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Evaluation of the susceptibility of *An. gambiae* s.l. to deltamethrin, chlorfenapyr and clothianidin in four agricultural areas of Benin

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Abstract
This study evaluates the susceptibility of vectors to deltamethrin, chlorfenapyr (CFP) and clothianidin (CTD) in different agro-ecological zones of the country. Populations of *An. gambiae* s.l. derived from larvae collected between April and September 2022 in 04 cropping zones: vegetable, cereal, rice and cotton were exposed to deltamethrin, chlorfenapyr and clothianidin. Biochemical and target-modification resistance mechanisms were detected in a sample of each mosquito population. All populations of *An. gambiae* s.l. tested with deltamethrin showed mortality that varied between 9 and 60%. Overall, the mean frequency of the kdr L1014F mutation across all localities was 86% followed by overproduction of detoxification enzymes. As for the new insecticides, the mortality rate was 98%-100% for the CFP and for the CTD regardless of the locality and the agro-ecological zone tested. Chlorfenapyr and clothianidin are viable alternatives that could be used for improved control of pyrethroid-resistant populations of *An gambiae* s.l**.**

Keywords: Resistance, chlorfenapyr, clothianidin, *Anopheles gambiae* s.l, Benin

1. Introduction
Recent successes in malaria control are largely attributed to the use of vector control tools, in particular long-lasting insecticidal nets (LLINs) and Indoor Residual Spraying (IRS) [1]. According to a report by the World Health Organization (WHO), out of 663 million cases of malaria averted in sub-Saharan Africa between 2001 and 2015, 69% were by LLIN [2]. In recent years, Benin made substantial efforts to make LLINs accessible to communities. For several years, pyrethroid insecticides were the only class of insecticides recommended for the treatment of nets due to their effectiveness in killing mosquitoes, repellent property that provides users with personal protection during sleeping hours, long residual activity, and low cost [3, 4]. Unfortunately, the widespread vector resistance to insecticides contributed to the reduced effectiveness of control tools incorporating these chemicals. This results in the persistence of the disease in endemic countries [5]. In 2007, N' Guessan *et al.* [6] reported on the reduced efficacy of pyrethroid-impregnated LLINs in an area of high vector resistance. Trials conducted in some regions of Benin [7, 8, 9, 10, 11] have confirmed the occurrence of the phenomenon and its spread in time and space. The use of the same classes of insecticides in both public health and agriculture were highly suspected to be the cause of the increase in the frequency of resistance mutations in Benin [12, 11]. Furthermore, the causes of the reduced effectiveness of LLINs are attributed to the presence of the Kdr gene, as well as the overproduction of metabolic enzymes that confer resistance to pyrethroids in Anopheles populations [13, 6]. With this situation, the implementation of a resistance management plan was necessary [14]. For this, LLINs that incorporate a pyrethroid and a synergist, piperonyl butoxide (PBO) that inhibits oxidases have been developed. These LLINs include Olyset® Plus and Perma Net® 3.0, all of which are WHO-prequalified.

However, the effectiveness of these LLINs in a given area depend on the level of activity of the mono-oxygenase enzymes within the vector populations. In Benin, the addition of the synergist PBO did not fully restore the susceptibility of vectors to pyrethroids in some locations [15]. Recent research exploring other classes of insecticides has identified clothianidin and chlorfenapyr as promising options for vector control. Thus, nicotinoid-based insecticides such as Sumi Shield® 50WG and Fludora Fusion®WP-SB, have been developed and pre-qualified for IRS in 2017 [16]. The clothianidin binds to the nicotinic receptor, blocks the transmission of nerve impulses, and causes the death of the insect [17]. As for chlorfenapyr, it has low toxicity for mammals and is classified as a low-hazard insecticide by the WHO. Due to its novel mode of action, chlorfenapyr is unlikely to exhibit cross-resistance with standard neurotoxic insecticides, as observed in *Anopheles* mosquito populations [18]. Several studies conducted in Côte d'Ivoire, Burkina Faso, Tanzania, and Benin [19-23], have evaluated the efficacy of chlorfenapyr-based IRS or LLINs through laboratory bioassays and experimental hut trials, with results suggesting that this molecule is promising [24]. However, these different studies did not take into account the insecticide pressure that each agro-ecological zone is subjected to.

