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Susceptibility of the Punctulatus group (Diptera: Culicidae) to insecticide carbamate in Papua, Indonesia

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Abstract. *Syahrani L, Dwiranti A, Asih PBS, Rozi IE, Permana DH, Bowolaksono A, Syafruddin D. 2025. Susceptibility of the Punctulatus group (Diptera: Culicidae) to insecticide carbamate in Papua, Indonesia. Biodiversitas 26: 1376-1383.* Insecticide Treated Nets (ITNs) and Indoor Residual Spraying (IRS) are the primary vector control tools recommended for reducing malaria transmission in endemic areas. However, the emergence and spread of pyrethroid resistance among the *Anopheles punctulatus* group poses a significant challenge for malaria vector control in Papua, Indonesia. While carbamate-based IRS is a potential strategy to mitigate resistance, its effectiveness within malaria control programs requires continuous monitoring. This study assessed the susceptibility of the Punctulatus group to bendiocarb (0.1%) and analyzed the underlying resistance mechanisms. Bioassays were conducted in three sub-districts of Keerom District, Papua. Additionally, biochemical assays were performed to measure the activity of detoxifying enzymes, including esterases, Glutathione S-Transferases (GSTs), and acetylcholinesterase. Ace-1 fragments were amplified using Polymerase Chain Reaction (PCR), followed by sequencing to detect nucleotide substitution at codon 119. Results indicated full susceptibility (100%) of the Punctulatus group to bendiocarb at all study sites. Compared to unexposed controls, bendiocarb-exposed populations exhibited significantly higher GST (p: 1.403e-06), elevated esterase activity (p: 0.247), and reduced acetylcholinesterase inhibition (p: 0.002). PCR sequencing of 29 samples confirmed that all carried the wild-type *Ace-1* allele (GGG, GGC, and GGT). These findings provide crucial evidence for malaria control programs in selecting appropriate vector control products and managing insecticide resistance. Despite full susceptibility to bendiocarb, elevated detoxification enzyme activity suggests potential early-stage metabolic resistance. Further molecular studies are recommended to investigate metabolic resistance mechanisms, along with rigorous monitoring using bioassays, biochemical assays, and molecular tools to track insecticide susceptibility trends over time.

Keywords: Ace-1, *Anopheles*, carbamate, resistance

INTRODUCTION

Papua Province, located in eastern Indonesia, reports the highest malaria burden in the country, with an Annual Parasite Incidence (API) of 169.25 in 2023 (Indonesian Ministry of Health 2024). The primary malaria vector in this region belong to the Punctulatus group, which has been well documented across eastern Indonesia, including the Mollucas and Papua (Nurwidayati et al. 2024; Rozi et al. 2024), as well as extending throughout New Guinea Island and Solomon Islands (Beebe et al. 2015; McLaughlin et al. 2019). There are 13 *Anopheles* members in the Punctulatus group, includings *Anopheles punctulatus*; *An. koliensis*; *An. clowi*; *An. rennellensis* (formerly *An. sp. nr punctulatus*); *An. farauti*; *An. farauti* (formerly *An. farauti* 1); *An. hinesorum* (formerly *An. farauti* 2); *An. torresiensis* (formerly *An. farauti* 3); *An. farauti* 4; *An. farauti* 5; *An. oreios* (formerly *An. farauti* 6); *An. irenicus* (formerly *An. farauti* 7) and *An. farauti* 8 (https://wrbu.si.edu/timeline-farauti).

The Punctulatus group's resistance to Dichloro-Diphenyl-Trichloroethane (DDT) and reduced susceptibility to pyrethroid (Katusele et al. 2022) indicated that this species has developed mechanisms to resist pyrethroid, which are commonly used in malaria control programs. The potential cross-resistance between DDT and pyrethroid is a significant concern for malaria vector control in Papua, due to their similar modes of action. To mitigate this issue, carbamate-class insecticides offer a promising alternative due to their distinct mode of action compared to pyrethroids. Pyrethroids mainly target sodium channels, while carbamates inhibit acetylcholinesterase (Fang et al. 2019). Indonesia has implemented a nationwide malaria control strategy based on large-scale, integrated intervention, combining Insecticide Treated Nets (ITNs) treated with pyrethroid and Indoor Residual Spraying (IRS) using both pyrethroid and carbamate insecticide (WHO 2019). The use of carbamates in IRS is being considered as a key measure to counteract the growing threat of pyrethroid resistance and sustain malaria control efforts in

high-burden regions like Papua.

