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Randomized detection of *kdr* allele frequencies in wild populations of *Aedes aegypti* (Diptera, Culicidae) in Colombo District, Sri Lanka

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ABSTRACT: Sri Lanka is one of the most affected countries in South Asia by dengue fever, with the number of dengue cases increasing over the last five years. The main strategy for managing disease outbreaks is to reduce infected vector populations with pyrethroid insecticides. However, extensive pyrethroid exposure has resulted in an increase in the selection of knockdown resistance mutations in *Aedes aegypti* (Linnaeus) (Diptera, Culicidae) voltage-gated sodium channel (*vgsc*) gene that confer pyrethroid resistance. Colombo district records the highest dengue incidence across the country each year, thus a failed vector control program will be a major threat to public health. Multiplexed Allele-specific PCR was used to genotype *kdr* alleles in wild *Ae. aegypti* mosquitoes obtained via random sampling from Wellawatte, Borella, and Battaramulla areas in the Colombo district. This study presents the co-occurrence of F1534C and V1016G *kdr* mutations from a randomized population in the Colombo district. 1534C mutant allele was predominant (with a 56.7% frequency) and 1016G was prevalent in 32.5 per cent of the population. The heterozygous mutant 1016VG genotype showed the highest distribution (with a 65% frequency) and the incidence of 1534FC was 56.7 per cent. Interestingly, 1016GG was completely absent and the FC/VG mutation combination had the highest incidence with 46.7 per cent. Furthermore, 82.36 per cent of individuals with the 1534FC genotype also had the 1016VG genotype, indicating a high prevalence of pyrethroid resistance in the studied population.

KEYWORDS: Pyrethroid, mutations, knockdown resistance, alleles, genotype

INTRODUCTION

Dengue, a prevalent arboviral disease in Sri Lanka, has reported approximately 65,000 cases annually over the past decade (Epidemiology Unit, 2022). Notably, the Colombo district, an urbanized area with unplanned human constructions, consistently reports the highest number of dengue cases in the island (Malavige *et al.,* 2021). The main vector responsible for transmitting the dengue virus

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(DENV) is *Aedes aegypti* (Linnaeus) (Diptera, Culicidae) (WHO, 2009). In the absence of an effective drug or vaccine for DENV infection, vector control is the main strategy in controlling the infections (WHO, 2009). Sri Lanka primarily employs space spraying using pyrethroids as the main vector control strategy (Karunaratne *et al*., 2013). Pyrethroids can bind to voltage-gated sodium channels (*vgsc)* in the nervous system of *Ae. aegypti*, thus hindering the maintenance of voltage difference across the membrane. Consequently, the mosquito will be imperiled to rapid paralysis and sudden death. This phenomenon is often referred to as "Knockdown" (Dong, 2014). However, the emergence of resistance to pyrethroids in *Aedes* species has been documented in various regions globally, including Asia, the Americas, the Middle East, and Africa (Moyes *et al*., 2017; Mashlawi *et al*., 2022). Thus, the efficacy of this approach has been compromised due to the widespread occurrence of knockdown resistance (*kdr*) mutations in *Ae. aegypti* populations (Fernando *et al*., 2018, 2020). Notably, a total of 15-point mutations occurring at the *vgsc* have been associated with *kdr* globally, with five confirmed to confer pyrethroid resistance, namely S989P, I1011M, V1016G/I, and F1534C, along with the recently discovered V410L (Du *et al*., 2016; Haddi *et al*., 2017).

