Brief Report

Insecticide Resistance in Aedes aegypti Mosquitoes: First Evidence of Kdr F1534C, S989P and V1016G Triple Mutation in Benin, West Africa

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Insecticide Resistance in *Aedes aegypti* Mosquitoes: First Evidence of kdr F1534C, S989P and V1016G Triple Mutation in Benin, West Africa

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Simple Summary: The effectiveness of chemical control of *Aedes aegypti* is threatened by the increasing frequency of insecticide resistance. This study aimed to determine in 2 cities of Benin the insecticide resistance profiles of *Aedes aegypti*, the presence of detoxification enzymes and the frequency of *kdr* mutations. *Ae. aegypti* eggs were collected in the study areas using Gravid *Aedes* traps. CDC bottle bioassays were used to assess the susceptibility status of adult female *Ae. aegypti,* followed by *kdr* screening using allele-specific PCR. Activity levels of key detoxification enzymes were measured among individual unexposed, un-engorged adult female *Ae. aegypti*. Among both study sites, *Ae. aegypti* were resistant to the pyrethroids deltamethrin and permethrin, but susceptible to the carbamate bendiocarb. Significant overexpression of glutathione-*S*-transferases and under expression of α and β esterases was observed in these vector populations. Three *kdr* mutations (F1534C, S989P and V1016G) were present in resistant *Ae. aegypti* at high frequencies, including the simultaneous occurrence of all three mutations in individual mosquitoes. Study findings will be used to inform prospective vector control strategies in Benin.

Abstract: Epidemics of arboviruses in general, and dengue fever, in particular, are an increasing threat in areas where *Aedes (Ae.) aegypti* is present. The effectiveness of chemical control of *Ae. aegypti* is threatened by the increasing frequency of insecticide resistance. The aim of this study was to determine the susceptibility status of *Ae. aegypti* to public health insecticides and assess the underlying mechanisms driving insecticide resistance. *Ae. aegypti* eggs were collected in two study sites in the vicinity of houses for two weeks using Gravid *Aedes* Traps (GATs). After rearing mosquitoes to adulthood, female *Ae. aegypti* were exposed to the diagnostic doses of permethrin, deltamethrin and bendiocarb, using Centers for Disease Control and Prevention (CDC) bottle bioassays. Unexposed, un-engorged female *Ae. aegypti* were tested individually for mixed-function oxidase (MFO), glutathione-*S*-transferase (GST) and esterase activity. Finally, allele-specific PCR (AS-PCR) was used to detect *kdr* mutations (F1534C, S989P and V1016G) in the voltage-gated sodium channel gene in insecticide-exposed *Ae. aegypti.* Most traps were oviposition positive; 93.2% and 97% of traps contained *Ae. aegypti* eggs in the 10ème arrondissement of Cotonou and in Godomey-Togoudo, respectively. Insecticide bioassays assays detected resistance to permethrin and deltamethrin in both study sites and complete susceptibility to bendiocarb. By comparison to the insecticide-susceptibility Rockefeller strain, field *Ae. aegypti* populations had significantly higher levels of GSTs and significantly lower levels of α and β esterases; there was no significant difference between levels of MFOs. AS-PCR genotyping revealed the presence of the three *kdr* mutations (F1534C, S989P and V1016G) at high frequencies; 80.9% (228/282) of *Ae. aegypti* tested had at least

one mutation, while the simultaneous presence of all three *kdr* mutations was identified in 13 resistant individuals. Study findings demonstrated phenotypic pyrethroid resistance, the overexpression of key detoxification enzymes and the presence of several *kdr* mutations in *Ae. aegypti* populations, emphasizing the urgent need to implement vector control strategies, targeting arbovirus vector species in Benin.

Keywords: *Aedes aegypti*; pyrethroid resistance; kdr mutations; detoxification enzymes; 10ème arrondissement of Cotonou; Godomey-Togoudo; Benin

1. Introduction

Aedes (Ae.) aegypti mosquitoes are the main vector species of dengue viruses worldwide. The incidence of this disease has increased dramatically worldwide over the past decades. The number of dengue fever cases reported to the World Health Organization (WHO) has increased by more than eightfold over the previous twenty years, from 505,430 cases in 2000 to over 2.4 million cases in 2010 and 5.2 million cases in 2019 [1]. *Ae. aegypti* is present in Benin and proliferating globally due to the development of trade, rapid urbanization, and high frequency of international travel [2]. Between 2010 and 2019, dengue fever cases have been diagnosed in Benin, resulting in at least one death [2].