The widespread use of insecticide for public health and agricultural pest control has increased selection pressure, leading to physiological and behavioral insecticide resistance (Sternberg and Thomas 2017). The primary resistance mechanisms include metabolic detoxification and target-site insensitivity. Target site insensitivity occurs when an insecticide can no longer effectively bind to its target due to structural changes, altered accessibility, or binding site mutations. This can result from various mechanisms, including insensitive Acetylcholinesterase (AChE), Gamma-Aminobutyric Acid (GABA) receptor mutation, and Voltage-Gated Sodium Channel (VGSC) mutation (You et al. 2020; Keïta et al. 2021; Ahmed-Yusuf et al. 2021). In contrast, metabolic resistance involves the enzymatic detoxification of insecticides. Three major enzyme families are involved: glutathione S-transferases, esterases, and cytochrome P450 monooxygenases, are responsible for breaking down toxic substances (Balabanidou et al. 2016; Safi et al. 2017; Pavlidi et al. 2018). These detoxification processes include hydrolysis, reduction, oxidation, and further detoxification, facilitated by metabolic enzymes and ATP-Binding Cassette (ABC) transporters (Ye et al. 2020; Aioub and Ashour 2023). Studies have confirmed that insecticide resistance in *Anopheles* sp. is driven by a combination of enzymatic detoxification and target-site insensitivity mechanisms (Machani et al. 2020; Tchouakui et al. 2022).

Resistance to carbamate and organophosphate insecticides has been linked to mutation in the *Ace-1* gene, particularly the G119S substitution, which results in AChE insensitivity (Dahan-Moss and Koekemoer 2016). AChE, which hydrolyzes the neurotransmitter acetylcholine at synapses, becomes less effective in resistant mosquitoes due to this mutation. The G119 mutation has been identified in *An. gambiae* (Elanga-Ndille et al. 2019; Fagbohun et al. 2020), and *An. sinensis* (Feng et al. 2015). Additionally, overproduction of detoxification enzymes due to increased transcription has been observed in resistant *Anopheles* sp. population (Ingham and Nagi 2024). Although resistance to pyrethroid and organochlorine has been documented in several Indonesian districts (Asih et al. 2012; Syahrani et al. 2024), there is currently no data on susceptibility of the Punctulatus group to bendiocarb, a carbamate insecticide in Papua. Given that bendiocarb is still used for IRS in Papua, monitoring insecticide resistance is crucial for early detection and management of resistance in vector populations. Reliable data on resistance status and underlying mechanism are essential towards developing appropriate and targeted intervention strategies. This study aims to examine the susceptibility status, metabolic resistance mechanism, and distribution of the *Ace-1* mutation among the population of the Punctulatus group in Keerom, Papua Province, Indonesia.

MATERIALS AND METHODS

Ethical statements

The Ethics Committee of Research in Health, Medical Faculty of Universitas Hasanuddin, Makassar, Indonesia, approved this study, No. 265/UN4.6.4.5.31/ PP36 /2023.

Study site and mosquito sampling

The study was conducted between May and July 2023 in three sub-districts: Arso Barat, Arso, and Arso Timur, Keerom District, Papua Province, Indonesia, i.e. (i) Sanggaria and Yatuharja located at Arso Barat (2°47'46"S 140°45'28"E) Sub-district, (ii) Sawanawa and Ubiyau located at Arso Sub-district (2°59'21"S 140°41'17"E) and (iii) Pitewi located at Arso Timur Sub-district (2°55'54"S 140°53'47"E) (Figure 1).

