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One Vector, Two Faces: Pyrethroid Resistance and Susceptibility to New-Generation Insecticides in *Anopheles gambiae* s.l. from Northern Benin

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Abstract

Background: The increasing resistance of malaria vectors to pyrethroids poses a major threat to the effectiveness of vector control interventions in sub-Saharan Africa. The introduction of new-generation insecticides, such as clothianidin and chlorfenapyr, offers a promising strategy for improving the control of resistant mosquito populations. This study aimed to assess the susceptibility status of *Anopheles gambiae* sensu lato populations to pyrethroids, clothianidin, and chlorfenapyr in northern Benin, while determining species composition and the frequency of the kdr L1014F mutation. **Methods:** Larvae of *Anopheles gambiae* s.l. were collected from the communes of Gogounou, Banikoara, and Boukombé between August and October 2024 and reared to adulthood under insectary conditions. Larvae were collected from all types of breeding sites encountered within each commune, without a systematic count of the number of sites sampled; larvae from the different breeding sites of a given commune were pooled prior to rearing and bioassays. Susceptibility to pyrethroids (deltamethrin and alpha-cypermethrin) was assessed using the World Health Organization (WHO) tube bioassay protocol, whereas susceptibility to clothianidin (4 µg/bottle) and chlorfenapyr (100 µg/mL) was evaluated using the Centers for Disease Control and Prevention (CDC) bottle bioassay method. Species within the *Anopheles gambiae* complex were identified

by polymerase chain reaction (PCR), and the kdr L1014F mutation was detected using allele-specific PCR. **Results:** High levels of pyrethroid resistance were observed in all three study sites, with mortality rates below 60% for all pyrethroids tested. In contrast, complete susceptibility to clothianidin was recorded, with 100% mortality 24 hours after exposure in all mosquito populations. Full susceptibility to chlorfenapyr was also observed, with 100% mortality achieved within 48 hours post-exposure. Of the 149 specimens analyzed, 68.45% were identified as *Anopheles gambiae* sensu stricto, 30.20% as *Anopheles coluzzii*, and 1.34% as *Anopheles arabiensis*. The kdr L1014F mutation was detected at high allelic frequencies, ranging from 83% to 95% among *A. gambiae* and *A. coluzzii*, and 75% in the small *A. arabiensis* subsample (n = 2; 95% CI: 19% - 99%) from Gogounou. **Conclusion:** *Anopheles gambiae* s.l. populations from northern Benin exhibit high levels of pyrethroid resistance associated with a high frequency of the kdr L1014F mutation. In contrast, clothianidin and chlorfenapyr remain highly effective against these resistant populations, supporting their use as valuable tools for malaria vector control. These findings highlight the importance of deploying new-generation insecticides while reinforcing continuous insecticide resistance surveillance to preserve their long-term effectiveness.

Keywords

Anopheles gambiae s.l., Pyrethroids, Clothianidin, Chlorfenapyr, Insecticide Resistance, kdr L1014F, Benin

1. Introduction

Vector control remains one of the cornerstones of malaria prevention, alongside early diagnosis and effective case management. It relies primarily on the large-scale deployment of long-lasting insecticidal nets (LLINs) and, to a lesser extent, indoor residual spraying (IRS), two interventions that have substantially contributed to reducing malaria morbidity and mortality across sub-Saharan Africa [1] [2]. For more than two decades, pyrethroids were the only class of insecticides approved for LLIN impregnation because of their rapid knockdown effect, low mammalian toxicity, and excito-repellent properties against mosquitoes [3] [4]. However, the almost exclusive reliance on this single class of insecticides, combined with the extensive use of pyrethroids in agriculture, has driven the emergence and rapid spread of insecticide resistance in the major malaria vector species [4] [5].

In *Anopheles gambiae* complex populations, pyrethroid resistance is mediated by two major mechanisms that frequently coexist within the same mosquito population. The first mechanism, known as target-site resistance, results from point mutations in the voltage-gated sodium channel gene (knockdown resistance, kdr), among which the L1014F substitution is by far the most prevalent in West Africa [5] [6]. The second mechanism involves metabolic resistance resulting from the

overexpression or enhanced activity of detoxification enzymes, including cytochrome P450 monooxygenases, glutathione S-transferases (GSTs), and non-specific carboxylesterases, which detoxify or sequester insecticides before they reach their target sites [7] [8]. The combined action of these mechanisms explains the high levels of resistance frequently observed in malaria vector populations and highlights the multifactorial nature of insecticide resistance, thereby complicating resistance management strategies.

