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Identification of the knockdown resistance (Kdr) mutations in *Anopheles gambiae s.l.* in the Mouila area, Southwest Gabon

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Abstract
In Gabon, members of the *Anopheles gambiae* complex are the reference vectors of *Plasmodium falciparum*. In Libreville and Port-Gentil, anopheles found resistant to organochlorines and pyrethroids. This resistance was related to the presence of the Kdr-east and Kdr-west genes. Otherwise, mechanisms of resistance in mosquitoes are still poorly known in Gabon. This study reports the situation of insecticide resistance and underlying mechanisms in *An. gambiae s.l.* populations from palm oil plantations from Mouila. All the anopheline populations tested showed resistance to almost all insecticides except organophosphates and carbamates families, which remain the only lethal molecules. Molecular identification of specimens revealed the presence of *An. gambiae s.s. An. gambiae s.s.* (93.14%) and *An. coluzzii* (6.86%). *Kdr* genotyping showed the presence of the L1014F mutation (*kdr*-West) as well as L1014S (*kdr*-East). This L1014F mutation was found at very high frequencies (0.99) in almost all sites surveyed, and in association with the L1014S (0.20). Results showed the mechanisms, which confer pyrethroids and organochlorines resistance to *An. gambiae s.l.* from Mouila area. These data, although preliminary, stress the need for monitoring of *An. gambiae s.l.* populations of these agricultural zones for a suitable insecticide resistance management system and vector control.

Keywords: Kdr mutations, Ace 1^R, *Anopheles gambiae s.l.*, PCR, Mouila, Gabon

1. Introduction
In sub-Saharan Africa, malaria is transmitted by about twelve species with highly variable competence [1]. *Anopheles* of the *Anopheles gambiae* complex are the most widespread vectors because of their size and wide geographical range [2]. Some of the anopheline species in this complex have an exceptionally high vectorial capacity for human malaria [3]. Currently, this group consists of eight members: *Anopheles gambiae* sensu stricto, *Anopheles coluzzii*, *Anopheles arabiensis*, *Anopheles quadriannulatus* A, *Anopheles quadriannulatus* B, *Anopheles bwambae*, *Anopheles melas* and *Anopheles merus*. Five of these species are found in Central Africa: *An. gambiae s.s.*, *An. coluzzii*, *An. melas*, *An. arabiensis* and *An. merus* [4]. These species are well adapted to natural and anthropogenic collections of water [5].
In Gabon, members of the *Anopheles gambiae* complex are the reference vectors of *Plasmodium falciparum*, the most common and deadly species [6]. For several years, cases of resistance to pyrethroids and DDT have been reported in anopheles populations [6, 7]. In Gabon, Mourou *et al.* [6] found organochlorines and pyrethroids resistance in the cities of Libreville and Port-Gentil. Resistance was related to the presence of the Kdr-east and Kdr-west genes. Otherwise, mechanisms of resistance in mosquitoes are still poorly known in Gabon. In the case of the Mouila zone, a heterogeneous climate combined with landscape changes may present a different situation of resistance. Landscape changes are recent and have been the result of human encroachment and development activities launched by the Government in 2010. According to Akono *et al.* [8, 9], each bioclimatic zone probably may display particular mechanisms of resistance. Altogether, the study of insecticide resistance is of paramount importance because it provides information on the evolutionary processes that allow insects to adapt to changes in their environment. To better understand the genetic mechanisms responsible for insecticide resistance in these agro-ecozones, a study was carried out in palm

oil plantation sites in the Mouila area (southwestern Gabon). The objective was to survey for the resistance mutations Kdr-e, Kdr-w and / or Ace 1^R mutations in *Anopheles gambiae s.l.* populations at the agricultural sites.

2. Materials and Methods

2.1 Study sites

This study was conducted in August 2017 (dry season) and in October 2017 (rainy season) in the Mouila area (Ngounié province), particularly in Mboukou and Moutassou (Fig. 1). The Mboukou site is at 1°39'06 "S and 10°49'42.6" E. It is located in the Tsamba-Magotsi division, about 35 kilometers from the city of Mouila. It is limited to the east by the Ngounié River and the villages of Saint-Martin and Migabe, then to the west by the Douya, Doubou, Mboukou and Rembo villages. The Moutassou site is found in the Douya-Onoye division, about 13 kilometers from Mouila. This site is at 1°59'33.8 "S and 11°02'25.2" E. It is limited to the north by the town of Mouila, to the east by the Moulandoufouala, Mbengui and Mbadi villages, and to the west by the Moutassou, Koumbanou, Ikolo-Ikolo and Digabosse villages. It is an ecosystem dominated by savanna, forest-savanna mosaics occupying about 75% of the area.

