚24'W) in the Sokone health district (**Figure 1**).

The Nioro and Ndoffane health districts are located in the Kaolack administra-

tive region, whereas Sokone is situated in the Fatick region. The study area is characterized by a Sudano-Sahelian climate, with annual rainfall ranging from 700 to 1300 mm. Temperatures typically vary between 18˚C and 40˚C, with peak temperatures recorded in May and the lowest in January [14]. The region experiences two distinct seasons: a rainy season from June to October and a long dry season from November to May. The hydrographic network of the study sites includes permanent water bodies bordered by dense aquatic vegetation, predominantly *Typha australis*, creating favourable breeding habitats for *An. funestus* larvae. Indoor-resting, blood-fed, and gravid female mosquitoes were collected from human dwellings using mouth aspirators and torches and transferred into holding cups covered with untreated netting. Mosquitoes were transported alive to the insectary, where they were maintained on a 10% sugar solution for 3 - 4 days until they became fully gravid. Individual oviposition was then induced in Eppendorf tubes, after which the egg batches from individual females were pooled for larval rearing and transferred into plastic trays for larval rearing [15]. After oviposition, all F0 females were individually preserved in labelled 1.5 ml Eppendorf tubes containing silica gel and stored at −20˚C for subsequent molecular analysis. Larvae from hatched eggs were reared under standard insectary conditions (25˚C ± 2˚C and 70% ± 10% relative humidity), and emerging F1 adults were pooled and randomly maintained in rearing cages for subsequent WHO susceptibility tests.

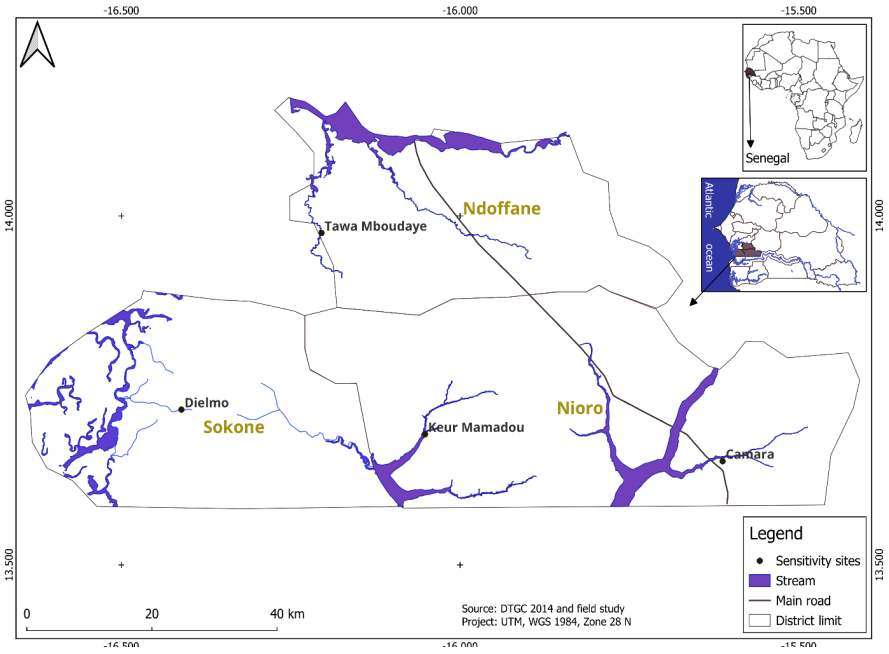

Figure 1. Geographic location of the study sites in central Senegal.

2.2. Molecular Species Identification

Wild-caught female mosquitoes were initially morphologically identified as members of the *Anopheles funestus* group using standard mosquito identification keys [16] [17]. Following oviposition, species identification was further confirmed us-

ing a multiplexed polymerase chain reaction (PCR) assay specific to the *An. funestus* group as described by Koekemoer *et al.* [18]. Genomic DNA was first extracted from individual mosquitoes following the method previously described by Collins *et al.* [19]. The amplified PCR products were visualized under UV illumination following gel electrophoresis to determine species-specific banding patterns.