In Benin, pyrethroid resistance was first reported in the late 1990s [9] and has subsequently spread throughout the country over the past two decades. Numerous studies conducted in different ecological settings have documented widespread resistance in *Anopheles gambiae* sensu lato populations, characterized by high frequencies of the kdr L1014F mutation and, in several areas, an increasing contribution of metabolic resistance mechanisms [10]-[13]. This situation directly compromises the effectiveness of pyrethroid-only LLINs and represents one of the major threats to the sustainability of malaria control achievements in the country.

In response to the progressive decline in pyrethroid efficacy, the World Health Organization now recommends the use of insecticides with different modes of action as part of insecticide resistance management strategies based on rotations, mosaics, or combinations of insecticides. Among the most promising alternatives are clothianidin, a neonicotinoid acting on nicotinic acetylcholine receptors, and chlorfenapyr, a pyrrole pro-insecticide whose active metabolite disrupts mitochondrial oxidative phosphorylation. Because these insecticides target different molecular pathways from pyrethroids and from each other, they have demonstrated high efficacy against pyrethroid-resistant Anopheles populations in a variety of epidemiological settings [14]-[16]. They are now incorporated into several next-generation vector control tools, including dual-active ingredient LLINs and IRS formulations.

In northern Benin, where malaria transmission remains among the highest in the country, up-to-date information on the susceptibility of *Anopheles gambiae* s.l. populations to these new-generation insecticides remains scarce, despite its importance for guiding evidence-based vector control decisions. Likewise, the species composition of the *Anopheles gambiae* complex and the frequency of target-site resistance mechanisms are continuously evolving and require regular monitoring to detect early changes that could compromise the effectiveness of current and future malaria vector control interventions.

The present study aimed to assess the susceptibility status of *Anopheles gambiae* sensu lato populations from three communes in northern Benin (Gogounou, Banikoara, and Boukombé) to pyrethroids (deltamethrin and alpha-cypermethrin), clothianidin, and chlorfenapyr. In addition, the study characterized the species composition of the vector populations and determined the frequency of the kdr L1014F mutation. By simultaneously documenting resistance to conventional insecticides and the efficacy of new-generation insecticides, this study provides evidence to support the Benin National Malaria Control Programme in se-

lecting the most appropriate vector control tools for the epidemiological and ecological context of northern Benin.

2. Materials and Methods

2.1. Study Area

The study was conducted between August and October 2024 in three communes located in northern Benin: Gogounou, Banikoara, and Boukombé (Figure 1). These communes were selected because of their high malaria transmission intensity and their importance for malaria vector surveillance [17].

Gogounou and Banikoara are located in the Alibori Department, whereas Boukombé belongs to the Atacora Department. These predominantly rural areas rely mainly on agriculture and livestock farming as their principal economic activities. The local ecological conditions, characterized by cultivated fields, temporary breeding sites, and seasonal water bodies, provide favorable habitats for malaria vector proliferation.

