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RESEARCH ARTICLE

Uncovering pyrethroid resistance mechanisms in *Anopheles punctulatus* group mosquitoes: A novel insight from Keerom, Papua, Indonesia

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ABSTRACT

Background and Aim: Malaria control programs in Indonesia heavily rely on insecticide-based interventions such as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS). However, the effectiveness of these strategies is increasingly compromised by the emergence of insecticide resistance, particularly in high-transmission regions like Papua Province. The mechanisms underlying pyrethroid resistance in local vector populations remain inadequately characterized. This study aimed to assess the permethrin resistance status and characterize the frequency of L1014F/S knockdown resistance (*kdr*) alleles in *Anopheles punctulatus* group mosquitoes in Keerom, Papua, to inform region-specific malaria vector control strategies.

Materials and Methods: An entomological and molecular investigation was conducted in five villages across three districts of Keerom Regency. Larval and adult *Anopheles* mosquitoes were collected through standard World Health Organization techniques. Insecticide susceptibility was evaluated using 0.75% permethrin bioassays. Molecular identification and detection of *kdr* mutations (L1014F/S) were performed using species-specific polymerase chain reaction and sequencing protocols. Allele frequencies were analyzed using Hardy–Weinberg equilibrium models, and statistical comparisons were made using one-way analysis of variance.

Results: Among 163 mosquitoes tested, permethrin resistance was confirmed across all districts, with mortality rates ranging from 50% to 68.66%. Molecular analyses identified *Anopheles koliensis*, *A. punctulatus*, and *A. peditaeniatus*. High frequencies of the L1014S mutation were detected in *A. koliensis* (0.87) and *A. punctulatus* (0.66), whereas *A. peditaeniatus* exhibited only wild-type alleles. No L1014F mutations were observed. The high prevalence of homozygous resistant genotypes indicates intense selection pressure, potentially linked to LLIN and agricultural insecticide use.

Conclusions: This study provides the first molecular evidence of widespread permethrin resistance mediated by L1014S mutations in the *A. punctulatus* group in Papua. These findings underscore the urgent need for enhanced resistance monitoring and the integration of alternative insecticides and non-chemical vector control methods to sustain malaria control efforts in the region.

Keywords: *Anopheles punctulatus*, *knockdown resistance*, L1014S, malaria vector control, Papua, pyrethroid resistance.

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INTRODUCTION

Malaria remains a major global public health concern, with Indonesia reporting 443,530 clinical cases in 2022, of which 89% were concentrated in Papua Province. This underscores a significant gap in the understanding of localized vector resistance mechanisms [1, 2]. Notably, South Sorong was the only regency among 43 (2%) in Papua declared malaria-free, whereas Keerom exhibited a high annual parasite incidence of 507.75 in the same year. Although the *Anopheles punctulatus* group is recognized as the predominant malaria vector in Papua, specific resistance mechanisms – particularly those involving knockdown resistance (*kdr*) mutations – remain under-investigated, potentially undermining the effectiveness of pyrethroid-based control strategies. The *Punctulatus* group comprises 13 recognized species, eight of which belong to the *Anopheles farauti* complex [3, 4]. The existence of such species complexes complicates accurate identification due to morphological similarities. The group exhibits heterogeneous malaria transmission patterns influenced by climatic conditions, anthropophilic behavior, and vector longevity. In addition, *Anopheles* larvae occupy diverse aquatic habitats that vary in size, permanence, vegetation cover, and water quality [5].

In the absence of a fully effective malaria vaccine, vector control strategies primarily depend on long-lasting insecticide-treated nets (LLINs) treated with pyrethroids and indoor residual spraying (IRS) using bendiocarb, a carbamate insecticide [6]. Organophosphates are commonly employed as larvicides to target immature mosquito stages [7]. Pyrethroids remain the insecticide class of choice for adult mosquito control due to their potent knockdown effect, prolonged residual activity, repellent properties, and low toxicity to mammals [8]. Globally, pyrethroids have become the most rapidly expanding class of insecticides, especially in agriculture. However, excessive reliance on them has contributed to the selection of resistant mosquito populations, thereby diminishing their utility in malaria control. Resistance to insecticides has become a growing global concern, jeopardizing the success of existing vector control measures [9–11].