2.3. Adult Mosquito Susceptibility Assays

Insecticide susceptibility assays were conducted on 3 - 5 day-old unfed F_1 females of *An. funestus* s.s. in accordance with standard WHO [20]. Mosquitoes were exposed to the WHO diagnostic concentration of insecticides from the four major classes used in public health, including pyrethroids (permethrin 0.75%, cyfluthrin 0.15%, deltamethrin 0.05%, lambda-cyhalothrin 0.05% and alpha-cypermethrin 0.05%), organochlorine (dichlorodiphenyltrichloroethane or DDT 4%), the carbamate (bendiocarb 0.1%), and the organophosphates (pirimiphos-methyl 0.25% and fenitrothion 1%). For each insecticide, approximately 100 mosquitoes were tested, divided into four replicates of 25 individuals each. A control group exposed to untreated papers was included for each experiment. Following exposure, mosquitoes were maintained under standard insectary conditions, and mortality was recorded 24 hours later. Resistance status was interpreted according to WHO criteria [20]. All bioassays were carried out under controlled laboratory conditions at 25˚C ± 2˚C and 70% ± 10% relative humidity.

2.4. Piperonyl Butoxide (PBO) Synergist Tests

The potential involvement of cytochrome P450s monooxygenases in insecticide resistance was investigated using PBO synergist assays following WHO guidelines [20]. Unfed F_1 *Anopheles funestus* s.s. adult females aged (3 - 5 days) were first exposed to PBO 4% impregnated papers for 1 hour and then immediately transferred to test tubes containing insecticide-treated papers for an additional 1-hour exposure. The insecticides tested included deltamethrin (0.05%), alpha-cypermethrin (0.05%), lambda-cyhalothrin (0.05%), permethrin (0.75%), cyfluthrin (0.15%), DDT (4%), and bendiocarb (0.1%). Mosquito mortality was recorded 24 hours post-exposure. Two controls were included in each experiment: one exposed only to PBO papers and the other exposed to untreated papers. The contribution of cytochrome P450-mediated metabolic resistance was inferred by comparing mortality rates between mosquitoes exposed to insecticide alone and those pre-exposed to PBO prior to insecticide exposure [20].

2.5. Assessment of Resistance Intensity

The intensity of resistance to pyrethroids and carbamate was assessed using WHO resistance intensity assays performed at 5x and 10x the diagnostic concentrations [20]. The insecticides tested included permethrin (3.75% and 7.5%), deltamethrin (0.25% and 0.5%), alpha-cypermethrin (0.25% and 0.5%), lambda-cyhalothrin

(0.25% and 0.5%), and bendiocarb (0.5% and 1%). Results were interpreted according to WHO criteria for resistance intensity [20]. Following the assays, dead mosquitoes were preserved individually in silica gel, while surviving mosquitoes were stored in RNALater® to maintain RNA integrity for subsequent molecular and genomic analyses. All samples were kept at −80˚C until processing.

2.6. Data Analysis

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, California USA) with significance set at 5% and confidence intervals (CI) calculated at 95%. Mortality results were interpreted according to WHO criteria (≥98%: susceptible; 90% - 97%: possible resistance; <90%: confirmed resistance). No additional statistical tests were applied, as WHO thresholds alone were considered sufficient for classification.

3. Results

3.1. Field Collection

Among mosquitoes collected indoors, a total of 600 females (F_0) were morphologically identified as members of the *Anopheles funestus* group. Subsequent molecular characterization confirmed that all identified specimens belonged to *Anopheles funestus* sensu stricto, which will hereafter be referred to as *An. funestus*.

3.2. WHO Susceptibility Tests

No mortality was recorded in either control group exposed to PBO-only papers or untreated papers, confirming the validity and reliability of the bioassays.

3.2.1. Phenotypic Resistance of *Anopheles funestus* to Standard Insecticide Concentrations

A total of 10,834 F_1 *Anopheles funestus* adults aged 3 - 5 days from the study sites were tested against different classes of insecticides at WHO diagnostic concentrations.