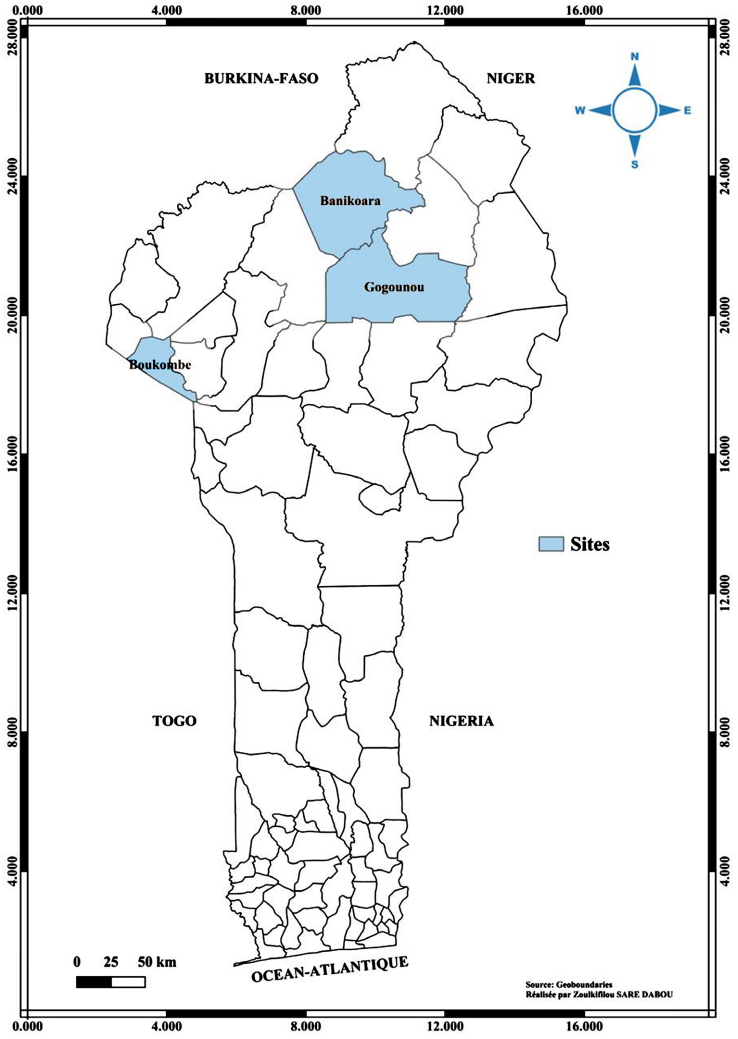

Figure 1. Map of Benin showing the study districts.

Malaria transmission in these areas is primarily sustained by members of the *Anopheles gambiae* sensu lato complex, particularly *Anopheles gambiae* sensu stricto and *Anopheles coluzzii*, which are the principal malaria vectors in Benin. The selection of these study sites was also motivated by the need to generate updated entomological data from high-transmission settings and to characterize vector populations within the current context of malaria vector control interventions in Benin [17].

2.2. Insecticide Susceptibility Bioassays

2.2.1. WHO Tube Susceptibility Bioassays

Insecticide susceptibility tests were performed according to the standard World Health Organization (WHO) tube bioassay procedures [18]. For each study site, batches of 20 - 25 non-blood-fed female *Anopheles gambiae* sensu lato mosquitoes aged 3 - 5 days were exposed for 1 hour to filter papers impregnated with deltamethrin (0.05%) and alpha-cypermethrin (0.05%). Two control batches of the same size were exposed to untreated filter papers to assess natural mortality. Knockdown (KD) was recorded at 15, 30, 45, and 60 minutes during the exposure period. At the end of the exposure, surviving mosquitoes were transferred to holding tubes and provided with cotton pads soaked in 10% sugar solution. Final mortality was recorded 24 hours after exposure.

2.2.2. CDC Bottle Bioassays with Clothianidin

Susceptibility to clothianidin was assessed using the Centers for Disease Control and Prevention (CDC) bottle bioassay method in accordance with World Health Organization (WHO) recommendations [19]. Glass bottles (250 mL) were coated with a clothianidin solution prepared in acetone supplemented with MERO®, used as the solvent and adjuvant, respectively. The diagnostic concentration was 4 µg per bottle. Bottles were coated by trained technicians and left to dry for 24 hours before use, and were used once (single-use bottles). Insecticide purity, MERO® concentration, and coating volume followed the manufacturer's instructions. Batches of 20 - 25 non-blood-fed wild female *Anopheles gambiae* sensu lato mosquitoes aged 2 - 5 days were introduced into the clothianidin-treated bottles and exposed for 60 minutes. Control bottles coated only with the solvent mixture (acetone plus MERO®) were included in each experimental series to monitor natural mortality. Following exposure, mosquitoes were transferred into paper holding cups covered with untreated netting and maintained under standard insectary conditions (25˚C ± 2˚C and 75% ± 10% relative humidity). Cotton pads soaked in 10% sugar solution were provided throughout the holding period. Mortality was recorded 24 hours after exposure.