In the absence of effective vaccines and available treatments, vector control remains the main strategy for dengue virus prevention. Vector control relies on the use of insecticides, such as pyrethroids, for house spraying and personal protection [3]. As a result of the strong selection pressures exerted by the use of insecticides in agricultural practices and for malaria control, insecticide resistance among *Ae. aegypti* populations is common and widespread worldwide. According to the WHO, insecticide resistance is defined as "the ability of mosquitoes to survive exposure to a standard dose of insecticide; this ability may result from physiological or behavioural adaptation". Two of the main mechanisms underlying insecticide resistance in mosquitoes are alterations in the insecticide target site, including knock-down resistance (*kdr*) mutations in the voltage-gated sodium channel (*vgsc*) gene and increased metabolic activity [4,5]. Metabolic resistance to pyrethroids is mediated by glutathione *S*-transferases (GSTs), esterases and mixed-function oxidases (MFOs) [6–8].

Kdr mutations have been studied in *Ae. aegypti* extensively; these mutations confer cross-resistance to pyrethroids and DDT by altering the structure of the *vgsc*, thus decreasing the affinity of target insecticides to bind [9]. Several *kdr* mutations have been identified in *Ae. aegypti* populations worldwide, including V1016I, V410L, S989P, I1011V, V1016G, I1011M and F1534C [9–11]. In Africa, F1534C, V1016I, V410L and S989P have been associated with pyrethroid resistance in *Ae. aegypti* [12–16].

The emergence and re-emergence of dengue fever epidemics requires effective vector control responses, including monitoring of vector population insecticide susceptibility. To date, there is a considerable paucity of insecticide resistance information for *Ae. aegypti* populations in Benin.

2. Materials and Methods

2.1. Study sites and mosquito sampling

Adult *Ae. aegypti* mosquitoes were reared from eggs collected over a 2-week period in the 10ème arrondissement of Cotonou and in Godomey-Togoudo, Abomey-Calavi from 31st December 2021 (Figure 1). This collection period coincided with the dry season, which is a period characterized by the scarcity of *Aedes* breeding sites. During this sampling period a total of 73 gravid *Aedes* traps were set in the 10ème arrondissement of Cotonou and 74 in Godomey-Togoudo. The traps used are black plastic pots that can contain half a liter of water, in which wooden egg-laying supports have been immersed. The traps were hung on the box or wall with a nail and a wire. The eggs carriers were removed from each trap after 7 days, eggs were counted and then hatched according to standard insectary rearing

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procedures for *Aedes* species. Larval hatching rate was measured by visually counting the number of larvae per bowl using a ladle. Adult emergence rate was measured by an aspirator.

