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Original article

Genes involved in multiple insecticide resistance in Anopheles gambiae and Anopheles coluzzii from Kpomé a tomatoes growing area in the southern Benin

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ABSTRACT

Background and objectives: Long lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS) are used to control malaria vectors in Benin. It is known that the main threat to effective malaria vector control is the selection of insecticide resistance in field Anopheles population. This study aimed to generate baseline data on the mechanisms involved in insecticide resistance in An. gambiae s.l. population from Kpomé. This information is useful for a proper evaluation of new formulations of vector control tools expected to be deployed in resistance management. Methods: Indoor-resting Anopheles mosquitoes were collected using electric aspirators. The insecticide susceptibility level of F1 adult offspring of An. gambiae s.l. was assessed using the WHO standard protocol. Genotyping of insecticide resistant alleles and Plasmodium detections were carried out using TaqMan assays. Results: WHO susceptibility test showed that An. gambiae s.l. from Kpome is highly resistant to DDT and permethrin. Moderate resistance level was recorded with deltamethrin and dieldrin, whereas full susceptibility was observed with bendiocarb and malathion. Molecular analysis of Plasmodium infections showed an infection rate of 13.2 % for An. gambiae s.l. Both L1014F and L1014S kdr mutations were found in this population of An. gambiae s.l. with high distribution of the L1014F resistant allele. Rdl and GSTe2 mutations were also detected in this population. The allelic frequencies of 22% and 37.5% were recorded for Rdl mutation (A296S) in An. coluzzii and An. gambiae s.s. respectively. In these same species, the allelic frequencies of GSTe2 mutation (L119S) were 26.47% and 7.14% respectively. Interpretation and conclusion: The observed co-occurrence of L1014F, L1014S, A296S and L119S mutations in both An. coluzzii and An. gambiae s.s. is worrisome. The presence of L1014S allele in this mosquito population suggested the spreading of this gene across Benin. The operational impact of these resistance genes on malaria control strategies needs further exploration in other malaria endemic areas.

Introduction

Malaria is still an important health concern among children under five and pregnant women, and responsible for up to 40% of outpatient visits and 30% of hospitalizations in Benin [1]. As in other African countries, malaria control programs are based on the application of chemical insecticides through either Long Lasting Insecticide-treated Nets (LLINs) or Indoor Residual Spraying (IRS). However, the establishment of insecticide resistance in vector populations could threaten the success of such malaria control programs in areas where the disease is endemic. In West Africa, the resistance of malaria vectors to the four major classes of insecticides used in public health interventions has been reported [2-6]. Because of the relative safety for humans at low dosage, the excito-repellent properties, the rapid rate of knock-down and killing effects, pyrethroid insecticides are the only class used for net treatment [7]. Today, the resistance to pyrethroids is widespread in the main malaria vectors An. gambiae s.l., Anopheles funestus and Anopheles arabiensi [8-12]. Two resistance mechanisms have been described including mutations in the gene encoding the voltage-gated sodium channel and enhanced detoxification [13-14]. In Central and West Africa regions, the substitution of Leucine to Phenylalanine at position 1014 (L1014F) appeared predominant [15-18], whereas the substitution of Leucine to Serine (L1014S) [19], was established in the central region [20-22]. Today, the serine substitution (L1014S) has been reported in some West African countries including Benin [5] and Burkina-Faso [23-24].

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Between 2007 and 2010, cross-resistance to DDT and pyrethroids was reported in An. gambiae s.l. through entomological surveys with strong geographical variations in a South-North transect [25-26]. Gene expression analysis with other molecular techniques revealed the over-expression of two P450 and one GST genes (CYP6M2 & CYP6P3; GSTe2) in addition to the kdr L1014F mutation potentially involved in DDT and pyrethroids resistance [6, 27]. Carbamates and organophosphates resistance due to the substitution of Glycine to Serine at position 119 in the oxyanion hole of the acetylcholinesterase enzyme [28] has also been detected in Côte d'Ivoire, Burkina-Faso, and Benin [29-31]. While the epidemiological consequences of pyrethroid resistance remain to be established, the rapid evolution of insecticide resistant alleles over the past decade is a real cause for concern for vector control management [32]. Significant advantages can be obtained for the insecticide resistance management by monitoring these markers of pyrethroid resistance. A better acknowledge of the genetic mechanisms involved in insecticide resistance and the occurrence of Plasmodium could be an important step to achieving success with insecticide resistance and malaria transmission management strategies. This study aimed to investigate the status of insecticide resistance genes and the level of Plasmodium infection in the An. gambiae population from Kpome, a rural area of tomatoes cultivation, through a combination of toxicological test and TaqMan assays.