Mosquito resistance generally arises through two primary mechanisms: Metabolic resistance and target-site resistance involving mutations at insecticide-binding sites. *Kdr*, which involves point mutations in the voltage-gated sodium channel (*VGSC*) gene, underlies resistance to both DDT and pyrethroid compounds [12, 13]. The amino acid substitutions L1014F (leucine to phenylalanine) and L1014S (leucine to serine) are strongly associated with *kdr*-mediated resistance in *Anopheles* species [13, 14]. In Indonesia, *kdr* alleles have been identified in *Anopheles sundaicus*, *Anopheles aconitus*, *Anopheles subpictus*, *Anopheles vagus*, *Anopheles tesselatus*, *Anopheles annularis*, and *Anopheles flavirostris* – species which inhabit diverse ecological zones, including coastal, lowland, and upland regions [15–17].

Despite the substantial burden of malaria in Papua Province, where nearly 90% of Indonesia's cases were reported in 2022 [1, 2], limited research has focused on the molecular mechanisms underlying insecticide resistance in local *Anopheles* populations. While the *A. punctulatus* group is recognized as the primary malaria vector in this region, the extent and distribution of *kdr* mutations – particularly L1014F and L1014S – remain inadequately characterized. Most previous studies have concentrated on morphological identification and entomological surveillance, with few incorporating molecular diagnostics to detect target-site mutations. Furthermore, the coexistence of morphologically similar sibling species within the *Punctulatus* group complicates vector identification and hinders effective resistance monitoring. Given the reliance on pyrethroid-based interventions such as LLINs and IRS, the absence of region-specific resistance data poses a major obstacle to designing targeted and sustainable vector control strategies.

This study aimed to investigate the status of permethrin resistance and determine the prevalence of L1014F/S *kdr* alleles in *A. punctulatus* group mosquitoes from Keerom Regency, Papua Province, Indonesia. By integrating entomological sampling with molecular diagnostics, the study seeks to provide critical evidence on species composition, resistance phenotypes, and *kdr* genotype distributions. The findings are intended to support the refinement of malaria vector control programs by informing the selection of effective insecticides and promoting the implementation of resistance management strategies tailored to the local vector population.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Ethics Committee of Research in Health, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia (No. 265/UN4.6.4.5.31/PP36/2023).

Study period and location

A cross-sectional entomological and molecular investigation was conducted between May and July 2023 in five villages across three districts of Keerom Regency, Papua Province: Sanggaria and Yatuharja (Arso Barat District); Sawanawa and Ubiyau (Arso District); and Pitewi (Arso Timur District). This study integrated field mosquito collection with laboratory analyses. Sanggaria, Yatuharja, and Pitewi are migrant residential areas situated near palm oil plantations, whereas Sawanawa and Ubiyau are inhabited by indigenous Papuans (Orang Asli Papua, OAP) and are located adjacent to primary forests and partially deforested areas (Figure 1) [18]. Malaria transmission in this region

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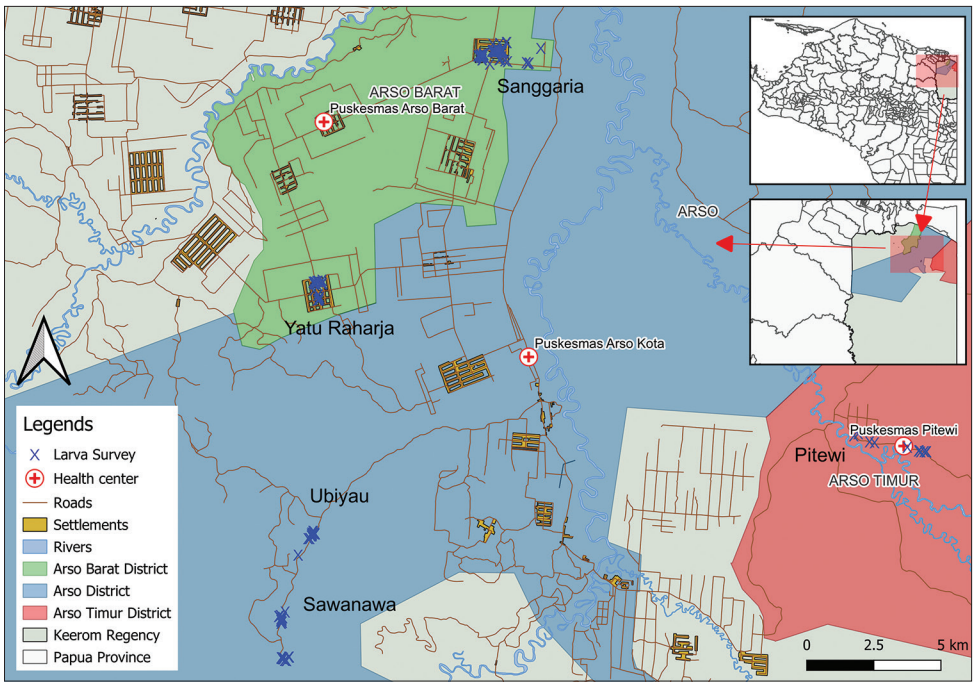