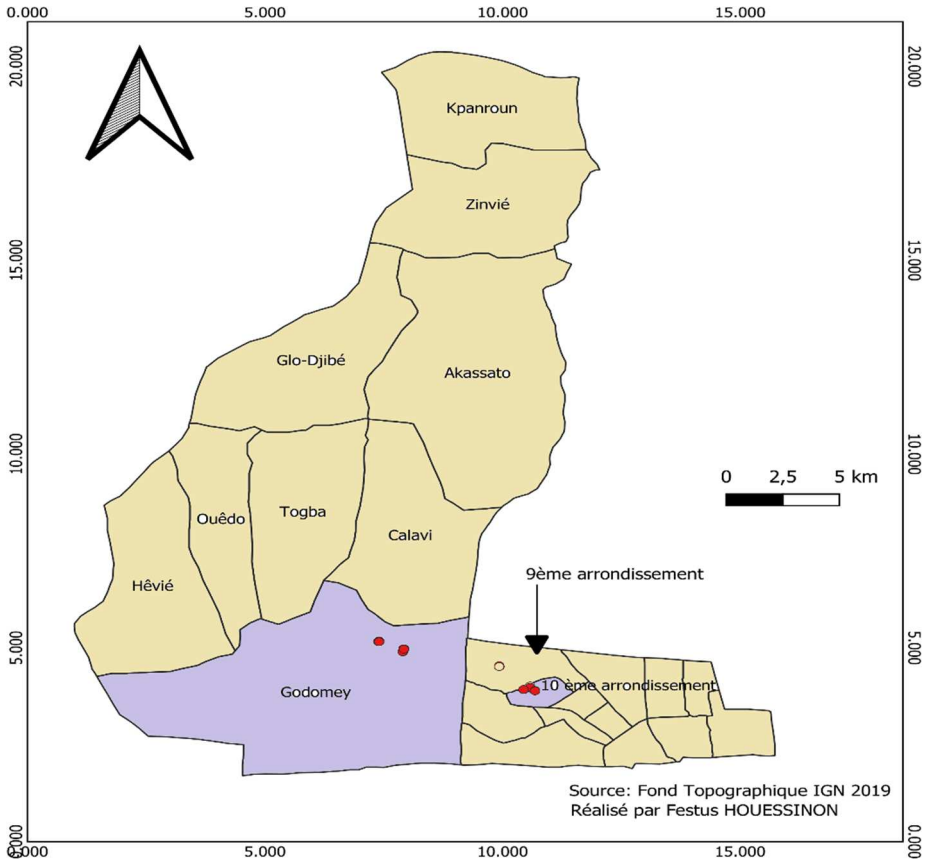

Figure 1. Map of trapping sites in Cotonou and Godomey-Togoudo, Benin.

2.2. Insecticide resistance bioassays

Insecticide resistance profiles for *Ae. aegypti* field populations were measured using Centers for Disease Control and Prevention (CDC) bottle bioassays [17]. Two-to-five-day old female *Aedes* mosquitoes were exposed to the diagnostic doses of deltamethrin (10 µg/bottle) and permethrin (10 µg/bottle) and bendiocarb (12.5 µg/bottle) in Wheaton 250 ml bottles, alongside an acetone-treated control bottle. In each bioassay, 10-25 unfed mosquitoes were introduced into each bottle and knock-down was scored every 15 minutes until all were dead, or up to two hours.

2.3. Aedes morphological identification

Adult *Aedes* spp. tested in insecticide resistance bioassays were identified using Fontenille's taxonomic keys [18] by visualisation under a dissecting microscope. *Ae. aegypti* and *Ae. albopictus* can be recognised by their characteristic white stripes on their legs. Then the thorax can be used to differentiate the two species; *Ae. aegypti* has two thin white median lines with a lyre pattern, whereas *Ae. albopictus* has only one distinct white central line.

2.4. Measurement of detoxification enzyme activity

Biochemical tests were performed to quantify the activity of families of detoxification enzymes, including non-specific esterases (α and β esterases), mixed-function oxidases (MFOs), glutathione *S*-transferases (GSTs) and total proteins in 3-5-day old female *Aedes* mosquitoes; a total of 80 non-insecticide exposed mosquitoes were tested per study site.

Individual mosquitoes were ground in 200 µl of distilled water using sterile pestles, after which the lysate was centrifuged at 14,000 rpm for two minutes. For non-specific esterases, per mosquito,

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10 µl of lysate was added to two wells (technical duplicate) of a 96-well ELISA plate, followed by 90 µl of 1% Triton Phosphate Saline (TBS) buffer was added. The plate was then incubated at 25 °C for 10 minutes, after which 100 µl of a solution of 500 µl α-naphthyl acetate or β-naphthyl acetate + 2.5 ml 1X Triton PBS buffer (pH 6.5) + 7 ml H_2O, was added to each well and the plate was again incubated at 25 °C for 30 minutes. Finally, 100 µl of a solution of 8 mg Fast Garnett Salt dissolved in 12 ml distilled water, was added to each well and the plate was incubated at 25 °C for 10 minutes. Plate absorbance values were read on an ELx808 spectrophotometer at 550 nm. Esterase activity of each mosquito was expressed as µmol α- or β-naphthol produced/min/mg protein and calculated according to: α- or β-naphthol in µmol per ml/amount of protein in mg per ml/30.

