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AEDES AEGYPTI KNOCKDOWN RESISTANCE MUTATIONS AND DENGUE VIRUS INFECTION IN HAITI

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ABSTRACT. Haiti is home to approximately 11 million people and has a high incidence of vector-borne disease, including more than 70,000 cases of dengue per year. Vector control is difficult in Haiti and adulticide spray of malathion is the main method of control employed during the outbreak of disease although pyrethroids are used in both bed net campaigns and in widely available aerosol cans for personal use. However, limited pathogen or insecticide resistance surveillance data are available for making operational decisions. In this study, we assessed *Aedes aegypti* from serial surveillance collections from 3 locations for the presence of dengue virus serotypes 1-3 (DENV1-3) by polymerase chain reaction and assessed, by melt curve analysis, samples from 10 locations in 2 departments for the presence of two mutations (V1016I and F1534C), that in combination, are linked to strong pyrethroid insecticide resistance. Only one of the 32 tested pools was positive for the presence of dengue virus. The two *knockdown resistance* (*kdr)* mutations were present in all locations. The 1016I mutation frequency varied from 0.29 to 0.91 and was in all sites lower than the 0.58–1.00 frequency of the 1534C mutation. We also observed that the genotype homozygous for both mutations (IICC), which has been linked to strong pyrethroid resistance, varied from 13 to 86% in each population. Notably, 3 locations - Ti Cousin and Christianville in Ouest department and Camp Coq in Nord department had more than 30% of the tested population without the presence of *kdr* mutations. These results indicate that the *kdr* markers of pyrethroid resistance are present in Haiti, at high frequency in several locations and, based on previous studies linking *kdr* genotypes and phenotypic resistance, that operational interventions with pyrethroids are not likely to be as effective as expected.

KEY WORDS *Aedes aegypti*, dengue, Haiti, *kdr*, knockdown resistance

INTRODUCTION

Vector-borne diseases such as dengue fever (DF) are endemic in Haiti with tens of thousands of cases reported annually and transmission rates over 500 cases per 100,000 (Institute for Health Metrics and Evaluation [IHME] 2019). With a population of approximately 11 million people, DF appears to be minimally important to native Haitians as they have acquired immunity to the disease, but to non-immune visitors, transmission of DF and the potentially deadly hemorrhagic fever and dengue shock syndrome are a major problem although the number of cases of these more severe forms are not available. All 4 dengue virus (DENV) serotypes (DENV1-4) have been reported in Haiti (Ventura and Ehrenkranz 1976, Halstead et al. 2001). Factors responsible for the high dengue transmission rates include lack of and/or poor adherence to individual protection measures such as bed nets and a weak government infrastructure for vector control activities at the national and district level. With little to no active mosquito control operations in Haiti, the probability of acquiring dengue is extremely high. Limited operational efforts do occur in direct response to severe disease outbreaks and relies on adulticide sprays, primarily pyrethroids. Much of this effort is focused on malaria eradication and anophelines with little focus on *Aedes* species (McAllister et al. 2012).

Integrated vector management (IVM) has been shown to be the most effective means to control vectors over the long-term (Centers for Disease Control and Prevention [CDC] 2023, World Health Organization [WHO] 2012), but an IVM approach is not possible in Haiti at this time due to governmental and economic insecurity. Control of dengue transmitting mosquitos by source reduction, as an element of an IVM program, is also a proven and effective method in many areas but the logistics of implementation in Haiti is daunting and not feasible given the weak infrastructure in comparison to the ease of adult vector control with insecticides. The findings of an early 2000s study that permethrin-treated bet nets could impact dengue transmission suggests that *Aedes* species were susceptible to permethrin, and therefore adult mosquito vector control by insecticide application might reduce *Aedes* population densities in Haiti (Lenhart et al. 2008). Millions of these treated bed nets have been distributed in Haiti and pyrethroid aerosol spray cans are widely available for use by residents suggesting that significant selective pressure for *Aedes aegypti* (L.) to develop pyrethroid resistance is possible (Steinhardt et al. 2017). However, very little information is available on insecticide resistance (IR) of mosquitoes in Haiti or the presence of IR genotypes that might impact success if vector control was implemented.