2.2.3. CDC Bottle Bioassays with Chlorfenapyr

The susceptibility of *Anopheles gambiae* s.l. populations to chlorfenapyr was evaluated using the standard CDC bottle bioassay protocol [19]. A stock solution of chlorfenapyr was prepared and diluted in acetone supplemented with MERO® to

obtain the diagnostic concentration of 100 µg/mL. As for clothianidin, bottles were coated by trained technicians, dried for 24 hours before use, and used once (single-use bottles); insecticide purity, MERO® concentration, and coating volume followed the manufacturer's instructions. Batches of 25 wild female *Anopheles gambiae* s.l. mosquitoes were introduced into chlorfenapyr-treated bottles (100 µg/mL) and corresponding control bottles. Mosquitoes were exposed for 60 minutes. After exposure, they were transferred into holding cups covered with untreated netting and maintained with cotton pads soaked in 10% sugar solution, which was renewed daily.

Mortality was recorded immediately after the exposure period and subsequently at 24, 48, and 72 hours post-exposure to account for the delayed toxic effect characteristic of chlorfenapyr [19].

2.3. Molecular Species Identification and Detection of the kdr L1014F Mutation

A total of 149 live specimens were randomly selected from the mosquitoes tested in the bioassays for species identification and kdr genotyping, distributed as follows: 49 in Banikoara, 50 in Boukombé, and 50 in Gogounou. Species belonging to the *Anopheles gambiae* complex were identified by polymerase chain reaction (PCR) following the protocol described by Santolamazza *et al.* [20]. The kdr L1014F mutation was detected in *Anopheles gambiae* s.l. populations using the allele-specific PCR method described by Martinez-Torres *et al.* [21].

2.4. Data Analysis

Data generated from the bioassays were recorded on standardized data collection forms and entered into Microsoft Excel for data cleaning, organization, and preparation for statistical analysis. Mortality rates were calculated for each insecticide as the proportion of dead mosquitoes relative to the total number of mosquitoes exposed. Mortality was assessed 24 hours after exposure for pyrethroids and clothianidin and up to 72 hours after exposure for chlorfenapyr because of its delayed mode of action. For the CDC bottle bioassays (clothianidin and chlorfenapyr), a total of 100 mosquitoes were tested per site-insecticide combination, distributed across four replicate batches of 25 mosquitoes each. Control mortality remained below 5% in all bioassays; Abbott's correction was therefore not applied.

The susceptibility status of *Anopheles gambiae* s.l. populations was determined according to World Health Organization (WHO) criteria [16]. The CDC bottle bioassay manual [19] specifies the same mortality thresholds and interpretation criteria as the WHO tube test, and these were applied identically to both bioassay types:

- mortality rates of 98% - 100% were classified as susceptible;
- mortality rates of 90% - 97% indicated possible resistance requiring confirmation;
- mortality rates below 90% were considered confirmed resistance.

The allelic frequency of the kdr resistance mutation was calculated according to the formula proposed by Martinez-Torres *et al.* [21]:

$$F(R) = [2nRR + nRS] / [2(nRR + nRS + nSS)]$$

where *nRR*, *nRS*, and *nSS* represent the numbers of homozygous resistant, heterozygous, and homozygous susceptible mosquitoes, respectively.

All statistical analyses were performed using R software (version 4.1.3). Mortality rates were compared between sites and insecticides using proportion tests and chi-squared tests, and statistical significance was determined at $P < 0.05$. Confidence intervals for mortality rates and for kdr allele frequencies were calculated using the chi-squared-based method for binomial proportions.

3. Results

3.1. Resistance Status of *Anopheles gambiae* s.l. to Pyrethroids

High levels of pyrethroid resistance were observed in *Anopheles gambiae* s.l. populations from all study sites, with mortality rates below 60% for all insecticides tested. The highest mortality rates following exposure to alpha-cypermethrin were recorded in Gogounou and Banikoara, reaching 48.28% and 12.20%, respectively. In contrast, the highest mortality rates following exposure to deltamethrin were observed in Gogounou and Boukombé, with mortality rates of 27.85% and 17.58%, respectively (Figure 2).