WHO susceptibility bioassays revealed varying levels of resistance across insecticide classes and study sites. Overall, *An. funestus* populations from Nioro and Ndoffane were resistant to pyrethroids, with mortality rates ranging from 46.7% to 88.3% (**Figure 2**).

Anopheles funestus populations were susceptible to DDT (100%) in Camara (Nioro) and Dielmo (Sokone), but resistance (88.3%, n = 103) to that organochlorine was found in Keur Mamadou (Nioro), whereas in Tawa Mboudaye (Ndoffane), suspected resistance (91.4%, n = 116) was observed.

Regarding organophosphates, *An. funestus* populations were fully susceptible to pirimiphos-methyl. However, resistance to fenitrothion was detected in Tawa Mboudaye and Keur Mamadou, with mortality rates of 86.4% (n = 103) and 76.1% (n = 109), respectively.

For carbamates, full susceptibility to bendiocarb was observed in Tawa Mboudaye and Dielmo, with mortality rates of 99.1% (n = 107) and 100% (n = 110), respec-

tively. In contrast, populations from Nioro were resistant, with mortality rates of 83.0% (n = 105) in Camara and 68.2% (n = 107) in Keur Mamadou (Figure 2).

Overall, *An. funestus* populations from central Senegal showed high levels of resistance to pyrethroids and carbamates, along with reduced susceptibility to organochlorines, while remaining fully susceptible to pirimiphos-methyl. These resistance patterns may be associated with local selection pressures, including agricultural insecticide use.

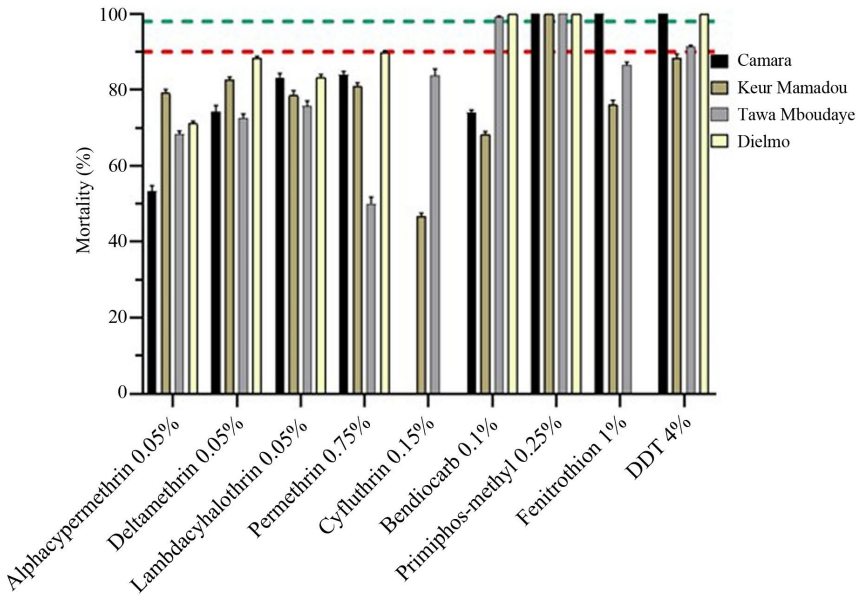

Figure 2. Susceptibility profile of F_1 mosquitoes across the four studied sites, showing mortality rates recorded 24 h post-exposure in *An. funestus* s.s. The red dotted line indicates resistance (90%) and the green dotted line indicates the susceptibility 98% threshold.

3.2.2. Resistance Intensity to Insecticides

Resistance intensity bioassays conducted using increased concentrations (5× and 10×) of alpha-cypermethrin, deltamethrin, lambda-cyhalothrin, permethrin, and bendiocarb revealed spatial variation in resistance levels across study sites, likely reflecting differences in local environmental conditions and insecticide use practices, particularly in agriculture. Overall, resistance intensity varied across study sites and insecticides, ranging from low to high levels. High intensity to pyrethroids was observed in some locations, while low to moderate intensity predominated for other compounds (Figure 3).