In Benin, introducing new generation vector control tools could help to well-manage, and overcome vector resistance to insecticides. The present study was undertaken as part of monitoring insecticide resistance in different agro-ecological zones of the country in preparation for the distribution of chlorfenapyr-incorporated LLINs. This will help the National Malaria Control Program (PNLP) to make decisions about the areas to target and also provide susceptibility information of vectors to the two new insecticides.

2. Methods

2.1. Study sites

This study was conducted between April and September 2022 in four cropping areas of Benin. The communes selected according to the climate were:

-Porto-Novo, Bohicon, Lokossa and Ifangni in the regions of humid savannah and degraded forests, Southern Benin. The climate is sub-equatorial with rainfall varying between 1100 to 1200 mm per year [25-27].

-Gogounou, Banikoara and Malanville in the dry savannah region of Northern Benin (Ahoyo & Guidibi 2006) with a sudanese climate that has a single rainy season (May to October) and a dry season (November to April). The average temperature and relative humidity are 28 °C and 70%, respectively [28, 29].

- Glazoué in central Benin, a transition zone located between the dry and humid savanna region. This area enjoys a Sudano-Guinean climate with two seasons. The rainy season extends over 6 months (mid-April to mid-October). The average rainfall is between 1200 and 1300 mm. The average temperature is around 27 °C [30].

In terms of distribution by crop zone, the communes of Ifangni and Porto-Novo practice market gardening as a growing activity, facilitated by the presence of lagoons and marigots in this region. The communes of Bohicon and Lokossa are cereal-growing zones, while the communes of Glazoué and Malanville are renowned for rice production in Benin. These areas sometimes use fertilizers and pesticides for better yield. The communes of Banikoara and Gogounou are considered the cotton-growing areas of Northern Benin, with large quantities of insecticides used to control cotton pests [31] (Fig. 1).

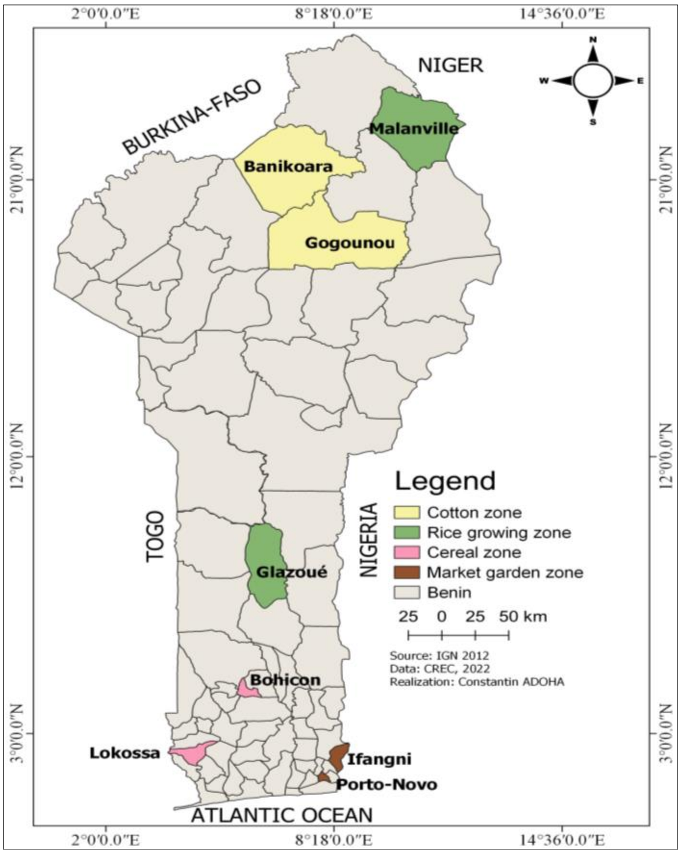

Fig 1: Study site

2.2 Collection and rearing of mosquito larvae
Larvae of *Anopheles gambiae* s.l. were collected in eight communes of Benin (Ifangni, Porto-Novo, Bohicon, Lokossa, Glazoué, Malanville, Banikoara and Gogounou). They were sampled from the positive breeding sites using a dipper and transported to the insectary of the Center for Research in Entomology of Cotonou (CREC) for rearing. The pupae obtained were grouped together in different cages for the emergence of adult mosquitoes. After morphological identification using the Coetzee determination key [32], only specimens of *Anopheles gambiae* s.l. were used for testing.