For MFOs, 20 µl of mosquito lysate was added to two wells (technical duplicate) of a 96-well ELISA plate, followed by 80 µl of 0.0625M Potassium Phosphate Buffer ($KHPO_4$; pH 7.2), then 200 µl of a solution of 10 mg of 3,3,5,5-tetramethylbenzidine or TMBZ in 4 ml ethanol, and 15 µl of sodium acetate buffer. After adding 25 µl of 3% hydrogen peroxide, the plate was incubated for 30 minutes, and plate absorbance values were read on an ELx808 spectrophotometer at 630 nm. Oxidase activity of each mosquito was expressed as nmol P450/mg protein and calculated according to: nmol P450/2*amount of protein in mg in 10 µl of lysate.

For GSTs, 10 µl of mosquito lysate was added to two wells (technical duplicate) of a 96-well ELISA plate, followed by 200 µl of a solution of 60 mg of reduced glutathione (GSH), 20 ml of 0.1M Sodium Phosphate Buffer (pH 6.5) and 13 mg of CDNB (1-chloro-2,4-dinitrobenzene) dissolved in 1 ml of methanol. Plate absorbance values were read kinetically on an ELx808 spectrophotometer at 340 nm for 5 minutes. GST activity of each mosquito was expressed as nmol GSH conjugate/min/mg of protein and calculated according to: (MilliDO*0.21)/(5.76*1000)/(amount of protein in mg in 10 µl of lysate.

For total protein, 10 µl of mosquito lysate was added to two wells (technical duplicate) of a 96-well ELISA plate, followed by 290 µl of a solution of Coomassie Plus Protein Assay diluted by half in distilled water. Plate absorbance values were read on an ELx808 spectrophotometer at 590 nm after incubating the plate for 5 minutes at 25 °C.

2.5. Ae. aegypti kdr genotyping

After grinding each mosquito in 200µl of 2% CTAB, it is then placed in a water bath or heating block at 65 °C for 05 minutes. Thereafter 200µL of chloroform is added and centrifuged at 12,000 rpm for 05 minutes at room temperature after mixing by inversion at least 10 times. The supernatant collected in well-labeled tubes is mixed with 200µl of isopropanol and centrifuged at 12,000 rpm at room temperature for 10 minutes. The isopropanol is drained and centrifuged for 05 minutes at 12,000 rpm after adding 200µl of 70% ethanol. After emptying the ethanol the resulting DNA pellet is dried for 05 minutes at speed-va or half a day on the bench. We add 140µl of sterile H_2O to the DNA pellets using a new cone for each tube which is left hanging on the bench overnight or half a day.

An allele-specific PCR (AS-PCR) was used to detect the presence of S989P, V1016G and F1534C *kdr* mutations. Each individual mosquito was tested by AS-PCR twice, the first PCR used a primer specific to the susceptible wild-type and the second PCR used a primer specific to the mutant. The primers used for the genotyping were: S989PF: 5′AATGATATTAACAAAATTGCGC3′ and S989PR: 5′GCACGCCTCTAATATTGATGC; V1016GF: 5′GCCACCGTAGTGATAGGAAATC3′ and V1016GVal-R: 5′CGGGTTAAGTTTCGTTTAGTAGC3′; F1534CF: 5′GGAGAACTACACGTGGGAGAAC3′ and F1534CR:5′CGCCACTGAAATTGAGAATAGC3′.

Each reaction was performed in a final volume of 25 µl containing 2.5 µl of 10X buffer, 1.5 µl of $MgCl_2$, 1 µl of dNTPs, 2.5 µl of forward primer, 2.5 µl of reverse primer, 2.5 µl of the susceptible or mutant primer, 0.2 µl of *Taq* polymerase, 6.4 µl pf H_2O and 6 µl of gDNA. PCR reaction conditions were: one cycle at 94 °C for 3 minutes, then 35 cycles of 94 °C for 30 seconds, 60 °C (for F1534C and V1016G) or 62 °C (for S989P) for 30 seconds and 72 °C for 1 minute, followed by one cycle of 72 °C for 7 minutes.