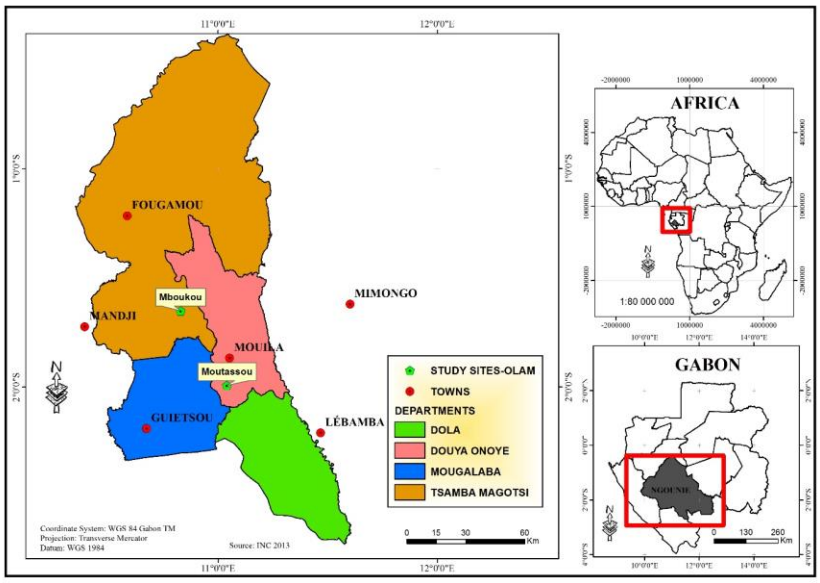

Fig 1: Geographical location of the study sites in Mouila

2.2 Mosquito collection and detection of Kdr and Ace 1^R mutations

The *Anopheles* used for insecticide susceptibility tests were collected as larvae in favorable natural (rivers, ponds, etc.) and artificial (pits, rills, wells, etc.) breeding sites. Larvae were collected using the dipping method using a 300-mL entomological cup [10]. Larvae of *Anopheles* were placed in trays and transported in coolers to the laboratory [8]. In the laboratory, they were maintained in breeding tanks containing water from their respective natural breeding sites and covered with mosquito net. Larvae were fed every day, *ad libitum* with finely ground aquarium fish food (*Sera vipagran*). On a daily basis, nymphs were separated from larvae and transferred to the breeding cages until the emergence of imagos. The resulting adults were fed with 10% sugar solution before susceptibility testing.

2.3 Susceptibility tests

Susceptibility tests were performed on non-blood fed adult females at 2-4 days of age, originating from larvae collected in the field. Bioassays were performed according to the standard protocols of the World Health Organization [11]. For this purpose, papers impregnated with deltamethrin (0.05%), permethrin (0.75%), lambdacyalothrin (0.05%), DDT (4%), bendiocarb (1%) and malathion (5%) were used. These insecticide-impregnated papers were provided by the WHO-Gabon. Tests were performed with batches of 25 *An. gambiae s.l.*, with four batches tested against each insecticide. Mosquitoes were exposed to insecticide-impregnated filter paper for 1 hour at 25-27 °C and 80% relative humidity.

2.4 *Anopheles* species identification

Mosquitoes used in the bioassays were morphologically identified using the key to determine *Culicidae* from Central Africa and Gabon by Baldacchino and Paupy [12]. For molecular identification of *Anopheles gambiae* mosquitoes, the total DNA of viable and dead mosquitoes was extracted according to Collins *et al.* [13] and diluted 1/15 in sterile water. Identification of the molecular forms of *Anopheles gambiae s.l.* was performed using the method of Favia *et al.* [14]. This Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP) diagnostic test consists of amplifying a DNA fragment of approximately 1500 base pairs (bp) using two oligonucleotides (AO and A 1.3). Subsequently, this sequence is digested with a restriction enzyme Hin61 or Tru9 making it possible to identify the molecular forms M and S henceforth called *Anopheles coluzzii* and *Anopheles gambiae s.s.* respectively.

