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Susceptibility of *Anopheles gambiae* sensu lato to different insecticide classes and mechanisms involved in the South-North transect of Benin

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Abstract
Monitoring vector resistance to insecticides is a major component of resistance management. Adults of *Anopheles gambiae* s.l. from larvae collected at thirteen sites were tested with papers impregnated with bendiocarb, permethrin; deltamethrin; pirimiphos-methyl and induced bottles of insecticide including bendiocarb; deltamethrin, permethrin and pirimiphos-methyl. Molecular analyses were performed and detoxification enzyme levels were determined for each study site. This study revealed a generalized distribution of resistance of *An. gambiae* s.l. to pyrethroids (permethrin and deltamethrin) and a clear resistance to carbamates (bendiocarb) especially in the northern region of the country. This vector is still susceptible to organophosphates, especially pirimiphos-methyl, but resistance is a dynamic phenomenon and it would be necessary to monitor the susceptibility of these anopheles to pyrimiphos-methyl and to find alternative vector control methods to slow down the spread of resistance genes.

Keywords: insecticide resistance, *Anopheles gambiae* s.l., pyrethroids, bendiocarb

Introduction
The wide expansion of vector resistance to pyrethroids [1] and carbamates [2, 3] is a major threat to the success of insecticide based malaria vector control programs. In 2007, N'Guessan *et al* (2007) [4] reported on the declining efficacy of pyrethroid-treated nets in experimental huts in Ladji, an area of high pyrethroid resistance in malaria vectors located in southern Benin. Studies on insecticide resistance conducted in some regions of Benin [1-2, 5-8] have confirmed the presence of this resistance and its evolution in time and space. In this context of searching for alternatives to overcome the resistance of vectors to pyrethroids in Africa, two mechanisms have been the subject of sustained research for quite some time. These are metabolic resistance, which is expressed by an increase in the activity of detoxification enzymes (Oxidases, Esterases and Glutathione-s-transferases) [9-12] and the *kdr L1014F* gene-related resistance frequently found in *An. gambiae* s.l. populations in Benin [2, 15-18] and which is due to the substitution of leucine for phenylalanine at position 1014. This presence of the kdr gene associated with the overproduction of metabolic enzymes, and the recent appearance of carbamate-resistant Anopheles populations require studies on the level of susceptibility of *An. gambiae* to the different insecticide classes on the south-north Benin transect.
Over the last decade, Benin has been confronted with the recurrent problem of vector resistance to insecticides. All pyrethroids used for impregnation of mosquito nets are concerned: permethrin (Olyset Net), deltamethrin (Perma Net, Dawa), alpha cypermethrin (Duranet). Carbamates, in particular bendiocarb, already used for IRS, are also concerned in some areas, including the Atacora department. Organophosphates are not excluded from this list because the decrease in sensitivity of *An. gambiae* to fenitrotion and propoxur has been recorded. However, no resistance to pyrimiphos-methyl used in IRS has been recorded in Atacora but since resistance is a dynamic process, it cannot be excluded that resistance to this insecticide will emerge in the future. Thus, the evolution of the situation must be monitored every year and must serve as a guide for the National Malaria Control Programs.

Therefore, we initiated this study to verify the level of susceptibility of *An. gambiae* s.l. in thirteen sites in order to determine the evolution of resistance in these vectors.

Methods

Study area

The resistance status of *An. gambiae* s.l, a major malaria vector in Africa, was determined in 13 communes selected along the North-South transect of Benin, in order to take into account all the different geo-climatic, ecological and epidemiological facies of the country. The selection of the sites is random but in such a way as to take into account the ecosystem, the climate, the geographical environment and the malaria data encountered in different areas of Benin. Mosquito larvae, whose adults were used as test, were collected from September 2017 to November 2018. The present study takes into account the thirteen (13) sites and provides information on the level of susceptibility of vectors to insecticides in all eco-epidemiological zones of Benin (Figure 1).

- **Cotton growing area (Kandi, N'Dali and Parakou):** This area is characterized by a high use of pesticides against cotton pests. In this zone, intensive cotton cultivation is associated with the use of several families of insecticides.
- **Rice-growing area (Malanville):** The Malanville perimeter is a rice-growing area of 70 hectares. Two rice crops are grown per year, one of which is grown in the off-season. It is therefore an off-season rice crop.
- **Urban market area:** An area that has not undergone any insecticide treatment but where people use impregnated mosquito nets, aerosol cans and smoke coils. It is defined by the cities of Cotonou and Porto-Novo.
- **Cereal area:** The localities of Missérété, Bantè, Ouidah and Allada located respectively in the departments of Ouémé, Collines and Atlantique.
- **Forest area:** Bohicon located in the department of Zou. This is an area where millet and corn are grown.
- **Hill area:** Dassa and Savè.

