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Insecticide Susceptibility of *An. gambiae* and *An. coluzzii* in Congo Brazzaville

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Abstract

Vector control against malaria in Congo is mainly achieved through the use of long-lasting insecticide treated nets (LLINs). However, the encouraging results achieved through the use of LLINs are threatened by the proven resistance of *Anopheles gambiae* s.l. to pyrethroids. The aim of this study was to assess the sensitivity of *Anopheles gambiae s.l.* to DDT and pyrethroids, and to identify the kdr mutation and the enzymes involved in the resistance to these insecticides. Two methods were used to collect *Anopheles* from 2016 to 2018, 2 methods were used: collection by dipping of larvae and collection of resting females. Susceptibility testing was performed according to the WHO protocol with unfed females aged 2 - 5 days old. The insecticides used were: DDT 4%, permethrin 0.75%, deltamethrin (0.05% and 0.5%) and lambda-cyhalothrin 0.05%. The synergist piperonyl butoxide (PBO) was used to identify the enzymes involved in resistance. Polymerase chain reaction was used to identify anopheline species and kdr mutations. The tests were performed at room temperature and relative humidity of 25˚C ± 2˚C and 70% ± 10% RH respectively. Susceptibility tests showed high resistance of Anopheles to DDT and pyrethroids. Two species belonging to the *Anopheles gambiae s.l.* complex were identified: *An. gambiae* (91.4%) and *An. coluzzii* (8.6%). The mechanism of resistance was attributed to the kdr (knockdown resistance) L1014F mutation which confers cross-resistance to DDT and pyrethroids with a frequency ranging from 0.68 to 0.86. Monooxygenase has been identified as the enzyme involved, as it is the main enzyme involved in metabolic resistance to pyrethroids. This resistance could compromise malaria control efforts based on the use of insecticide-treated nets. However, PBO incorporated into LLINs could be effective in managing the established resistance.

Keywords

Malaria, *Anopheles gambiae*, *Anopheles coluzzii*, Resistance, Insecticides, KDR Mutation, Monooxygenase, Congo

1. Introduction

Malaria is the most common parasitic disease in the world, despite all the efforts made to control and eliminate it. In 2023, 263 million cases of malaria were recorded worldwide, including 597,000 deaths [1].

In the Republic of Congo, malaria is a real public health problem. In 2023, the number of malaria cases recorded in health facilities was 1,327,964 [2].

The two main methods of malaria vector control in Africa are long-lasting insecticide-treated mosquito nets (LLINs) and Indoor Residual Spraying (IRS) [3]. The use of LLINs alone prevented 69% of cases in sub-Saharan Africa [4].

In Congo in particular, LLINs are the main method of vector control. However, the major challenge of LLINs in malaria control is the spread of vector resistance to pyrethroids used to impregnate bed nets [5]. Entomological studies of resistance in *Anopheles gambiae* s.l. species have demonstrated resistance to pyrethroids and DDT in many locations in the country [5]-[8]. In addition, resistance of *An. gambiae* s.l. to pyrethroids and DDT has been reported in many other countries in Africa [9]-[13]. Globally, among the 88 countries that reported insecticide resistance data to WHO, the proportion attributable to pyrethroids and organochlorines was 87% and 82%, respectively [13].

The main mechanisms identified are mutations of the target sites particularly those located at the level of voltage-gated sodium channels [14] [15], and metabolic resistance involving detoxifying enzymes [16]. Indeed, a study conducted in 2009 in Boutoto and its surroundings in Congo, showed that DDT resistance was linked to the presence of the L1014F mutation, while pyrethroid resistance was due to monooxygenases and esterases [6]. This mutation is particularly prevalent in Central Africa, in countries such as Gabon, DRC, Central Africa, Cameroon and Angola, to name but a few. It is in this context that only this mutation has been identified in this study. Resistance to pyrethroids is a worrying phenomenon as it could compromise the effectiveness of LLINs, which is the main vector control strategy of the National Malaria Control Program (NMCP) in Congo. Therefore, in order to maintain the achievements and continue the use of LLINs, it seemed essential to have data on the level of sensitivity of vectors to insecticides.

The objectives of the present study carried out in this context are to update the level of resistance to pyrethroids and DDT, to identify the species of the *Anopheles gambiae* s.l. complex and the KDR mutation (L1014F) involved in the resistance to these insecticides and the metabolic mechanisms.

2. Material and Method

2.1. Study Sites

The study sites were the city of Brazzaville, the urban commune of Kintele and the village of Djoumouna as shown in **Figure 1**.

The city of Brazzaville (4˚15'S and 15˚16'E) is the political capital of the Republic of Congo. It is situated at an average altitude of 326 meters [17]. The climate is equatorial, with a dry season from June to September and a rainy season from October to May. The climatic parameters for the last 5 years (2015 to 2019) showed an average temperature of 26.08˚C and rainfall of 127.22 mm of water on average per month. The average maximum and minimum relative humidity were 89.57% and 55.63% respectively [18]. Brazzaville is irrigated by numerous rivers, including the Djoue, Djiri, Tsieme, Mfoa, Madoukou-tsekele, Mfilou and Kelekele.

The vegetation of the city and its surroundings consists of forest and savanna. With its 9 districts, the city is home to more than half of the Congolese population. Studies carried out in Brazzaville have shown that species of the *Anopheles gambiae* s.l. complex are mainly involved in malaria transmission [19]-[23]. Regarding the intensity of transmission, the study by Trape and Zoulani in 1989 showed that in the densely populated districts (Poto-Poto, Moungali and Ouenze), one person received an infectious bite every three years. It was also shown that in a recently urbanized district, such as Mfilou-Ngamaba in those years, one person received more than 100 infectious bites per year [21].