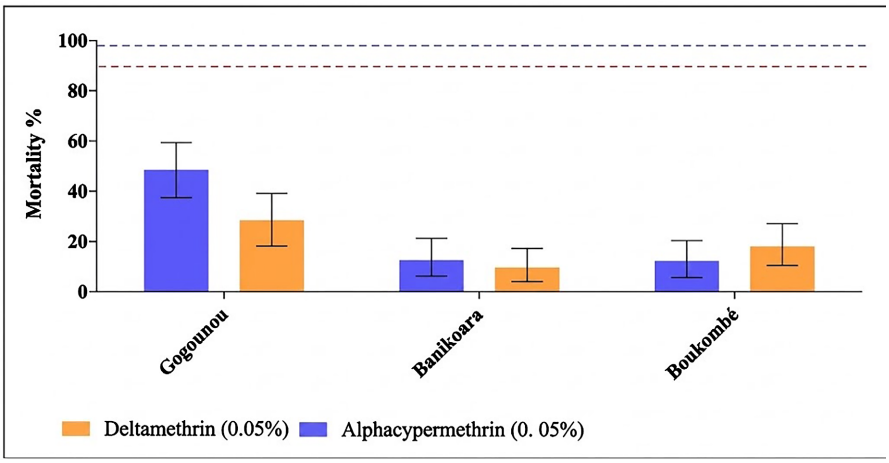

Figure 2. Mortality rate after exposure of *A. gambiae* s.l. to deltamethrin and alpha-cypermethrin using the WHO tube test.

3.2. Susceptibility of *Anopheles gambiae* s.l. to Clothianidin (4 µg/Bottle)

Complete susceptibility of *Anopheles gambiae* s.l. populations to clothianidin (4 µg/bottle) was observed across all study sites. A mortality rate of 100% was recorded 24 hours after exposure in all mosquito populations, indicating full susceptibility to this insecticide (Figure 3).

3.3. Susceptibility of *Anopheles gambiae* s.l. to Chlorfenapyr (100 µg/mL)

Complete susceptibility of *Anopheles gambiae* s.l. populations to chlorfenapyr was observed in all study sites. Twenty-four hours after exposure, the WHO susceptibility threshold (mortality rate ≥ 98%) was achieved in all communes except Gogounou, where mortality remained slightly below this threshold. However, 48 hours after exposure, mortality reached 100% in all study sites, confirming the full efficacy of chlorfenapyr against the tested mosquito populations (Figure 4).

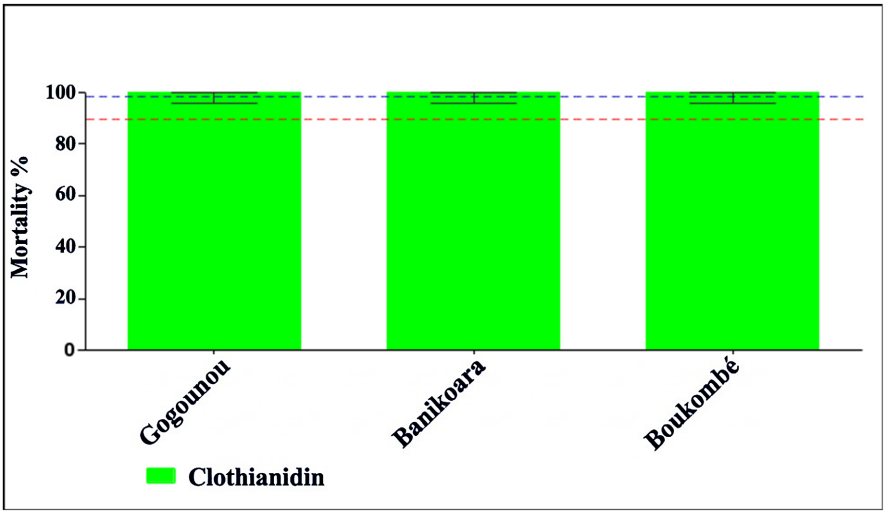

Figure 3. Mortality rates of *Anopheles gambiae* s.l. populations after exposure to clothianidin (4 µg/bottle) using the CDC bottle assay.

