



Detection of *kdr* and *ace-1* mutations in wild populations of *Anopheles arabiensis* and *An. melas* in a residual malaria transmission area of Senegal

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ABSTRACT

In the central western Senegal, malaria transmission has been reduced low due to the combination of several effective control interventions. However, despite this encouraging achievement, residual malaria transmission still occurring in few areas, mainly ensured by *An. arabiensis* and *An. melas*. The resurgence or the persistence of the disease may have originated from the increase and the spread of insecticide resistance genes among natural malaria vectors populations. Therefore, assessing the status and mechanisms of insecticides resistance among targeted malaria vectors is of highest importance to better characterize factors underlying the residual transmission where it occurs. Malaria vectors were collected from three selected villages using nocturnal human landing catches (HLC) and pyrethrum spray collections (PSC) methods. *An. gambiae* s.l. specimens were identified at the species level then genotyped for the presence of *kdr*-west (L1014F), *kdr*-east (L1014S) and *ace-1*^R mutations by qPCR. *An. arabiensis* (69.36%) and *An. melas* (27.99%) were the most common species of the *Gambiae* complex in the study area. Among *An. arabiensis* population, the allelic frequency of the *kdr*-east (22.66%) was relatively higher than for *kdr*-west mutation (9.96%). While for *An. melas* populations, the overall frequencies of both mutations were very low, being respectively 1.12% and 0.40% for the L1014S and L1014F mutations. With a global frequency of 2%, only the heterozygous form of the G119S mutation was found only in *An. arabiensis* and in all the study sites. The widespread occurrence of the *kdr* mutation in both *An. arabiensis* and *An. melas* natural populations, respectively the main and focal vectors in the central-western Senegal, may have contributed to maintaining malaria transmission in the area. Thus, compromising the effectiveness of pyrethroids-based vector control measures and the National Elimination Goal. Therefore, monitoring and managing properly insecticide resistance became a key programmatic intervention to achieve the elimination goal where feasible, as aimed by Senegal. Noteworthy, this is the first report of the *ace-1* mutation in natural populations of *An. arabiensis* from Senegal, which need to be closely monitored to preserve one of the essential insecticide classes used in IRS to control the pyrethroids-resistant populations.

1. Introduction

In the central-western Senegal, the implementation of several preventive antimalarial measures, such as the Indoor Residual Spraying

(IRS), the Long-Lasting Insecticide-treated Nets (LLINs), the Seasonal Malaria Chemoprevention (SMC) and cases management has contributed to significantly reduce malaria transmission across the area. Indeed, the large-scale implementation of the SMC targeting children

Abbreviations: IRS, Indoor Residual Spraying; SMC, Seasonal Malaria Chemoprevention; ITNs, insecticide-treated nets; LLINs, Long Lasting Insecticide-treated Nets; NMCP, National Malaria Control Programme; PMI, President's Malaria Initiative; WHO, World Health Organisation; HLC, human landing catches; PSC, and pyrethrum spray collections; DDT, dichlorodiphenyltrichloroethane; Kdr, knockdown resistance; Vgsc, voltage-gated sodium channel gene; SS, homozygote susceptible; Rws, hertzogous *kdr*-west; ReS, hertzogous *kdr*-east; RWRw, homozygous *kdr*-west resistant; RWRr, heterozygous hybrid *kdr*-east & *kdr*-west; ReRe, homozygous *kdr*-east resistant; Ace1, acetylcholinesterase; qPCR, quantitative polymerase chain reaction; ATL, Animal Tissue Lysis; Buffer ATL, is a tissue lysis buffer for use in purification of nucleic acids.

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under-ten was highly effective in reducing the malaria burden among young and old children by at least 60% (Cisse et al., 2016). In addition, between 2013 and 2014, IRS interventions were implemented in the area, targeting 30% of the population living in hotspots to complement the SMC (Sy et al., 2015). The combination of these effective interventions led to a significant decrease of the *Anopheles gambiae* s.l. human-biting rates as well as the levels of transmission in the targeted villages (Sy et al., 2018). Nevertheless, the widespread of resistance of parasites to antimalarial drugs (Ridley, 2002; Miotto et al., 2015) and of main vectors to insecticides (Carnevale and Mouchet, 2001; Hemingway et al., 2016) may hamper malaria control efforts and ultimately jeopardize the achievement of elimination goals in most of eligible areas, if not properly managed.