In Keur Mamadou, *An. funestus* populations exhibited low resistance intensity to alpha-cypermethrin and permethrin, moderate resistance intensity to deltamethrin, and high resistance intensity to lambda-cyhalothrin. In Camara, low resistance intensity was observed for bendiocarb, deltamethrin, and permethrin, whereas moderate to high resistance intensity was recorded for alpha-cypermethrin (5×) and lambda-cyhalothrin (5×), with mortality rates of 95.2% and 92.2%, respectively.

In the Ndoffane health district, high resistance intensity was detected in Tawa

Mboudaye, with approximately 85% mortality at 10× for alpha-cypermethrin. In the same locality, moderate resistance intensity was observed for deltamethrin, lambda-cyhalothrin, and permethrin, with complete mortality (100%) recorded at 5×. This resistance profile may reflect selective pressure associated with the intensive use of agricultural insecticides in this horticultural area. In Dielmo, resistance intensity was low for lambda-cyhalothrin and moderate for alpha-cypermethrin (Figure 3). Overall, resistance intensity varied according to study sites and insecticides tested, ranging from low to high levels. High-intensity resistance to pyrethroids was observed in some locations, whereas low to moderate resistance intensity predominated for other insecticides (Figure 3).

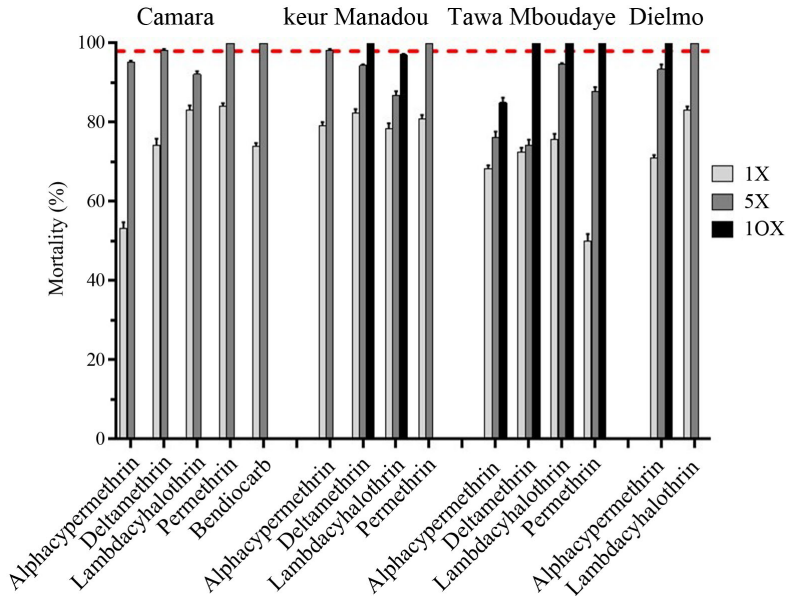

Figure 3. Resistance intensity profiles of resistance *Anopheles funestus* populations to pyrethroids and bendiocarb. Red dotted line indicates the threshold of 98% mortality.

3.2.3. Bioassays, Synergist PBO (Piperonyl Butoxide)

Synergist assays using piperonyl butoxide (PBO) 4% were conducted to investigate the potential involvement of cytochrome P450 monooxygenases in the observed resistance to pyrethroids, bendiocarb, and DDT across the study sites.

Pre-exposure of *An. funestus* s.s. to PBO resulted in a complete restoration of susceptibility to type I pyrethroids (permethrin and cyfluthrin), bendiocarb, and DDT ($p < 0.001$), indicating a predominant role of cytochrome P450 monooxygenases in metabolic resistance in these populations.

In contrast, a partial recovery of susceptibility was observed for type II pyrethroids (alpha-cypermethrin, deltamethrin, and lambda-cyhalothrin) following PBO pre-exposure ($p < 0.001$) (Table 1). This partial restoration suggests that, in addition to cytochrome P450 monooxygenases, other resistance mechanisms may contribute to the observed phenotypic resistance.

No mortality was recorded in the control groups (PBO-only exposure and untreated papers), confirming the validity of the bioassays.