2.3 WHO susceptibility tube test
Females *Anopheles gambiae* s.l. aged 2-5 days were used for the tests. Thus, batches of 20-25 female mosquitoes were exposed to 0.05% deltamethrin for 60 minutes. During the test, the number of mosquitoes knock down by the insecticide was recorded every 15 minutes. Other batches of 20 to 25 mosquitoes exposed to insecticide-free papers served as controls. After exposure, the mosquitoes were transferred to the observation tubes where they were fed with a 10% sugar solution for 24 hours. Mortality rates were determined 24 hours after testing [33].

2.4 WHO bottle bio-assay?
The chlorfenapyr (CFP) solution used for coating the bottles was prepared from the stock solution diluted with acetone. Glass Wheaton bottles (250 ml) and their caps were coated with 1 ml of CFP (100 µg/ml). In parallel, bottles coated with 1 ml of acetone served as controls. The exposure of different mosquito populations to CFP was performed over 60 minutes. Thereafter, mosquitoes were transferred to a paper cup with free access to a 10% sugar solution. The knockdown rate was recorded at 60 min and the mortality at 24 h, 48 h and 72 h after exposure. One hour after insecticide exposure, dead mosquitoes were preserved on RNA Later and stored in a -80°C freezer. Mosquitoes that died after 24 h, 48 h and 72 h were collected and stored in silica gel [34]. The same test was performed with the *Anopheles gambiae* Kisumu susceptible strain. Results obtained with the wild mosquitoes was compared to those of of the susceptible strain. The tests were conducted at 26 °C±2 °C (temperature) and 78% ±10% (relative humidity).

2.5. Modified WHO susceptibility tube test
The ability of SumiShield® 50WG to kill wild populations of *Anopheles gambiae* s.l. was evaluated through WHO susceptibility tube testing with some minor modifications from the standard guidelines [34]. 12 cm by 15 cm Whatman® filter papers were treated with the candidate a diagnostic dose of Sumi Shield® 50 WG (containing 50% CTD) diluted in distilled water. A stock solution was prepared by diluting 264 mg of Sumi Shield® 50 WG in 20 ml of distilled water. Two milliliters of the mixed solution was pipetted evenly onto each filter paper and stored at 4 °C until use. Filter paper treated with 2 ml of distilled water was used as a negative control. The duration of the exposure to CTD was set at 60 minutes. After exposure, mosquitoes were transferred to untreated paper-lined observation tubes (25 °C and 80% humidity) with free access to 10% sugar solution. The knockdown was recorded after 30 min and 60 min. Mortality was recorded at 1, 2, 3, 4, 5, 6 and 7 days after exposure [35].

2.6. Data analysis
The mortality rates recorded 24 hours after exposure of mosquito populations to the diagnostic dose of the different insecticides were interpreted according to the criteria of the World Health Organization [34]:

- Mortality rate ≥98%: susceptible mosquito population
- Mortality rate between 90 and 97%: possible resistance to that requires further investigation.
- Mortality rate ≤ 90%: Resistant mosquito population

The formula F= (2RR+RS)/ (2(RR+RS+SS)) was used to calculate Kdr mutation frequencies. With RR: the number of homozygous mosquitoes; RS: the number of resistant mosquitoes and SS: the number of homozygous mosquitoes. The binomial exact test for comparison of proportions was used to generate confidence intervals for mortality rates and Kdr mutation frequencies.
The overproduction of detoxification enzymes by the wild-type strain was compared with that of the sensitive laboratory strain (Kisumu) using the Mann-Whitney U test. R software was used for all statistical analyses.