MATERIAL AND METHODS

Ethical Clearance and consent to participate

No ethical clearance was required. However, consent of the community leaders was sought prior to adult mosquito's collection in the community. In addition, consent of household heads was sought prior to using the house for mosquito collection.

Study area and mosquito collection

Blood fed adult females An. gambiae s.l resting indoor were captured in houses between 6:00 and 9:00 a.m in the Toffo District at a village named Kpomé (6°55′N, 2°19′E) in Southern Benin, a rural area where people use significant amounts of pesticides for tomatoes protection (Fig 1). Mosquito sampling was conducted in December 2013. Blood-fed and gravid An. gambie resting indoors were collected using electric aspirators and immediately transported to the insectariums of the International Institute of Tropical Agriculture (IITA, Benin). The collected females were stored in cages and provided with 10% sugar solution on cotton. They were maintained for 5 days to allow them to fully reach the gravid stage. The gravid mosquitoes were then individually placed into 1.5 ml Eppendorf tubes containing one centimeter square piece of filter paper inserted into the bottom of the tube. The cap of the Eppendorf tube was pierced with 2 holes to allow air into the tube. Eggs were checked of daily in the tube, and females that laid eggs were carefully removed from the tubes and after death, were transferred into Eppendorf tubes containing silica gel. Eggs were stored at 25°C for up to 2 days before being allowed to hatch in small cup. They were later transferred into larvae bowls for rearing. Egg batches obtained from 100 females were pooled and reared together. Larvae were fed daily with Tetramin (TM) Baby Fish Food. To reduce larvae mortality, the breeding water was changed every two days. F1 adult mosquitoes were randomly mixed in cages for bioassays.Molecular identification

Genomic DNA was individually extracted from all females that were used for individual oviposition using the Livak extraction method [33]. The Anopheles gambiae species were identified using polymerase chain reaction (PCR) [34].

Insecticide susceptibility test

Susceptibility tests were carried out on 3-5 days female's progeny generated from larva rearing. Standard WHO test kits and impregnated papers (from Malaysia) at diagnostic doses were used [35]. The test papers included 4% DDT and 4% dieldrin (organochlorine), 0.1% bendiocarb (carbamate), 0.75% permethrin and 0.05% deltamethrin, and 5% malathion (organophosphate). Briefly, for each tested insecticide, four replicates of 20–25 unfed females were exposed to an impregnated paper for 60 min, after which they were transferred in tubes with untreated papers and placed under observation at 25°C and 80% relative humidity (RH) with sugar solution.. Mortality was recorded 24h after exposure to each insecticide. Tests with untreated papers were run in parallel as controls.

Kdr genotyping

DNA extracts from females that lay eggs were subsequent to TaqMan analysis to screen the L1014F and L1014S mutations as previously described [23, 36]. Forward and reverse primers and three minor groove binding (MGB) probes (Applied Biosystems) were designed using the Primer Express™ Software Version 2.0. Primers kdr-Forward (5'-CATTTTTCTTGGCCACTGTAGTGAT-3'), and kdr-Reverse (5'- CGATCTTGGTCCATGTTAATTTGCA-3') were standard oligonucleotides with no modification. The probe WT (5'-CTTACGACTAAATTTC-3') was labelled with VIC at the 5' end for the detection of the wild type allele, while the probes kdr-w (5'-ACGACAAAATTTC-3') and kdr-e (5'-ACGACTGAATTTC- 3') were labelled with 6-FAM for detection of the kdr-W and kdr-E alleles respectively.

Detection of L119F-GSTe2 and A296S-rdl mutations in Anopheles gambiae population

To detect the L119F and GSTe2 mutations, 100 females of field-collected An. gambiae s.l population were genotyped by TaqMan assays in the Agilent MX3005P machine (Stratagene Mx3005P, Agilent Technologies) as previously described [37]. For the probes used, the wild type allele was labeled VIC while the mutant allele was labeled FAM.

TaqMan for Plasmodium quantification

The Plasmodium infection rate was determined on wild mosquito using the TaqMan assay [38]. The real-time PCR machine (Stratagene Mx3005P, Agilent Technologies) was used for the amplification

according to the protocol previously described [38]. Primers were used together with two probes labelled with fluorophores, FAM to detect Plasmodium falciparum, and HEX to detect mix infection of P. ovale, P. vivax and P. malariae. Two P. falciparum samples and a mixture of P. ovale, P. vivax and P. malariae were used as positive controls.