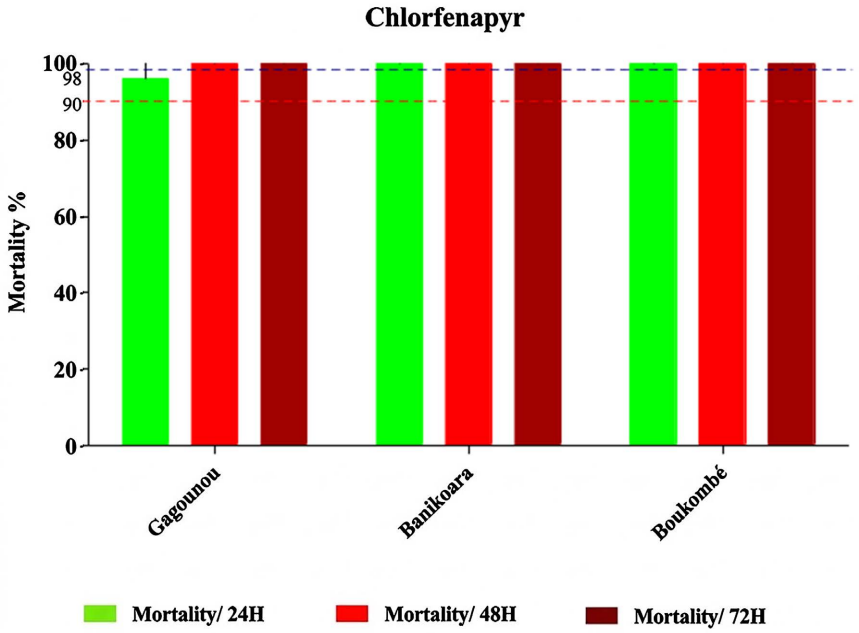

Figure 4. Mortality rate after exposure of *A. gambiae* s.l. to chlorfenapyr using the CDC bottle test.

3.4. Molecular Identification of Species within the *Anopheles gambiae* Complex

Among the 149 mosquito specimens analyzed, 68.45% (102/149) were identified as *Anopheles gambiae* sensu stricto, 30.20% (45/149) as *Anopheles coluzzii*, and 1.34% (2/149) as *Anopheles arabiensis*. *Anopheles gambiae* s.s. was the predominant species across the three study communes, followed by *Anopheles coluzzii*, whereas *Anopheles arabiensis* was detected at a very low frequency (Figure 5).

3.5. Frequency of the kdr L1014F Mutation in *Anopheles gambiae* s.s., *Anopheles coluzzii*, and *Anopheles arabiensis*

The kdr L1014F mutation was detected at high frequencies in *Anopheles gambiae* s.l. populations from the three study communes, with genotypic frequencies of 94%, 86%, and 87% in Banikoara, Boukombé, and Gogounou, respectively. Among *Anopheles gambiae* sensu stricto, the allelic frequency of the L1014F mutation was 95% (95% CI: 89 - 99) in Banikoara, 86% (95% CI: 78 - 92) in Boukombé, and 94% (95% CI: 73 - 100) in Gogounou. In *Anopheles coluzzii*, the corresponding allelic frequencies were 83% (95% CI: 52 - 98) in Banikoara and 86% (95% CI: 76 - 93) in Gogounou. Overall, these findings demonstrate a high prevalence of the kdr L1014F mutation among malaria vector populations in the study communes (Table 1).

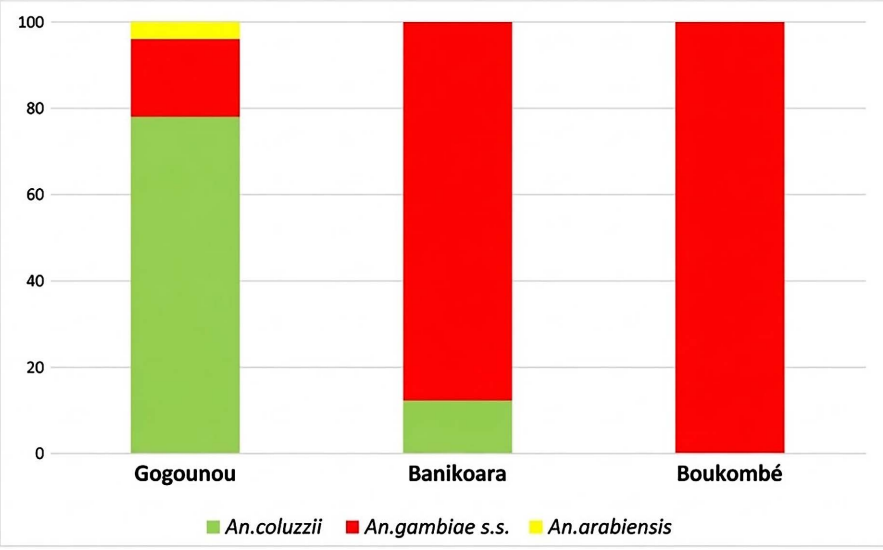

Figure 5. Distribution of molecular species in the *Anopheles gambiae* sensu lato at the different study communes.