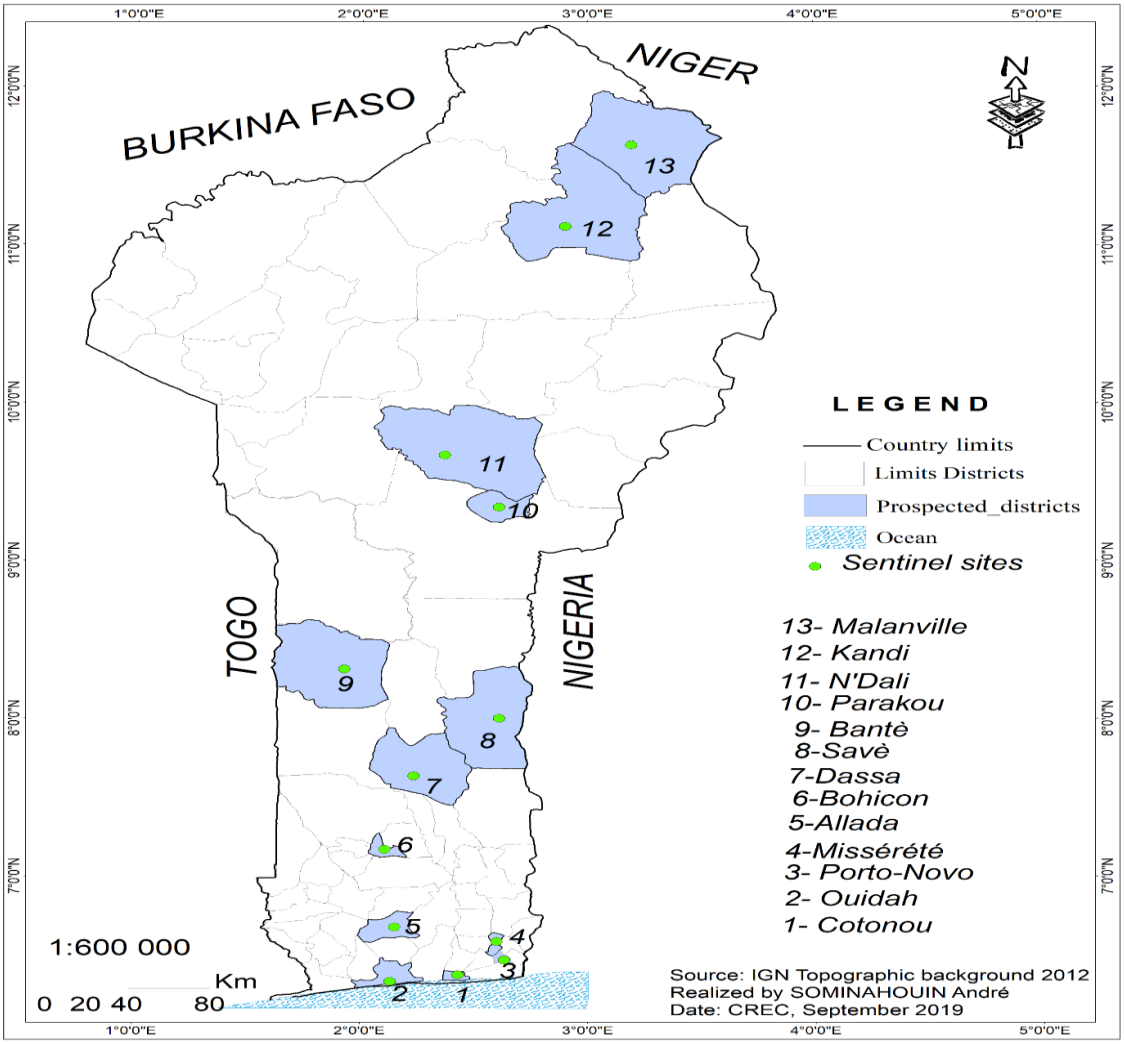

Fig 1: Map of Benin showing the communes where larvae were collected.

Mosquito collection

An. gambiae s.l. larvae were collected during the study period from different larval sites in the communes of Cotonou, Porto-Novo, Missérété, Ouidah, Bantè, Allada, Bohicon, Malanville, Kandi, N'Dali, Parakou, Dassa and Savè using standard dippers and containers. The collected larvae were sorted (figure 1), kept in labeled jars, and transported to the insectary of the Center for Entomological Research of Cotonou (CREC) for rearing. Emerging adults from the field larval collections which were placed in cages were fed on 10% honey solution and kept at 27 ± 2 °C and relative humidity of 72 ± 5%. Morphologically identified 2-5-day old adult females were used for susceptibility testing to various insecticides and biochemical analyses.

Susceptibility testing of collected *An. gambiae* s.l. to insecticides using WHO method

Female mosquitoes aged 2-5 days, morphologically identified as *An. gambiae* s.l. were exposed to different doses of various insecticides for susceptibility testing using the insecticide-impregnated papers as described by the standard WHO testing protocol [19]. Female mosquitoes aged 2-5 days, morphologically identified as *An. gambiae* s.l. were exposed to different doses of various insecticides for susceptibility testing using insecticide-impregnated papers as described by the standard WHO testing protocol. These mosquitoes were exposed to insecticide-impregnated papers :

- Deltamethrin (Pyrethroids): 0.05% (Delta 1x);
- Permethrin (Pyrethroids) : 0.75% (Per 1x) ;
- Bendiocarb (Carbamates): 0.1% (Bendio 1x) ;
- Pirimiphosmethyl (Organophosphates) : 0.25% (P.M 0.25%)

In the tests, batches of 20 to 25 mosquitoes were introduced into each tube with impregnated paper. During the one-hour exposure, the number of mosquitoes that were knocked down by the insecticide at different time intervals (5, 10, 15, 20, 30, 45, 60 minutes) was recorded. Batches of mosquitoes subjected to the unimpregnated papers served as controls. After 60 minutes of exposure, mosquitoes were transferred to observation tubes lined with untreated papers (25°C and 80% humidity) with free access to sweet juice (10% honey juice). Mortality after 24 hours was determined according to the WHO protocol. Phenotypic resistance to insecticides was assessed by the estimated mortality rate as follows: Mortality rate (%) =Number of dead individuals x 100 / Number of individuals tested. We did not use Abbot's formula for corrected mortality since the observed mortality rates in the controls were less than 5%. Live and dead specimens from each locality from the tests were subjected to PCR for species identification and determination of resistance mechanisms (*kdr* and *ace-1R*).

Susceptibility testing of collected *An. gambiae* s.l. to insecticides using CDC method

The principle of the CDC bottle bioassay is to determine the time required for an insecticide to enter an arthropod, pass through its intermediate tissues, reach the target site, and act on that site relative to a susceptible control. Anything that prevents or delays the achievement of the goal of killing arthropods contributes to resistance. The diagnostic dose applied in this study was the CDC recommended dose [20]. These doses were first verified with the susceptible reference strain of *An. gambiae* Kisumu before applying them to field populations. The different insecticide doses used per bottle for the different tests were:

- Deltamethrin (Pyrethroids) : 12.5µg (Delta 1x) ;
- Perméthrin (Pyréthrinoïdes) : 21.5µg (Per1x) ;
- Bendiocarb (Carbamates) : 12.5µg (Bendio 1x) ;
- Pirimiphos-méthyl : 20µg (P.M 1x)

For each dose of insecticide, the different populations were exposed for a diagnostic exposure time of 30 minutes. The solution was prepared and the bottles were sensibilized according to the CDC protocol [20]. Fifteen to twenty-five fasting female *An. gambiae* s.l. mosquitoes were introduced into four 250 ml Wheaton bottles coated with insecticide and one control bottle coated with acetone only. The number of dead and live mosquitoes was monitored at different time intervals (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 minutes).

Identification of An. gambiae complex species and molecular characterization of kdr L1014F and ace-1 G119S resistance alleles

Using previously established protocols of Santolamazza *et al* (2008), live and dead mosquitoes from the susceptibility tests from all doses were analyzed by PCR to determine the species of the *An. gambiae* s.l. complex [13].

The genotypes of the *L1014F kdr* (knock-down resistance) mutation in the sodium channel associated with resistance to pyrethroid insecticides and the *ace-1 G119S* (insensitive acetylcholinesterase**)** mutation associated with resistance to carbamates and organophosphates were determined according to the protocols of Martinez *et al*. (1998) and Weill *et al*. (2004), respectively [14, 15]. The allelic frequency of these two mutations was evaluated at each site to analyze correlations with phenotypic resistance.

Enzymatic tests on microplates

Approximately 30-50 *An. gambiae* s.l. (F1) females from each site, aged 2-5 days and not previously used for any insecticide test, were used for biochemical analyses. Before these analyses, these mosquito specimens were stored at -80 °C in dry microcentrifuge tubes. Biochemical enzyme assays [21] were carried out to compare the level of activity of mixed oxidases (MFO), non-specific esterases (α and β-esterases), and glutathione S-transferases (GST) in Parakou, Kandi, and Malanville mosquito populations with that of *An. gambiae* s.s. Kisumu, a susceptible laboratory strain. Since enzymes degrade rapidly at room temperature, mosquitoes were ground on ice in 200 µl of distilled water and the extract was centrifuged at 12,000 rpm for 2 min. For GST, 10 µl of mosquito grindings in two replicates were put into each Nunc plate well to which 200 µl of a solution of reduced glutathione (GSH) and CDNB (1-chloro-2,4-Dinitrobenzene) was added. Concerning MFOs, after putting 20 µl of crushed material in two replicates in each well, 80 µl of 0.0625M Potassium Phosphate buffer (KHPO4) pH=7.2 and 200 µl of 0.25M Tetramethyl Benzidine (TMBZ) solution pH 5.0 and, 25 µl of a 3% hydrogen peroxide solution were added in each well. For the non-specific esterases, 90 µL of two replicates of shredded material were added to each plate well, 90 µL of 1% Triton Phosphate Buffer (PBS) pH 6.5, 100 µL of a solution composed of 0.3M alpha-Naphthyl acetate (or beta-Naphthyl acetate) and Triton PBS pH 6.5, water and Fast Garnett Salt (FGBC) solution. Readings for each enzyme activity were taken as an endpoint at 340nm, 630nm and 550nm for GST, MFO and non-specific esterases respectively.