Figure 1: Map of the study area. The blue sign indicates the sampling area (Source: http://tanahair.indonesia.go.id/portal-web).

is stable and highly endemic, with both LLIN and IRS interventions implemented in all study villages [2].

Mosquito collection and morphological identification

Larval and adult *Anopheles* mosquitoes were collected following standard World Health Organization (WHO) methodologies, including larval sampling and human landing catches (HLC) conducted from 18:00 to 04:00. The collected specimens were morphologically identified using standard identification keys for female *Anopheles* mosquitoes [19, 20] and preserved for subsequent molecular analysis.

Insecticide susceptibility tests

Susceptibility testing was carried out in accordance with the WHO guidelines using standardized test kits for adult mosquitoes. Permethrin-impregnated papers (0.75%) were obtained from the Vector Control Research Unit, Universiti Sains Malaysia. A total of 163 female *Anopheles* mosquitoes collected through HLC were exposed to the insecticide-treated papers for 1 h. Control groups were exposed to untreated papers. Knockdown was recorded at 5-min intervals during exposure. Mosquitoes were then transferred to holding tubes and provided with a 10% sugar solution. Mortality was recorded 24 h post-exposure. Test results were discarded if control mortality exceeded 20% [21]. Dead and surviving mosquitoes were stored separately in 1.5 mL tubes containing silica gel and preserved at –20°C for molecular analysis.

DNA extraction

Genomic DNA was extracted from individual mosquitoes using a 2% Cetyltrimethyl Ammonium Bromide solution, following previously established protocols by Irfan *et al.* [22]. Extracted DNA was stored at 4°C for use in *kdr* allele amplification and molecular species identification.

Detection of the *kdr* allele mutation

Allele-specific polymerase chain reaction (PCR) was conducted on individual DNA samples to detect L1014F/S *kdr* mutations using a modified version of the standard protocol [16]. The primers AgFkdr (5′-GACCATGATCTGCCAAGATGGAAT-3′), AgRkdr (5′-GCAAGGCTAAGAAAAGGTTAAGCA-3′), and AnKdr (5′-GAGGATGAACCGAAATTGGAC-3′) were used in a semi-nested format. PCR reactions were performed in a total volume of 25 µL, comprising 12.5 µL MyTaq HS Red Mix, 0.25 µL of both forward and reverse primers at 20 pmol concentration, 0.2 µL of template DNA, and 11.8 µL of molecular grade water. PCR cycling conditions included an initial denaturation at 95°C for 1 min, followed by 29 cycles of denaturation at 95°C for 15 s, annealing at 51°C for 15 s, and extension at 72°C for 5 s, with a final extension at 72°C for 5 min. Amplification products were confirmed by Sanger sequencing to validate the presence of *kdr* mutations.

Molecular identification of the *A. punctulatus* group

Approximately 600–800 base pairs of the internal transcribed spacer 2 (ITS2) region were amplified using the primers ITS2F (5′-TGTGAACTGCAGGACACAT-3′) and ITS2-R (5′-TATGCTTAAATTCAGGGGGT-3′) [23, 24]. PCR amplification was conducted in a 25 µL reaction volume under the following conditions: Initial denaturation at 94°C for 5 min; 40 cycles of 94°C for 1 s, 53°C for 1 s, and 72°C for 2 min; followed by a final extension at 72°C for 7 min. PCR products were visualized using 2% agarose gel electrophoresis and subsequently sequenced.

Statistical analysis

Mosquito resistance status was determined following the WHO criteria [21]. Populations with ≥98% mortality were classified as fully susceptible; those with 90%–98% mortality were considered suspected resistant; and those with ≤90% mortality were categorized as resistant. A combination of statistical analyses was employed: One-way analysis of variance was used to compare mortality rates across districts, while Hardy–Weinberg equilibrium analysis was applied to assess *kdr* allele frequencies and infer resistance dynamics at the population level [25].