Regarding the sensitivity of Anopheles to insecticides, studies conducted in the 1950s and 1960s had shown that *An. gambiae* s.l. was resistant to organochlorines (DDT, HCH, dieldrin) and sensitive to malathion [20] [23]. In 2014, Nianga Bikouta showed that vector populations were resistant to DDT and pyrethroids (deltamethrin, permethrin, and lambda-cyhalothrin), but susceptible to bendiocarb and malathion [22].

During the present study, larvae were collected from six (6) sites in Brazzaville: three (3) integrated with horticultural activities (The bled, Test garden and Mayanga) with breeding grounds consisting of wells dug in the ground and used as water reserves for watering vegetables, and one (1) considered as polluted (Makelekele). Polluted and non-polluted breeding sites were distinguished by the presence of plastic and organic waste. Finally, three (3) consisted of puddles left over from the rainy season (Bacongo, Moungali and Madibou).

The commune of Kintele is located 25 km north of Brazzaville (4˚9'0"S, 15˚20'32"E) with an average altitude of 201 m [24]. The population is estimated at 11,105 inhabitants. The economic activity of the municipality of Kintele is flourishing. There are 265 small and medium-sized commercial enterprises, 64 small and medium-sized industries, mainly specialized in construction, and 6 small state markets. The houses are made of cement bricks with corrugated iron roofs. These dwellings generally have gaps that allow many arthropods, including mosquitoes, to pass through. The relief consists of valleys, plains, hills and plateaus. The climate is tropical and humid. With two seasons, a rainy season from October to May and a dry season from June to September, the average annual rainfall is 1370 mm and the average temperature is 25.5˚C [25]. Kintele is irrigated by two rivers, the Djiri and the Blue Chatelet. The vegetation is mainly wooded savannah, sometimes with scrub, where manioc and pineapple are grown. Vegetables are grown on the banks of the Congo River [26]. A study of malaria transmission in 1987 showed that the *An. gambiae* s.l. complex contained the main malaria vector species in Kintele [27].

Figure 1. Map of Congo including Brazzaville, Kintele and Djoumouna (CERGEC, 2020).

Djoumouna (4˚22'34"S, 15˚9'36"E) is a rural area located 25 km southeast of Brazzaville in the Pool department, with an average altitude of 217 m [28]. The population is estimated at 635 inhabitants. The main activities are agriculture, fish farming and cattle rearing. Djoumouna is located in an area of degraded secondary forest. The climate is similar to that of Brazzaville. The town is bordered by four rivers: the Lomba, the Kinkoue, the Loumbangala and the Djoumouna. These rivers supply water to a number of fish ponds. These ponds are permanent breeding grounds for the larvae proliferation of the Anopheles mosquito. In addition to these breeding sites, there are many other temporary breeding sites. Many studies were carried out in the village between the 1970s and 1990s [29]-[31]. These showed that species belonging to the *An. gambiae* s.l. complex were the main vector species of malaria and that the intensity of malaria transmission was expressed as more than 1000 ft/h/a [32]. In terms of insecticide resistance, *An. gambiae* s.l. was sensitive to deltamethrin [29].

2.2. Mosquito Sampling

Two sampling methods were used to obtain adults for susceptibility testing in the three study sites from May 2016 to December 2018.

In the first method, Anopheles larvae were collected by the dipping method according to the WHO protocol [33]. The larval sites consisted of unpolluted sites (river backwaters, water pools, ruts and ditches), a site polluted by the presence of numerous plastic and organic wastes (a decorative fountain) and sites in market gardens.

The mosquito larvae collected in the field were transferred to the insectarium of the National Institute for Research in Health Sciences (NIRHS), fed with fish food (Comipex®) and reared to adult stage.

The second method was to collect resting females in the bedrooms. Susceptibility tests were carried out using first generation (F1) females from eggs collected from wild females.

The female Anopheles used in the tests were identified morphologically identified as belonging to the *An. gambiae* complex according to the key of Gillies and De Meillon 1968).

2.3. Insecticide Sensitivity Tests

Susceptibility testing was performed using susceptibility test kits and the WHO standard protocol for adults [16] [34]. Testing was performed at an average room temperature and relative humidity of 25˚C ± 2˚C and 70% ± 10% RH, respectively.

For each test series, 4 insecticide exposure tubes and 2 control tubes were monitored in two phases. In the first phase, 4 and 6 batches of 25 mosquitoes each were placed in non-insecticide-impregnated paper tubes and observed for one hour. Insecticide-impregnated papers were obtained through WHO/AFRO from its reference center (Vector Control Research Unit, Sains Malaysia University, Penang, Malaysia).

Four insecticides belonging to two families were tested with pyrethroids including lambda-cyhalothrin (0.05%), deltamethrin (0.05% and 0.5%), permethrin (0.75%) and an organochlorine DDT (4%). The use of the 0.5% (10×) dose of deltamethrin made it possible to assess the intensity of resistance according to WHO criteria. These results were interpreted as an indicator of the selective force exerted on local populations, in comparison with the standard 0.05% test.