Among these mutations, F1534C and V1016G have shown extensive distribution in various populations worldwide, with a notable occurrence in Asian *Ae. aegypti* populations (Linss *et al*., 2014; Vera-Maloof *et al*., 2015; Al Nazawi *et al*., 2017; Brito *et al*., 2018; Fernando *et al*., 2018; Ranathunge *et al*., 2021). Furthermore, evidence suggests that their co-occurrence confers a higher level of pyrethroid resistance than when they occur singularly (Du *et al*., 2013, 2016; Linss *et al*., 2014; Vera-Maloof *et al*., 2015; Saavedra-Rodriguez *et al*., 2018). To monitor the efficiency of current vector control strategies, regular assessments of pyrethroid resistance in *Ae. aegypti* populations are essential (Kushwah *et al*., 2020; Wuliandari *et al*., 2020). Genotyping *kdr* alleles, especially those confirmed to confer pyrethroid resistance, is a valuable tool in predicting the efficacy of pyrethroids in the field (Du *et al*., 2013, 2016; Linss *et al*., 2014; Vera-Maloof *et al*., 2015; Saavedra-Rodriguez *et al*., 2018). However, most studies have focused on genotyping pyrethroid-resistant individuals, emphasizing the need for randomized sampling from wild-caught adult populations to provide an accurate representation of *kdr* mutation distribution in a specific area (Du *et al*., 2016; Linss *et al*., 2014). The present study documents the co-occurrence of F1534C and V1016G *kdr* mutations, their distribution, and mutation associations in randomized populations from the Colombo district, Sri Lanka.

MATERIALS AND METHODS

Mosquito sampling and rearing: Wellawatte (6.8741°N; 79.8605°E), Borella (6.9122°N; 79.8829°E), and Battaramulla (6.897994°N; 79.922287°E), localities in the Colombo district were selected for sample collection as they were among the areas with the highest reported dengue cases on the island during 2021 (Epidemiology Unit, 2022). Also, these sites are routinely sprayed with permethrin and deltamethrin (National Dengue Control Unit Sri Lanka, 2016). Preimaginal stages (eggs, larvae, and pupae) (F_0) of *Ae. aegypti* mosquitoes were collected from January to April 2022, by placing around 20-30 ovitraps in 20-25 randomly selected neighboring houses from each locality for 5-7 days. Each house was accommodated with a maximum of two ovitraps at a distance between 5 to 10m apart from each other based on the structural design of the house. Ovitraps were set up considering favored breeding places of *Ae. aegypti*, such as dark, shady places with more human presence and less exposure to direct sunlight (Brown *et al.,* 2011; Rakotoarivony and Schaffner, 2012). The collected samples were transported to the insectary at the Centre for Biotechnology, University of Sri Jayewardenepura. The eggs collected from all the localities were hatched in separate containers to avoid any contamination of samples. Subsequently, the emerging larvae were fed with high-protein fish feed, and once emerged; the adults were supplied with a 10 per cent sucrose solution. All larvae and adults (F_{-0}) were maintained at 28 ± 2^0C with a relative humidity of 75 ± 10 per cent. Female *Ae. aegypti* were morphologically

identified to the species level based on the thorax patterns (WHO, 2020). Identified female *Ae. aegypti* adults were killed by deep freezing, and DNA was extracted from single mosquitoes by modified phenol-chloroform DNA extraction protocol (Ballinger-Crabtree *et al.,* 1992).

Genotyping of F1534C and V1016G *kdr* mutations using Multiplex Allele-specific PCR: A total of 60 samples from three collection sites were screened for *kdr* mutations at 1016 and 1534 mutation sites following a multiplex allele-specific (MAS) PCR protocol developed by Saingamsook *et al.* (2017). The PCR primer pair used, the region amplified in the *vgsc* gene, and the product sizes are shown in Table 1. Each PCR reaction was performed in a 25µl volume containing: 5ng of DNA sample, 2µM of $MgCl_2$ (Promega®),7.7µl of *Taq* Ready mix (2X) (FastGene®), and primers (Sigma-Aldrich Solutions®): Gly1016f (1.25µM), Val1016r (0.625µM), Gly1016r (1.25µM), c1534-f (0.625µM), c1534-r (0.625µM), Ae1534F-r (0.25µM) and Ae1534C-f (1.25µM). The amplification consisted of 92 °C for a 2 min heat activation step, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s with a 2 min final extension step at 72 °C. PCR products were loaded onto 3 per cent (TBE) agarose gel and electrophoresis was conducted for 50min at 100V with a 50 bp DNA ladder (Promega USA). The scoring of *kdr* alleles was done according to Fig.1 and table 2.