PCR products were separated by gel electrophoresis in a 2% agarose gel, stained with ethidium bromide. PCR amplicon sizes for the detection of *kdr* mutations were 240 bp (S989P), 284 bp (F1534C)

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or 348 bp (V1016G). Non-allele specific external primers produced bands of 594 bp (S989P), 517 bp (F1534C) or 592 bp (V1016G).

2.6. Data analysis

Global Positioning System (GPS) coordinates of the gravid *Aedes* traps were recorded using the OSM Tracker for Android application. Insecticide susceptibility test results were recorded and analysed using Microsoft Excel 2016. Biochemical assay data were recorded and analysed using GraphPad Prism 5 software. All statistical analyses were conducted in Stata/SE 17.0, including Pearson's Chi-squared test to investigate deviations from Hardy-Weinberg equilibrium.

3. Results

3.1. Mosquito sampling

A total of 147 gravid *Aedes* traps were used in the two study areas, of which 142 were positive (i.e., female *Aedes* oviposited in them); yielding an attractiveness rate of 97%. The total number of eggs obtained was 15,844; 8846 eggs in the 10ème arrondissement of Cotonou and 6998 eggs in Godomey-Togoudo.

Of 8846 eggs obtained in the 10ème arrondissement of Cotonou, 3154 hatched (35%) and 1240 adult *Aedes* mosquitoes emerged (39%). Of 6998 eggs obtained in Godomey-Togoudo, 4918 eggs hatched (70%) and 2486 adult *Aedes* mosquitoes emerged (50%).

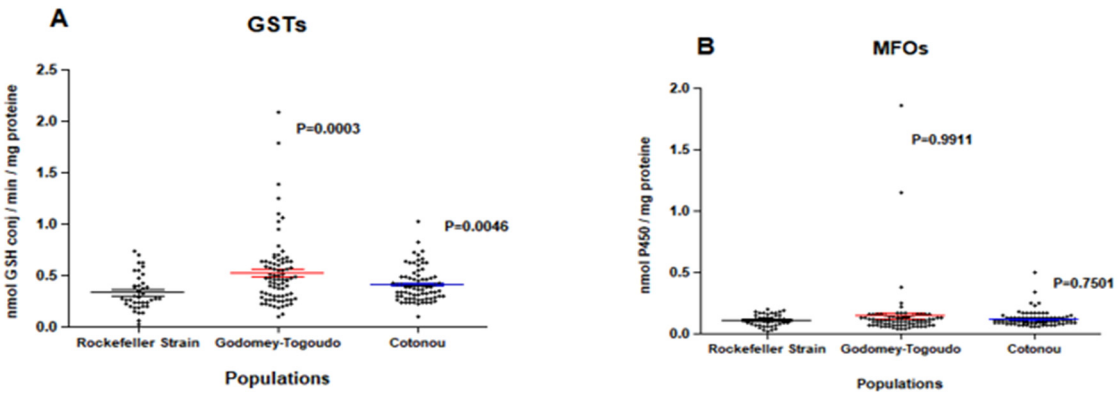
Of the 3726 total mosquitoes that emerged, 3723 were *Ae. aegypti* (>99%), while 3 were *Ae. albopictus* (<1%). One of the 3 *Ae.albopictus* comes from the 10ème arrondissement of Cotonou and the others from Godomey-Togoudo.

3.2. Insecticide resistance profiles

High resistance to permethrin and deltamethrin was evident in both populations of *Ae. aegypti.* Mosquito mortality was 85.7% and 82.7%, following exposure to the diagnostic dose of deltamethrin or permethrin after 30 mins, respectively, in Godomey-Togoudo. Similarly, in the 10ème arrondissement of Cotonou, mortality was 88% with both deltamethrin and permethrin. By comparison, complete susceptibility to bendiocarb was observed in both vector populations.

3.3. Expression of detoxification enzymes

Field insecticide-resistant *Ae. aegypti* populations had significantly higher median levels of GSTs compared to the insecticide-susceptible Rockefeller reference strain, with p = 0.0003 in Godomey-Togoudo and p = 0.0046 in the 10ème arrondissement of Cotonou (Figure 2A). By comparison, field *Ae. aegypti* did not differ in their expression levels of MFOs, compared to the susceptible strain (Figure 2B) and had significantly lower median levels of non-specific esterases (α and β esterases) (Figure 2C,D.)