Statistical analysis

World Health Organization criteria [35] used to determine the resistance status of mosquito population is described:

Mortality rate > 98% = susceptible mosquito population

Mortality rate between 90 -98% = suspected resistance in the mosquito population

Mortality rate < 90% = resistant mosquito population

The frequency of resistant alleles L1014F, L1014S, A296S and L119F was compared using GENEPOP software between An. coluzzii and An.gambiae s.s. The level of significance was set at $p<0.05$.

RESULTS

Species composition

A total of 821 mosquitoes were caught over ten days out of which 736 (89.64%) were Anopheles. Among the 736 anopheles mosquitoes, 410 (55%) belonged to the Anopheles gambiae group and 326 (45%) to the Funestus group. Of the 100 anopheles mosquitoes subjected to PCR species identification, 80 were successfully identified including 70 An. coluzzii, 8 An. gambiae s.s and 2 An. coluzzii/An. gambiae s.s. hybrids (Fig 2).

Insecticide susceptibility status of An. gambiae s.l

WHO bioassays carried out using adult female mosquitoes revealed that An. gambiae s.l. population of Kpome was highly resistant to DDT and permethrin with mortality rates of 8% and 20% respectively. Resistance was also recorded against deltamethrin and dieldrin with 62% and 73% mortality rates respectively, whereas full susceptibility was observed with bendiocarb (100%) and malathion (99%). Detailed results of the susceptibility tests are given in Fig 3. In all cases, control mortality rate amounted to <5% and therefore no correction with Abbott's formula was required [35].

Anopheles gambiae s.l. infection to Plasmodium species

Genomic DNA of 70 identified An. coluzzii, 8 An. gambiae s.s and 2 hybrids were used to test for sporozoite infection using TaqMan assay. From the 80 mosquitoes tested, 10 (13%) exhibited Plasmodium infections. Out of the 10 that scored positive by TaqMan, 50% were identified as infected by P. falciparum and the remaining 50% by P. vivax /P. ovale /P. malariae. Nine of the ten mosquitoes harboring malaria sporozoites belonged to An. coluzzii, whereas only one belonged to An. gambiae s.s./An. coluzzii hybrid.

Target-site mutations and frequencies of resistance alleles in An. gambiae s.l population

Kdr L1014F and L1014S

The TaqMan assay was successful in detecting kdr L1014F genotypes in 57 out of 70 (81%) An. coluzzii analysed, all 8 (100%) An. gambiae s.s. and in 1 of 2 (50%) hybrids. The L1014F kdr mutation was detected at frequencies of 50% and 51% in the total An. coluzzii and An. gambiae s.s. respectively (Table 1). These frequencies were similar between the two species ($p=0.86$). Analysis of the distribution of the kdr L1014F genotypes showed that the mutation was mainly present at the heterozygote state in both species, showing the RS genotype in 98% and 100% of An. coluzzii and An. gambiae s.s. mosquitoes, respectively, whereas the homozygote resistant (RR) genotype appeared in only 2% of An. coluzzii mosquitoes (Table 2).

For the kdr L1014S, TaqMan assay was successful in 59 out of 70 (84.2%) An. coluzzii and 6 out of 8 (75%) An. gambiae s.s. The allelic frequencies of L1014S mutation were 18% and 25% in An. coluzzii and An. gambiae s.s. respectively (Table 2). The frequency was higher in An. gambiae s.s. than in An. coluzzii ($p=0.00001$). The distribution of this kdr genotype revealed that the mutation was also predominant at the heterozygote state in both species showing the RS genotype in 22.03% and 50% of An. coluzzii and An. gambiae s.s. respectively (Table 2).

Rdl and GSTe2 mutations

Of the 70 An. coluzzii and 8 of An. gambiae s.s. tested, the TaqMan assay was successful in detecting A296S genotypes in 48 (68.57 %) of the An. coluzzii and in all the 8 (100%) of the An. gambiae s.s. The allelic frequencies of A296S were significantly higher in An. gambiae s.s. (37.5%) than in An. coluzzii (22%) ($p=0.0021$) (Table1). This mutation was present at heterozygote state in both species with RS genotypes in 40% and 75% of An. coluzzii and An. gambiae s.s., respectively, whereas the homozygote resistant (RR) genotype appeared in only 2% of An. coluzzii mosquitoes (Table 2).