Data analysis

The resistance status of the tested mosquito populations was determined according to WHO criteria [19] for tests performed with WHO tubes and according to CDC criteria (Brogdon et Chan, 2010). Thus, for WHO tube testing the resistance status is determined as follows:

- A mortality rate between 98% and 100% indicates full sensitivity
- A mortality rate between 90% and 97% indicates suspected resistance to be confirmed
- When the mortality rate is less than 90%, the population is considered resistant to the insecticides tested.

For sensitivity testing in CDC bottles, the resistance status is determined as follows

- Mortality rate = 100%: the population is fully susceptible
- Mortality rate < 100% : the population is considered resistant to the tested insecticides

Mortality rates of *An. gambiae* s.l. populations at different sites were compared using a 2 × 3 stratified contingency table and Pearson's $\chi 2$ test in the statistical software, R 2.15. The strata in the 2 × 3 contingency table included insecticide and dosage. The allelic frequencies of the *kdr L1014F* and *ace-1 G119S* genes were analyzed to assess their variability in mosquito populations. Using SPSS® (SPSS Inc. Published in 2009. PASW Statistics for Windows, version 18.0. Chicago: SPSS Inc.), a comparative measure of mean enzyme activities between study sites was performed to assess the variation in enzyme activity of mosquito populations in each locality using a one-way analysis of variance (ANOVA). Tukey's test was used to compare means. An independent samples test was performed to compare enzyme activity between susceptible mosquitoes in the field and in the laboratory (Kisumu).

Results

Susceptibility testing of collected *An. gambiae* s.l. to insecticides

The results obtained with the different tests using the WHO tube method and the CDC bottle method are shown in the table below:

Table 1 : Mortality rates at diagnostic doses of deltamethrin, permethrin, pirimiphos-methyl and Bendiocarb in 13 communes of Benin according to the WHO tube test and CDC bottle test methods

	Insecticides	Localities	Cotonou	P-Novo	Bohicon	Allada	Missérété	Dassa	Savè	Parakou	Kandi	Malanville	Bantè	Ouidah	N'Dali	P-value
WHO tests	Deltaméthrin	Nbr tested	85	94	94	96	92	83	92	90	89	91	96	86	89	< 0,0001
		%Mortality	16,5^{a}	14,9^{ab}	11,7^{ab}	43,8^{c}	30,4^{abc}	45,8^{c}	21,7^{abc}	40^{c}	15,7^{ab}	14,3^{ab}	22,9^{abc}	22,1^{abc}	36^{ac}	
	Perméthrin	Nbr tested	96	85	102	96	88	87	88	84	96	93	98	88	87	< 0,0001
		%Mortality	43,8^{acd}	14,1^{bc}	21,6^{abc}	17,7^{bc}	27,3^{abc}	51,7^{ad}	37,5^{acd}	47,6^{ad}	52,1^{ad}	16,1^{bc}	17,3^{bc}	17^{bc}	24,1^{abc}	
	Bendiocarb	Nbr tested	90	99	86	91	99	94	99	87	87	98	94	99	93	< 0,0001
		%Mortality	96,7^{ac}	94,9^{ac}	96,5^{ac}	100^{ac}	99^{ac}	83^{a}	91,9^{ac}	97,7^{ac}	94,3^{ac}	94,9^{ac}	95,7^{ac}	99^{ac}	87,1^{ac}	
	Pirimiphos-méthyl	Nbr tested	89	96	92	95	89	97	98	96	87	88	92	93	87	0,6049
		%Mortality	100	100	100	100	100	100	100	99	99	100	98,9	100	100	
CDC tests	Deltaméthrin	Nbr tested	90	82	63	74	85	75	74	89	77	65	65	70	62	< 0,0001
		%Mortality	88,9^{ab}	79,3^{abd}	73^{abcd}	75,7^{abcd}	74,1^{abcd}	50,7^{cd}	54,1^{bcd}	75,3^{abcd}	72,7^{abcd}	66,2^{abcd}	61,5^{bcd}	60^{bcd}	73,8^{abcd}	
	Perméthrin	Nbr tested	81	79	88	79	79	85	82	60	74	73	73	75	78	< 0,0001
		%Mortality	69,1^{a}	34^{b}	61,4^{ab}	44,3^{ab}	44,3^{ab}	55,3^{ab}	58,5^{ab}	43,3^{ab}	71,6^{a}	65,8^{ab}	68,5^{a}	49,3^{ab}	64,1^{ab}	
	Bendiocarb	Nbr tested	85	84	99	73	77	81	74	79	86	67	77	69	87	0,1114
		%Mortality	98,8	100	100	100	100	97,5	94,6	97,5	96,5	97	97,4	100	95,4	
	Pirimiphos-méthyl	Nbr tested	83	90	82	86	85	91	84	83	81	80	79	73	82	NA
		%Mortality	100	100	100	100	100	100	100	100	100	100	100	100	100	

Effect of deltamethrin on *An. gambiae* complex populations

Using the WHO tube method, *An. gambiae* s.l. populations in the thirteen localities showed very low mortality rates (< 100%), 24 hours after exposure to the diagnostic dose of deltamethrin (0.05%) suggesting a generalized resistance of *An. gambiae* populations at these localities. Indeed, these mortality rates varied between 11% and 22% for the localities of Bohicon, Porto-Novo, Cotonou, Kandi, Malanville, Savè, Bantè and Ouidah while in the communes of Missérété, Allada, Parakou, N'Dali and Dassa, the mortality rates recorded were between 30% and 45%. (Table 1, Figure 2).

Using the CDC bottle test method, *An. gambiae* s.l. complex populations at the thirteen localities also showed low mortality rates (<100%) after the diagnostic time (30 minutes) of their exposure to the diagnostic dose of deltamethrin (12.5µg) suggesting widespread resistance of An. gambiae complex populations at these localities. However, the recorded mortality rates were higher than those obtained with the WHO tube method and ranged from 50% to 88% (Tableau 1, Figure 2).