2.5 Identification of the insecticide resistance genes

The protocol used to detect the L1014S (kdr-east) or L1014F (kdr-west) alleles was adapted from Martinez-Torres *et al.* [15] and Ranson *et al.* [16]. Similarly, the determination of the Kdr mutation was adapted from Martinez-Torres *et al.* [15] and Ranson *et al.* [16]. The PCR-Polymerase of Allelic Specific Amplification (PCR-PASA) diagnostic test consisted of the use of four oligonucleotides or primers called
Agd1 (5'-ATAGATTCCCCGACCATG-3'),
Agd2 (5'-AGACAAGGATGATGAACC-3'),
Agd3 (5' -AATTTGCATTACTTACGACA-3'),
Agd4 (5'-CTGTAGTGATAGGAAATTTA-3') and Taq polymerase to search by amplification for resistant or

sensitive alleles on a DNA fragment coding for "sodium channel voltage-dependent" in each tested mosquito. This test therefore makes it possible to define homozygous resistant (RR), homozygous sensitive (SS) and heterozygous sensitive (RS) genotypes. The frequency of the Kdr mutation at each site was calculated with "genepop 2.0" software [17].

The Ace 1^R (G119S) mutation in mosquitoes identified as *An. gambiae s.l.* was made using PCR-RFLP by applying the protocol described by Weill *et al*. [18] with some modifications. This PCR reaction was based on the use of two specific primers whose base sequences were:

Ex3AGdir GATCGTGGACACCGTGTTCG, Ex3AGrev AGGATGGCCCGCTGGAACAG [18].

3. Results

3.1 *Anopheles* species abundance

All *Anopheles* adults that emerged after rearing (N=423) were morphologically identified as belonging to the *An. gambiae complex* (Table 1).

Table 1: Species composition of the *An. gambiae complex* in the study area

	An. gambiae s.l.	*An. gambiae s.s.*	*An. coluzzii*
Number	423	394 (93.14%)	29 (6.86%)

The PCR sequences showed that two anopheline species were present, namely *An. gambiae s.s*. (n = 394; 93.14%) and *An. coluzzii* (n = 29; 6.86%). *An. gambiae s.s*. was collected at all sites and in both seasons, whereas *An. coluzzii* was collected only from certain points at Mboukou in the rainy season.

3.2 Mechanisms of insecticide resistance

A total of 234 living and dead mosquitoes were selected for the identification of the Kdr and Ace 1^R genes. The Kdr mutation was present in *An. gambiae s.l.* with a high frequency of resistant homozygous genotypes (RR). The frequency of the Kdr-w gene (0.99) was much higher than the kdr-e gene (0.20) (Table 2).

Table 2: Frequency of the occurrence of the Kdr resistant genes in identified samples

Collection sites	N	Species		*Kdr* West (1014F)				*Kdr* Est (1014S)			
		An. gambiae s.s	*An. coluzzii*	RR	RS	SS	F (R)	RR	RS	SS	F (R)
Mboukou	80	80	0	79	1	0	0.99	21	1	58	0.27
Ngounie	21	20	1	20	1	0	0.98	0	0	21	0.00
Doubou	13	12	1	13	0	0	1.00	3	1	9	0.27
Mavassa	18	18	0	17	1	0	0.97	1	6	11	0.22
Moutassou	52	52	0	52	0	0	1.00	3	9	40	0.14
Moutambe Sane Foumou village	15	15	0	15	0	0	1.00	5	9	1	0.63
Mboukou village	35	35	0	35	0	0	1.00	0	0	35	0.00
Total	234	232	2	231	3	0	0.99	33	26	175	0.20

RR = resistant homozygotes, RS = heterozygotes for resistance, SS = Susceptible homozygotes; F(R) = Frequency of the resistance allele = [RS + 2x (RR)] / [2x (RR + RS + SS)]

The Kdr-w frequencies were ranged between 0.97 and 1. Their highest values were recorded at Mboukou village, Moutassou, Doubou and Moutambe Sane Foumou village (RF = 1), while the weakest ones were recorded at Mavassa (RF = 0.97). The Kdr-e frequencies ranged from 0 to 0.63 (Table 2). High kdr-e frequencies were observed at Moutambe Sane Foumou village (RF = 0.63), whereas low frequencies were observed at Ngounie (RF = 0). The Ace 1^R resistance gene was not detected, i.e. all mosquitoes tested were susceptible homozygotes (SS) (Table 3).