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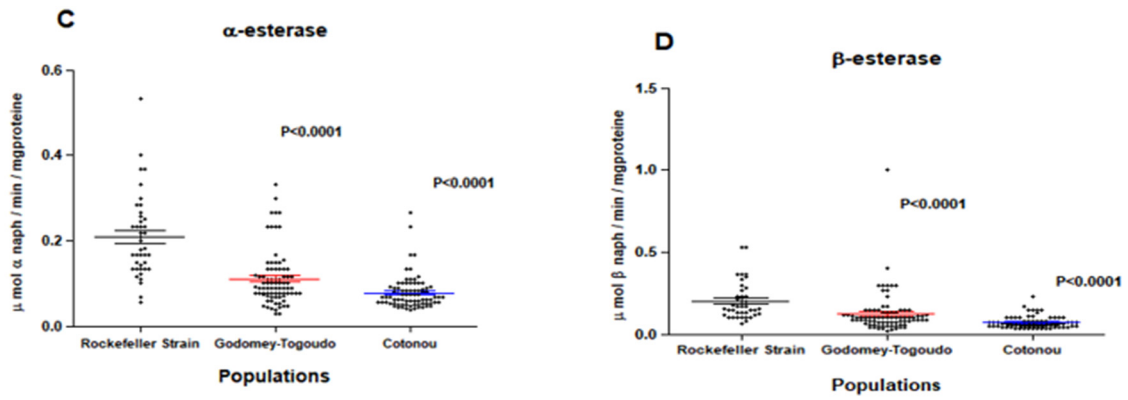

Figure 2. Expression levels of glutathione-*S*-transferases (**A**), mixed-function oxidases (**B**), α (**C**) and and β (**D**) esterases among *Ae. aegypti* Rockefeller strain (insecticide-susceptible) and two insecticide-resistant *Ae. aegypti* field populations collected from Godomey-Togoudo and in the 10ème arrondissement of Cotonou (Vedoko). Medians for each group are represented by solid-coloured lines, accompanied by 95% confidence intervals.

3.4. Kdr mutation screening

Three *kdr* mutations (S989P, V1016G and F1534C) were identified in 82.9% (262/316) pyrethroid-resistant *Ae. aegypti* from both study sites (Table 1). S989P ranged in frequency from 0.63 to 0.78 and was under significant selection in Godomey-Togoudo ($\chi2$ = 6.89; p = 0.0087; Table 2). F1534C was present in the highest frequencies (0.64-0.75), with significant deviations from Hardy-Weinberg equilibrium in both study sites ($\chi2$ = 6.21; p = 0.013 and $\chi2$ = 14.27; p = 0.00016, in the 10ème arrondissement of Cotonou and Godomey-Togoudo, respectively). V1016G ranged in frequency from 0.55 to 0.69, with no evidence for ongoing selection in either site (Table 1). We identified 13 insecticide-resistance *Ae. aegypti* with the simultaneous presence of all three *kdr* mutations.

Table 1. *kdr* mutation (S989P, V1016G and F1534C) allele frequencies in Benin.

kdr Mutation	Study Site	# Mosquitoes Tested	Homozygote Mutation (RR)	Heterozygote Mutation (RS)	Homozygote Wild Type (SS)	Allele Frequency R	Allele Frequency S	χ^2 test	p-value
S989P	10ème arrondissement of Cotonou	24	9	12	3	0.63	0.37	0.107	0.74
	Godomey-Togoudo	54	36	12	6	0.78	0.22	6.89	**0.0087**
F1534C	10ème arrondissement of Cotonou	78	48	21	9	0.75	0.25	6.21	**0.013**
	Godomey-Togoudo	111	54	33	24	0.64	0.36	14.27	**0.00016**
V1016G	10ème arrondissement of Cotonou	33	12	12	9	0.55	0.45	2.35	0.13
	Godomey-Togoudo	16	9	4	3	0.69	0.31	2.80	0.094