3. Results
3.1. Susceptibility of *An. gambiae* s.l. populations to deltamethrin in different agro-ecological zones
Table 1 presents the mortality rates of *An. gambiae* s.l. after exposure to deltamethrin in the different agro-ecological zones. These rates were less than 80% in all sites, indicating resistance to deltamethrin. They are significantly lower in the cotton (Banikora: 9.20% [4.05-17.32]) and rice (Malanville: 17.58% [10.40-26.98]) growing areas compared to all the other surveyed sites.

Table 1: Susceptibility of different populations of *An. gambiae* s.l. to deltamethrin

Deltamethrin				
Study areas	**Sites**	**Number tested**	**Mortality 24H % [CI]**	**Status**
Market gardening	Ifangni	99	35.35 [26,01-45,60]	RR
	Porto-Novo	80	47.50 [36,21-58,98]	RR
Cereal growing	Bohicon	101	39.60 [30,01-49,83]	RR
	Lokossa	86	55.81 [44,70-66,52]	RR
Rice growing	Glazoué	88	37.50 [27,40-48,47]	RR
	Malanville	91	17.58 [10,40-26,98]	RR
Cotton growing	Gogounou	79	27.85 [18,35-39,07]	RR
	Banikoara	87	9.20 [4,05-17,32]	RR

%: Percentage; CI: Confident interval

3.2 Resistance mechanisms of *An. gambiae* populations in different agro-ecological zones

Table 2 shows the frequency of the L1014F kdr mutation in sibling species of the *Anopheles gambiae* complex in the four agro-ecological zones surveyed. Overall, the mean frequency of the kdr L1014F gene for all sites was 86% [84–89]. This frequency was similar in *An. gambiae* (91% [87-94]), and *An. coluzzii* (84% [80 - 87] at all sites. The resistant kdr L1014S allele was not detected in our samples.

Table 2: *Kdr* L1014F frequencies observed in different species of the *An. gambiae* complex

Sites/Species	Number tested	Genotypes 1014F/1014F	1014F/1014L	1014L/1014L	Kdr Frequency (%)	CI 95%
Ifangni	50	38	8	4	84	[75 - 91]
An. coluzzii	23	17	4	2	83	[69 - 92]
An. gambiae	27	21	4	2	85	[73 - 93]
Porto-Novo	50	39	7	4	85	[76 - 91]
An. coluzzii	50	39	7	4	85	[76 - 91]
Bohicon	50	35	15	0	85	[76-91]
An. coluzzii	26	19	7	0	87	[74-94]
An. gambiae	24	16	8	0	83	[70-93]
Lokossa	99	80	13	6	87	[82 - 92]
An. coluzzii	50	37	8	5	82	[73 - 89]
An. gambiae	49	43	5	1	93	[86 - 97]
Malanville	50	35	11	4	81	[72 - 88]
An. arabiensis	2	1	1	0	75	[19 - 99]
An. coluzzii	48	34	10	4	81	[72 - 88]
Gogounou	50	40	7	3	87	[79 - 93]
An. arabiensis	2	1	1	0	75	[19 - 99]
An. coluzzii	39	31	5	3	86	[76 - 93]
An. gambiae	9	8	1	0	94	[73 - 100]
Banikoara	49	44	4	1	94	[87 - 98]
An. coluzzii	6	4	2	0	83	[52 - 98]
An. gambiae	43	40	2	1	95	[89 - 99]
All area	398	311	65	22	86	[84 - 89]
An. coluzzii	242	181	43	18	84	[80 - 87]
An. gambiae	152	128	20	4	91	[87 - 94]
An. arabiensis	4	2	2	0	75	[37 - 95]