Despite significant progress in reducing malaria incidence (from 34 to 23‰) and mortality (from 3.52 to 2.11%) between 2015 – and 2016, Senegal, as most of the sub-Saharan Africa countries, belongs to the endemic area where malaria remains a major public health problem (PNLP, 2017). In the central-western part of the country, successful control interventions have led to reducing malaria transmission, no longer occurring with high level apart in residual foci (hotspots). The residual transmission recorded in the hotspots has been shown to be maintained by *An. arabiensis*, *An. coluzzii* and *An. melas* (Sy et al., 2018). In such situation, there is an urgency to characterize the insecticide resistance status and putative underlying mechanisms of the vectors involved in the hotspots to provide useful information to target and implement the most-effective malaria control and elimination strategies. The widespread distribution of the *kdr*-west (L1014F) and the *kdr*-east (L1014S) mutations have been previously reported in *An. gambiae* s.l.

populations from several areas in Senegal (Niang et al., 2016; Dia et al., 2018; Sy et al., 2020), while the *ace-1* mutation (G119S) was not yet found in the country. The *kdr* mutations confer cross-resistance to DDT and pyrethroids whilst the *ace-1^R* mutation confer cross-resistance to organophosphates and carbamates.

This study aims to investigate the prevalence of the *kdr* and *ace-1^R* mutations among natural populations of *An. arabiensis* and *An. melas* in a residual malaria transmission area in the central-western Senegal.

2. Methods

2.1. Study area

The study was carried out from July 2017 to December 2018 in three villages located in the central-western Senegal. The estuary village of Mbind Coly (14° 17'10" N; 16° 54'30" W), surrounded by swamps, belongs to the coastal area of Mbour department. While the villages of Diofior (13° 58'41" N; 16° 45'45" W) and Keur Martin (14° 24'29" N; 16° 34'29" W), both in the department of Fatick, are respectively located in the delta area of the Saloum river and in the western mainland of the department. All the three study villages are characterized by widespread presence of salty soils and brackish water bodies (Fig. 1).

2.2. Mosquito sampling and processing

2.2.1. Mosquito collections and species identification

Adult mosquitoes were collected by Human-Landing Catches (HLC), indoor and outdoor during two successive nights in three rooms chosen