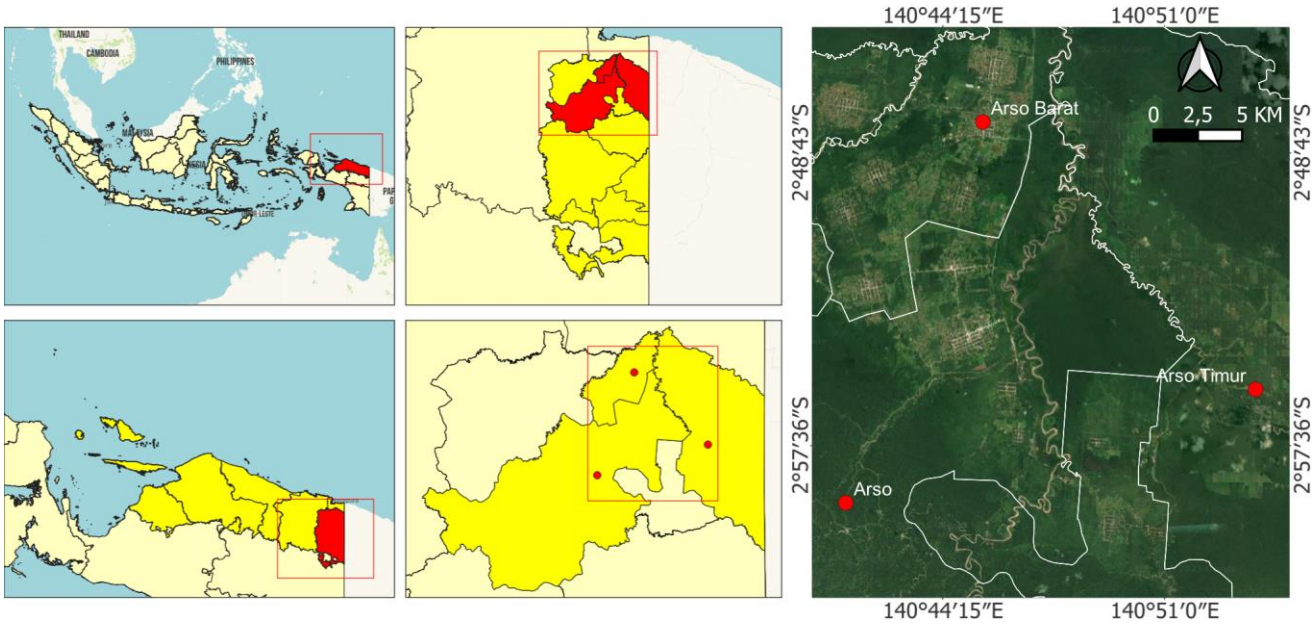

Figure 1. Location map of the study area in Keerom District, Papua Province, Indonesia, i.e.: (i) Sanggaria and Yatuharja located at Arso Barat Sub-district, (ii) Sawanawa and Ubiyau located at Arso Sub-district and (iii) Pitewi located at Arso Timur Sub-district

Malaria transmission in this area is both stable and high, with both ITNs and IRS being used in all villages. Mosquitoes were collected using both larval and adult collection. The dipping technique collected larvae in different breeding habitats, including ponds/lakes, ditchs/gutters, seepages, rain pools, stream margins, and swamps. Anopheles late-stage larvae were collected and transported in a labeled container to the field insectary. The larvae were allowed to emerge into adults, and the emerged adults were fed on 10% sucrose solution. Since the larval sites may not have provided enough larvae for the insecticide tests, adult mosquitoes were also collected through Human Landing Catch (HLC) and indoor resting collections. The adult collections were conducted in randomly selected houses across the community between 6:00 PM and 04.00 AM using aspirators. Captured mosquitoes were kept in paper cups and kept in a cool place before being transported to the laboratory.