In the second phase, mosquitoes were exposed to the insecticide in tubes with impregnated paper, while control mosquitoes were placed in tubes containing un-impregnated paper. During the 60 minutes exposure period, the knock-down effect was observed every 5 minutes from the 5th minute of exposure, and the number of knocked-out mosquitoes was recorded. At the end of the exposure period, females were transferred to observation tubes. Mortality was assessed 24 hours after exposure to the insecticide [16].

2.4. Bioassays with Synergists

In order to assess the involvement of detoxifying enzymes in resistance to pyrethroids (deltamethrin), tests were conducted with 4% PBO (piperonyl butoxide), a monooxygenase inhibitor [16].

The PBO tests were conducted according to WHO protocols with four batches of 25 female *An. gambiae s.l.* aged 2 to 5 days. Two batches were pre-exposed for 1 hour to papers impregnated with 4% PBO, then immediately transferred to 0.05% deltamethrin papers for 1 hour of exposure. One batch was exposed only to 0.05% deltamethrin and a control batch was kept on untreated paper. Mortality was recorded 24 hours after exposure. Finally, 1 to 10 exposed Anopheles mosquitoes (survivors and dead) were stored individually in 1.5 ml Eppendorf tubes containing silica gel and cotton wool at −20˚C in order to identify the different species and the kdr mutation.

These tests were limited to the Makelekelé site, chosen because of its high level of domestic pollution, which favors the selection of metabolic resistance mechanisms. This choice was justified by the desire to document the potential role of detoxifying enzymes in a context where resistance is particularly pronounced.

2.5. Criteria for Validity of Susceptibility Testing

The data collected were analyzed according to WHO criteria. The parameters measured were the knockdown times or the time required to knock down 50% (KD50) and 95% (KD95) of the specimens tested. The knockdown effect of the insecticide was assessed during the 1 h exposure period and mortality was assessed 24 h after the exposure period. Mortality was interpreted according to WHO criteria for determining susceptibility status [16].

The susceptibility, probable resistance, and resistance status of the tested mosquitoes were evaluated according to the following criteria: a mortality rate ≥ 98% indicates that the tested population is susceptible; a mortality rate between 90% and 97% indicates the presence of probable resistance; and a mortality rate < 90% corresponds to a population resistant to the insecticide used.

For PBO tests to be valid, there must be no mortality in batches exposed to PBO alone. Second, the mortality rates of the PBO and then deltamethrin tests must be greater than the mortality rates of deltamethrin alone, according the criteria are the same as those for the tests with papers impregnated with insecticides by comparing the tests with PBO-insecticide and the tests with insecticides alone [16].

2.6. DNA Extraction

DNA was extracted from mosquitoes preserved with silica gel according to the protocol described by Morlais *et al.*, (2004) [35]. The method involved grinding the legs and wings in 200 µl of 2% CTAB (Cetyl Trimethylammonium Bromide) in an Eppendorf tube. The tubes were then incubated for at least 5 minutes at 65˚C in a dry bath and then placed on ice for 3 minutes. To each tube, 200 µl of chloroform was then added. After manual agitation, the tubes were centrifuged at 12,000 rpm for 5 minutes. The supernatant from each tube was then pipetted into a new tube to which 200 µl of isopropanol was added. The new tubes were again centrifuged at 12,000 rpm for 15 minutes, then emptied and drained. The resulting pellet was washed with 200 µl of cold 70˚ ethanol and then the tubes centrifuged at 12,000 rpm for 5 minutes. The drained was then removed, drained and placed in a Speed-vac to dry the DNA pellet for 5 minutes. The resulting DNA was solubilized in 20 µl of bi-distilled water and stored at −20˚C until PCR testing.

2.7. Identification of Species by PCR

Molecular identification of species belonging to the *An. gambiae* s.l. complex was performed according to the method developed by Wilkins *et al.*, (2006) [36]. PCR was performed using Thermus aquaticus polymerase (Sigma, USA). The reaction volume was 12.5 µl with the following composition: 6.4 µl of water; 1.25 µl of 10X buffer; 1.25 µl of 2mM dNTP; 0.5 µl of each primer (UN, AR, ML, M1, and S1); 0.1 µl of Taq, 1 µl of DNA template.

The reaction mixture was initially incubated at 94˚C for 5 minutes followed by 30 cycles of amplification at 94˚C for 30 seconds for denaturation to 58˚C for 30 seconds for hybridization and 72˚C for 30 seconds for extension, followed by final extension at 72˚C for 5 minutes.

The size of the amplified DNA sequences was assessed after electrophoresis on a 2% agarose gel. The agarose gel was prepared by dissolving 2 g of agarose in 100 ml of 0.5X TBE buffer to which 5 µl of ethidium bromide (BET) was added. The gel was then migrated for 20 minutes, in a tank connected to 170 volts. Visualization was performed under UV light. The size of the bands obtained was estimated using a 1 kb scale; 221 bp for *An. gambiae*, 333 bp for *An. coluzzii* and 387 bp for *An. arabiensis*.

2.8. Detection of the KDR Mutation

Detection of the Kdr mutation (L1014F) was performed according to the method developed by Huynh *et al.*, (2007) [37]. The L1014F (Kdr-w) mutation was detected using IMP PCR primers. PCR was performed using Taq DNA polymerase. An 11.5 µl reaction mixture was prepared with the following composition: 3.95 µl of water; 2.5 µl buffer without MgCl2; 0.7 µl of 25 mM $MgCl_2$; 1.25 µl of 2 mM dNTP; 0.5 µl of each primer (IPCF, ALTRev, WT and West); 0.1 µl of Go Taq; and 1 µl of DNA template. The reaction mixture was initially incubated at 95˚C for 5 minutes, followed by 35 cycles of amplification (95˚C for 30 seconds, 59˚C for 30 seconds, and 72˚C for 30 seconds), and a final extension at 72˚C for 5 minutes.