For the L119S, TaqMan assay was successful in detecting L119S genotypes in 34 out of 70 (48.5%) and 7 out of 8 (87.5%) An. coluzzii and An. gambiae s.s. respectively. The allelic frequency of L119S mutation was higher in An. coluzzii (26.47%) than An. gambiae s.s. (7.14%) ($p=0.00001$) (Table 1). Analysis of the distribution of the L119F genotype shows that the mutation was mainly present in the heterozygote state in both forms. The RS genotype appeared in 41% and 14.3% of An. coluzzii and An. gambiae mosquitoes respectively, whereas the homozygote resistant (RR) genotype appeared in only 6% of An. coluzzii (Table 2).

Table 1: Frequencies of kdr (L1014F/S), Rdl (A296S) and GSTe2 (L119S) alleles in *An. coluzzii* and *An. gambiae s.s.*.

Species	Molecular resistance markers											
	kdr-west genotype			kdr-east genotype			Rdl-genotype			GSTe2-genotype		
	N (alleles)	f(L1014F)	f(L1014L)	N (alleles)	f(1014S)	f(1014L)	N (alleles)	f(296S)	f(296A)	N (alleles)	f(119F)	f(119L)
An. coluzzii	114	0.51[a]	0.49[a]	118	0.18[e]	0.82[b]	96	0.22[f]	0.78[c]	68	0.26[f]	0.74[c]
An. gambiae s.s.	16	0.5[a]	0.5[a]	12	0.25[f]	0.75[c]	16	0.38[h]	0.62[c]	14	0.07[i]	0.93[b]
An. coluzzii/An. gambie s.s.	4	0.5[a]	0.5[a]	4	0.00	1.00[d]	4	0.00	1.00[g]	-	-	-

Numbers sharing the same letter are not different significantly; N, number of alleles; f, allelic frequency.

Table 2: Genotyping of kdrs, Rdl and GSTe2 alleles in *An. coluzzii* and *An. gambiae s.s.*.

Alleles	*An.coluzzii*			*An. gambiae s.s.*		*An.coluzzi/An. gambiae s.s.*	
	RR (%)	RS (%)	SS (%)	RS (%)	SS (%)	RS (%)	SS (%)
L1014F	2 (1/57)	98 (56/57)	0	100 (8/8)	0	100 (1/1)	0
L1014S	0	22.03 (13/59)	77.97 (46/59)	50 (3/6)	50 (3/6)	0	100 (2/2)
A296S	2.08 (1/48)	39.58 (19/48)	58.33 (28/48)	75 (6/8)	25 (2/8)	0	100 (1/1)
L119F	5.88 (2/34)	41.17 (14/34)	52.94 (18/34)	14.3 (1/7)	85.7 (6/7)	0	0

NB: Values are given in percentage (%) with positive and tested numbers in brackets; RR, RS and SS are homozygote resistant, heterozygote resistant and homozygote susceptible genotypes respectively.

Figure 1: Geographic location of Toffo district (Kpome) in Southern Benin.

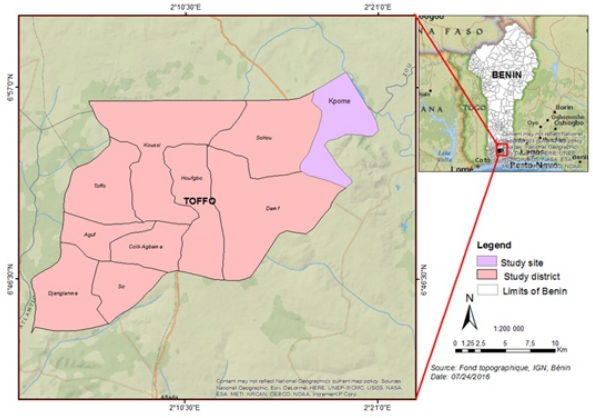


Figure 2: Distribution of Anopheles gambiae s.s. and An. coluzzii species in Kpome.