%: Percentage; CI: Confident interval

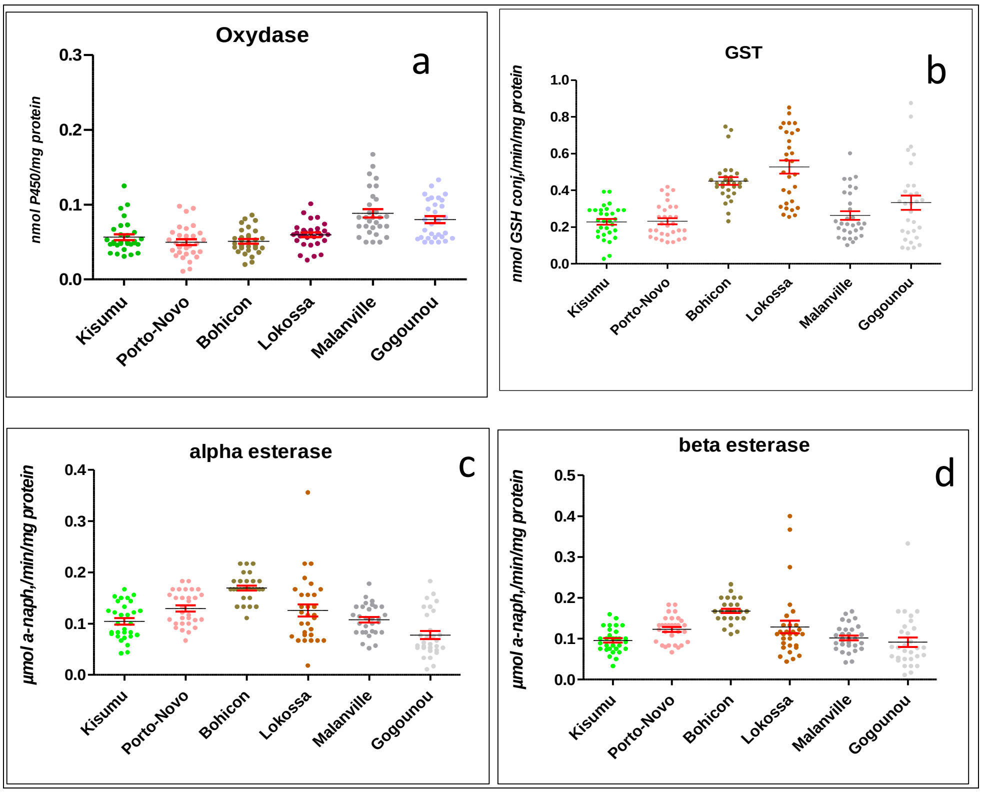

Fig 2: Mono-oxygenase (a), glutathione-S-transferase (b), α-esterase (c) and β-esterase (d) activities of *Anopheles gambiae* s.l. populations.

Figure 2 shows the mean levels of enzymatic activities in the wild and susceptible populations of mosquitoes. In all the surveyed sites, at least one detoxifying enzymes showed high activity compared to the Kisumu strain. Oxidase activity was significantly elevated in the cotton (Gogounou) and rice (Malanville) growing areas compared to the Kisumu susceptible strain (Fig. 2a). The highest glutathione-S-transferase (GST) activities were observed in populations from cereal-growing areas (Bohicon and Lokossa) with a significant difference compared to the Kisumu susceptible strain (Fig. 2b). As for α esterases and β esterases, their activity was significantly higher in the populations of the cereal (Bohicon and Lokossa) and market gardening (Porto-Novo) areas (Fig. 2c & d).

3.3. Susceptibility level to chlorfenapyr

A full susceptibility (mortality of 100%) of all wild populations of *An. gambiae* s.l. to chlorfenapyr was observed at 24-48 hours post-exposure (Table 3).

Table 3: Susceptibility of different *An. gambiae* s.l. populations to chlorfenapyr

Chlorfenapyr						
Study areas	Sites	Number tested	Mortality % [CI]			Status
			24 H	48 H	72 H	
market gardening	Ifangni	100	99 [94, 55-99, 97]	99 [94, 55-99,97]	100 [96, 38-100]	SS
	Porto-Novo	102	100 [96,45-100]	100 [96, 45-100]	100 [96, 45-100]	SS
Cereal growing	Bohicon	100	100 [96, 38-100]	100 [96, 38-100]	100 [96, 38-100]	SS
	Lokossa	107	100 [96, 61-100]	100 [96, 61-100]	100 [96, 61-100]	SS
Rice growing	Glazoué	107	100 [96, 61-100]	100 [96, 61-100]	100 [96, 61-100]	SS
	Malanville	94	100 [96, 15-100]	100 [96, 15-100]	100 [96, 15-100]	SS
Cotton growing	Gogounou	115	100 [96, 84-100]	100 [96, 84-100]	100 [96, 84-100]	SS
	Banikoara	117	100 [96, 90-100]	100 [96, 90-100]	100 [96, 90-100]	SS