The size of the amplified DNA sequences was assessed after electrophoresis on a 2% agarose gel. The agarose gel was prepared by dissolving 2 g of agarose in 100 ml of 0.5X TBE buffer to which 5 µl of ethidium bromide (BET) was added. Gel migration was performed for 20 min in a 170-volt connected vessel. The bands were visualized under UV light. The size of the bands obtained was estimated using a 1 kb scale; 156 bp for the resistant band, 214 bp for the susceptible band and 314 bp for the common band.

2.9. Statistical Analysis of the Data

Data for the present study were entered into a Microsoft Office Excel 365 file and analyzed using R software (version 8.4.1). Mortality rates were calculated for each location and study site, but also for anopheline species and insecticide type. For pyrethroids, the 50% and 95% knockdown times with their confidence intervals were determined using the log-time probit model of the Dose Effect Function package on XLSTAT 2020 software. Mortality rates were compared using Pearson's or Fisher's Chi2 tests or non-parametric Kruskal-Wallis and Mann-Whitney tests for numbers less than 5. The significance level of the tests was set at 0.05 (p value < 0.05).

3. Results

3.1. Sensitivity to Insecticides

3.1.1. Knock-Down Effects

The KD50 and KD95 times for DDT of *An. gambiae* s.l. in 5 sites from 2016 to 2018 were not determined.

KD50 knockdown times ranged from 10 to 60 min and KD95 ranged from 15 to 60 min in the 3 locations (**Tables 1-5**), mainly for deltamethrin. In Brazzaville, the KD50 knockdown time was between 30 and 60 min for deltamethrin 0.05% (**Table 2**). For DDT, lambda-cyhalothrin and permethrin, the knock-down times were indeterminate because they exceeded 60 min. In Djoumouna, the KD50 and KD95 knockdown times were calculated only for deltamethrin, which were 25 min and 45.5 min respectively. For deltamethrin 0.5% (10× diagnostic dose) the KD50 was 10 and 30 min and the KD95 was 10 and 40 min.

The highest KD50 and KD95 knockdown times were recorded at the market garden site The bled, where they were 30 and 40 min respectively. At Kintele, these times were the lowest observed, being 10 min each. At Djoumouna, the KD50 and KD95 were 10 and 15 minutes respectively. For DDT, permethrin and lambda-cyhalothrin, the KD50 and KD95 knockdown times were not determined.

Regarding the tests with the synergist PBO, the KD50 and KD95 knockdown times for deltamethrin alone were 33.3 and 51 min respectively. For the PBO + deltamethrin test, the KD50 and KD95 knockdown times were 23.3 and 33.3 min, respectively (**Table 4**).

3.1.2. Mortality Rates

Figures 2-7 show mortality rates (%) of *An. gambiae* s.l. after 24 h exposure to insecticides.

Table 1. KD50 and KD95 times for deltamethrin with and without PBO in Makelekele in 2018.

Locality	KD	deltamethrin 0.05% only	PBO+ deltamethrin 0.05%
Makelekele	tested number	75	75
	KD50 (min)	33.3 (19.2 - 52.9)	23.3 (19.5 - 29.1)
	KD95 (min)	61 (59.7 - 64.1)	33.3 (28.7 - 38.2)

Table 2. KD50 and KD95 times for DDT 4%, deltamethrin 0.05%, deltamethrin 0.5%, lambdacyhalothrin 0.05% and permethrin 0.75% for *An. gambiae s.l.* in the 5 sites from 2016 to 2018.

Insecticide and diagnos-tic dos	Years	KD	Bacongo	Djoumouna	Madibou	Test garden	The bled	Makelekele	Kintele	Moungali	Mayanga
DDT 4%	2016	Tested number	75								
		KD50 (min)	375 (352.8 - 387.2)								
		KD95 (min)	712 (709.2 - 734.2)								
	2017	Tested number		100	100						
		KD50 (min)		500 (487.7 - 507.2)	204.5 (201.8 - 206.2)						
		KD95 (min)		950 (937.5 - 952.2)	388.6 (376.8 - 390.8)						
	2018	Tested number				100	100				
		KD50 (min)				865 (853.1 - 876.2)	754 (747.8 - 759.2)				
		KD95 (min)				1352 (1339.7 - 1354.2)	1126 (1117.8 - 1134.2)				
Deltamethrin 0.05%	2016	Tested number	75								
		KD50 (min)	30 (29.5 - 30.7)								
		KD95 (min)	60 (59.9 - 62.5)								
	2017	Tested number			75					100	
		KD50 (min)			30 (27.7 - 32.3)					23.9 (21.5 - 26.3)	
		KD95 (min)			60 (57.8 - 62.2)					37.1 (34.5 - 39.4)	
	2018	Tested number		100			100	100			100
		KD50 (min)		25 (19.2 - 30.8)			59 (58.6 - 65.2)	30 (27.7 - 32.3)			52 (49.8 - 54.2)
		KD95 (min)		45.5 (44.9 - 50.6)			96.2 (93.8 - 98.1)	40 (37.7 - 42.3)			91.9 (89.8 - 94.1)
Deltamethrin 0.5%	2018	tested nomber		100		100	100	100	75		100
		KD50 (min)		10 (9.9 - 10.3)		15 (12.3 - 16.4)	30 (28.4 - 34.2)	15 (14.9 - 15.8)	10 (9.9 - 10.8)		10 (8.3 - 14.7)
		KD95 (min)		15 (14.7 - 15.8)		21.3 (19.5 - 24.5)	40 (37.2 - 43.9)	21.7 (16.2 - 30.9)	10 (9.9 - 10.8)		18.8 (14.9 - 22.3)