Table 3: Frequency of the Ace1^R genes expressed in the identified samples

Collection sites	N	Species		Ace 1^R (G119S)			Ace 1^R
		An. gambiae s.s.	*An. coluzzii*	RR	RS	SS	F (*Ace.1^R*)
Mboukou	80	80	0	0	0	80	0
Ngounie	21	20	1	0	0	21	0
Doubou	13	12	1	0	0	13	0
Mavassa	18	18	0	0	0	18	0
Moutassou	52	52	0	0	0	52	0
Moutambe Sane Foumou village	15	15	0	0	0	15	0
Mboukou village	35	35	0	0	0	35	0
Total	234	232	2	0	0	234	0

4. Discussion

In this study, two sympatric species of *Anopheles* were identified from Mouila: *An. gambiae s.s*. (which dominated) and *An. coluzzii* [19]. Several culicid species, particularly those of the *An. gambiae* complex, can share the same breeding habitats [20]. Both *An. gambiae s.s.* and *An. coluzzii* may be encountered in small transitional habitats because they appear to have similar requirements [19]. However, the distribution of these two species varied between the sampling periods *An. gambiae s.s*. was observed in both seasons, whereas *An. coluzzii* was collected only during the rainy season. According to Della-Torre *et al.* [21], *An. gambiae s.s.* will breed in many anthropogenic habitats; its requirements are less demanding than *An. coluzzii*.

In sympatry, *An. gambiae s.s*. appears to survive better than *An. coluzzii*, as it can survive in polluted anthropogenic habitats [22]. Altogether, intra and inter-specific competition appears to favour *An. gambiae s.s*. over *An. coluzzii*, which prefers cleaner water more prevalent in the rainy season. Cannibalism also influences intra and inter-species competition within populations of the *An. gambiae* complex [23]. Regardless, several species of *Anopheles* are present in various localities of Gabon, e.g. *An. melas*, *An. coluzzii* and *An. gambiae s.s.* are present in Libreville and Port-Gentil [6]. *An. melas* was likely not detected at Mouila as it is typical of coastal zones characterized by high salinity.

PCR results revealed the ubiquitous presence of the Kdr resistance mutations, and the absence of the Ace 1^R mutation. Both the Kdr-w and Kdr-e mutations were common in the *Anopheles gambiae* s.s., with a particularly high frequency of Kdr-west homozygotes. As Ace 1^R / Ace 1^R individuals were not observed; multi-resistance has not yet developed within the *An. gambiae s.l.* in the Mouila area.

The identification of Kdr resistance mutations correlates with decreased susceptibility to pyrethroids and DDT in the *Anopheles* of the Mouila area [24]. Martinez-Torres *et al.* [15] showed that cross-resistance to pyrethroids and DDT is due to the Kdr mutation in insects as a whole. The Ace 1^R mutation, although not yet detected at Mouila, was confirmed in *An. gambiae* in the Ivory Coast many years ago [25]. Moreover, this resistance profile is not necessarily specific to our study area. An analogous situation has been described by Pinto *et al.* [7] for *Anopheles* at Libreville, and more recently by Mourou *et al.* [6] for Libreville (Estuaire) and Port-Gentil (Ogooué-Maritime). These authors believed that insecticide resistance in Libreville has arisen from the domestic use of insecticides.

The resistance observed in this study in *An. gambiae s.s.* at Mouila has most likely originated from the use of insecticides by palm oil plantations workers and other inhabitants. Indeed, the protection of palm oil industry has largely pushed the Olam Palm Company to use higher doses of insecticides in their palm oil plantations. In addition, palm plantations are routinely sprayed with several agricultural insecticides of different and largely unmonitored chemical composition. The heavy use of insecticides in this agro-industrial area has clearly set up ideal conditions for selection pressure on anopheline populations that favors individuals with the resistance gene. Our results corroborate a similar situation that has evolved in the Ivory Coast. There, Konan *et al.* [26] reported *An. gambiae* resistance to pyrethroids and DDT in Tiassalékro, an irrigated rice-growing village in the southern forest zone.