Table 1. Frequency of the kdr L1014F mutation in *Anopheles gambiae* s.s. and *Anopheles coluzzii*.

Districts/ Species	N tested	1014F/ 1014F	1014F/ 1014L	1014L/ 1014L	kdr Frequency (%)	95% CI
Banikoara	49	44	4	1	94	87 - 98

Continued

A. coluzzii	6	4	2	0	83	52 - 98
A. gambiae	43	40	2	1	95	89 - 99
Boukombé	**50**	**39**	**8**	**3**	**86**	**78 - 92**
A. gambiae	50	39	8	3	86	78 - 92
Gogounou	**50**	**40**	**7**	**3**	**87**	**79 - 93**
A. arabiensis	2	1	1	0	75	19 - 99
A. coluzzii	39	31	5	3	86	76 - 93
A. gambiae	9	8	1	0	94	73 - 100

4. Discussion

This study provides updated information on the insecticide resistance status of *Anopheles gambiae* sensu lato populations from three communes in northern Benin, a region where information on susceptibility to new-generation insecticides has remained limited. Three major findings emerged from this study: (i) high and widespread resistance to pyrethroids associated with a high frequency of the kdr L1014F mutation; (ii) complete susceptibility to clothianidin and chlorfenapyr; and (iii) a clear predominance of *Anopheles gambiae* sensu stricto within the *Anopheles gambiae* complex, with only a marginal contribution of *Anopheles arabiensis*. These findings have important implications for selecting appropriate vector control tools in northern Benin.

The mortality rates recorded following exposure to deltamethrin and alpha-cypermethrin, all below 60%, were well below the WHO threshold for confirmed resistance (<90%), indicating high levels of resistance rather than a simple reduction in susceptibility. These findings are consistent with the progressive decline in pyrethroid efficacy reported across Benin over the past two decades [6] [7] [10] and agree with the recent study by Hougbe *et al.* [22], which documented widespread pyrethroid resistance along the south-north transect of Benin. The consistency of these findings across different ecological settings and study periods suggests that pyrethroid resistance in *Anopheles gambiae* s.l. has reached an advanced stage throughout the country, likely exceeding the operational effectiveness of interventions relying solely on pyrethroid-based insecticides.

The high level of resistance observed may result from the combined selection pressure exerted by repeated nationwide LLIN distribution campaigns and the intensive agricultural use of pyrethroids, particularly in cotton and vegetable production systems that are widespread in the Alibori and Atacora Departments. Although this hypothesis has been proposed in several previous studies conducted in Benin and elsewhere in West Africa [10] [22], our study does not provide direct evidence because quantitative data on agricultural insecticide use were not collected, nor were comparisons made between areas of intensive agricultural activity and non-agricultural sites. Future studies integrating entomological surveillance with agricultural pesticide-use data would be valuable to better establish this relationship.

The high frequencies of the kdr L1014F mutation observed in both *Anopheles gambiae* s.s. and *Anopheles coluzzii* (83% - 95%) are consistent with the major contribution of target-site resistance to the pyrethroid resistance phenotype. The lower frequency (75%) observed in Anopheles arabiensis from Gogounou (**Table 1**) should be interpreted with caution given the very small sample size (n = 2) and the correspondingly wide confidence interval (19% - 99%). Similar frequencies, approaching fixation, have been reported elsewhere in Benin [13] [22] [23], suggesting a nationwide convergence toward high kdr allele frequencies. Nevertheless, the L1014F mutation alone is unlikely to account for the extremely low mortality rates observed in this study. The contribution of metabolic resistance mechanisms cannot be excluded and should be investigated through synergist bioassays using piperonyl butoxide (PBO), biochemical enzyme assays, and molecular analyses targeting cytochrome P450 monooxygenases, glutathione S-transferases, and esterases.