Insecticide susceptibility bioassays

Insecticide susceptibility bioassays were performed using World Health Organization (WHO) standard procedures (WHO 2022). Bendiocarb insecticide-impregnated paper at a diagnostic concentration of 0.1% and control-impregnated paper is placed in the holding and control tubes. A total of 125 female Punctulatus group from the wild-type adult collection were exposed to insecticide-impregnated paper for 1 hour; likewise, female *Anopheles* sp. from wild-type larva reared were exposed to control-impregnated paper. The number of knocked-down mosquitoes were recorded during one hour of insecticide exposure. Further, the mosquitoes were transferred back to the holding tube with a piece of cotton wool soaked in a 10% sugar solution and maintained at 26±2°C and 80±10% relative humidity. Mortality rates were recorded 24 hours after exposure. WHO criteria was used to assess the resistance status (WHO 2022). After the assay, mosquito specimens were morphologically identified using taxonomic keys and combined with parallel ecologic and bionomic data to improve the accuracy of species identification. Some members of the Punctulatus group can be distinguished through different morphological characteristic such as type of proboscis (Rozeboom et al. 1946). Following the test, representative samples were maintained at -70 °C for metabolic enzyme analysis and *Ace-1* molecular detection.

Biochemical assays

The biochemical assay was performed according to the WHO protocol (WHO 1998) and commercial kit. The enzyme activity of GST, esterase, and inhibition rates of AChE were measured. A representative sample of individual adult mosquitoes were homogenized using a pestle in phosphate buffer and centrifuge at 3000 rpm at 4°C for 20 min; the resulting supernatant was used as the enzyme source.

Esterase assay

Alpha-naphthyl acetate (Sigma) was used as a substrate to assess esterase activity. With a micropipette, 20 µL of test samples homogenate were transferred into a 96 wells flat-bottom microplate, of 200 µL working solution (consists of 1mL naphthyl acetate in 99 mL of phosphate buffer) was added and followed by a 50 µL coupling reagent consists of fast blue b salt in sodium lauryl sulfate (SDS). Similarly, the control blank contains a working solution, coupling reagent and 20 µL ddH_2O. After incubating the mixtures at room temperature for 15 minutes, the absorbance was measured at 570 nm.

Glutathione S-Transferase (GST) assay

The GST measurement also followed assay kit procedures (MyBioSource, MBS9718984). Assay buffer, chromogen, and substrate working reagent were added to the test well and blank well. The homogenate samples were transferred to the test well, and ddH_2O was added to the blank well. The mixture was thoroughly mixed, and the absorbance value was measured at 340 nm at 10 seconds and 310 seconds.

Acetylcholinesterase assay

The acetylcholinesterase assay kit colorimetric (Abcam, ab138871) was used, and 50 uL of the acetylthiocholine reaction mixture was added to 50 uL of test samples. The reaction was incubated for 30 min at room temperature and protected from light. A specific acetylcholinesterase inhibitor, donepezil hydrochloride (ab 120763) was added to the test sample. The absorbance was measured at 410 nm.

Detection of Ace-1 allele and species confirmation

Deoxyribonucleic Acid (DNA) was extracted from individual adult mosquitoes using Chelex-100 (BioRad Laboratories, Hercules, CA, USA) with slight modifications to the protocol. The Polymerase Chain Reaction (PCR) diagnostic test was used to detect the presence of G119S mutation (Aïkpon et al. 2014). The reaction mixture was conducted on a total volume of 25 µL containing 40 pmol of each primer Ex3Agdir (5'GATCGTGGACACCGTGTTCG3') and Ex3Agrev (5'AGGATGGCCCGCTGGAACAG3'). The PCR conditions were an initial cycle at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 20 seconds, followed by a final extension at 72°C for 5 minutes. The PCR fragment was visualized on 2% agarose gel stained with floroSafe stain. PCR product amplicons were sequenced to confirm the presence of the G119S mutation. Molecular species confirmation was conducted using ITS2 barcoding (St. Laurent et al. 2016).