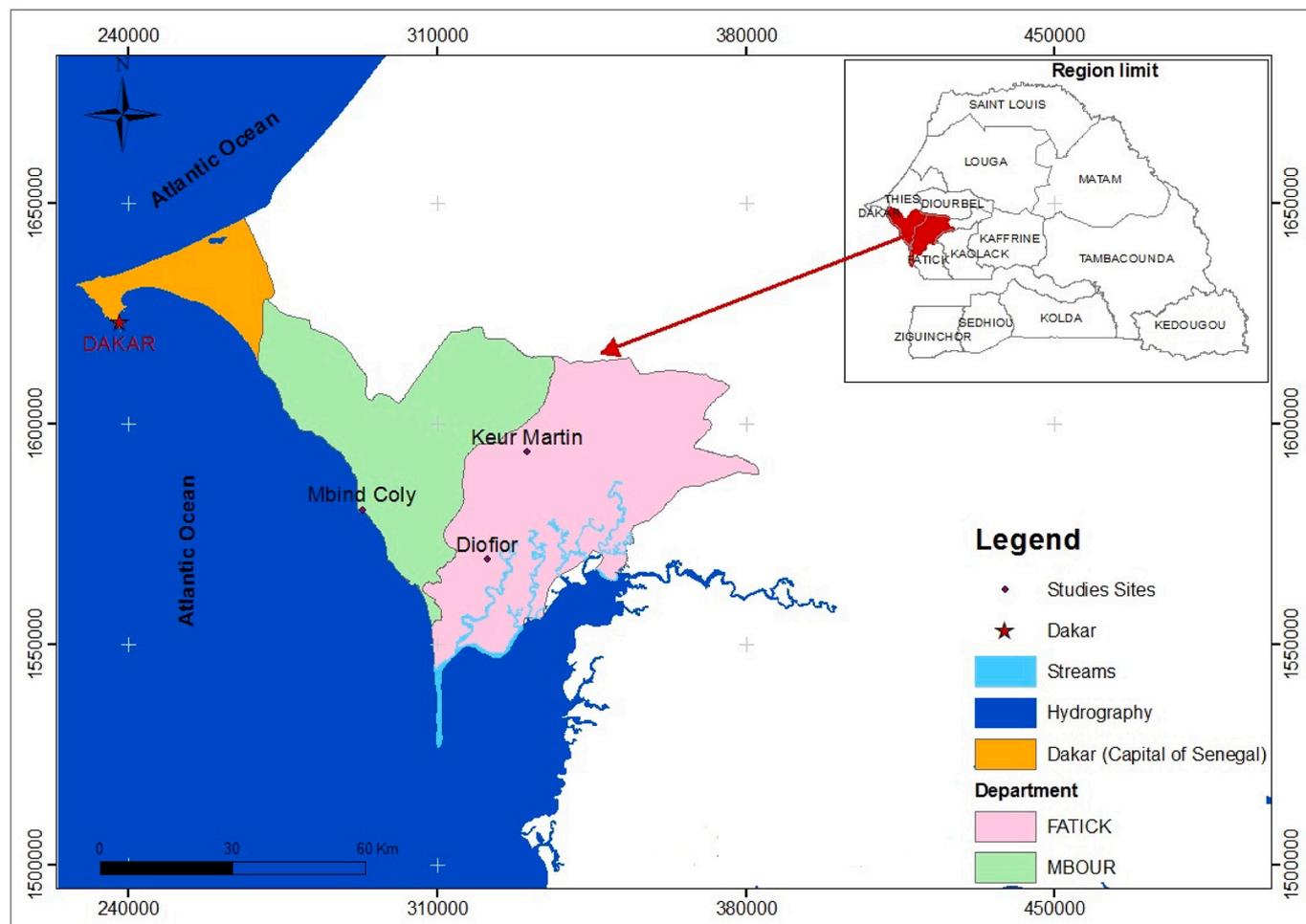


Fig. 1. Study area (central-western Senegal).

throughout the village, and by Indoor Pyrethrum Spray Collections (PSC) early on the morning in 30 randomly selected rooms per village. Upon collection, mosquitoes were morphologically identified to genus level, and anophelines were subsequently identified to species level using a classical morphological key (Gillies and De Meillon, 1968). All specimens (100%) of *An. gambiae* s.l. collected from Diofior (131) and Mbine coly (374) and 50% of those randomly selected from Keur martin (1238) were subsequently identified by PCR as described by Wilkins et al. (2006). Prior to the PCR assays, the genomic DNA was extracted from individual mosquito specimen using the automated QIA cube Extractor robot (Qiagen). Head and thorax were grinded then incubation in a mixture of proteinase K and buffer ATL solution overnight at 56 °C for tissues lysis. The digest eluates were transferred into lysis 96 well plate and placed inside the extraction robot. The DNA was eluted in 80 µl of water and stored at -20 °C.

2.2.2. Screening for *kdr* and *ace-1* mutations

The presence of *kdr*-east and *kdr*-west mutations on the voltage-gated sodium channel gene (*vgsc*), and G119S mutation on the *ace-1* gene was assessed using the TaqMan qPCR methods described by Bass et al. (2007, 2010).

Overall, 1209 *Anopheles arabiensis* (91 from Diofior, 871 from Keur Martin and 247 from Mbine Coly) and 488 *Anopheles melas* (34 from Diofior, 346 from Keur Martin and 108 from Mbine Coly) were respectively screened to detect the presence of both *kdr* mutations.

Subsamples of 1192 *Anopheles arabiensis* (91 from Diofior, 860 from Keur Martin and 241 from Mbine Coly) and 482 *Anopheles melas* (34 from Diofior, 345 from Keur Martin and 103 from Mbine Coly) were respectively genotyped for the target site G119S mutation.

2.3. Statistical analysis

At each target site mutation locus, the genotypic and allelic frequencies were estimated in conformity to Hardy–Weinberg equilibrium using Genepop v.3.2. (Raymond and Rousset, 1995). Data were compared using the pairwise fisher test, the Kruskal–Wallis test, the chi-square, or the Fisher exact tests where applicable with the statistic significant threshold set at P value ≤ 0.05 . All statistical analyses were performed using R software (version 3.0.2).

3. Results

3.1. *Anopheles arabiensis* and *Anopheles melas* were dominant in the study sites

Overall, 3575 specimens of *An. gambiae* s.l. were collected (1215 by HLC and 2360 by PSC) during the study period. Of these, 92, 93 and 1030 were caught on human respectively in Mbine Coly, Diofior and Keur Martin. While 693, 63 and 1604 were collected from resting places inside human dwelling in the same study sites.