Only sporadic surveillance of *Ae. aegypti* has been conducted in Haiti even though there is substantial dengue transmission. Early studies found dichloro-diphenyl-trichloroethane (DDT) resistance in an *Ae. aegypti* population derived from Port-au-Prince, but we could find no other publication of efficacy studies in Haiti (Busvine and Coker 1958). When pyrethroids became available in the 1970s, they were widely adopted in the

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Caribbean and the Americas and reports of resistance began within just a few years (Chadwick et al. 1977, Prasittisuk and Busvine 1977). Phenotypic resistance to pyrethroids is now widely reported in the Americas and has been recently reviewed, though notably, these reviews do not include data from Haiti (Smith et al. 2016, Guedes et al. 2020). The 2010 earthquake response resulted in the first published assessment of pyrethroid IR in 2 *Ae. aegypti* populations from Port-au-Prince, Haiti (McAllister et al. 2012). This study found susceptibility to permethrin and deltamethrin and very low presence of the 1016 valine to isoleucine (V1016I) single nucleotide polymorphism (SNP) that is one of two primary markers of strong pyrethroid resistance in *Ae. aegypti*. Enzymatic activity levels were also higher in these 2 field populations, but this was not observed as actual phenotypic resistance in bottle bioassay testing. We could find no other published IR testing in Haiti or the Dominican Republic apart from a report that examined four populations from Haiti finding pyrethroid resistance and some malathion resistance (Ledoux et al. 2020). Notably, nearby locations like Cuba, Mexico, and Florida have shown frequent and strong IR to pyrethroids and that both the V1016I and a phenylalanine to cysteine SNP (F1534C), jointly known as *knockdown resistance* (*kdr*) mutations, are quite common in *Ae. aegypti* (Flores-Suarez et al. 2016, Estep et al. 2018, Rodríguez et al. 2020). Several studies have thoroughly examined the relationship between these mutations and have found strong associations between ensembles of SNPs and resistance as well as a pattern of sequential coevolution (Vera-Maloof et al. 2015, Saavedra-Rodriguez et al. 2018, Fan et al. 2020, Cosme et al. 2020). Notably, the infiltration and spread of the 1534C mutation and subsequent rapid spread the 1016I was confirmed in a nearly 15-yr retrospective study in Iquitos, Peru and happened within just a few years of operational pyrethroid use where efficacy was lost as the frequency of the 1016I and 1534C SNPs increased (Baltzegar et al. 2021).

In this study, we derived pathogen and IR information from *Ae. aegypti* collected from 13 locations in Haiti during 2017 and 2018. We examined sequential pooled samples from 3 locations for the presence of DENV1-3 collected during 2 months in 2017 and we also assessed populations from 10 locations for the presence of the 1016I and 1534C *kdr* mutations of the voltage gated sodium channel that have been correlated with IR to pyrethroids in field populations (Flores-Suarez et al. 2016, Estep et al. 2018, Mack et al. 2021).

MATERIALS AND METHODS

Dengue virus testing from mosquito pools

Aedes aegypti samples for DENV testing were collected from three locations in the arrondissement of Leogane (Fig. 1, Table 1) during February and March of 2017, using 3 trap types, BG-Sentinel traps (Biogents, Martinsburg, WV) baited with octenol, CDC light traps baited with octenol, or CDC gravid traps baited with synthetic hay infusion to increase the chances of collecting a variety of mosquito species. Traps were run for approximately 24 h for BG-Sentinel and 12 h for the CDC traps. *Aedes aegypti* were pooled (1–20 females per pool) by date and trap type for pathogen analysis and then whole RNA was extracted using a Qiagen QIAamp viral RNA mini kit (Qiagen, Germantown, MD). Two microliters of isolated RNA from each pool was tested for the presence of DENV1-3 using TaqMan 1-Step Fast Virus reagents (Thermo Fisher Scientific, Waltham, MA) and previously published primers (0.9 µM DenS_F: 5'-ggatagaccagagatcctgctgt-3', 0.9 µM DenAs_R: 5'-cattccattttctggcgttc-3', 0.25 µM Plus: 5'-cagcatcattccaggcacag-3') in 20 µl reactions (Drosten et al. 2002, Liu et al. 2016). Primers amplify DENV1-3 and are not serotype specific. Negative controls of nuclease free water and positive controls containing diluted purified DENV3 RNA were included in the appropriate assay. Purified DENV RNA was obtained through BEI Resources (BEI Resources, NIAID, NIH: Dengue Virus Nucleic Acid Panel, NR-32847, Manassas, VA). Thermocycling conditions were: 5 min at 50°C, 30 sec at 95°C, then 40 cycles of 3 sec at 95°C and 30 sec at 60°C on a QuantStudio 6 Flex real-time polymerase chain reaction (RT-PCR) System (Thermo Fisher Scientific, Waltham, MA). Exponential amplification of a target or the positive control with a cycle threshold (Ct) of less than 35 was considered positive.