Continued

lambda-cyhalothrin 0.05%	2018	tested nomber		100		100				
		KD50 (min)		76.9 (74.7 - 79.1)		71.4 (69.2 - 73.6)				
		KD95 (min)		145 (142.8 - 147.2)		136 (133.9 - 138.2)				
		tested nomber		0	75					
		KD50 (min)			30 (29.5 - 30.7)					
		KD95 (min)			60 (59.9 - 62.5)					
		tested nomber	75							100
		KD50 (min)	170.4 (168.2 - 172.6)							24.9 (22.5 - 27.2)
		KD95 (min)	323.9 (321.7 - 326.1)							38.3 (36.0 - 40.6)
		tested nomber				100				
		KD50 (min)				429 (426.8 - 431.2)				
		KD95 (min)				814 (811.8 - 816.2)				

KD50: time required to knock out 50% of mosquitoes; KD95: time required to knock out 95% of mosquitoes; min: minute. Susceptibility testing conducted in Bacongo and Madibou in 2016 showed that, resistance is not homogeneous within a locality.

Table 3. Mortality for DDT 4%, deltamethrin 0.05%, deltamethrin 0.5%, lambdacyhalothrin 0.05% and permethrin 0.75% for *An. gambiae s.l.* in the 5 sites from 2016 to 2018.

Sites	Year	Insecticide tested	Number of females tested	Number of replicates (lots de 25)	Mortality (%)
Bacongo	2016	DDT 4%	75	3	12
The garden	2018		100	4	37
The Bled			100	4	5
Madibou	2017		100	4	41
Djoumouna			100	4	33
Bacongo	2016	Deltamethrin 0.05%	75	3	98.6
Madibou			100	4	98.6
Moungali	2017		75	3	48
Makelekele	2018		100	4	84
The Bled			100	4	59
Mayanga			100	4	50
Djoumouna			100	4	82
Kintele	2017	Deltamethrin 0.05%	75	3	100
The Bled			100	4	98
Mayanga			100	4	85
Makelekele			100	4	90
Djoumouna			100	4	100

Continued

Makelekele	2018	Deltamethrin 0.05% only	75	3	78.6
		Deltamethrin 0.05% and PBO	75	3	98.6
Madibou	2016	Permethrin 0.75%	75	3	85.3
Bacongo	2017		100	4	14
Moungali			75	3	73.3
The garden	2018		100	4	1
The garden	2018	Lambdacyalothrin 0.05%	100	4	49
Djoumouna			100	4	29

Table 4. Frequencies of the kdr L1014F allele in survivors, deaths and controls of *An. gambiae* and *An. coluzzii* between 2016-2018.

	An. gambiae					*An. coluzzii*				
	N	SS	RS	RR	F[R]	N	SS	RS	RR	F[R]
Survivor	81	1	42	38	0.73	3	0	2	1	0.66
Death	79	3	41	35	0.7	8	0	4	4	0.75
Total	**160**	**4**	**83**	**73**	**0.72**	**11**	**0**	**6**	**5**	**0.72**

N: number tested; SS: sensitive; RS: resistant heterozygous; RR: homozygous resistant; F [R]: frequency of the allele.

Table 5. Frequencies of the kdr L1014F allele from *An. gambiae* and *An. coluzzii* in the 8 sites between 2016-2018.

Site	Species	N	SS	RS	RR	F[R]	P(HW)
Bacongo	*An. gambiae*	27	1	17	9	0.7	0.048
	An. coluzzii	1	0	0	1	1	--
Makelekele	*An. gambiae*	31	0	16	15	0.7	0.052
	An. coluzzii	1	0	0	1	1	--
Test garden	*An. gambiae*	55	0	38	17	0.6	<0.001
	An. coluzzii	3	0	0	3	1	--
The bled	*An. gambiae*	14	0	3	11	0.9	0.653
	An. coluzzii	1	0	0	1	1	--
Madibou	*An. gambiae*	13	1	2	10	0.8	0.1402
	An. coluzzii	1	0	0	1	1	--
Mayanga	*An. gambiae*	32	3	15	14	0.7	0.7210
	An. coluzzii	0	0	0	0	0	--
Kintélé	*An. gambiae*	8	0	7	1	0.6	0.0278
	An. coluzzii	2	0	2	0	0.5	--
Djoumouna	*An. gambiae*	19	0	13	6	0.7	0.0234
	An. coluzzii	9	0	8	1	0.6	--

N: number sampled; SS: sensitive; RS: resistant heterozygous; RR: homozygous resistant; F [R]: frequency of the allele; P (HW): P. value provided by the Hardy-Weinberg test.