Data analysis

World Health Organization criteria (WHO 2022) were used to determine the resistance status of the mosquito population as follows: mortality rate is >98%: susceptible mosquito population; mortality rates ranged between 90-98%: suspected resistance in the mosquito population; mortality rates <90%: resistant mosquito population to the insecticide. Abbot's formula corrected mortality was conducted if the unexposed control mortality ≥5%.

$$\text{Corrected mortality (AI)} = \frac{(\%)\text{observed mortality} - \%\text{ control mortality}}{(100 - \%\text{ control mortality})} \text{ X } 100$$

The enzyme activity was read in a microtitre plate reader (Multiskan Go), and the results data were directly extracted to Microsoft Excel for further analysis. Mean values of activity of each enzyme of unexposed control and bendiocarb exposed treatment populations were compared employing ANOVA or Kruskal-Wallis. Prior to these tests, normality test was performed to assess the distribution of the absorbance value data. The ClustalW program as implemented in BioEdit software, was used to align the multiple DNA sequence based on similarities and evolutionary relationship with the Anopheles strain susceptible and mutant exported from GenBank, National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genbank/) and was used as reference sequences. Hardy-Weinberg equilibrium for observed genotyped frequencies for S (susceptible) and R (resistant) alleles.

RESULTS AND DISCUSSION

Insecticide susceptibility

Table 1 displays the bioassay results after 24 hours. A standard dosage of 0.1% bendiocarb was administered to 125 females within Punctulatus groups from Arso Barat, Arso, and Arso Timur. The test sample needed to be corrected since the percentage of unexposed control mortality was higher than 5%. The mortality rate was 100% in all sites, indicated that the Punctulatus group was fully susceptible to bendiocarb at 0.1%.

The knockdown assessment, conducted over one hour, showed that the Punctulatus group population from Arso Kota experienced a faster knockdown than those from other study sites. In contrast, the Punctulatus group from Arso Timur had the slowest knockdown rate (Figure 2).

Biochemical assays

The samples from bioassay phenotypes were divided into two groups: unexposed (control) and bendiocarb exposed (treatment). The non-specific enzyme activity of GST and esterase in the control and susceptible populations were successfully measured. The mean activity of metabolic enzymes in the susceptible population were higher than the control (Figure 3). An active unit of GST activity, defined as 1 µL/L CDNB per mL per minute, was catalyzed to bind glutathione (GSH). The mean activity level in the control population was 0.001978 (U/mL), while in the susceptible population was 0.01087 (U/mL). Kruskal Wallis, a statistical non-parametric test on data with a non-normal distribution demonstrated that there were significant differences in GST activity between two populations (p-value: 1.403e-06).

At the same time, the mean activity of esterase, using alpha-naphthyl acetate substrate, was higher in the treatment population than the control population, 0.263 and 0.209 (U/mL), respectively. The statistical analysis, ANOVA of data with normal distribution indicated the differences in mean activity between the two populations were not statistically significant (p-value: 0.247).

Table 1. Mortality percentage and susceptibility status of bendiocarb 0.1%

Location	n	% mortality after 24 h	% control	Corrected mortality	Status
Arso Barat	37	100	100	100	S
Arso	54	100	93.3	100	S
Arso Timur	34	100	91.3	100	S