Table 1. Effect of PBO pre-exposure on the susceptibility profile of mosquitoes to pyrethroids, bendiocarb, and DDT.

Localities	Insecticide one time dose	Insecticide only mortality rate (%)	$CI_{95\%}$	N	PBO + insecticide mortality rate (%)	$CI_{95\%}$	N
Camara	Alphacypermethrin	51.8	(40.5 - 63.1)	81	80	(69.6 - 88.1)	80
	Deltamethrin	72.1	(61 - 81.6)	79	92.6	(84.4 - 97.2)	81
	Lambdacyhalothrin	83.9	(74.1 - 91.2)	81	90.1	(81.5 - 95.6)	81
	Permethrin	83.1	(72.9 - 90.7)	77	100	(95.4 - 100)	78
	Bendiocarb	81.5	(71.3 - 89.2)	81	100	(95.6 - 100)	82
Keur Mamadou	Alphacypermethrin	81	(70.6 - 89)	79	94	(86.5 - 98)	83
	Deltamethrin	80.3	(69.5 - 88.5)	76	96.2	(83.3 - 99.2)	79
	Lambdacyhalothrin	78.5	(69.5 - 85.8)	107	93	(86.1 - 97.1)	100
	Permethrin	79.2	(68.5 - 87.6)	77	100	(95.5 - 100)	80
	Cyfluthrin	46.7	(36.9 - 56.6)	105	92.3	(85.4 - 96.6)	104
	DDT	86.1	(76.4 - 92.8)	79	100	(95.4 - 100)	78
	Bendiocarb	42.8	(31.6 - 54.6)	77	98.8	(93.4 - 99.9)	82
Tawa Mboudaye	Alphacypermethrin	77.6	(66.6 - 86.4)	76	94.8	(87.2 - 98.6)	77
	Deltamethrin	70.4	(59.2 - 80)	81	96.1	(89.2 - 99.2)	78
	Lambdacyhalothrin	71.6	(60.5 - 81.1)	81	84.5	(74.9 - 91.5)	84
	Permethrin	48.2	(37.1 - 59.4)	83	100	(95.4 - 100)	79
	Cyfluthrin	80	(69.9 - 87.9)	85	100	(95.7 - 100)	84
Dielmo	Alphacypermethrin	66.7	(55.1 - 76.9)	78	84.2	(74 - 91.6)	76
	Deltamethrin	83.13	(73.3 - 90.4)	83	89.6	(80.5 - 95.4)	77
	Lambdacyhalothrin	84.1	(74.3 - 91.3)	82	92	(83.4 - 97)	75
	Permethrin	92.5	(84.4 - 97.2)	80	100	(95.3 - 100)	77

N: number of mosquitoes exposed, CI: confidence interval, and PBO: piperonyl butoxide.

4. Discussion

The WHO Global Plan for Insecticide Resistance Management underscores the importance of continuous monitoring of insecticide resistance and characterization of underlying mechanisms to preserve progress achieved in malaria control [21]. In this context, the present study represents the first comprehensive evaluation of insecticide resistance patterns *in An. funestus s.s.* populations from central Senegal.

Our findings reveal widespread resistance to multiple classes of insecticides, including pyrethroids, carbamates, organophosphates, and organochlorines (DDT). The comparable resistance profiles observed across Nioro, Ndoffane, and Sokone districts suggest that multiple insecticide resistance is broadly distributed among *An. funestus* natural populations across central Senegal. This situation is particularly concerning given that pyrethroids remain the principal class of insecticides used in long-lasting insecticidal nets (LLINs), including both PBO-based and dual

active ingredient (AI) nets.

Resistance intensity assays further demonstrated predominantly moderate to high levels of resistance to pyrethroids across study sites, consistent with previous reports from northern Senegal [7] and other regions of Africa, including West, Central, and Southern Africa [7] [12] [22]-[27]. Notably, exposure to type II pyrethroids (alpha-cypermethrin, deltamethrin, and lambda-cyhalothrin) showed higher resistance intensity than type I pyrethroids (permethrin), a pattern also reported elsewhere [7] [13] [28]. This variation may reflect differences in the specificity and expression levels of metabolic enzymes, particularly cytochrome P450 monooxygenases, which are known to metabolize pyrethroid subclasses differently [29].