Knockdown resistance genotyping

Samples for assessment of the V1016I SNP and the F1534C SNP were collected from 10 locations—7 in the arrondissements of Leogane and Gressier in the Ouest department and 3 in the Limbe arrondissement in the Nord department collected during January to June of 2018 (Fig. 1 Inset A and B). A minimum of 26 individual *Ae. aegypti* from each location were homogenized in 200 µl of deionized water and then tested using previously described methods and primers in a melt curve assay (Saavedra-Rodriguez et al. 2007; Yanola et al. 2011; Estep et al. 2018, 2023). Assays were assembled in 384-well plates on an epMotion 5750 workstation (Eppendorf, Hamburg, Germany) in 10 µl volumes. Reactions were subjected to standard "FAST" cycling of 40 cycles on the QuantStudio 6 Flex (Thermo Fisher Scientific, Waltham, MA) with a final melt curve ramp (continuous data acquisition over 60-95°C) to assess melting temperature. Controls were included on each plate and consisted of a nuclease free water negative control, the ORL1952 strain (no *kdr* mutations, genotype VVFF), the dilocus *kdr* mutant Puerto Rico strain (genotype IICC) and an artificial heterozygote created by homogenizing a single ORL1952 and single PR together (genotype VIFC) (Estep et al. 2017). Allele presence and zygosity for each organism were determined based on the characteristic melting temperature (T_m) peaks of the melt curves resulting from the controls as previously described (Estep et al. 2018). The susceptible valine allele (1016V) was indicated by a T_m of 85.9 ± 0.4C and the resistant isoleucine allele (1016I) by a T_m of 77.3 ± 0.4C. For position 1534, the

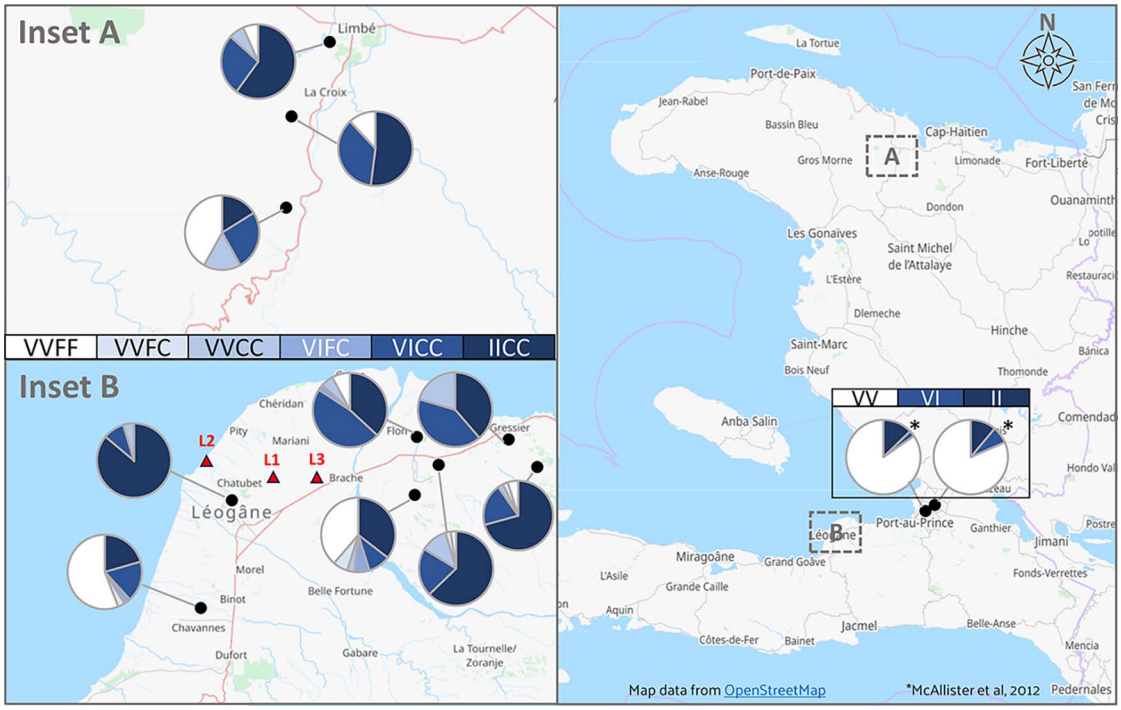

Fig. 1. Sampling locations for dengue testing (red triangles) and knockdown resistance mutation genotyping (black circles). Pie charts for each location represent the various *kdr* genotypes as noted in the legend bar.