RESULTS

Mosquito collection and morphological identification

Larval surveys indicated that ditches located in front of houses were the most common breeding sites across all three districts. Other identified breeding habitats included rain pools, wells, groundwater sources, artificial ponds, puddles, and open swamps. A total of 181 potential larval habitats were surveyed, with *Anopheles* larvae detected in 18.8% of them (Table 1).

Morphological and molecular identification of 163 adult *Anopheles* mosquitoes confirmed the species composition of the *Punctulatus* group as follows: *Anopheles* koliensis (68.44%), *A. punctulatus* (29.57%), and *Anopheles peditaeniatus* (1.99%).

Permethrin susceptibility status

Bioassays revealed substantial levels of permethrin resistance among *Anopheles* populations. The recorded mortality rates were 50% in Arso Timur, 60.71% in Arso, and 68.66% in Arso Barat (p = 0.208), indicating widespread resistance across the study sites (Table 2).

Species confirmation

A total of 51 *Anopheles* specimens, comprising both larvae and adults, underwent molecular species confirmation. The analysis identified *A. koliensis* (n = 30) and *A. punctulatus* (n = 8) with 98% sequence similarity to reference strains, along with the rare detection of *A. peditaeniatus* (n = 13), which showed greater than 95% similarity. These findings underscore the species diversity of the *Punctulatus* group in the region.

Kdr allele frequency

Among 135 representative *Anopheles* specimens (both larvae and adults), the L1014S (*kdr*-east) mutation was detected. Of these, 30 were homozygous for the susceptible wild-type allele (TTA/TTA), 96 were homozygous for the resistant allele (TCA/TCA), and 9 were heterozygous (TTA/TCA). The L1014S mutation was present in both *A. koliensis* and *A. punctulatus*, while only the wild-type genotype (TTG/TTG) was identified in *A. peditaeniatus* (Figure 2). The frequency of the L1014S allele was notably high, recorded at 0.87 in *A. koliensis* and 0.66 in *A. punctulatus*. No *kdr* mutation was found in *A. peditaeniatus*. This study provides the first documentation of L1014S at such high frequencies in Papua, suggesting a distinct evolutionary trajectory of resistance in local vector populations. The distribution of *kdr* genotypes and allele frequencies is presented in Table 3.

DISCUSSION

Understanding the extent of pyrethroid resistance among malaria vector species in Keerom offers critical insights into the efficacy of insecticide-based vector control strategies. This study contributes to clarifying the current status and underlying mechanisms of pyrethroid resistance while also enabling the assessment of potential risks associated with the emergence of localized resistance. To date, this is the first report providing molecular evidence of widespread permethrin resistance in the *Punctulatus* group in Keerom, highlighting the genetic basis of resistance through the detection of the L1014S mutation.

The *kdr* observed is attributed to target-site insensitivity caused by the L1014S mutation in the *VGSC* gene, which diminishes the binding affinity of pyrethroids, thereby compromising malaria control effectiveness. Among *Anopheles* species, the most frequently documented *kdr* mutation conferring resistance is L1014F, a leucine-to-phenylalanine substitution, which has been reported in *Anopheles gambiae*, *Anopheles arabiensis*, *Anopheles stephensi*, and *Anopheles sinensis* [10, 11, 26, 27]. In Indonesia, L1014F has been identified as the predominant mutation in South Lampung, while both L1014F and L1014S have been reported in Sumba [16, 17]. In contrast, only the L1014S mutation was detected in the *Punctulatus* group specimens analyzed in the present study. Both homozygous and heterozygous genotypes were observed in *A. punctulatus* and *A. koliensis*. The

Table 1: Anopheles larva habitat index at the study sites.

Habitat type	Study sites					Total (%)
	Sanggaria (%)	Yatuharja (%)	Sawanawa (%)	Ubiyau (%)	Pitewi (%)	
Pond/lake	20.0 (10)	0.0 (4)	28.6 (14)	66.7 (6)	0.0 (3)	27.0 (37)
Ditch/gutter	5.0 (40)	6.3 (16)	57.1 (7)	0.0 (4)	42.9 (7)	13.5 (74)
Seepage/spring/well	0.0 (6)	-	-	0.0 (3)	-	0.0 (9)
Rain pools	6.3 (16)	18.2 (11)	29.2 (24)	25.0 (4)	50.0 (2)	21.1 (57)
Stream margin	-	-	0.0 (1)	-	-	0.0 (1)
Swamp	66.7 (3)	-	-	-	-	66.7 (3)
Total	9.3 (75)	9.7 (31)	32.6 (46)	29.4 (17)	33.3 (12)	18.8 (181)

The numbers in brackets indicate the total number of water bodies

codon encoding L1014 is TTA, and the L1014S mutation results from a T>C substitution at the second nucleotide position of this codon. The *kdr* mutation frequency was notably high, with the majority of alleles found in the homozygous resistant form. These findings suggest that the elevated frequency of L1014S may be driven by selective pressure resulting from extensive pyrethroid use, as well as genetic drift within geographically isolated mosquito populations in Papua. Shifts in allele frequencies may also be influenced by changes in sibling species composition or varying levels of insecticide exposure [28–30].