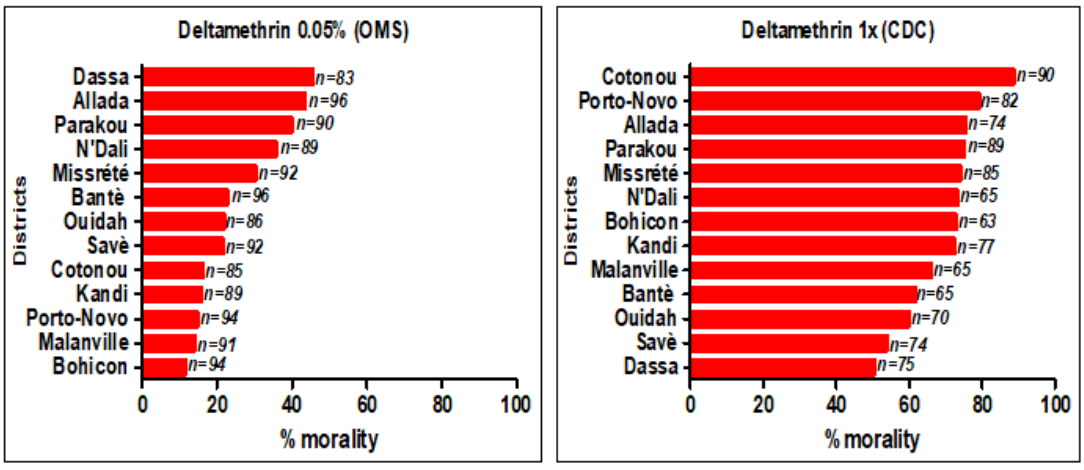

Fig 2: Mortality rate of *An. gambiae* s.l. from the thirteen communes after exposure to deltamethrin 0.05% with the WHO test and deltamethrin 12.5µg with the CDC test

Effect of permethrin on *An. gambiae* complex populations

Using the WHO tube method, *An. gambiae* s.l. populations in the thirteen localities showed very low mortality rates (<100%) 24 hours after exposure to the diagnostic dose of permethrin (0.75%) suggesting widespread resistance of *An. gambiae* populations at these localities. Mortality rates ranged from 14% to 27% in Bohicon, Porto-Novo, Malanville, Ouidah, Bantè, Allada, N'Dali and Missérété, while in Cotonou, Kandi, Savè, Dassa and Parakou mortality rates ranged from 37% to 52% (Table 1, Figure 3).

With the CDC bottle test method, *An. gambiae* s.l. complex populations in the thirteen localities also showed mortality rates less than 100% after the diagnostic time (30 minutes) of their exposure to the diagnostic dose of permethrin (21.5 µg) suggesting a generalized resistance of An. gambiae complex populations at these localities. Mortality rates recorded were less than 50% for the localities of Porto-Novo, Ouidah, Allada, Parakou and Missérété while vector populations in Bohicon, Cotonou, Kandi, Malanville, Savè, Bantè, Dassa and N'Dali showed mortality rates between 50% and 69% (Table 1, Figure 3).

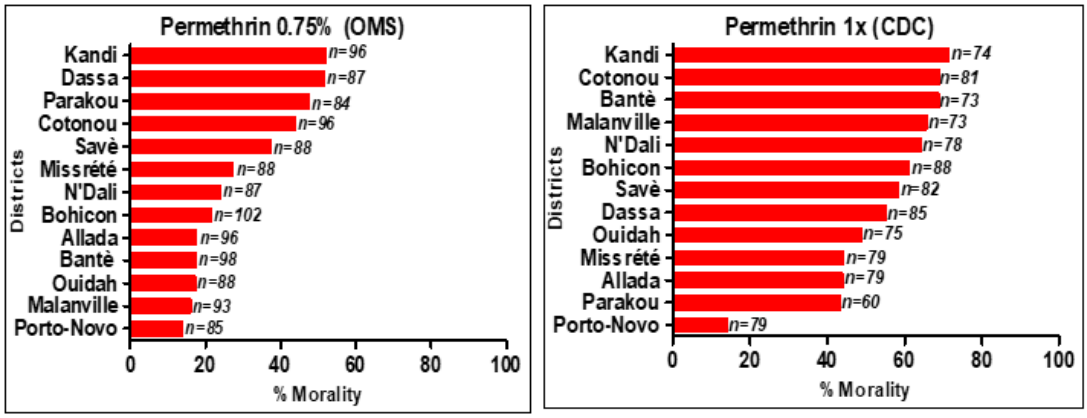

Fig 3 : Mortality rate of *An. gambiae* s.l. from the thirteen communes after their exposure to deltamethrin 0.05% with the WHO test and 12.5µg with the CDC bottle test.

Effet du bendiocarb sur les populations du complexe *An. gambiae*

Using the WHO tube method, *An. gambiae* s.l. populations in thirteen localities showed mortality rates higher than 98% with bendiocarb (0.1%) in Ouidah (98.98%), Missérété (98.99%) and Allada (100%) suggesting a sensitivity of these populations to bendiocarb. However, resistance was recorded with the vector populations of N'Dali (87.98%) and Dassa (83.49%) while the vector populations of Cotonou, Porto-Novo, Bohicon, Savè, Parakou, Malanville, Bantè, and Kandi showed mortality rates between 90% and 97%. (Table 1, Figure 4)

Using the CDC bottle method, only the populations of Bohicon, Porto-Novo, Ouidah, Allada, and Cotonou showed full sensitivity (100%) to bendiocarb (12.5µg). The localities of Kandi, Malanville, Savè, Bantè, Dassa, Parakou, N'Dali and Missérété showed mortality rates between 94% and 97% indicating a suspicion of resistance to bendiocarb in these localities (Table 1, Figure 4).

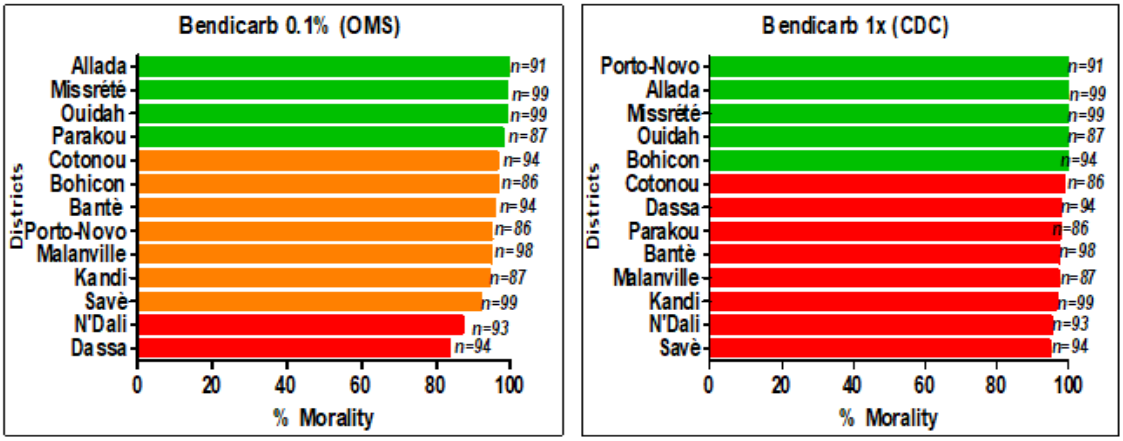

Fig 4 : Mortality rate of *An. gambiae* s.l. from the thirteen communes after exposure to bendiocarb 0.1% with the WHO test and bendiocarb 12.5µg with the CDC test

Effect of pirimiphos-methyl on populations of *An. gambiae* complex

Using the WHO tube method, *An. gambiae* s.l. populations in the thirteen localities showed mortality rates higher than 98% with pirimiphos-methyl (0.25%) suggesting a sensitivity of these populations to pirimiphos-methyl. Mortality rates were 100% in all localities except Parakou (98.95%), Kandi (98.85%) and Bantè (98.90%). (Table 1, Figure 5).