The molecular identification of *An. gambiae* s.l. revealed the presence of *An. arabiensis*, *An. coluzzii*, *An. gambiae*, *An. melas* and *gambiae-coluzzii* hybrids. With the respective proportion of 69.36% (1209/1743) and 27.99% (488/1743), *An. arabiensis* and *An. melas* were the most frequent

species of the complex in the study area during the study period. By study village, *An. arabiensis* represented 69.47% (91/131), 70.36% (871/1238) and 66.04% (247/374) respectively in Diofior, Keur Martin and Mbine Coly, while *An. melas* accounted for 25.95% (34/131), 27.95% (346/1238) and 28.88% (108/374) respectively in the same villages. In addition to these, *An. coluzzii* was found at lesser proportion (2.29%) as well as *An. gambiae* and *gambiae-coluzzii* hybrids representing less than 1% of the complex members in the area (Table 1).

3.2. Genotypic and allelic frequencies of the *kdr* and *ace1^R* mutations in natural *Anopheles arabiensis* and *Anopheles melas* populations

3.2.1. Genotypic and allelic frequencies of the *kdr* mutations

All the six genotypes – L1014L (SS), F1014F (RwRw), L1014F (RwS), S1014S (ReRe), L1014S (ReS) and F1014S (ReRw) – described at 1014 locus of the Voltage-gated Sodium Channel were found among all the studied populations of *An. arabiensis* across the study area (Table 2). While only four of them were found in the natural population of *An. melas*.

Overall, the homozygous wildtype genotype (SS) was the most predominant in both *An. melas* (97.54%) and *An. arabiensis* (42.93%). Noteworthy, all the homozygous and heterozygous *kdr* genotypes were found in almost all the studied population of *An. arabiensis*, with the overall predominance of the heterozygous *kdr*-east genotype (ReS = 34.08%), followed by the heterozygous *kdr*-west genotype (RwS = 14.81%), and the heterozygous *kdr*-east/west genotype (ReRw = 4.63%). The *kdr*-east homozygous ReRe genotype was found in a relative higher proportion (3.31%) compared to the *kdr*-west homozygous RwRw genotype (0.25%).

No significant difference ($p = 0.42$) was found when comparing all the different genotypes using the Kruskal–Wallis test.

On the other hand, in *An. melas* populations, the heterozygous ReS *kdr* genotype was the most frequent one (1.02%), followed by the heterozygous RwS genotype (0.82%), while the homozygous ReRe genotype was the less frequent (0.61%) genotype found. Whilst no specimen of *An. melas* was found carrying the RwRw or the ReRw genotypes across the area over the study period.

Among the *An. arabiensis* populations, the overall allelic frequency of *kdr*-east (22.66%) was relatively higher compared to the *kdr*-west (9.96%) mutation, without any significant difference in the allelic frequencies between the study sites (Pairwise.Fisher.Test; p -value > 0.05), neither for the *kdr*-east nor for the *kdr*-west (Fig. 2).

For *An. melas*, the allelic frequencies of the *kdr* genes were very low (Fig. 3).

3.2.2. Genotypic and allelic frequencies of the *ace1^R* mutation

A total of 1192 and 482 specimens of *Anopheles arabiensis* and *Anopheles melas* were respectively screened for the presence of the G119S mutation. The *ace 1^R* mutation was found only among *An. arabiensis* populations, and only at the heterozygous form (RS) with an overall genotypic frequency of 4.11%. The frequency of the mutation was relatively the highest in Keur Martin (5.23%) and Diofior (3.3%) (Table 3).

With a respective frequency of 1.64, 2.61 and 0.2 in Diofior, Keur

Table 1

Specific identification of the members of *Anopheles gambiae* complex collected in the different study sites.

Species	Diofior (DELTA)		Keur Martin (CONTINENTAL ZONE)		Mbine Coly (COASTAL ZONE)		Total	
	N	%	N	%	N	%	N	%
<i>An. arabiensis</i>	91	69.47%	871	70.36%	247	66.04%	1209	69.36%
<i>An. coluzzii</i>	5	3.82%	20	1.61%	15	4.01%	40	2.29%
<i>An. gambiae</i>	0	0.00%	1	0.08%	4	1.07%	5	0.28%
<i>An. melas</i>	34	25.95%	346	27.95%	108	28.88%	488	27.99%
Hybrid <i>coluzzii-gambiae</i>	1	0.76%	0	0.00%	0	0.00%	1	0.05%
Total	131	100.00%	1238	100.00%	374	100.00%	1743	100.00%

Table 2
Genotypic frequencies of *kdr* mutation in *An. arabiensis* and *An. melas* at the three study sites.