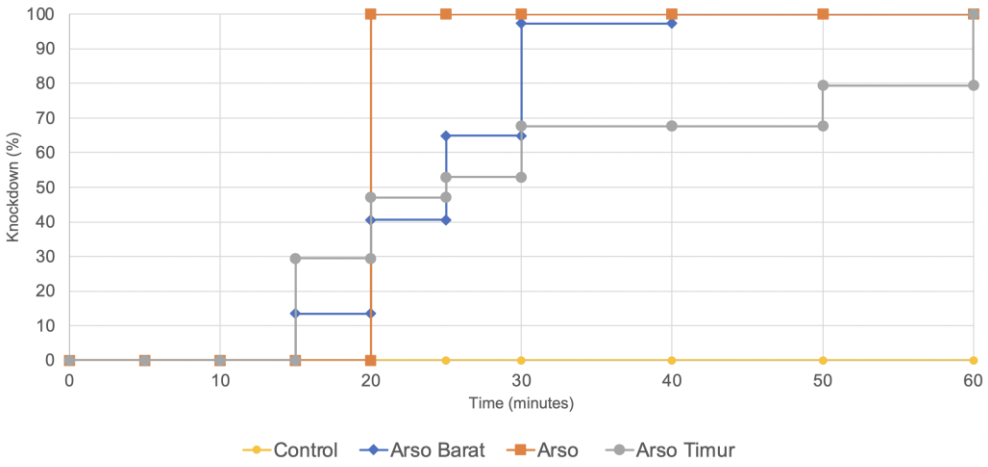

Figure 2. Knockdown (%) and knockdown times (in minutes) profile of the Punctulatus group in the study area. The knockdown percentage of control population was 0 during observation

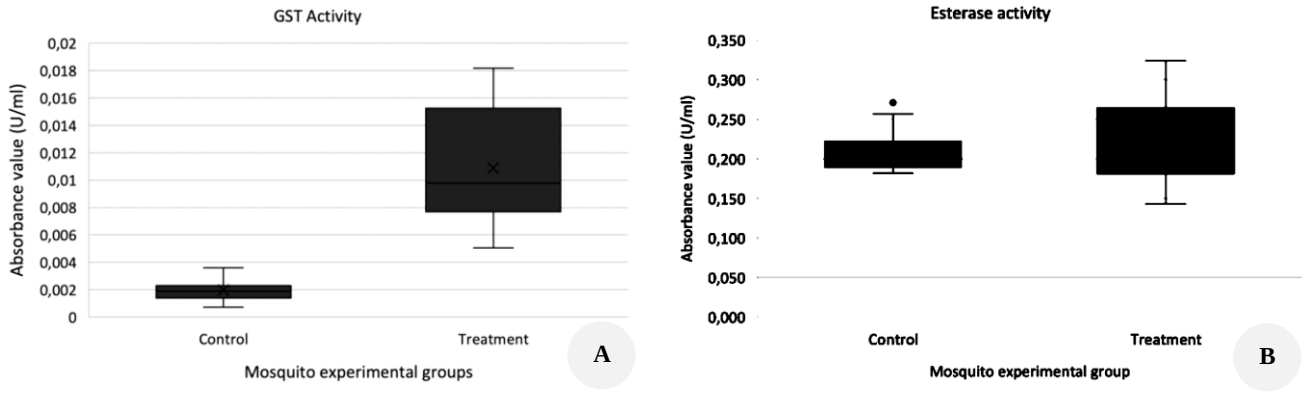

Figure 3. Detoxifying enzymes of (A) GST activity and (B) Esterase activity in control and treatment populations. The mean of GST and esterase activity in treatment was higher than control population. The error bar was represented minimum and maximum absorbance value, the asterisks indicated outlier values

Insensitive acetylcholinesterase

The mean activity of AChE was higher in the control and treatment groups-without inhibitor than with inhibitor groups. (Figure 4). Post-inhibition of donepezil shows that the activity of AChE decreased in both the control and treatment population. Moreover, ANOVA on data with normal distribution revealed the activity of AChE in the control population was reduced but not statistically significant (p-value: 0.630). However, AChE activity was significantly decreased in the treatment group, according to statistical tests employing Kruskal Walis on data with non-normal distribution (p-value: 0.002).

Genotypes and the Ace-1 allele frequencies

The 510 bp DNA fragment for *Ace-1* was successfully amplified. The sequence alignment (Figure 5) shows the G119 wild type appeared in three alleles, GGG, GGC, and GGT, which were homozygous. The detection of the G119S *Ace-1* allele in 29 samples revealed that the mutation was absent (Table 2). Using ITS2 barcoding, species confirmation of 11 samples revealed a 99.7% similarity to *An. koliensis* (Accession OR289964.1).