Using the CDC bottle test method, all populations of *An. gambiae* complex in the thirteen localities of our study showed total lethality (100%) to pirimiphos-methyl (20µg) suggesting total sensitivity of these populations to pirimiphos-methyl (20µg) (Tableau 1, Figure 5).

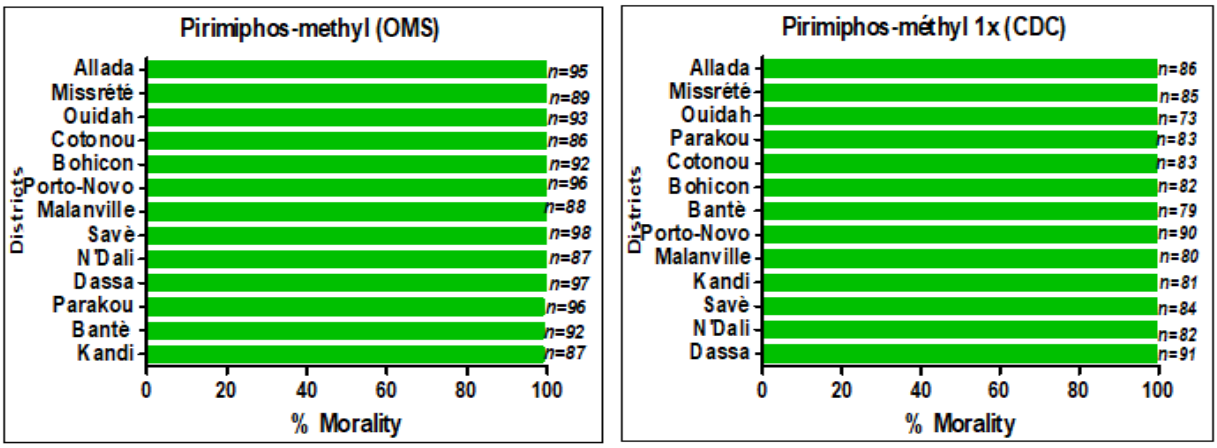

Fig 5: Mortality rates of *An. gambiae* s.l. from the thirteen communes after their exposure to pirimiphos-methyl 0.25% with the WHO test and pirimiphos-methyl 20µg with the CDC test.

Mapping insecticide resistance

Resistance mapping (Figure 6) shows a generalized distribution of resistance to pyrethroids (deltamethrin and permethrin) from the South to the North of Benin. There is also a resistance of vectors to bendiocarb in the Center and North and a wide distribution of their sensitivity to pirimiphos methyl.

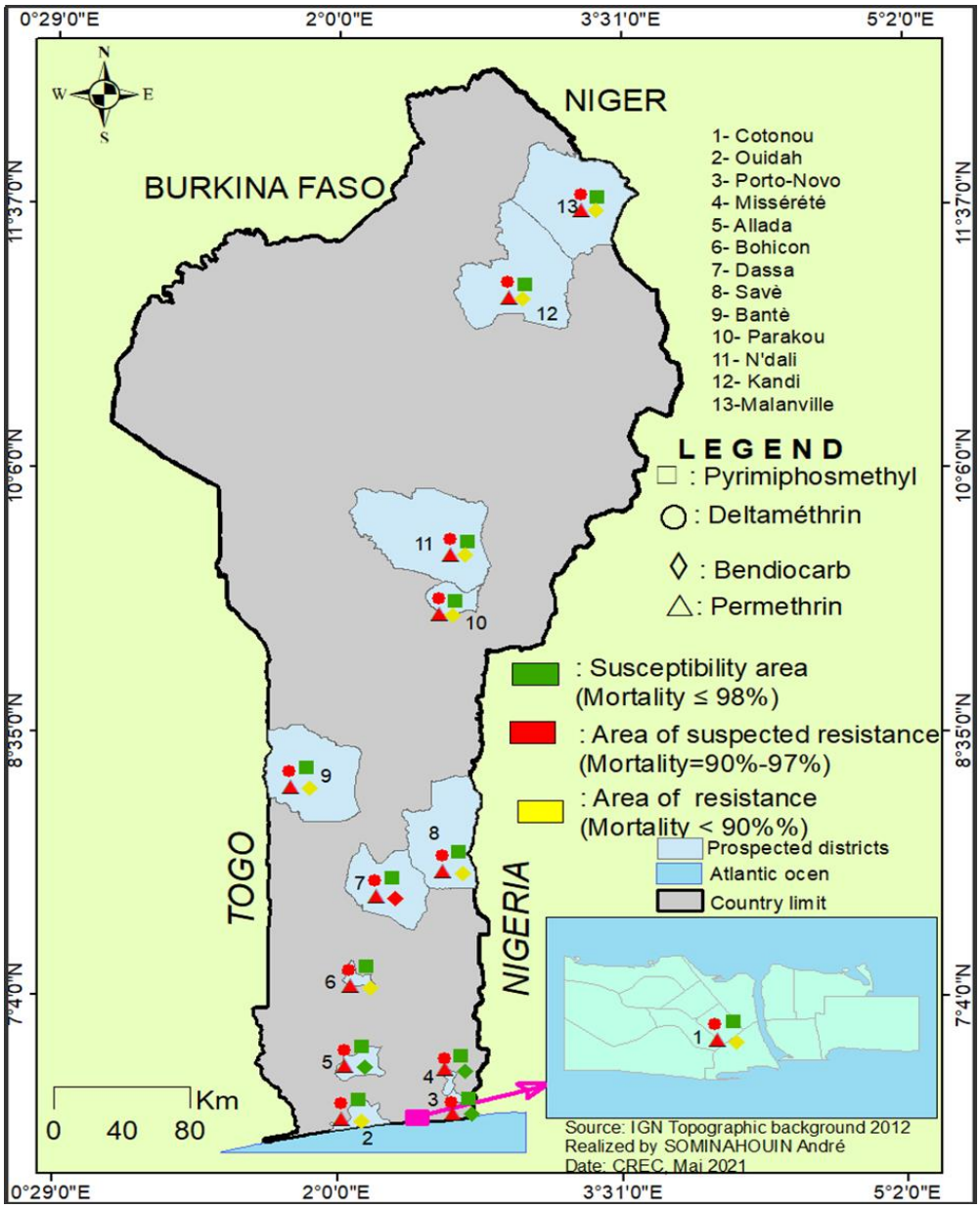

Fig 6: Distribution of vector resistance to insecticides

Resistance status of *An. gambiae* s.l. to the diagnostic dose of deltamethrin, permethrin, bendiocarb and pirimiphos-methyl by WHO and CDC test methods

The results of susceptibility testing of *An. gambiae* s.l. using the WHO tube test method or the CDC bottle test method to the diagnostic dose of permethrin and deltamethrin show widespread resistance to these insecticides in all the communes where larval surveys were conducted (Table 2). For Bendiocarb and using the WHO tests, only the Anopheles populations of the communes of Missérété, Allada and Ouidah were found to be sensitive, the vector populations of Dassa and N'Dali showed resistance, while a suspicion of resistance was noted in the communes of Cotonou, Porto-Novo, Bohicon, Savè, Parakou, Kandi, Malanville and Bantè (Table 2). The same trends were observed with CDC bottle tests for bendiocarb with total susceptibility (100% mortality) observed in Bohicon, Allada, Missérété, Ouidah and Porto-Novo. On the other hand, with pirimiphos-methyl, all *An. gambiae* populations in the thirteen communes were found to be susceptible with both the WHO tube test and the CDC bottle test methods (Table 2).