Statistical Analysis: Allele frequencies, genotype frequencies, and Hardy-Weinberg equilibrium of F1534C and V1016G loci were calculated using the HW-test software (Santos *et al.,* 2020). Hardy–Weinberg equilibrium (HWE) of *kdr*-alleles in a population was tested using Fisher's exact test. Wright's inbreeding coefficient was calculated using the formula F = (He - Ho/He), where 'He' is expected heterozygosity and 'Ho' is observed heterozygosity. Pairwise linkage disequilibrium coefficients and associated chi-squared tests between F1534C and V1016G loci were calculated using LINKDOS (Garnier-Gere and Dillmann, 1992) following previously described guidelines (Saavedra-Rodriguez *et al.,* 2018; Vera-Maloof *et al.,* 2015). Genotype data from adjacent sites Wellawatte and Borella were pooled to increase the sample size for detecting linkage disequilibrium. The prevalence of all six genotype combinations was calculated and graphed by Microsoft Excel (2018). The proportion of 1016VG genotype distribution in individuals who are heterozygous mutants for F1534C mutation was calculated and visualized by R Studio Team (2022).

RESULTS

MAS PCR genotyping of *kdr* mutations of *Ae. aegypti* populations:

Genotyping results of *kdr* alleles at loci 1534 and 1016 carried out on 60 field-collected F_0 populations from all collection sites, indicated that F1534C was the most widespread *kdr* point mutation with 56.7 per cent of the individuals having the heterozygous mutant genotype 1534FC and 28.3 per cent of them being homozygous mutant 1534CC while only 15 per cent were homozygous wild type 1534FF. Overall, the V1016G mutation was less common compared to F1534C. Among the individuals 65 per cent had the heterozygous mutant genotype 1016VG, while only 35 per cent of them had homozygous wild type 1016VV and surprisingly, homozygous mutant genotype 1016GG was absent in all collection sites (Table 3).

Mutation combinations:

Six out of nine possible genotype combinations were observed in the 60 samples from all three locations. Frequency of each of the nine bi-locus combinations is depicted in Fig. 2. Only 10 per cent of the population had wild-type alleles at both 1534 and 1016 sites. Double mutant combinations were found only as FC/VG and CC/VG. The most common bi-locus genotype combination was heterozygous double mutant FC/VG with a percentage of 46.7 per cent (28 out of 60 individuals). Homozygous mutant 1534CC with homozygous wild type 1016VV (CC/VV) was the second most common combination (15%); and 13.3 per cent of the individuals had a double mutant CC/VG combination. Both FF/VV and FC/VV were present in 10 per cent while 5 per cent of the population had FF/VG combinations. No individual

Table 1. Sequences of Primers used in this study (Saingamsook *et al.,* 2017)

Primer	Primer sequence (5'-3')	Product size	[a]Exon
1016 genotyping			
Gly1016f	ACCGACAAATTGTTTCCC		15-16[b]
Val1016r	[short GC tail][c]AGCAAGGCTAAGAAAAGGTTAATTA	60	16
Gly1016r	[long GC tail][d]AGCAAGGCTAAGAAAAGGTTAACTC	80	16
1534 genotyping			
c1534-f	GCGTACCTGTGTCTGTTCCA	368	23
c1534-r	GGCTTCTTCGAGCCCATCTT		24
Ae1534F-r	GCGTGAAGAACGACCCGA	232	24
Ae1534C-f	CCTCTACTTTGTGTTCTTCATCATCTG	180	24

[a]Exon from the *Ae. aegypti* VGSC gene. This transcript corresponds to VectorBase Transcript ID AAEL006019
[b]Intron between exon 15 and 16
[C]Short GC tail sequence: 5^2 -GCG GGC-3'
[d]Long GC tail sequence: 5^2 -GCG GGC AGG GCG GCG GGG GCG GGG CC-3^2

was found to be a homozygous mutant for both F1534C and V1016G mutations. Homozygous 1534CC was only found in conjunction with homozygous wild type 1016VV and heterozygous mutant 1016VG at a combined frequency of 28.33 per cent (17 out of 60 individuals). No individual had a homozygous mutant 1016GG genotype from any collection site. Heterozygous mutant 1534FC was found only in combination with heterozygous mutant 1016VG and homozygous wild type 1016VV at a combined frequency of 56.7 per cent. In contrast, the heterozygous mutant 1016VG genotype was found in combination with all three genotypes of the 1534 mutation including 1534FF, 1534FC, and 1534CC at a combined frequency of 65 per cent from all collection sites. Also, VG was the most expressed genotype with a 65 per cent frequency.