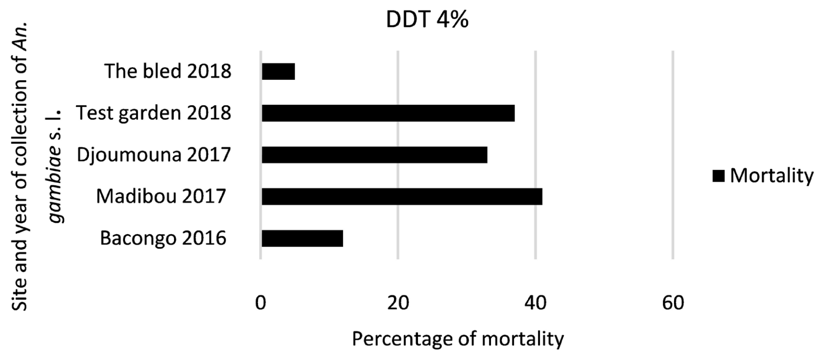

Figure 2. Mortality to DDT 4% 24 hours after exposure of *An. gambiae s.l.* in 4 sites in Brazzaville and in Djoumouna between 2016 and 2018.

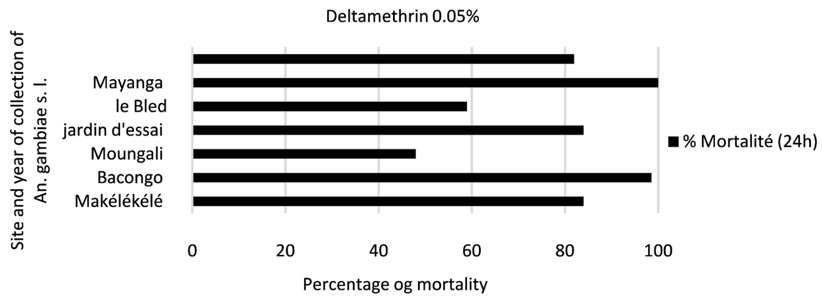

Figure 3. Mortality to 0.05% deltamethrin 24 hours after exposure *of An. gambiae s.l.* in 6 sites in Brazzaville and Djoumouna between 2016 and 2018.

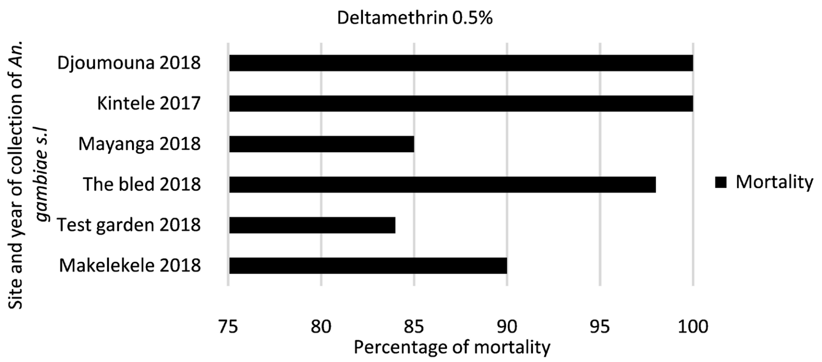

Figure 4. Mortality to 0.5% deltamethrin 24 hours after exposure of *An. gambiae s.l.* in 4 sites in Brazzaville, in Kintele and in Djoumouna in 2017 and 2018.

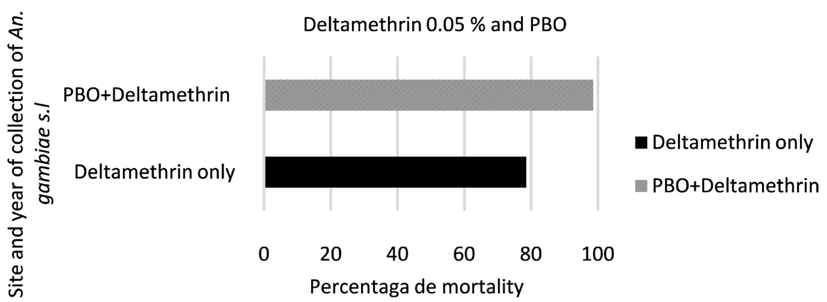

Figure 5. Mortality of *An. gambiae s.l.* with deltamethrin 0.05% with or without PBO in Makelekele in 2018.

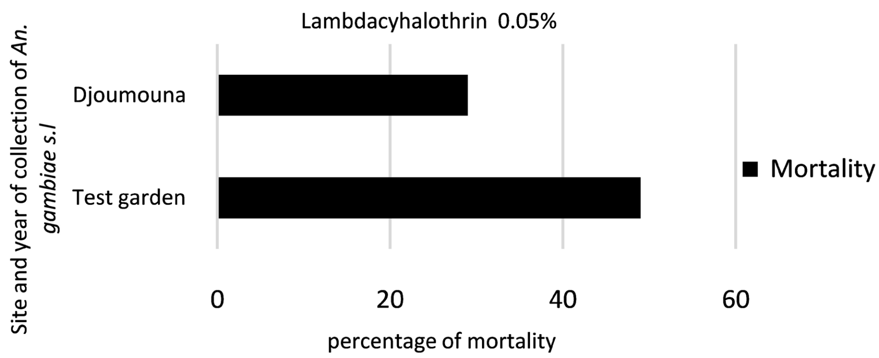

Figure 6. Mortality to lambdacyhalothrin 0.05% 24 hours after exposure of *An. gambiae s.l.* at the Test garden and Djoumouna 2018.