Table 2: Resistance status to the diagnostic dose of deltamethrin, permethrin, pirimiphos-methyl and Bendiocarb in 13 communes of Benin according to WHO tube and CDC bottle test methods.

	WHO test								CDC test							
	Deltaméthrin		Perméthrin		Bendiocarb		Pirimiphos-méthyl		Deltaméthrin		Perméthrin		Bendiocarb		Pirimiphos-méthyl	
Localities	Nbr tested	Status of R	Nbr tested	Status of R	Nbr tested	Status of R	Nbr tested	Status of R	Nbr tested	Status of R	Nbr tested	Status of R	Nbr tested	Status of R	Nbr tested	Status of R
Cotonou	85		96		90		89		90		81		85		83	
Porto-Novo	94		85		99		96		82		79		84		90	
Bohicon	94		102		86		92		63		88		99		82	
Allada	96		96		91		95		74		79		73		86	
Missérété	92		88		99		89		85		79		77		85	
Dassa	83		87		94		97		75		85		81		91	
Savè	92		88		99		98		74		82		74		84	
Parakou	90		84		87		96		89		60		79		83	
Kandi	89		96		87		87		77		74		86		81	
Malanville	91		93		98		88		65		73		67		80	
Bantè	96		98		94		92		65		73		77		79	
Ouidah	86		88		99		93		65		73		69		73	
N'Dali	89		87		93		87		65		73		87		82	

= Population résistante; = Population suspectée de résistance ; =Population sensible

Characterization of molecular forms and resistance genes (kdr and ace-1) in communes

The distribution of *An. gambiae* s.l shows the presence of three main species of the *An. gambiae* complex: *An. gambiae*, An. Coluzzii and *An. arabiensis* with very high proportions of *An. gambiae* in almost all localities except Malanville and Allada where all Anopheles analyzed are Coluzzii. As for the distribution of resistance genes, the frequency of the *L1014F* allele of the kdr gene is high in all localities. The lowest frequency of the kdr resistance gene was observed in Malanville (0.64) and the highest in Cotonou (0.916). In contrast to the L1014F allele of the kdr resistance gene, the frequency of the G119S allele of the ace-1R gene is very low in all surveyed locations (Tables 3 et 4).

Table 3: Distribution of species of the An. gambiae complex and frequency of the resistant *kdr* allele (*L1014F*) in the communes of Cotonou, Porto-Novo, Bohicon, Allada, Missérété, Dassa, Save, Parakou, Kandi, Malanville, Bante, Ouidah and N'Dali

Localities	Species	Number tested	Genotypes			f (1014F)	df	p-value*
			1014F	*1014L*	*1014L*			
			1014F	*1014F*	*1014L*			
Cotonou	*An. coluzzii*	50	36	12	2	0,84	NA	NA
Porto-Novo	*An. gambiae* s.s.	13	10	2	1	0,84	1	0.591
	An. coluzzii	37	23	11	3	0,77		
Parakou	*An. gambiae* s.s.	47	31	12	4	0,79	1	0.8548
	An. coluzzii	3	2	0	1	0,67		
Kandi	*An. gambiae* s.s.	44	28	12	4	0.77	1	0.9172
	An. Arabiensis	6	4	2	0	0.83		
Dassa	*An. gambiae* s.s.	49	31	12	6	0,76	1	1
	An. coluzzii	1	1	0	0	1		
Savè	*An. gambiae* s.s.	35	21	12	2	0,77	2	0,8395
	An. coluzzii	3	2	0	1	0,67		
	An. Arabiensis	12	7	4	1	0,75		
Bantè	*An. gambiae* s.s.	50	23	20	7	0,66	NA	NA
Bohicon	*An. gambiae* s.s.	22	12	7	3	0,7	1	1
	An. coluzzii	28	14	12	2	0,71		
N'Dali	*An. gambiae* s.s.	30	19	8	3	0,77	2	0,5234
	An. coluzzii	8	5	3	0	0,81		
	An. Arabiensis	2	2	0	0	1		
Allada	*An. coluzzii*	50	34	10	6	0,78	NA	NA
Ouidah	*An. gambiae* s.s.	42	22	16	4	0,71	1	1
	An. coluzzii	8	4	3	1	0,69		
Malanville	*An. coluzzii*	50	22	20	8	0,64	NA	NA
Missérété	*An. gambiae* s.s.	43	23	15	5	0,71	1	1
	An. coluzzii	7	4	2	1	0,71		

*p-value: based on the χ2-square test comparing frequencies between the two species by locality; f=frequency

Table 4: Distribution of species of the An. gambiae complex and frequency of the resistant *ace-1* allele (*G119S*) in the communes of Cotonou, Porto-Novo, Bohicon, Allada, Missérété, Dassa, Save, Parakou, Kandi, Malanville, Bante, Ouidah and N'Dali

Localities	Species	Number tested	Genotypes			f (119S)	df	p-value*
			119S	*119G*	*119G*			
			119S	*119S*	*119G*			
Cotonou	*An. coluzzii*	50	0	6	44	0,06	NA	NA
Porto-Novo	*An. gambiae* s.s.	13	0	2	11	0,0769	1	0,5925
	An. coluzzii	37	0	2	35	0,027		
Parakou	*An. gambiae* s.s.	47	0	5	42	0,0532	1	1
	An. coluzzii	3	0	0	3	0		
Kandi	*An. gambiae* s.s.	44	0	4	40	0,0454	1	1
	An. Arabiensis	6	0	0	6	0		
Dassa	*An. gambiae* s.s.	49	0	8	41	0,0816	1	1
	An. coluzzii	1	0	0	1	0		
Savè	*An. gambiae* s.s.	35	0	3	32	0,0429	2	0,8752
	An. coluzzii	3	0	0	3	0		
	An. Arabiensis	12	0	1	11	0,0417		
Bantè	*An. gambiae* s.s.	50	0	7	43	0,07	NA	NA
Bohicon	*An. gambiae* s.s.	22	0	3	19	0,068	1	0,7815
	An. coluzzii	28	0	2	26	0,036		
N'Dali	*An. gambiae* s.s.	30	0	6	24	0,1	2	0,0731
	An. coluzzii	8	0	1	7	0,06		
	An. Arabiensis	2	0	0	2	0		
Allada	*An. coluzzii*	50	0	3	47	0,03	NA	NA
Ouidah	*An. gambiae* s.s.	42	0	5	37	0,06	1	0,7073
	An. coluzzii	8	0	0	8	0		
Malanville	*An. coluzzii*	50	0	4	46	0,04	NA	NA
Missérété	*An. gambiae* s.s.	43	0	7	36	0,08	1	1
	An. coluzzii	7	0	1	6	0,07		

*p-value: based on the χ2-square test comparing frequencies between the two species by locality; f=frequency

Enzymatic tests on microplates

Biochemical assays showed significantly elevated enzyme activities in some mosquito populations (Figure 7).