The observed correlation between *kdr* genotype and phenotypic resistance within the *Punctulatus* group suggests similar exposure across mosquito populations, thereby exerting strong selection pressure favoring *kdr*. Since pyrethroid-treated LLINs remain the primary malaria intervention in Papua, a national mass distribution was implemented in Keerom by local village malaria cadres. A prior study by Rozi *et al.* [5] reported that 84% of households in Keerom possessed and regularly used LLINs [5]. Furthermore, the extensive application of pyrethroids in agriculture – particularly on palm oil plantations – as well as domestic insecticide usage, has likely contributed to the escalation of resistance levels [8, 31, 32].

The absence of *A. peditaeniatus* from adult mosquito collections and the lack of detectable resistance in this species may be explained by behavioral characteristics, such as a preference for outdoor feeding and zoophilic tendencies, which reduce contact with insecticide-treated nets [5, 33]. While this study provides robust evidence for the presence of the L1014S mutation in the dominant malaria vectors in Papua, it does not directly attribute the persistently high malaria incidence in Keerom to insecticide resistance alone. Other contributing factors must also be considered. Moreover, resistance to type I pyrethroids such as permethrin may not extend to type II pyrethroids (e.g., cypermethrin, deltamethrin, α-cyhalothrin) or mixed type I/II compounds (e.g., esfenvalerate, fenpropathrin) [34, 35]. Nonetheless, increasing pyrethroid resistance elevates the risk of malaria transmission. High frequencies of *kdr* alleles may compromise the efficacy of LLINs and IRS in regions where *Anopheles* species harbor such mutations [36–38]. Consequently, type II pyrethroids remain viable alternatives for use in LLINs and IRS interventions in Keerom.

Table 2: Mortality and susceptibility status of the *Punctulatus* group.

Districts	n[a]	% Mortality after 24 h[b]	% Control[c]	Status[d]
Arso Barat	67	68.66	90	R
Arso	56	60.71	81.82	R
Arso Timur	40	50	91.3	R

[a]Total sample size. [b]A mortality percentage of test mosquitoes. [c]Percentage of alive mosquitoes. [d]Confirmed resistance as determined by the WHO

Table 3: Genotype and allele frequency of *knockdown resistance* in the *Punctulatus* group.

Species	Total number of samples analyzed	Susceptible genotypes	L1014S genotypes		Allelic frequency	
		SS	RS	RR	S	R
Anopheles koliensis	94	8	8	78	0.13	0.87
Anopheles peditaeniatus	13	13	0	0	1	0
Anopheles punctulatus	28	9	1	18	0.34	0.66