Synergist bioassays using PBO provided additional evidence on the mechanisms underlying insecticide resistance among natural populations of *An. funestus* in the study area. The complete restoration of susceptibility to permethrin, cyfluthrin, bendiocarb, and DDT following pre-exposure to PBO indicates a predominant role of cytochrome P450-mediated metabolic resistance in these studied wild *An. funestus* populations. In contrast, only partial restoration of susceptibility was observed for type II pyrethroids, indicating that additional mechanisms, such as the involvement of other detoxification enzymes or target-site mutations, may also contribute to the observed resistance phenotype. These findings are consistent with previous studies highlighting the major role of P450 enzymes in *An. funestus* resistance across Africa [22] [27]. The high levels of resistance observed in *An. funestus* s.s. populations are likely driven by multiple selective pressures. The widespread use of LLINs, which primarily rely on pyrethroids, is widely recognized as a major factor contributing to the selection of resistance [5] [27] [28]. In addition, agricultural practices in the study areas may play a substantial role in resistance development. The application of insecticides, including lambda-cyhalothrin-based products, organophosphates such as dimethoate, diazinon, and organochlorines such as endosulfan, may lead to contamination of mosquito breeding sites through runoff, thereby maintaining continuous selection pressure on vector populations. Similar associations between agricultural insecticide use and the selection of insecticide resistance among malaria vectors have been reported from several African settings [30]-[34]. Moreover, the domestic use of insecticides and informal vector control practices may further exacerbate this selection pressure.

The detection of bendiocarb resistance in Keur Mamadou and Camara in the Nioro health district is of particular concern, given its importance as an alternative insecticide for IRS in areas with high pyrethroid resistance. Although bendiocarb has demonstrated effectiveness in malaria control programs in Senegal and other endemic settings [35]-[37], the emergence of resistance may compromise its long-term operational effectiveness. In contrast, the full susceptibility observed to pirimiphos-methyl suggests that organophosphates may remain effective for IRS-based interventions in the study area. However, the detection of resistance to

fenitrothion in some localities underscores the importance of careful insecticide selection and continuous resistance monitoring.

At the molecular level, previous studies conducted in Senegal have reported overexpression of cytochrome P450 genes, including CYP6M7 and CYP6Z1, in association with resistance to pyrethroids and carbamate [7] [38]. Our results are consistent with these findings and further support the dominant role of metabolic resistance in *An. funestus* wild populations. Nevertheless, the incomplete restoration of susceptibility observed for type II pyrethroids indicates that additional resistance mechanisms may be involved, highlighting the need for further investigations to better characterize the full range of mechanisms contributing to resistance. A limitation of this study is that resistance mechanisms were inferred from bioassays and synergist tests, without direct gene expression or target-site data.

5. Conclusion

This study provides the first comprehensive assessment of susceptibility and resistance to public health insecticides in *An. funestus* natural populations from the study site in central Senegal. The results reveal moderate to high levels of resistance to pyrethroids, together with emerging resistance to carbamates and organochlorines, predominantly driven by metabolic mechanisms involving cytochrome P450 monooxygenases. These findings raise concerns regarding the sustainability of current insecticide-based vector control interventions, particularly LLINs that primarily depend on pyrethroids. The deployment of next-generation vector control tools, such as PBO-based or dual-insecticide LLINs, should therefore be considered to improve vector control intervention efficacy in these areas. In addition, strengthening insecticide resistance monitoring, together with comprehensive characterization of resistance mechanisms and their geographical distribution range, will be critical to inform evidence-based resistance management strategies and sustain malaria vector control efforts in Senegal.

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Author Contributions

BS conceived, designed, and supervised the study. EAN, SD, ID, and LK contributed to supervision. MBF, BMS, AFD, MF, and OT carried out the field collection and performed the laboratory analysis. MBF and BS analyzed the data and wrote the manuscript. AKD contributed to the draft of the manuscript. All authors have read, edited, and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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