%: Percentage; CI: Confident interval

3.4. Susceptibility level of populations of *An. gambiae* s.l. to clothianidin

Mortality rates observed to CTD, 24 hours after exposure, were less than 80% in all surveyed sites. They were significantly lower in the rice (Glazoué: 21.97% [13.97-31.88]) and cotton (Banikoara: 31.86% [22.49-42.47]) growing areas compared to the market gardening (Ifangni: 77.27% [67.11-85.53]); Porto-Novo: 68.88 [58.26-78.23]), and cereal (Bohicon: 89.65% [81.27-95.16]; Lokossa: 35.64 [26.36-45.79]) areas (Table 4). On the other hand, they were 58.09% [48.07-67.66] and 55.33% [45.22-65.14] in Gogounou (cotton zone) and Malanville (rice-growing zone), respectively (Table 4). These rates increased as time goes in all surveyed sites. The susceptibility level was reached on the fourth day in Ifangni, Bohicon, Porto-Novo, and Gogounou (mortality rate≥ 98%). Between the 6th-7th day, all the remaining wild populations reached the susceptibility level (Table 4).

Table 4: Resistance status of different populations of *An. gambiae* s.l. to clothianidin

Clothianidin										
Study areas	Sites	Number tested	% Dead							Status
			Day 1% [CI]	Day 2% [CI]	Day 3% [CI]	Day 4% [CI]	Day 5% [CI]	Day 6% [CI]	Day 7% [CI]	
Market gardening	Ifangni	88	77, 27 [67, 11-85, 53]	95, 45 [88, 77-98, 75]	96, 59 [90, 36-99, 29]	100 [95, 89-100]	100 [95, 89-100]	100 [95, 89-100]	100 [95, 89-100]	SS
	Porto-Novo	90	68, 88 [58, 26-78, 23]	84, 44 [75, 28-91, 23]	93, 33 [86, 05-97, 51]	98, 89 [93, 96-99, 97]	100 [95, 98-100]	100 [95, 98-100]	100 [95, 98-100]	SS
Cereal growing	Bohicon	87	89, 65 [81, 27-95, 16]	93, 1 [85, 59-97, 43]	96, 55 [90, 25-99, 28]	100 [95, 85-100]	100 [95, 85-100]	100 [95, 85-100]	100 [95, 85-100]	SS
	Lokossa	101	35, 64 [26, 36-45, 79]	55, 45 [45, 22-65, 34]	74, 26 [64, 6-82, 44]	88, 12 [80, 17-93, 71]	94, 06 [87, 52-97, 79]	97, 03 [91, 56-99, 38]	99, 01 [94, 61-99, 97]	SS
Rice growing	Glazoué	91	21, 97 [13, 97-31, 88]	42, 86 [32, 53-53, 66]	60, 44 [49, 64-70, 54]	85, 71 [76, 81-92, 17]	97, 8 [92, 29-99, 73]	100 [96, 03-100]	100 [96, 03-100]	SS
	Malanville	103	55, 33 [45, 22-65, 14]	75, 73 [66, 29-83, 64]	89, 32 [81, 69-94, 55]	96, 12 [90, 35-98, 93]	100 [96, 48-100]	100 [96, 48-100]	100 [96, 48-100]	SS
Cotton growing	Gogounou	105	58, 09 [48, 07-67, 66]	84, 76 [76, 44-91, 03]	93, 33 [86, 75-97, 28]	99, 05 [94, 81-99, 98]	100 [96, 55-100]	100 [96, 55-100]	100 [96, 55-100]	SS
	Banikoara	91	31, 86 [22, 49-42, 47]	65, 93 [55, 25-75, 55]	83, 52 [74, 27-90, 47]	92, 31 [84, 79-96, 85]	95, 6 [89, 13-98, 79]	100 [96, 03-100]	100 [96, 03-100]	SS