SNP for phenylalanine (1534F) was indicated by a T_m of 79.5 ± 0.4C and the resistant cysteine (1534C) by a T_m of 84.2 ± 0.4C. Wells that failed to amplify or that gave indeterminate results were excluded. A minimum of 25 results for each location were used for analysis (Table 2). Genotype percentages were calculated by (shown for IICC genotype),

$$IICC\% = \frac{(N(IICC))*100}{N(total\ tested)}$$

and allele frequencies were calculated using the following equation (shown for the 1016I allele),

$$f(1016I) = \frac{(2*N(IICC)) + (1*N(VIFC + VICC))}{(2*N(total\ tested))}.$$

RESULTS

Pathogen testing controls produced the expected results with the spiked DENV3 positive control sample resulting in a C_t of ~33 and the negative controls failing to amplify. Of the field collected samples, only 1 pool from Sigueneau, collected from a CDC gravid trap, was positive for the presence of DENV1-3 with a Ct of ~29 (Table 1). This overall sample positivity rate was approximately 3%. We did not further assess which specific DENV serotype was present.

Results of the *kdr* genotyping assay showed both the 1016I and 1534C SNPs were present in all 10 locations, but the frequencies of the two mutations were variable (Fig. 1, Table 2). The frequency of the 1534C SNP ranged from 0.41-1.00. In Gressier town and Leogane town, 1534C was at fixation and the 1534F allele was not detected. In Merger, Lacolline, Ravine des Roches, and La Salle, the frequency of the 1534C mutation was not fixed, but was greater than 0.90. The Camp Coq and Christianville sites had frequencies of 1534C below 0.60. The lowest 1534C frequency was 0.41 in Ti Cousin.

The frequency of the 1016I mutation was also widely variable ranging from 0.29 to 0.91. In contrast to the 1534C frequency, the towns of Leogane and Gressier had very different frequencies of the 1016I mutation at 0.91 and 0.59 respectively. The 4 locations with 1534C frequencies above 0.90 also had relatively high 1016I frequencies (greater than 0.61) (Table 2). We observed that in all 10 locations, the frequency of the 1534C mutation exceeded the frequency of the 1016I mutation by 0.09–0.41.

Considering these two loci together as a genotype, the dilocus mutant homozygote (IICC) was found in all 10 *Ae. aegypti* populations but varied from 86% of the Leogane mosquito population to only 16% of the Camp Coq mosquito population. The percentage of the VICC and VIFC genotypes, each with one copy of the recessive IC allele, accounted for 9–50% of each mosquito population. The Christianville School, Ti

Table 1. Sample data and dengue testing results for serial samples from three locations in Haiti.

GPS	Site	Date	Trap type	Result
18.522, –72.649	Ca Ira	2/2/2017	CDC Light	–
18.522, –72.649	Ca Ira	2/7/2017	BG Sentinel	–
18.519, –72.599	Sigueneau	2/7/2017	CDC Gravid	–
18.519, –72.599	Sigueneau	2/8/2017	BG Sentinel	–
18.519, –72.599	Sigueneau	2/8/2017	CDC Gravid	–
18.518, –72.632	Belval	2/13/2017	CDC Light	–
18.518, –72.632	Belval	2/13/2017	CDC Gravid	–
18.519, –72.599	Sigueneau	2/13/2017	CDC Gravid	–
18.519, –72.599	Sigueneau	2/13/2017	CDC Light	–
18.518, –72.632	Belval	2/14/2017	BG Sentinel	–
18.522, –72.649	Ca Ira	2/15/2017	BG Sentinel	–
18.518, –72.632	Belval	2/20/2017	CDC Gravid	–
18.522, –72.649	Ca Ira	2/20/2017	CDC Gravid	–
18.522, –72.649	Ca Ira	2/20/2017	BG Sentinel	–
18.519, –72.599	Sigueneau	2/20/2017	BG Sentinel	–
18.519, –72.599	Sigueneau	2/20/2017	CDC Light	–
18.519, –72.599	Sigueneau	2/20/2017	CDC Gravid	–
18.522, –72.649	Ca Ira	2/21/2017	CDC Gravid	–
18.519, –72.599	Sigueneau	2/21/2017	CDC Gravid	+ (C_t=28.9)
18.518, –72.632	Belval	2/22/2017	CDC Gravid	–
18.518, –72.632	Belval	2/22/2017	CDC Light	–
18.519, –72.599	Sigueneau	2/22/2017	CDC Gravid	–
18.519, –72.599	Sigueneau	3/6/2017	CDC Gravid	–
18.522, –72.649	Ca Ira	3/7/2017	BG Sentinel	–
18.522, –72.649	Ca Ira	3/7/2017	CDC Gravid	–
18.522, –72.649	Ca Ira	3/8/2017	BG Sentinel	–
18.519, –72.599	Sigueneau	3/8/2017	CDC Gravid	–
18.519, –72.599	Sigueneau	3/8/2017	BG Sentinel	–
18.522, –72.649	Ca Ira	3/13/2017	BG Sentinel	–
18.518, –72.632	Belval	3/14/2017	CDC Gravid	–
18.518, –72.632	Belval	3/14/2017	BG Sentinel	–
18.519, –72.599	Sigueneau	3/21/2017	BG Sentinel	–
	DENV3 positive control			+ (C_t=33.9)
	Nuclease free water			–