Discussion

Information on the resistance status of the primary malaria vectors in Keerom is crucial for guiding insecticide selection by The National Malaria Control Programme. Given the widespread pyrethroid resistance in Keerom, it is essential to monitor carbamate susceptibility, as carbamates are the standard insecticide used in the IRS program. Bendiocarb, which has a different mode of action from pyrethroids, has been the insecticide choice for IRS in Keerom. This study found that Punctulatus group populations collected from three districts in Keerom were fully susceptible to 0.1% bendiocarb. This is the first report of bendiocarb susceptibility assay in Keerom, Papua. The study also examined potential resistance mechanisms by assessing the frequency of the *Ace-1* allele (insensitive acetylcholinesterase), as well as GST and esterase metabolic enzyme activity.

Differences in metabolic enzyme groups activities between control and treatment populations indicate the involvement of metabolic enzymes in insecticide resistance mechanisms. Increased GST, esterase, and acetylcholinesterase activities indicate overproduction of metabolic enzymes in bendiocarb-exposed populations. In many cases, resistance has been attributed to increases in the amount of one or more metabolic enzymes, either due to gene amplification or, more commonly, through increases in transcriptional rate (Ndo et al. 2019; Adedeji et al. 2020).

Acetylcholinesterase inhibitors or anti-cholinesterases inhibit acetylcholine breaks down or hydrolyzes (Ach). Donepezil was used in this study as a reversible cholinesterase inhibitor (resembling carbamate action), inhibiting AChE activity, maintaining Ach level by decreasing its breakdown rate, and accumulating in the brain and skeleton (Luo et al. 2021). In this study, the AChE reduction as a results of donepezil inhibition revealed the effectiveness of donepezil or carbamate in control and treatment population. In response to insecticide exposure, mosquitoes overproduce critical detoxification enzymes to enhance their survival while conserving energy by reducing the production of less essential enzymes.

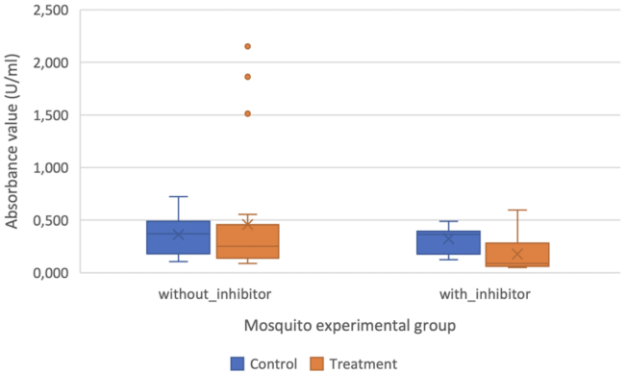

Figure 4. AChE enzyme activity in control and treatment population. Donepezil inhibiton in the control and treatment groups showed a decrease in AChE activity. The error bar was represented minimum and maximum absorbance value, the asterisks indicated outlier values