In contrast, the complete susceptibility to clothianidin observed across all three study sites, despite advanced kdr-mediated resistance, highlights the absence of cross-resistance between pyrethroids and neonicotinoids, which act on distinct molecular targets—the voltage-gated sodium channel and the nicotinic acetylcholine receptor, respectively. These findings are consistent with those recently reported by Hougbe *et al.* [24] across eighteen sites distributed along the south-north transect of Benin, where clothianidin remained fully effective despite widespread pyrethroid resistance. However, although both studies cover similar geographical areas and relatively close time periods, continued monitoring remains essential because susceptibility to neonicotinoids may evolve under increasing selection pressure, particularly as agricultural use of this insecticide class expands.

Complete susceptibility to chlorfenapyr, achieved within 48 hours in all study sites, is consistent with the delayed mode of action of this pyrrole insecticide, whose insecticidal activity depends on metabolic activation that disrupts mitochondrial oxidative phosphorylation. The slightly lower mortality observed at 24 hours in Gogounou, which reached 100% after 48 hours, reflects the expected delayed toxic effect of chlorfenapyr rather than evidence of emerging resistance. These findings are consistent with reports from other West African countries demonstrating high chlorfenapyr efficacy against pyrethroid-resistant Anopheles populations [14] [15] [25] and further support the deployment of dual-active ingredient LLINs incorporating this insecticide.

Molecular species identification revealed that *Anopheles gambiae* sensu stricto remained the predominant species in the study area, followed by *Anopheles coluzzii*, whereas *Anopheles arabiensis* was detected only at a very low frequency. This species composition is consistent with previous studies conducted in northern Benin [13] [23], where ecological conditions, particularly the abundance of temporary larval habitats associated with agricultural activities, favor the predominance of *Anopheles gambiae* s.s. Continued monitoring of species composition

remains important because shifts in the relative abundance of sibling species may occur in response to insecticide pressure or environmental changes, potentially influencing resistance dynamics and malaria transmission.

This study has several limitations that should be considered when interpreting the findings. Only the kdr L1014F mutation was investigated among target-site resistance mechanisms. Other resistance-associated mutations, including L1014S, N1575Y, and ace-1 G119S, were not assessed. Likewise, metabolic resistance mechanisms were not characterized. Despite these limitations, our findings clearly demonstrate that pyrethroids alone are no longer an optimal option for malaria vector control in northern Benin. In contrast, the sustained susceptibility to clothianidin and chlorfenapyr strongly supports the deployment of new-generation insecticides through dual-active ingredient LLINs and indoor residual spraying. Preserving the long-term efficacy of these insecticides will require continuous entomological surveillance, regular monitoring of resistance mechanisms, and the implementation of integrated insecticide resistance management strategies in accordance with WHO recommendations.

5. Conclusions

This study demonstrates that *Anopheles gambiae* sensu lato populations from Gogounou, Banikoara, and Boukombé exhibit high levels of pyrethroid resistance associated with a high frequency of the kdr L1014F mutation, while remaining fully susceptible to clothianidin and chlorfenapyr, two insecticides belonging to different chemical classes with modes of action distinct from those of pyrethroids.

These findings confirm the potential of clothianidin and chlorfenapyr as effective alternatives for malaria vector control in areas where pyrethroid resistance is widespread and support the deployment of next-generation LLINs and IRS formulations incorporating these insecticides. Maintaining their effectiveness will require continuous entomological surveillance integrating phenotypic monitoring with molecular and biochemical characterization of resistance mechanisms to detect early changes in vector susceptibility and guide national insecticide resistance management strategies. The evidence generated by this study provides valuable information to support decision-making by the National Malaria Control Programme and to optimize malaria vector control interventions in Benin.

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Availability of Data and Materials

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

The original study was conducted by C.D.K., A.S.S., A.O.D.C., S.S. and R.A., who also supplied the data. The idea for the study was conceptualized and generated by C.D.K., A.S.S., R.A. and A.O.D.C. Data were collected by C.D.K., R.A. and S.S. P.F.L. and O.D. drafted the manuscript. Statistical data analysis was performed by A.S.S. and R.A. P.F.L., S.S. and R.A.O. provided intellectual criticism on the content of the manuscript. All authors have read and approved the final submitted manuscript.

Conflicts of Interest

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

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Abbreviations

kdr: knock-down resistance;

NMCP: National Malaria Control Program;

CI: confidence interval;

CDC: Centers for Disease Control and Prevention;

WHO: World Health Organization.