SS=Susceptible, RS=Heterozygous resistance, RR=Homozygous resistance

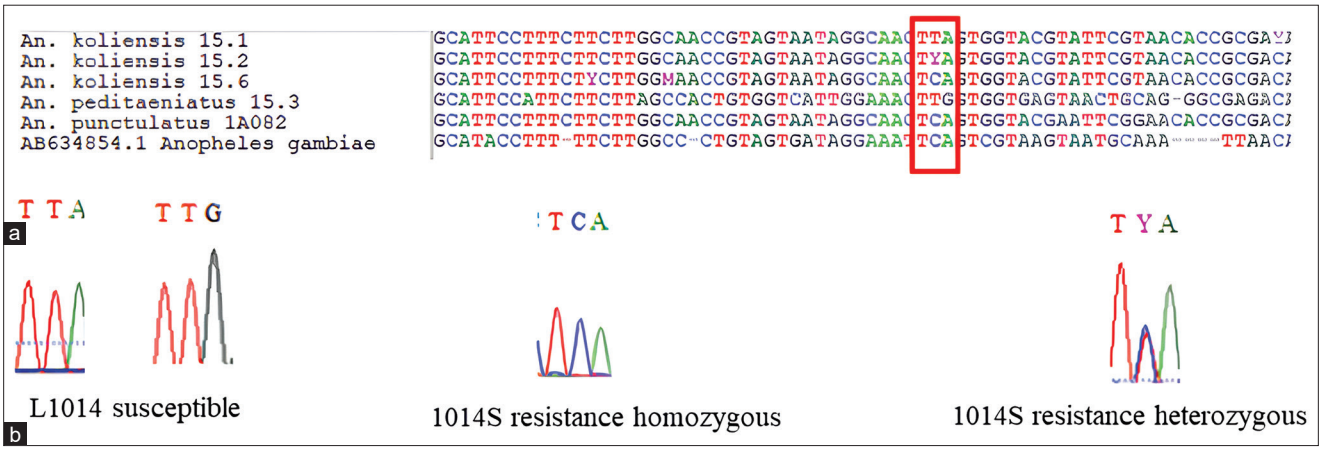

Figure 2: *Knockdown resistance* allele detected on segment 6 of the voltage-gated sodium channel (*VGSC*) gene of *Anopheles* spp. (a) DNA alignment of the *VGSC* genes *of Anopheles punctulatus, Anopheles koliensis, Anopheles peditaeniatus*, and *Anopheles gambiae* as sequence references. The red box encloses the polymorphic site (codon 1014). (b) Electropherograms of *dr* alleles in the *VGSC* gene of the *Punctulatus* group.

The findings of this study hold significant implications for malaria vector control strategies. There is a pressing need to strengthen fundamental research in insecticide resistance surveillance and elucidate resistance mechanisms to ensure informed decision-making. Future studies should include broader geographic sampling and incorporate additional insecticides to comprehensively map the distribution of *kdr* alleles and support the development of regionally tailored mosquito control programs.

CONCLUSION

This study provides the first molecular evidence of widespread permethrin resistance in the *A. punctulatus* group from Keerom Regency, Papua Province, Indonesia. Bioassays revealed confirmed resistance to 0.75% permethrin in all surveyed districts, with mortality rates below 70%. Molecular identification confirmed the predominance of *A. koliensis* and *A. punctulatus*, with the rare detection of *A. peditaeniatus*. Notably, a high frequency of the L1014S (*kdr*-east) mutation was detected in *A. koliensis* (0.87) and *A. punctulatus* (0.66), whereas no *kdr* mutation was found in *A. peditaeniatus*. These findings suggest an evolving resistance profile likely driven by selective pressure from prolonged and widespread pyrethroid use in both public health and agricultural settings.

The results highlight a critical challenge for malaria control programs in Papua, where LLINs and IRS using pyrethroids remain central components of vector management. The detection of high L1014S frequencies underscores the need for evidence-based rotation of insecticides and the strategic integration of non-pyrethroid or type II pyrethroid compounds to sustain intervention efficacy. Furthermore, the species-specific differences in resistance genotypes suggest that behavioral and ecological traits influence selection pressure and exposure.

A major strength of this study lies in its integration of entomological surveillance with molecular diagnostics, enabling precise species identification and detection of resistance mechanisms. This approach provides a more accurate resistance profile compared to morphology-based methods alone, particularly in regions with cryptic species complexes like the *Punctulatus* group.

However, the study is not without limitations. The sample size, while representative, was restricted to a single regency and limited time frame, potentially overlooking seasonal or spatial variations in resistance dynamics. In addition, the study focused solely on the L1014F/S mutations in the *VGSC* gene, without assessing other resistance mechanisms such as metabolic enzyme overexpression or cuticular resistance.

Future studies should aim to expand geographical and temporal coverage across Papua and neighboring regions to determine the full extent of resistance patterns. Integrating additional molecular markers, such as those involved in metabolic resistance (e.g., P450s, glutathione S-transferases, esterases), and conducting longitudinal monitoring will provide a more comprehensive understanding of resistance evolution. Moreover, the development and deployment of integrated vector management strategies – including larval source management, insecticide rotation, and non-chemical interventions – are essential to mitigate the impact of resistance and achieve sustained malaria control.

AUTHORS' CONTRIBUTIONS

PBAS, LS, and DS: Conceptualized the study and drafted the manuscript. LS, PBAS, AB, AD, and DS: Methodology. LS, DHP, IER, and II: Investigation. LS, PBAS, AB, AD, and DS: Edited the manuscript. DS, PBAS, AB, and AD: Supervised the study. All authors have read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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