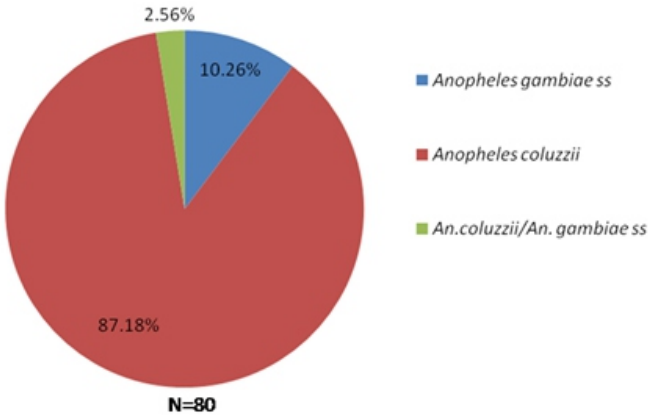


Figure 3: Insecticide resistance profiles of An. gambiae s.l. in Kpome. Histograms are represented with error bars at 5%.

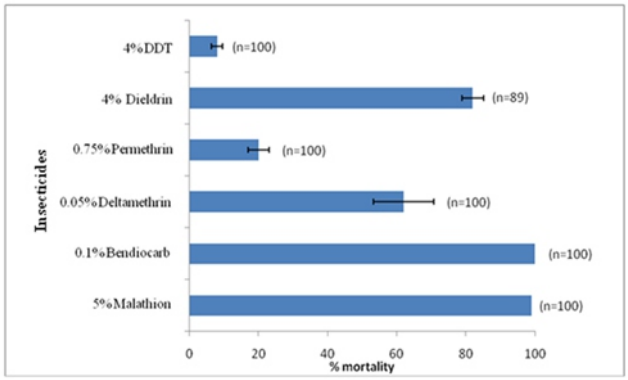
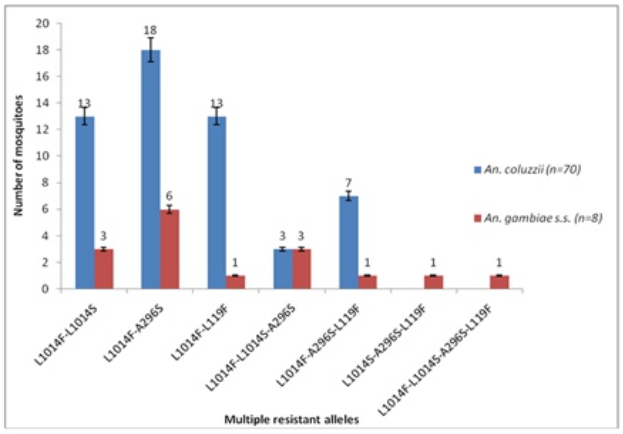


Figure 4: Co-occurrence of resistant alleles in Anopheles gambiae s.s. and An. coluzzii in Kpome. Histograms are represented with error bars at 5%.



DISCUSSION

In the present study, the main malaria vector, An. gambiae s.l was collected at a big zone of tomatoes cultivation in southern Benin, to assess the insecticide resistance profile and the plasmodium infection prevalence.

The anopheline fauna was mainly dominated by An. gambiae s.s, An. coluzzii and An. funestus in the study site. This is consistent with previous studies carried out in the neighboring district, Torri-Bossito in 2008 [39] in southern Benin. The co-appearance of these three species (An. gambiae s.s., An. coluzzii and An. funestus s.s.) which exhibit different behavior is a serious concern to malaria vectors control programs.

High level of organochlorine (DDT/Dieldrin) and Pyrethroid (permethrin and deltamethrin) resistance was identified in An. gambiae s.l. This resistance profile could be attributed to a combination of the presence of target site mutations; L1014F, L1014S, L119S and A296S. The selective pressure behind this multiple-insecticide resistance in mosquitoes can be attributed to the uncontrolled use of insecticides to protect tomatoes against pests attack. Indeed, the use of insecticides in agriculture has been linked to resistance in malaria vectors by several authors [40-41]. This stands as the most plausible explanation in this district of intense agricultural practices. Kpome is a huge agricultural setting with intense production of tomatoes which mainly serve a commercial purpose. According to Yadouleton et al [42], the larval of all instars face more selection pressure towards the families of insecticides; pesticides treatment causes movement of chemical particles from pesticides residues to larval breeding sites and are the major causes leading to selection of resistance in mosquitoes. Concerning the household individual and collective protection, against mosquito nuisance, the use of insecticides products such as aerosol and mosquito coils could also affect field selection pressure towards the family of insecticides in the mosquito populations [43]. We cannot exclude the possibility that besides these targets site mutations, other genetic mechanisms and metabolic enzymes could be contributing to the phenotype observed as suggested from previous studies [44-45]. The emergence of a new mutation (termed N1575Y) within the linker domains III-IV of the VGSC was recently found in An. gambiae s.l. This N1575Y mutation inextricably occurs with the L1014F mutation on the same haplotypic background. Moreover, evidence suggests that a secondary selective sweep associated with resistance to pyrethroids/DDT is occurring throughout the West African region [27, 45]. Several metabolic enzymes including the CYP6M2, CYP6P3, GSTD3 and GSTE2, were also identified to be involved in pyrethroids/DDT resistance in Anopheles gambiae s.l and An. funestus populations from Benin [6, 27, 37, 46].