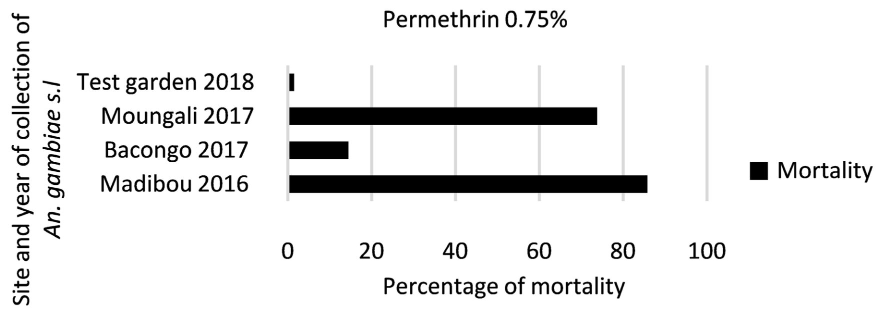

Figure 7. Mortality to permethrin 0.75% 24 hours after exposure of *An. gambiae s.l.* in 4 sites in Brazzaville between 2017 and 2018.

Mortality rates for DDT were less than 50% and ranged from 48% to 98.6%. For deltamethrin 0.05%, the highest mortality rates were recorded in the Bacongo and Madibou districts in 2016.

For deltamethrin 0.5%, the highest mortality rates (100%) were observed in the The bled neighborhood in Kintele and in Djoumouna. The lowest mortality rates (84% and 85%) were observed in the districts of Test garden and Mayanga. For permethrin 0.75%, mortality rates ranged from 1% to 85.3%, with the lowest (1%) was recorded in the Test Garden market garden area and the highest (85.3%) in Madibou. For lambda-cyhalothrin 0.05%, the lowest mortality rate (29%) was recorded in Djoumouna and the highest (49%) in Test garden. Statistical tests showed that there was a relationship between sensitivity to these insecticides and the collection site ($p < 0.001$).

Regarding the tests with PBO, there was a recovery of sensitivity to deltamethrin after the use of PBO. Statistical tests indicated a relationship between sensitivity to deltamethrin and the use of PBO at $p = 0.0054$.

3.2. Identification of Species in the *An. gambiae* Complex

Of the females tested for insecticide susceptibility, 244 females were sampled for species and kdr mutation identification. Two species were identified at the study sites, 223 *An. gambiae* and 21 *An. coluzzii*.

An. gambiae was the main species identified in the three sites, with frequencies of 94.8%, 80% and 67.9% in Brazzaville, Kintele and Djoumouna, respectively.

3.3. Detection of the Kdr Mutation

Of the 244 tested females tested, 239 carried the kdr L1014F mutation. Among the 223 *An. gambiae* females, 218 carried the mutation and 5 were susceptible. In addition, all 21 *An. coluzzii* carried the kdr L1014F mutation. The frequency of the kdr mutation varied between 0.5 and 1 in the three sites. In the Brazzaville sites, the frequency ranged from 0.6 to 1, in Kintele from 0.5 to 0.6 and in Djoumouna from 0.6 to 0.7 (**Table 4**).

Comparison of kdr frequencies between surviving and dead *An. gambiae* carrying the kdr mutation showed no difference between homozygous and heterozygous individuals. However, for *An. coluzzii*, there was a statistically significant difference between surviving and dead individuals carrying the kdr mutation in the homozygous and heterozygous states (**Table 5**).

4. Discussion

Molecular identification of the species of the *Anopheles gambiae* s.l. complex showed that it consists of 2 species: *An. gambiae* (91.4%) and *An. coluzzii* (8.6%) in the three study sites. The presence of these species confirms the geographical distribution revealed in previous studies [38]. These results are similar to those obtained in 2009 by Koekemoer and al. (2011), who showed that the main species collected in the village of Boutoto and its surroundings in the Kouilou department of Congo was *An. gambiae* (former molecular form S). Comparable results were obtained in Gabon and the Democratic Republic of Congo [39] [40]. The predominance of *An. gambiae* may be related to the nature of the larval sites encountered, which are temporary or permanent sunny sites without vegetation. Indeed, these larval sites are favorable for the development of this species [38] [39] [41] [42].

The presence of *An. coluzzii* would be linked to the existence of permanent larval sites such as, fish ponds in Djoumouna and market horticultural sites in Brazzaville as indicated in **Figure 1**; permanent sites are the preferred sites of this species whereas *An. gambiae* develops preferentially in temporary larval sites such as ponds. *An. coluzzii* was also collected in Kintele, and the presence of the above-mentioned sites in this commune cannot be excluded.

Susceptibility testing showed that the females tested were resistant to DDT and pyrethroids. The only populations susceptible to deltamethrin in 2016 were those in the Bacongo and Madibou districts. These results corroborate those obtained by other authors who demonstrated resistance of *An. gambiae* s.l. to DDT and pyrethroids in Pointe-Noire, Ouesso, Sibiti and Djambala [5] [43].

Resistance of *An. gambiae* s.l. to pyrethroids has also been reported in other African countries such as Angola [44], Cameroon [45], Gabon [39], Niger [46], Democratic Republic of Congo [47] and Senegal [13].

The resistance of *An. gambiae* s.l. to these insecticides could be attributed to the widespread use of LLINs and the uncontrolled use of pyrethroids in agriculture (against crop pests) and public health (against disease vectors and mosquito nuisance). In fact, 2,481,563 LLIN were distributed nationally between 2011-2012 [48]. The insecticide used to impregnate these LLINs is deltamethrin. The widespread use of these LLINs may have exerted a selection pressure leading to the development of insecticide resistance to this family. Pyrethroid and DDT susceptibility studies conducted prior to LLINs distribution between 2002 and 2010 showed that populations were susceptible or declining in susceptibility at 5 out of 8 sites [5] [6] [43]. Studies conducted after the distribution of LLIN between 2013 and 2016 [5] [7] showed that all populations tested were resistant to pyrethroids, similar to our study.