The activities of non-specific esterases (α and β esterases) are higher in the populations: Kandi, Parakou, Porto-Novo, Dassa, Savè, Bohicon and Cotonou than in the Kisumu strain ($p < 0.05$). The activity of mixed function oxidases is higher in the populations of Kandi, Cotonou, Dassa and Savè than in

Kisumu (p <0.05). Concerning glutathione S-transferase (GST) activity, the highest was observed in the populations of Kandi, Parakou, Allada, Bohicon, Missérété, Dassa and Cotonou compared to the Kisumu strain. (p <0.05) (Figure 8).

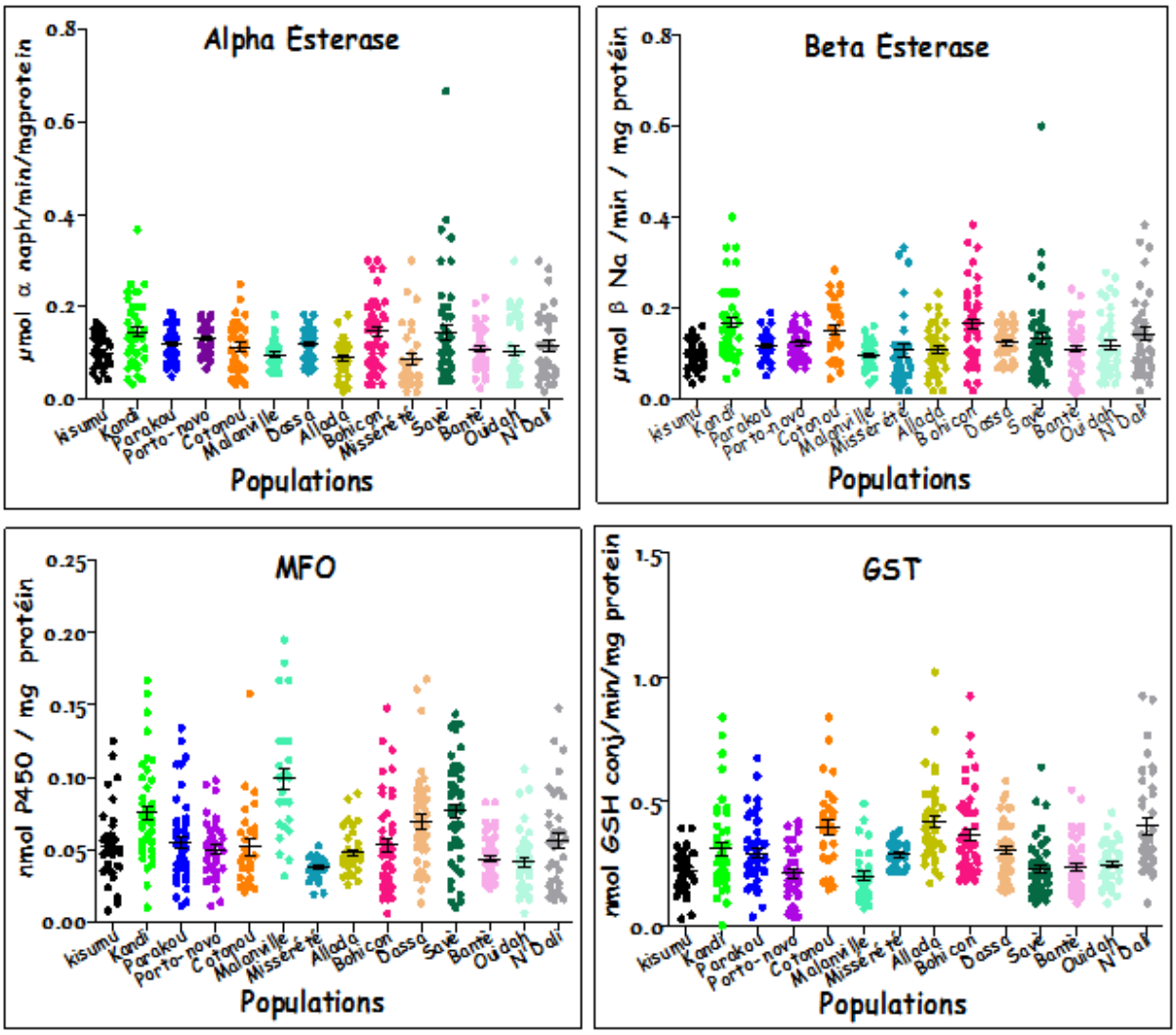

Fig 7: Mean and standard error of enzyme overexpression [α- and β- esterases, mixed-function oxidases (MFO) and glutathione-S-transferase (GST)] in *Anopheles gambi*ae s.l. compared with *An. gambiae* s.s Kisumu (laboratory reference strain) characterized by spectrophotometry

Discussion

Insecticide resistance monitoring is recommended by WHO in all countries that use long-lasting insecticidal nets (LLINs) or indoor residual spraying (IRS) for malaria control [22].

An. gambiae s.l. populations from the 13 localities showed high levels of resistance to the diagnostic dose of permethrin and deltamethrin from south to north confirming a generalization of malaria vector resistance to pyrethroids observed in Benin by several authors in the past [3, 8, 9, 23].

Using the WHO tube method, all thirteen populations of *An. gambiae* s.l. were resistant to permethrin with mortality rates ranging from 14% to 27% in Bohicon, Porto-Novo, Malanville, Ouidah, Bantè, Allada, N'Dali and Missérété while in Cotonou, Kandi, Savè, Dassa and Parakou mortality rates ranged from 37 to 52%. With deltamethrin, these mortality rates varied between 11% and 22% for the localities of Bohicon, Porto-Novo, Cotonou, Kandi, Malanville, Savè, Bantè and Ouidah while in the communes of Missérété, Allada, Parakou, N'Dali and Dassa, the mortality rates recorded were between 30% and 45%. Similarly, using the CDC bottle test method, mortality rates recorded with deltamethrin or permethrin were higher than those obtained with the WHO tube method and varied between 40% and 88% suggesting pyrethroid resistance in these localities.

For Bendiocarb and using WHO tests, only Anopheles populations in the communes of Missérété, Allada and Ouidah were found to be sensitive, the vector populations of Dassa and N'Dali showed resistance, while suspected resistance was noted in the communes of Cotonou, Porto-Novo, Bohicon, Savè, Parakou, Kandi, Malanville and Bantè. The same trends are observed with CDC bottle tests for bendiocarb with a total sensitivity (100% mortality) observed in Bohicon, Allada, Missérété, Ouidah and Porto-Novo. On the other hand, with pirimiphos-methyl, all *An. gambiae* populations in the thirteen communes were found to be susceptible with both the WHO tube and CDC bottle test methods.

Although two different protocols were used to determine susceptibility to the different insecticides (permethrin, deltamethrin, bendiocarb, and pirimiphos-methyl), the resistance status to these insecticides recorded with WHO tube tests or CDC bottle tests was the same. This result confirms the work of Aïzoun *et al* in Benin in 2013 and Omondi *et al* in 2017 in Kenya [24, 29]. The mortality rates recorded with the CDC bottle method were higher than those obtained with the WHO tube method. This would be due to the diagnostic time (30 minutes) observed with the CDC bottle tests during which all dropped mosquitoes are

considered dead compared to the WHO tube test which records mortality only 24 hours after exposure of mosquitoes to insecticide-impregnated papers [24].