%: Percentage; CI: Confident interval

4. Discussion

The susceptibility of malaria vectors from different agro-ecological zones of Benin was assessed to deltamethrin, clothianidin and chlorfenapyr. Findings from the present study showed that regardless of the cropping area considered, the mortality of the different mosquito populations observed with deltamethrin was less than 80%, which suggests that pyrethroid resistance is widespread in Benin as shown by several previous studies conducted about fifteen years ago [36, 37]. Recent studies provide evidence of the obstacle that constitutes the resistance phenomenon for the success of vector control strategies [38, 39- 41]. Four cropping areas (market gardening, cereal, rice, and cotton) were surveyed as part of the present study. Overall, deltamethrin mortality rates were significantly lower in cotton (Banikora) and rice (Malanville) areas compared to others. This high intensity of vector resistance to insecticides could be explained by the strong insecticide pressure that these areas undergo with the objective of protecting crops against pests and increasing productivity [31, 42-45]. The strong insecticide resistance observed in vectors was also associated with high frequencies of the kdr L1014F mutation, especially in the two cotton-growing areas (Banikoara: 94% and Gogounou: 87%). Similar results were recently observed by Sagbohan *et al.* [15], that incriminated cultivation practices as favoring gene selection and a rise in insecticide resistance intensity. The kdr L1014F frequency was similar in *An. gambiae* and *An. coluzzii* in all agro-ecological areas, which is contrary to the work of Gnanguenon *et al*. [46]; Yahouedo *et al.* [47] and Salako *et al*. [48]. This observed resistance is also reinforced by the overproduction of oxidases in cotton and rice-growing areas (Gogounou and Malanville), which suggests that pyrethroid insecticides are in use in cotton and rice fields [49].

Based on this information, it is clear that the effectiveness of control tools incorporating pyrethroids is questioned.

On the other hand, clorfenapyr and clothianidin, two new-generation insecticides recommended by the WHO, showed full susceptibility against all wild populations of *An. gambiae* s.l. (Mortality > 98%). This could be due to the differing mode of action of these insecticides [50]. While pyrethroids are neurotoxic, chlorfenapyr is an uncoupler of oxidative phosphorylation preventing the formation of the main energy reservoir ATP in insect mitochondria [51, 52]. Even though clothianidin is a neurotoxic insecticide, it has its site of action on nicotinic acetylcholine receptors [53, 54] whereas pyrethroids act on voltage-gated sodium channels [55, 56]. Given that these new insecticides are also in demand in agriculture, the susceptibility of all populations of *An. gambiae* s.l. observed in this study rules out the hypothesis of possible cross-resistance between these two products. Similar results showing perfect vector sensitivity to the new insecticides were observed with clothianidin by Oxborough *et al.* [35] in areas of high malaria vector resistance in Africa. According to the authors, sensitivity of wild populations of *An. gambiae* s.l. to clothianidin has been observed in 11 African countries. However, they pointed out that at least one site in 5 countries had vector mortalities below 98%. Two years later, in a similar study, the same author showed perfect sensitivity of wild populations of *An. gambiae* s.l. to chlorfenapyr at a concentration of 100 µg/ai/bottle in 10 African countries [57]. This information is in line with the idea of combining these new products with pyrethroids in the context of vector resistance management. Two new tools fit into this category: the mosquito nets with double active ingredients Interceptor G2 and the new insecticide formulation fludora fusion. Interceptor G2 is a new mosquito net incorporating chlorfenapyr and a pyrethroid insecticide (alphacypermethrin) being deployed in communities in Benin. Evidence of the community effectiveness of this net has already been provided by randomized controlled trials in Benin [58] and Tanzania [59]. In view of the susceptibility of vectors to this product, whatever the growing area, Interceptor® G2 LLINs could be considered a promising tool in the management of vector resistance to pyrethroids. However, the selection of the resistance mutation being a dynamic phenomenon, rigorous monitoring of the sensitivity of the vectors to these products is necessary over time.

5. Conclusion

The results of this study confirm the widespread resistance of *An. gambiae* s.l. to pyrethroid insecticides whatever the agro-ecological area is. However, all mosquito populations from market gardening, cereal, rice and cotton growing areas showed perfect susceptibility to CFP and CTD. These results suggest that these new molecules can be used to manage of vector resistance to insecticides in Benin.

Declaration of interest

The authors declare that they have no conflicts of interest.

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