Species	Genotypes	Study sites						TOTAL	
		Diofior (DELTA)		Keur Martin (CONTINENTAL ZONE)		Mbine Coly (COASTAL ZONE)		Number	genotypic frequency
		Number	genotypic frequency	Number	genotypic frequency	Number	genotypic frequency		
<i>Anopheles arabiensis</i>	SS	37	40.66%	366	42.02%	116	46.96%	519	42.93%
	RwS	11	12.09%	133	15.27%	35	14.17%	179	14.81%
	ReS	34	37.36%	300	34.44%	78	31.58%	412	34.08%
	RwRw	2	2.20%	1	0.11%	0	0.00%	3	0.25%
	RwRe	3	3.30%	49	5.63%	4	1.62%	56	4.63%
	ReRe	4	4.40%	22	2.53%	14	5.67%	40	3.31%
Total		91		871		247		1209	
<i>An. melas</i>	SS	34	100.00%	335	96.82%	107	99.07%	476	97.54%
	RwS	0	0.00%	4	1.16%	0	0.00%	4	0.82%
	ReS	0	0.00%	5	1.45%	0	0.00%	5	1.02%
	RwRw	0	0.00%	0	0.00%	0	0.00%	0	0.00%
	RwRe	0	0.00%	0	0.00%	0	0.00%	0	0.00%
	ReRe	0	0.00%	2	0.58%	1	0.93%	3	0.61%
Total		34		346		108		488	

SS: homozygote susceptible.
 RwS: hetrozygous *kdr* west.
 ReS: hetrozygous *kdr* east.
 RwRw: homozygous *kdr* west resistant.
 RwRe: Hybrid *kdr* east & *kdr* west.
 ReRe: homozygous *kdr* east resistant.