An important result of this study is the detection of the L1014S kdr mutation in wild An. gambiae s.l populations from Southern Benin. This resistance allele was previously found in the North (Malanville) and Central (Bohicon) regions of the country in An. arabiensis [5]. In the present study, 16 specimens of An. coluzzii and An. gambiae s.s. were found at the heterozygote state with up to 12% allelic frequency. Indeed, it is necessary to imagine the migration of the resistant anopheles strain from Bohicon or Malanville along the wind and means of road transport towards Cotonou the economic capital of Benin. This lead to dispersion of genes which is achieved by mixing the local strain of Kpome with the field ones coming from Bohicon and Malanville. To our knowledge, this study is the first showing the presence of L1014S kdr mutation in wild An. gambiae s.l from southern Benin. These results confirmed the previous study in Burkina-Faso, where high frequency of this kdr mutation was reported in An. gambiae s.l [23]. These findings provide additional evidence of the rapid spread of kdr mutations in An. gambiae s.l throughout Africa and will serve as baseline data for careful monitoring of this allele in West African countries. Further studies should be carried out to set the actual geographical distribution of L1014S kdr allele in West Africa, its role in phenotypic resistance to pyrethroids, and its impact on the efficacy of pyrethroid treated materials used in Public Health. The exhibition of multiple insecticide resistance mechanisms in malaria vectors is worrisome for malaria prevention and control strategies in Africa [47]. In Benin, reduced efficacy of LLINs

and IRS has been shown in areas of high frequencies of L1014F mutation [48, 49]. Interestingly, a full susceptibility was observed in An. gambiae against malathion and bendiocarb, hence confirming the data published by Djogbenou et al. [26], and contrasting recent studies (Gnanguenon et al.[4] and Aïkpon et al.[30]) which both reported the emergence of carbamate resistance in malaria vectors in Northern Benin.

Knowledge on the sporozoite rates in malaria vectors is an important component to assess the transmission risk and the effect of any interventions strategy. Thus, in the present study, using the TaqMan assay, we showed that An. coluzzii was highly infected by Plasmodium. In fact, 13% of tested mosquitoes were positive for sharing malaria parasite. This infection prevalence in southern Benin corroborates with other studies from Mali that reported a high infection rate in An. coluzzii [50]. However, this infection rate was higher than those usually reported in southern Benin [51]. Interestingly, out of the 10 positive mosquitoes found, 5 were identified to be infected with P. falciparum and 5 others with P. ovale/P. malariae/P. vivax. These results suggested that in this organochlorine/pyrethroids resistance area, there might be co-occurrence of four malaria parasites. These results agreed with those of Sandeu et al,[52] who found a mixed infection of P.malariae and/or P.vivax in An. gambiae and An. funestus in the DDT/pyrethroids-resistance area of Tori-Kpomassé and Ouidah district in the southern Benin. This co-occurrence of malaria parasites in the same area could complicate the chemoprophylaxis used to fight against malaria parasites in the community. The current malaria control strategy recommended by the World Health Organization (WHO) relies on the use of artemisinin-based combination therapy (ACT), the use of intermittent preventive treatment during pregnancy (IPTp) and the universal distribution of LLINs [53].

CONCLUSION

The present study highlights the high resistance profile of An. gambiae s.l. to synthetic pyrethroids and DDT, which are insecticides of choice in Public Health programmes. The spread of multiple resistance mechanisms observed in wild populations of An. gambiae s.l is worrisome as this could threaten the effectiveness of malaria vector control strategies in the study region. Routine insecticide resistance monitoring should therefore be implemented as well as new strategies for pyrethroids/DDT-resistance management at all levels of the vector control programs.

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Author's contribution: RD designed the study, coordinated laboratory works and analyzed the data. ID, RA and RA, conducted field sampling and contributed in laboratory analysis. VG mapped the study site conducted laboratory analysis which included advanced molecular assays drafted and reviewed the paper. All authors read and approved the final manuscript.

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