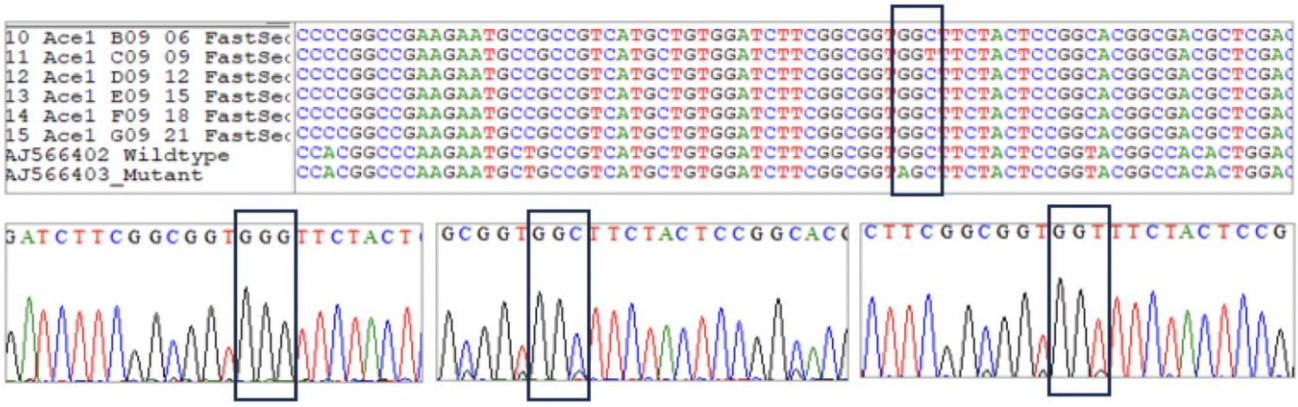

Figure 5. DNA alignment of the Ace-1 gene of the Punctulatus group compared to sequence reference; and electropherogram for Ace-1 wild-type alleles

Table 2. G119S frequency in the Punctulatus group examined exposed to bendiocarb 0.1%

Phenotype	Total of sample analysed	Genotype G119S				Allelic frequency	
		Susceptible			Resistant	S	R
Susceptible	29	GGG	GGC	GGT	AGC	1	0
		3	13	13	0		

Note: S: Susceptible; R: Resistant

A study from a resistant bendiocarb population demonstrated the order of importance of metabolic enzymes, esterase followed by GST as a secondary role in carbamate resistance and then P450 (Adhikari and Khanikor 2021; Ahmed-Yusuf et al. 2021). Since insecticide resistance mechanisms involving metabolic changes and enzyme threshold levels remain unclear due to numerous confounding factors, identifying molecular markers for metabolic resistance is crucial. Over-expressed in Rb-S/C-S, cytochrome P450 genes (cyp6z3, cyp12f2, cyp6m3, and cyp6m4) and one glutathione S-transferase (gstms3) are possibly linked with resistance to bendiocarb (Antonio-Nkondjio et al. 2016; Sandeu et al. 2020).

At the same time, the role of the *Ace-1* mutation in conferring bendiocarb resistance was assessed, and the nucleotide substitution causing G to S amino acid change at codon 119 was not found in the Punctulatus group examined. These results support bioassay susceptibility. Molecular assay performed on the Punctulatus group from the study area showed that the homozygous wild type was present in all susceptible genotypes (SS). According to a study on *An. gambiae* resistant to bendiocarb in Mali, the resistant allele of *Ace-1* consistently correlated with mosquito survivors following bendiocarb exposure. The susceptible population never detected the mutation (Keïta et al. 2021). Many studies have reported that in areas where *Ace-1* resistance is present, the heterozygous allele is the predominant, while homozygous resistant individuals are more likely to die during pupation (Keïta et al. 2021; Sy et al. 2021). In addition, mosquitoes with *Ace-1* resistance gene have a smaller body size than wildtype (Djogbénou et al. 2010).

Due to insufficient IRS coverage with bendiocarb compound, and uneven implementation in Keerom, along with a limited impact on adult mosquitoes (12.5% potential IRS impact) (Rozi et al. 2024), the Punctulatus group, particularly *An. koliensis*, has remained susceptible to bendiocarb. The Punctulatus group's low indoor resting behavior (2.1/night) is likely to result in lower selection pressure to the insecticide. Another factor is that agricultural use of carbamate cannot be linked to selection for carbamate resistance; this is solely because the study areas were non-agricultural.

Studies on bendiocarb in *Anopheles* sp. are rarely carried out (Keïta et al. 2021; Ahmed-Yusuf et al. 2021). As a carbamate class of insecticide widely used for public health programs, it is necessary to evaluate and monitor the resistance status of this insecticide so that concerned stakeholders can make informed and evidence-based decisions on vector control. In conclusion, bendiocarb is still effective against the Punctulatus group in Keerom. Elevated detoxification enzyme activities indicated that the Punctulatus group metabolic enzyme susceptibility has decreased. Molecular-based metabolic resistance analysis is advised, and rigorous monitoring of the susceptibility status by bioassay and biochemical and molecular assays is recommended.

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