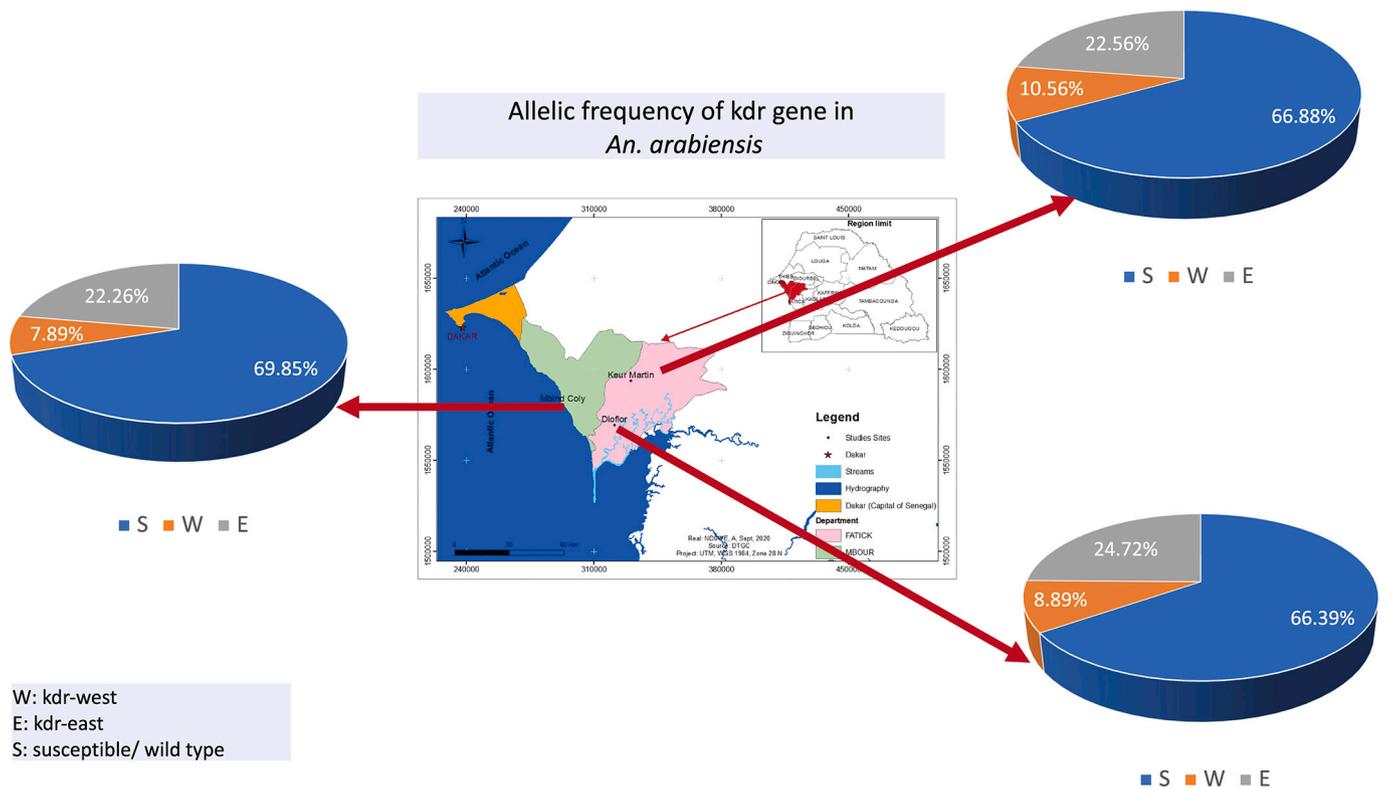


Fig. 2. Allelic frequencies of *kdr* mutation in *An. arabiensis* populations at the three study sites.

Martin and Mbine Coly, the allelic frequencies of the *ace-1^R* mutation was only significant different between Mbine Coly and Keur Martin (pairwise.fisher.test comparison, *p*-value = 0.00057).

4. Discussion

An. gambiae s.l. was the main malaria vector found in the study area during the study period, as frequently reported in the sub-Saharan Africa (Vercruyse and Jancloes, 1981; Gazin and Robert, 1987). Similarly to

previous study (Diagne et al., 1994; Niang et al., 2014), the members of the *An. gambiae* complex found in the study area are *An. arabiensis*, *An. coluzzii*, *An. gambiae*, and *An. melas*; with *An. arabiensis* and *An. melas* being the most predominant species during the study period whatever the surveyed sites. The highest frequency of *An. arabiensis* is consistent with its previously reported predominance in several parts of Senegal (Faye et al., 1994; Dia, 2014). This is likely due to its better adaptation to the most common dried condition found across the country, excepted the most humid southern regions of the country (Dukeen and Omer,