4. Discussion

To deploy appropriate control strategies targeting arbovirus vectors, it is crucial to understand the distribution of key mosquito species, their bionomics and insecticide resistance profiles. Study findings report *Ae. aegypti* in the 10ème arrondissement of Cotonou and Godomey-Togoudo during the dry season, which might be explained by climate change, transport development and increasing urbanisation. Both populations of *Ae. aegypti* were characterised by high pyrethroid resistance (to deltamethrin and permethrin), but complete susceptibility to bendiocarb. Pyrethroid resistance in these populations is not expected, given that insecticide treatment specifically targeting *Ae. aegypti* in Benin is rare; rather it may have been driven by several alternate factors. It is possible that insecticides used to control other vector species, e.g., long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS) or household mosquito repellents used to target *Anopheles* vectors of malaria, may have exerted indirect selection pressure on *Ae. aegypti* for the evolution of pyrethroid resistance. Furthermore, water contamination caused by pesticides used for agricultural practices may have also contributed to the development of resistance.

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Regarding insecticide resistance mechanisms, *Ae. aegypti* populations were characterised by overexpression of GSTs and a slight, but non-significant, increase in the activity of MFOs. It is likely that overexpression of both types of metabolic enzymes could confer pyrethroid resistance in these populations. Further investigation is warranted of molecular mechanisms involving the GSTe2 gene, which may be contributing to pyrethroid resistance in *Ae. aegypti* in Benin. By comparison, under expression of non-specific esterases (α and β esterases) observed in these same populations may explain the strong sensitivity to bendiocarb; therefore, this insecticide has the potential to be used in both areas to suppress *Ae. aegypti.*

Several mutations at nine different loci in *Ae. aegypti* have been identified, which are implicated in reduced insecticide susceptibility. Of these, F1534C, S989P and V1016I are widely reported *kdr* mutations and have been associated with DDT and pyrethroid resistance [12–15]. In this study, AS-PCR genotyping revealed the presence of S989P, F1534C and V1016G mutations in both populations of *Ae. aegypti* in Benin; 82.9% of genotyped mosquitoes carried at least one mutation. The frequencies of S989P in both study sites (63% and 78%) were much higher compared to that observed in Nigeria (7%) for the first time in Africa. To our knowledge, this is the first report of V1016G in Africa, and this is the first time all three *kdr* mutations have been detected in *Ae. aegypti* in Benin. We identified 13 insecticide-resistance *Ae. aegypti* with the simultaneous presence of all three *kdr* mutations. The co-occurrence of two or three *kdr* mutations has been previously reported in China, Nigeria and Malaysia, which results in highly intense pyrethroid resistance [11,15,20].

In this study the detection of multiple *kdr* mutations and overexpressed detoxification enzymes in the major dengue virus vector *Ae. aegypti* is concerning and cautions against the use of pyrethroids in arbovirus control programmes in Benin. In this part of West Africa, additional surveillance activities are needed to further investigate other co-occurring molecular and metabolic insecticide resistance mechanisms, as well as to assess the susceptibility of these *Ae. aegypti* populations to other pyrethroids (e.g., alpha-cypermethrin, cyfluthrin or lambda-cyhalothrin), with our without synergists (e.g., piperonyl butoxide), or classes of insecticides (e.g., organophosphates, neonicotinoids, pyrroles etc.).

5. Conclusions

This study reports resistance to deltamethrin and permethrin in *Ae. aegypti* populations, collected from the 10ème arrondissement of Cotonou and Godomey-Togoudo in Abomey-Calavi. Molecular and metabolic mechanisms associated with pyrethroid resistance included *kdr* mutations: F1534C, S989P and V1016G, and significant overexpression of certain detoxification enzymes. To our knowledge, this is the first report of V1016G in Africa, and this is the first time all three *kdr* mutations have been detected in *Ae. aegypti* in Benin, suggesting alternative vector tools may be required for arbovirus control in this part of West Africa. Study results highlight the importance of strengthening and scaling-up surveillance activities to respond to the growing global threat of insecticide resistance to the control of vector-borne diseases.

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Informed Consent Statement: Mosquito collections did not directly involve humans, but their living environments and permissions were requested and obtained prior to the placement of the mosquito traps.

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