These results obtained with the diagnostic doses of pyretrinoids (permethrin and deltamethrin) with both the WHO tube method and the CDC bottle method confirm the results of Aïzoun *et al.* (2013), Gnanguenon *et al.* (2015) and Salako *et al.* (2018) [7, 8, 24]. The low mortality rates recorded with the diagnostic dose of pyretroids in the center and north of the country (Dassa, Savè, Bantè, Parakou, N'Dali, Kandi and Malanville) would be due to the very strong insecticide selection pressure exerted on mosquitoes in these localities, particularly N'Dali, Kandi, Parakou and Malanville, which are characterized by a high level of agricultural production (cotton and rice) associated with a significant or even abusive use of chemical products to control the pests. This is due to the generalization of cotton cultivation in the north of the country as already reported by other authors [26, 27] but also to insecticide pressures resulting from the massive use of pyrethroid-impregnated mosquito nets [28, 29].

Levels of resistance to pyrethroids (permethrin and deltamethrin) varied from one commune to another. This variation in the level of resistance to deltamethrin or permethrin depends on the variation of selection pressures exerted on the different vector populations. Indeed, this class of insecticide (pyrethroids) is found on most nets distributed nationwide [30]. In addition, there are pressures from the use of agricultural insecticides in localities where agriculture and market gardening are the main activities [17, 26].

The high intensity of resistance recorded in the different agro-ecological zones is associated with the presence of the kdr gene which seems to be the main mechanism of insecticide resistance in these localities. This gene was found at very high allelic frequencies (0.70 on average) in all tested populations of *An. gambiae* s.l. The *kdr L1014F* frequency seems to be higher in *An. gambiae* s.s. than in *An. coluzzii* because in the communes where these two species occur simultaneously, the *kdr L1014F* frequency in *An. gambiae* is always higher than that observed in *An. coluzzii*. Recent work by Gnanguenon *et al*. (2015) and Yahouédo *et al*. (2016), on the north-south transect and in southern Benin have already confirmed these results [7, 31]. Previous studies have shown that the frequencies of the *kdr L1014F* gene are very high in *An. gambiae* in West and Central Africa [13, 33] compared to *An. coluzzii*, which has lower frequencies except in a few urban and peri-urban coastal areas [34]. This spread of the *kdr 1014F* gene in malaria vector populations in Benin could compromise the effectiveness of currently used vector control tools [35, 36].

In addition to resistance to pyrethroids, we noted resistance to bendiocarb (carbamates). It is associated with the presence of the ace-1R gene detected in the localities concerned. This mutation was previously reported by Corbel *et al.* (2007), Djènontin *et al*. (2010), Yadouleton *et al*. (2010) and Aïkpon *et al.* (2013) [3, 5, 9, 37]. This resistance to bendiocarb observed in the different localities is associated with low frequencies of the ace-1R gene (2 to 4.5% depending on the locality). Corbel *et al*. (2007), Djènontin *et al*. (2010), Aïzoun *et al*. (2013) and Gnanguenon *et al*. (2015) had recorded bendiocarb resistance associated with almost zero ace-1R gene frequencies (0 to 1%), which could mean that the ace-1 mutation is increasing in Benin [7, 9, 24, 37]. Recent results obtained by Aïkpon *et al.* (2013) in Atacora; Gnanguenon *et al.* (2015) in Kandi and Salako *et al.* (2018) in Alibori and Donga confirm the increase (1 to 6% depending on the locality) in *ace-1R* gene frequencies in Benin [3, 7, 8]. It is therefore urgent that continuous monitoring be carried out in environments treated with bendiocarb in order to follow the evolution of the *ace-1R* gene compared to the initial state. This monitoring will allow better management of insecticide resistance in malaria vectors for better control of malaria transmission.

However, while the *kdr* and *ace-1R* genes confer a significant effect on vector resistance, they do not fully explain the observed cases of resistance because many homozygous susceptible individuals survive exposure to pyrethroids and carbamates [34]. This suggests that other alternative resistance mechanisms such as metabolic mechanisms operate in these mosquito populations. In order to explore the metabolic resistance mechanisms involved in the resistance of these populations, a biochemical approach was used. Thus, the high activity of non-specific esterases noted in the communes of Kandi, Parakou and Dassa in particular shows that resistance to bendiocarb is not only linked to the presence of the ace-1R gene. Indeed, esterases can confer resistance to organophosphates and carbamates [38]. This overproduction of esterases is thought to be due to insecticidal pressure on mosquito larvae in cotton crops to control pests [25, 27].

No resistance to pirimiphos-methyl was observed at any of our sites for this study. However, it would be necessary to monitor the susceptibility of these anopheles to pyrimiphos-methyl and to find other alternative vector control methods to curb the spread of resistance genes. Rotation or combination of insecticides in IRS campaigns are strategies to be promoted for resistance management, which is becoming a concern.

Conclusion

This study shows the resistance pattern of malaria vectors to the different categories of insecticides used for vector control in Benin. It reveals a generalized distribution of resistance of *An. gambiae* s.l to pyrethroids and a clear resistance to carbamates (bendiocarb) especially in the northern region of the country. This vector is still susceptible to organophosphates, especially pirimiphos-methyl, but since resistance is a dynamic phenomenon, it would be necessary to monitor the susceptibility of these anopheles to pyrimiphos-methyl and to find other alternative vector control methods to slow down the spread of resistance genes. This has already started with fludora fusion and sumishield, two insecticides currently under evaluation in Benin. Thus, rotation or combination of insecticides in IRS campaigns are strategies to be promoted for resistance management, which is becoming a heavy burden for the malaria control program (NMCP). The kdr resistance gene was observed at high frequencies in all three species of the *An. gambiae* complex detected: *An. coluzzii*, *An. arabiensis* and *An. gambiae*. The ace-1R gene was also detected but at very low frequencies. The involvement of metabolic resistance mechanisms was also observed with an overexpression of the activity of these enzymes in some localities.

The most practical opportunity for vector control programs to manage resistance is through the appropriate use of insecticides. The timing of insecticide use, method of use (combination or single), frequency of use, and duration of use are factors that need to be planned for to delay the spread of resistance until other, more effective strategies are designed and implemented. However, it would be interesting to determine the intensity of vector resistance in each zone or commune given the variability in the level of resistance

observed in different localities in order to better guide resistance management strategies.

List of abbreviations

IRS: indoor residual spraying; PMI: President's Malaria Initiative; WHO: world health organization; LLIN: long lasting insecticidal nets; NMCP: national malaria control program; EIR: entomological inoculation rate; PCR: polymerase chain reaction; IRS: indoor residual spraying; CREC: Centre de Recherche Entomologique de Cotonou; RTI: research triangle international; ITN: insecticide treated net.

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Author's Contributions

MCA, RO, and GGP conceived the study. MCA, HWS, WS, RO, GGP, ASS and GGP have participated in the design of the study. Entomologic data was collected by RO, ASS, HWS, AAS and GGP and laboratory analysis was carried out by AS, RO, PGG and ASS. CDK drafted the manuscript. Statistical data analysis by ASS, BY, SC, IA, AJF, HWS, RO, PGG and MCA critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

The data supporting the conclusions of this article are included within the article. The raw data used and/or analyzed in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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