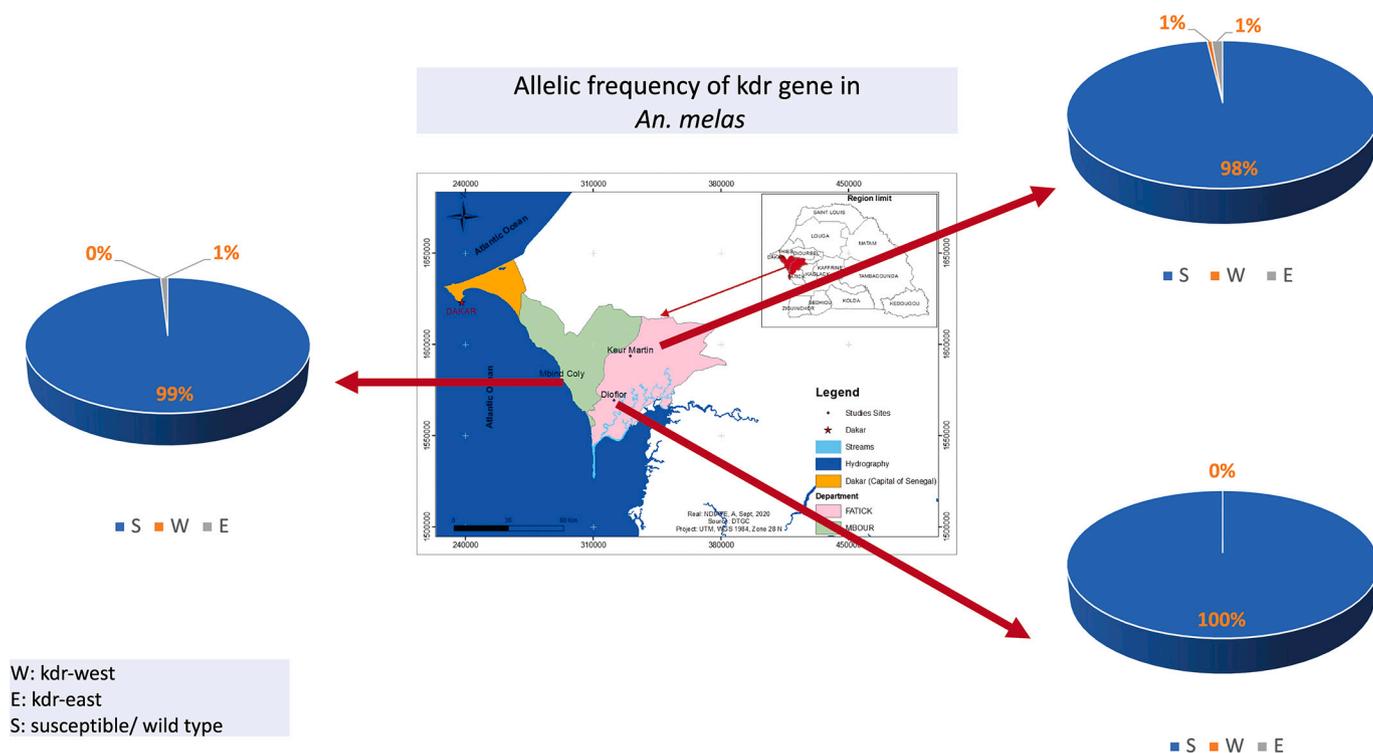


Fig. 3. Allelic frequencies of *kdr* mutation in *An. melas* populations at the three study sites.

Table 3
Genotypic frequencies of *ace-1* mutation in *An. arabiensis* and *An. melas* at the study sites.

Species	Genotypes	Study sites						TOTAL	
		Diofor (DELTA)		Keur Martin (CONTINENTAL ZONE)		Mbine Coly (COASTAL ZONE)		Number	genotypic frequency
		Number	genotypic frequency	Number	genotypic frequency	Number	genotypic frequency		
<i>Anopheles arabiensis</i>	SS	88	96.70%	815	94.77%	240	99.59%	1143	95.89%
	RS	3	3.30%	45	5.23%	1	0.41%	49	4.11%
	RR	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Total		91		860		241		1192	
<i>Anopheles melas</i>	SS	34	100.00%	345	100.00%	103	100.00%	482	100.00%
	RS	0	0.00%	0	0.00%	0	0.00%	0	0.00%
	RR	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Total		34		345		103		482	

SS: homozygote susceptible.
RS: heterozygous ace-1R.
RR: homozygous ace-1R.

1986). The particular ecological characteristic of the study area, located at the vicinity of Saloum Delta’s swamp with widespread presence of brackish water bodies, may explain the high proportions of *An. melas*, which is known as an important focal malaria vector where it is found in high proportion (Diop et al., 2002) as recorded here. Indeed, as one of the salty species of the Gambiae complex, *An. melas*, due to its larval ecological adaptation to brackish water, breeds in the common salty surface waters spread across the study area as already described by Diop et al. (Diop et al., 2002). Indeed, the creation of the brackish breeding sites suitable for *An. melas* larvae is strongly linked with the cyclic fluctuation of tidal movements. And the sympatric co-occurrence of *An. melas*, *An. arabiensis* and *An. coluzzii* has been previously reported in the Saloum delta area, where their respective densities fluctuate spatially and temporally depending to the season and tidal movements (Faye et al., 1994; Dia, 2014; Diop et al., 2002). As already known, *An. arabiensis* is the most predominant anopheline species in the Cape Verde Peninsula and in the northern and central regions of Senegal (Dia et al., 2018). Its widespread in the region may be explained by its better

adaptation to drought and arid environments. While *An. melas*, mostly found in the coastal part of Senegal and at a much lower density than *An. arabiensis*, is known to be exophilic and zoophagous (Diop et al., 2002; Bryan et al., 1987).

The molecular characterization of putative genetic mutations conferring target site insecticide resistances revealed the presence of the *kdr*-west and the *kdr*-east *kdr* mutations in the natural populations of *An. arabiensis* and *An. melas* previously in central western Senegal (Pagès et al., 2008; Namountougou et al., 2012). To our knowledge, this is the first report of the *ace-1^R* (G119S) mutation in the country, found so far only among the natural population of *An. arabiensis* (Weill et al., 2003).

Overall, the *kdr*-east was the most predominant *kdr* mutation whatever the study population of both species. The same observation has been previously reported for *An. arabiensis* in Dakar, where the *kdr*-east was almost fixed among the study populations (Niang et al., 2016). However, at the country level, the *kdr*-west was the most prevalent genotype (Niang et al., 2016). Given that previous studies have shown that *kdr*-west confers greater insecticide resistance compared to *kdr*-east

(Soderland et al., 1990; Lynd et al., 2010), further studies need to be undertaken to further determine their respective impact on the targeted vector populations of both *An. arabiensis* and *An. melas* in the study area, and how they may have contributed to the residual transmission in central western Senegal.