The resistance observed in our study site would imply that the Kdr gene is likely well established in a large geographical region in Africa. Indeed, resistance of *An. gambiae s.l.* to pyrethroids and insecticides in general has been reported wherever it has been recently studied, e.g. in the Ivory Coast, Cameroon, Benin, Burkina Faso, Equatorial Guinea, and Ghana [6, 7, 17, 27-31]. This resistance is thought to be primarily linked to the legacy of DDT resistance [9]. Resistance to DDT and pyrethroids insecticides on a larger scale is also occurring as several studies have reported cases elsewhere in sub-Saharan Africa [25, 28, 32-36].

According to Akogbeto *et al.* [37], some *An. gambiae s.l.* lay their eggs in branches contaminated with insecticide residues. The presence of these residues is related to their misuse in agriculture [38]. In the Gambia, DDT (4%) residues were found in soil samples [39]. It is therefore possible that the use of DDT in the past and, pyrethroids in more recent years, has resulted in cross-resistance within the *Anopheles* populations over time. This could explain the significant decrease in sensitivity observed in our parallel bioassays [6, 17]. The resistance observed in these bioassays has been validated by the high frequency of both Kdr resistance mutations in our molecular analysis. Kdr-w results from the substitution of leucine TTA, present on the wild-type Kdr allele with phenylalanine (TTA). This mutation is now widely distributed in West Africa [28, 33, 40]. The distribution and prevalence of the L1014F Kdr mutation in *An. gambiae* are now well documented in a few African countries, e.g. in Burkina Faso [35, 41]. Numerous countries have reported this high-frequency mutation in *An. gambiae*, particularly in the Sudan region where the frequencies are approaching fixation [35, 41, 42]. In recent years, the frequency of this mutation has increased in *An. coluzzii* and *An. arabiensis*, two species of the *An. gambiae* complex. Though the L1014F mutation remains widespread in the ecological regions of Sudan and has a relatively high frequency in all the three species (on average 50%), the frequencies reported in our study were nevertheless lower than in the ecological regions of the old cotton belt of the Sudan [41, 43, 44].

For the other bioclimatic zones, namely the central and northern regions, the allelic frequencies of L1014F varied among the three species with particularly high frequencies in *An. arabiensis*. The reasons for the reduction of the L1014F frequency in *An. gambiae* in the Sudan region are not known. However, a similar trend has recently been observed in the western region of Burkina Faso, where biological and transgenic control practices have been implemented for the protection of cotton crops over the last four years [43, 44]. The second type of mutation, Kdr-e, consists of the mutation of the leucine codon (TTA) to serine (TCA). This mutation has been described for the first time in East Africa [16]. This mutation was recently recorded at higher frequencies in *An. arabiensis* in central Burkina Faso and at 38% average frequencies in Bobo-Dioulasso [45, 46] and in north of Senegal at 89.53% frequency [47]. The low frequency of the Kdr-e mutation observed in our study corroborates with the work conducted in Burkina Faso by Namountougou *et al.* [44] who recorded only a few individuals of *An. gambiae* carrying this mutation. In West Africa, the origin of the L1014S mutation in *An. gambiae*, *An. coluzzii* and *An. arabiensis* is not as well understood. Indeed, the proximity of Burkina Faso to Benin where the L1014S mutation was reported for the first time in *An. arabiensis* [48] suggests that it arrived in Burkina Faso via the migration of this species (*An. arabiensis*) carrying the mutation of Benin. However, the presence of this mutation in *An. gambiae* and *An. coluzzii* in Burkina Faso remains to be elucidated.

This study is the first work that reports the coexistence of these resistance genes in this area of Gabon. This information is essential in order to select the best insecticide for vector control [49]. In the same way, this data is important because insecticide resistance has often been the origin of failed efforts to the fight against mosquitoes in certain countries. In our study area, resistance will clearly impact negatively on the effectiveness of preventive measures such as the deployment of long-lasting insecticide-treated bed nets (LLINs) or Intra-domiciliary insecticide spraying (IDIS).

5. Conclusion

The study showed that in the Mouila area, the *Anopheles gambiae* complex was mainly composed of *An. gambiae s.s.* and *An. coluzzii*. Populations of these species showed Kdr but not Ace 1^R resistance gene. The presence of the Kdr mutation in these populations is believed to be the cause of resistance to DDT and pyrethroids. The results of this research are currently being used to support the development of control strategies for these vectors, based on integrated resistance management and monitoring. This information should be taken into account by the National Malaria Control Program (NMCP) in the implementation of vector control strategies in agro-industrial zones and their environs.

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