Susceptibility testing conducted in Bacongo and Madibou in 2016 showed that resistance is not homogeneous within a site. It correlates with activities carried out in the study area, such as the use of pesticides in agriculture, the collection, storage and disposal of agricultural waste, insecticide the spraying of insecticides for culicidal pest control, the discharge of waste water and the dumping of waste in dumpsiteslandfills. These activities make it impossible to take a comprehensive view of resistance in a locality. Therefore, for effective vector control, it is essential to conduct susceptibility testing at different sites to obtain information essential for understanding resistance at the site and to develop appropriate control methods for each site.

Studies in other countries have shown that the extensive use of LLINs has been a major factor in the development of pyrethroid resistance [49] [50].

In agriculture, pesticides, in particular pyrethroids (cypermethrin, alphacypermethrin and lambda-cyhalothrin), are used continuously to control pests. This use exerts a selective pressure on the *Anopheles* populations that develop in this environment. As a result, individuals carrying the resistance genes have a selective advantage. These insecticides are toxic to both the crop pests and the *Anopheles* larvae that develop in them.

This is supported by mortality rates of less than 60% for DDT and pyrethroids (at diagnostic doses). For deltamethrin 0.5% (10x), mortality rates ranged from 84 to 100% in the market garden sites. This shows that there is significant selection pressure for the emergence or maintenance of resistance at these sites.

These results are consistent with those obtained by other authors in Africa. Indeed, these authors have shown that the use of insecticides on farms and in the storage of agricultural commodities is one of the main factors in the development of resistance in *An. gambiae* s.l. [8] [51]-[55].

As DDT is no longer used in Congo [56], resistance to this insecticide is thought to be related either to the use of organochlorines in horticulture, or to cross-resistance with pyrethroids with which it shares the same target site [57], or to the persistent selection pressure exerted by this insecticide in the 1950s and 1960s in the context of malaria control [23].

In the context of this selective pressure, the use of aerosols (Baygon®, Total®, Premium®...) and insecticide sprays against disease vectors may also have contributed to the development of resistance. These sprays contain cypermethrin, imiprothrin, permethrin, tetramethrin and deltamethrin.

The Kdr L1014F mutation, which confers resistance to pyrethroids, was detected in both *An. gambiae* and *An. coluzzii* at all sites studied. This mutation was initially described in Boutoto, where the L1014F and L1014S alleles coexisted [6]. It is widespread in Africa, particularly in Cameroon, Burkina Faso, Sierra Leone, and Benin [58]-[61]. However, during this study, the Kdr L1014F mutation was found indiscriminately in both dead and surviving mosquitoes, suggesting that it is only one of several resistance mechanisms involved.

Tests using PBO confirmed the major contribution of metabolic mechanisms. In Côte d'Ivoire, Kouassi *et al.* (2024) showed that pre-exposure to PBO significantly increases the mortality of *An. gambiae s.l.* exposed to deltamethrin, indicating the involvement of metabolic enzymes in resistance [62]. In Tanzania, Kabula *et al.* (2024) reported that a national survey revealed complete or partial restoration of sensitivity in the majority of sites after pre-exposure to PBO [63]. Similarly, in Gabon, Boussougou-Sambe *et al.* (2024) observed an almost complete restoration of sensitivity to deltamethrin in *An. gambiae s.s.*, confirming the key role of P450 monooxygenases and detoxification enzymes (esterases, GST) in resistance [10].

These results confirm that pyrethroid resistance in Africa is based on a synergy between target and metabolic mechanisms. Kdr mutations, although important, appear to be complemented by overexpression of cytochrome P450 genes, notably CYP6M2, CYP6P3, CYP6P4, and CYP6P5 [63] [64]. PBO inhibits these monooxygenases, insecticides (WHO, 2023). Thus, PBO-treated mosquito nets are a relevant operational option for countering oxidase-dominated resistance, as studies conducted in Tanzania and Gabon have also shown [10] [63].

Finally, it should be noted that this study was limited to the characterization of the Kdr L1014F gene, without analyzing other mutations such as L1014S or recent metabolic variants (e.g., E205D in CYP6P3). The future integration of these parameters, including the quantification of CYP450 overexpression (CYP6M2, CYP6P3, CYP6Z1) and cuticular genes (CYP4G16), will provide a more comprehensive and integrated view of resistance mechanisms in local Anopheles populations.

5. Conclusions

During this study, species of the *An. gambiae* s.l. complex were the main species collected. Two species belonging to this complex were identified: *An. gambiae* and *An. coluzzii*. The species tested were resistant to DDT and pyrethroids, with the exception of the Bacongo and Madibou sites in 2016. These results show that resistance can vary from one site to another, depending on the activities carried out there.

This resistance could be due to the use of insecticides in public health, in agriculture and in the widespread use of LLINs. The KDR L1014F mutation and monooxygenase enzymes are involved in resistance to these insecticides.

To ensure the effectiveness of insecticides against Anopheles, it is necessary to advocate for coordination between the Ministry of Health and other departments involved in the use of insecticides (agriculture, environment, trade, etc.).

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Conflicts of Interest

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

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