Cousin, and Camp Coq populations had greater than 38% of sampled *Ae. aegypti* without *kdr* mutations at 1534 or 1016.

DISCUSSION

This study, the largest to date of Haitian *Ae. aegypti*, gives a picture of an IR situation much different than that found after the earthquake in 2010. McAllister (2012) showed very few *kdr* mutations in the two locations examined. Seven years later we observed the common dilocus *kdr* mutation (IICC) in every location we sampled and found it represented more than 70% of the Leogane and Merger populations. If the 10 locations surveyed in the two departments examined in this study were representative of the overall situation in Haiti in 2018, the *kdr* mutations have spread rapidly in less than a decade from the survey of McAllister (2012). This rapid increase in resistance allele frequencies is in line with previous observations about the spread of *kdr* mutations in the Americas (García et al. 2009, Baltzegar et al. 2021). Iquitos, Peru, and several locations in Mexico have both seen rapid increases in the frequency of *kdr* mutations and, specifically, the increase in the portion of the population that has the very resistant IICC genotype (García et al. 2009, Baltzegar et al. 2021).

This dataset also supports a few other findings about *kdr* mutations that have been observed in other studies. As in this study, the 1534C and 1016I often occur as an ensemble and while the 1016V susceptible allele can occur with the 1534C, the reverse, where 1016I occurs with 1534F, is extremely uncommon in field populations (Estep et al. 2018, Baltzegar et al. 2021, Mack et al. 2021). We also observed fixation or near fixation of the 1534C allele in several locations in Haiti, just as seen in Florida, California, Mexico, and Brazil (García et al. 2009, Estep et al. 2018, 2023; Melo Costa et al. 2020; Mack et al. 2021). We also observed variability in *kdr* genotype frequency between locations here separated by kilometers and note that local variability in IR, possibly due to the limited dispersal of *Ae. aegypti*, has been observed to occur at finer scale within neighborhoods or even blocks within neighborhoods. (Estep et al. 2018, 2023; Mundis et al. 2020; Baltzegar et al. 2021).

This increase in the IICC genotype and fixation of the 1534C allele can be due to continual pressure

Table 2. Frequency of the 1016 isoleucine and 1534 cysteine mutations in populations from 10 locations in Haiti.

GPS	Location	Genotype percentage						Number tested	*f*(1534C)	*f*(1016I)
		IICC	VICC	VIFC	VVCC	VVFC	VVFF			
18.511, –72.634	Leogane	86	9.3	0	4.7	0	0	43	1.00	0.91
18.536, –72.518	Merger	70.7	19.5	0	2.4	2.4	4.9	41	0.94	0.80
18.541, –72.551	Lacolline	62.8	20.9	0	11.6	2.3	2.3	43	0.96	0.73
19.700, –72.427	Ravine des Roches	60	26.7	0	6.7	0	6.7	30	0.93	0.73
19.627, –72.429	Chobotte	52	36	0	0	0	12	25	0.88	0.70
18.542, –72.522	Gressier	38.6	40.9	0	20.5	0	0	44	1.00	0.59
18.541, –72.551	La Salle	36.8	47.4	2.6	5.3	0	7.9	38	0.91	0.62
18.524, –72.556	Christianville	35.5	9.7	6.5	3.2	6.5	38.7	31	0.55	0.44
18.470, –72.646	Ti Cousin	20.6	17.6	2.9	0	2.9	55.9	34	0.41	0.31
19.639, –72.424	Camp Coq	16.1	25.8	0	16.1	0	41.9	31	0.58	0.29