Given that *An. melas* is known to be much more exophilic and zoophilic than its others sibling, including *An. arabiensis* (Diop et al., 2002; Bryan et al., 1987), the latter is likely to be more exposed to pesticide given their larval ecological, biting behavior and host preferences differences. Indeed *An. melas* breeds in salty brackish water where few or no insecticide residuals are found while *An. arabiensis* breeds mainly in permanent rice paddies or other agricultural schemes, where it is highly exposed to insecticide pressure with the same chemicals used both in agriculture and public health. On the other hand, differences in their biting and resting behavior and host preferences are likely exposing them to different level of insecticide pressure. Indeed *An. melas* being more exophilic and zoophilic may escape the contact with the insecticide used in some insecticide-based vector control such as pyrethroids-impregnated LLINs. Therefore, high coverage of pyrethroid-impregnated nets in this region may expose additional pressure on *An. arabiensis* but at lesser extent on *An. melas* populations, which may explain the higher prevalence of the *kdr* mutations in *An. arabiensis* compare to *An. melas*. Following the report of both *kdr* mutations in wild population of *An. melas* but at lowest frequencies than in its sibling *An. arabiensis*, there is an urgent need to closely monitor the insecticide resistance to chemical used in the public health sector, in particular to pyrethroids mainly used for the impregnation of LLINs, among this focal malaria vector natural populations across their distribution range in the country. This is mandatory for the implementation of targeted insecticide resistance management tailored to *An. melas* biting and resting behavior. There is also a need to investigate the putative origins (introgression vs de novo apparition) and evolutionary histories of both the west and the east *kdr* mutations in its natural population in Senegal.

Furthermore, extensive studies are needed to determine the selection pressures (agricultural vs public health) involved in the selection of the observed *kdr* mutations and most importantly to the high frequencies of the *kdr*-east (Padonou et al., 2012).

While only found at very low frequencies and only in *An. arabiensis*, this is to our best knowledge the first report of the *ace-1^R* among natural populations of this major malaria vector in Senegal. The *ace-1* mutation results from the substitution of glycine (GGC) by serine (AGC) at 119 position of the gene coding for acetylcholinesterase (Weill et al., 2003). Conferring the cross-resistance to Carbamates and Organophosphates to specimens harboring the mutation, this is of the highest concern in the study area as well as for the country where both molecules are used recently for Indoor Residual Spraying (IRS) following the increase in several part of the country of pyrethroid-resistance populations as part of the pyrethroid resistance management (Sy et al., 2019; PMI, 2018). The *ace-1^R* has likely been selected following the recent extensive use of the Pirimiphos-methyl (Organophosphate) for IRS in the study area (Sy et al., 2019). Despite its low frequencies, the presence of the *ace-1^R* mutation even only in *An. arabiensis* so far is of the highest concerns. Thus, there is an urgent need to closely monitored its evolution to prevent the failure of current and future insecticide-based vector control interventions using these two chemicals families. Moreover, there is a critical need to carry out additional studies, notably by sequencing the *ace-1* gene wherever vectors populations are found carrying the mutation to better investigate its origin and evolutionary history among the natural malaria vectors populations all over the country.

5. Conclusion

This is the first report of the *ace-1^R* mutation (G119S) among natural population of *An. arabiensis* in Senegal. The study also showed the presence of the *kdr* genes among natural populations of both *An. arabiensis* and *An. melas*, sometime at very high prevalence. The presence of

these important target site mutations among natural populations of the two key malaria vectors in the study area stresses the urgent need to manage properly insecticide resistances and prevent the loss of gain obtained so far in the study area and the whole country. The relation between high frequency of the *kdr* mutation in the studied species and the residual malaria transmission in the hotspots of central-western Senegal needs also to be further investigate regarding its potential to compromise the elimination goal. As such, monitoring the resistance is of the highest priority for the implementation of current and future insecticides resistances management strategies to better control keys malaria vectors. Moreover, futures studies should also investigate the involvement of metabolic resistance mechanisms among the keys malaria vectors maintaining the residual transmission in the hotspots of central-western Senegal.

Authors' contributions

Conceptualization: OS, EAN, OG and OF. Methodology: OS, EAN, LK, OG and OF. Investigation: OS, AN, PCS and MAN. Supervision: OS, EAN, LK, OG and OF. Data curation: OS, EAN, BSA, LK and PCS. Formal analysis: OS, EAN, BSA, LK and PCS. Validation: OS, EAN, LK, OG and OF. Writing - original draft: OS, EAN, BSA and PCS. Writing - review & editing: MAN, OS, EAN, BSA, AKD, MN and OKG.

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Ethics approval

This study was approved by the Ethics Committee of University Cheikh Anta Diop of Dakar, Senegal.

Declaration of Competing Interests

The authors declare that they have no competing interests.

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