from vector control operations but evidence exists that it is not always the case (Mundis et al. 2020, Baltzegar et al. 2021). In Haiti, where vector control operations with pyrethroids are rare, pressure driving high levels could be due to private, household use of pyrethroids or establishment of populations by founders with high levels of *kdr* (Brennan et al. 2021).

Notably, the 2010 assessment found low levels of phenotypic resistance along with infrequent *kdr* mutations. By 2018, when the samples in this current study were collected, the level of the 1016 and 1534 mutations had increased substantially and, based on research published in the last 10 years, are indicative of increasingly high levels of pyrethroid resistance. With the caveat that using *kdr* genotype percentages have not been exhaustively demonstrated to quantify levels of pyrethroid IR, it is clear from both laboratory and field studies that a strong correlation exists. Generally, as the frequency of the IICC genotype rises in a Western hemisphere *Ae. aegypti* population, the level of resistance to pyrethroids increases, whether measured by topical application or by time to death against a specific dose (Brito et al. 2018; Estep et al. 2018, 20023; Mack et al. 2021; Scott et al. 2021). Applying this to our study, several of these Haitian *Ae. aegypti* populations likely have high resistance to pyrethroids. The 86% IICC Leogane mosquito population has higher IICC percentage than a Houston, TX population (MCA53) that was about 60% IICC and 39% VICC with a permethrin resistance ratio (RR) of about 35 and somewhat less than the Miami Beach strain (91% IICC, permethrin RR $\sim$ 55) or the Puerto Rico strain (>97% IICC, permethrin RR $>$ 60) (Estep et al. 2017, 2018, 2023). Based on these previously published studies, the Leogane mosquito population likely has a permethrin RR somewhere in the 40-60-fold range (Estep et al. 2017, 2018, 2023). Similarly, populations from Merger, Lacolline, Ravine des Roches, and Chobott had IICC percentages above 50% and are likely in the range of 25-35-fold permethrin resistance based on similar populations with similar levels of the IICC genotype (Estep et al. 2018, 2023). The mosquito populations with 16–40% IICC probably range from 11- to 20-fold resistance when compared to previously assessed field strains.

The primary response to outbreak of *Aedes* transmitted disease in Haiti is likely to be the adulticide spray of malathion rather than pyrethroids, but unfortunately, malathion IR is not rapidly assessable with a simple genetic assay for *Ae. aegypti* as it is for some species (Weill et al. 2004). While biochemical assays could possibly inform the potential for OP resistance, conclusive linkages between increased enzymatic activity or patterns of increased activity and phenotypic IR are still unclear as elevated levels are regularly found in strains without much IR (McAllister et al. 2012, Vontas et al. 2020). As such, the best way to measure OP resistance currently is by phenotypic bioassay, which these samples did not permit as they arrived as RNA (for DENV testing) or as non-viable specimens for genetic testing.

Unlike OP IR, assessing pyrethroid IR is not hampered by a lack of good markers. Thus the presence of a high percentage of the markers of pyrethroid resistance present in the samples we tested indicates that personal interventions like treated bed nets or aerosol spray cans of pyrethroids are not maximally effective. Resistant *Ae. aegypti* are willing to feed through permethrin treated fabrics so a treated bed net or room spray is likely to be compromised (Agramonte et al. 2017, Estep et al. 2020).

This study expands previous knowledge of pyrethroid IR in Haiti, but it is still very limited, including producing no information about IR to malathion, the primary adulticide intervention in use. Much more needs to be done. Many areas of the country, outside of the Departments tested here, have yet to be surveyed so the overall picture of IR is unclear, and this limits the usefulness of this information for helping other parts of Haiti make decisions about pesticide use. Developing a broader picture of IR in Haiti is likely to continue to be difficult, but it is a critical need for a country that faces constant problems from dengue with more than 83,000 cases in 2019 (IHME 2019). It is also critical to develop a better understanding of the operational efficacy of formulated pyrethroids and other interventions, like organophosphate sprays or novel formulations against these *Ae. aegypti*

so that an effective public health response can be implemented to limit disease transmission.

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