Three patterns of mutational associations were identified: i). Nearly all (82.36%) of heterozygous mutant 1534FC individuals were heterozygous mutant for 1016VG loci, while the remaining (17.64%) were homozygous wild type for 1016VV; ii). Almost half of the individuals with (47.06%) homozygous mutant 1534CC had heterozygous mutant 1016VG genotype whereas the rest (52.94%) of the 1534CC population were having homozygous wild type 1016VV; and iii). None of the individuals had homozygous mutant 1016GG genotypes in all three collection sites.

Table 2. Criteria for the scoring of multiplex PCR amplified 1534 and 1016 alleles using agarose gel electrophoresis

Genotype	PCR bands present and their size (bp)
1534	
FF	Only one band at 232bp
FC	Two bands at 232 and 180bp
CC	only one band at 180bp
1016	
VV	Only one band at 60bp
VG	Two bands at 60 and 80 bp
GG	Only one band at 80bp

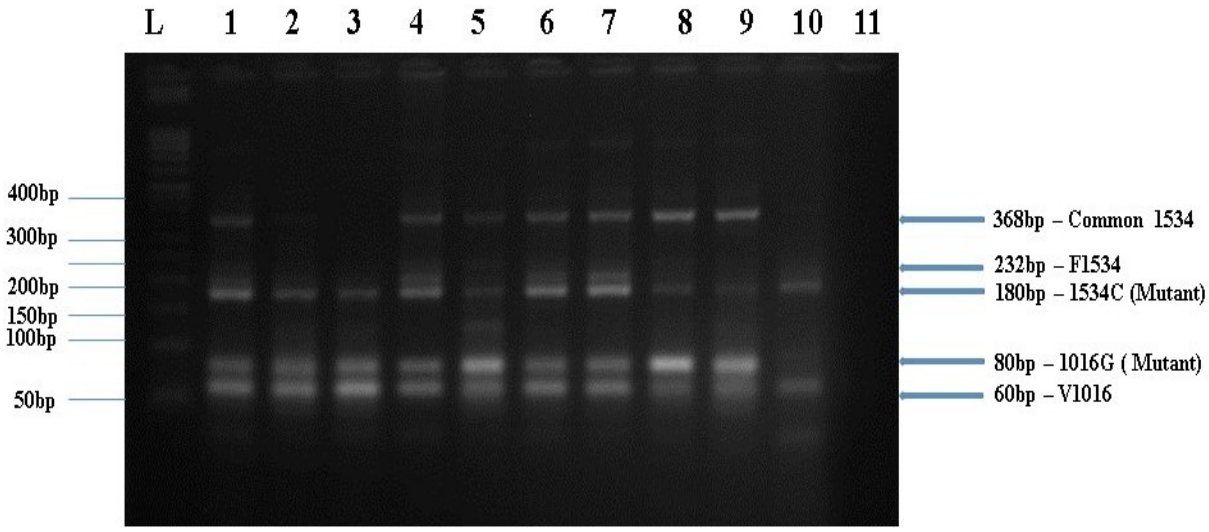

Fig. 1 Gel electrophoresis results. Heterozygous mutant genotypes for both F1534C and V1016G *kdr* mutations are shown in the 1st,4th,5th, 6th and 7th lanes. The 2nd,3rd,8th and 9th lanes show a genotype that is heterozygous mutant for the 1016 allele but homozygous mutant for the 1534 allele. The 10th lane contains homozygous mutant for 1534 allele and homozygous wildtype for 1016 allele. Lane L is the low molecular weight DNA ladder. The last lane (11) contains the negative control in which PCR water was used as the template in the multiplex PCR reaction

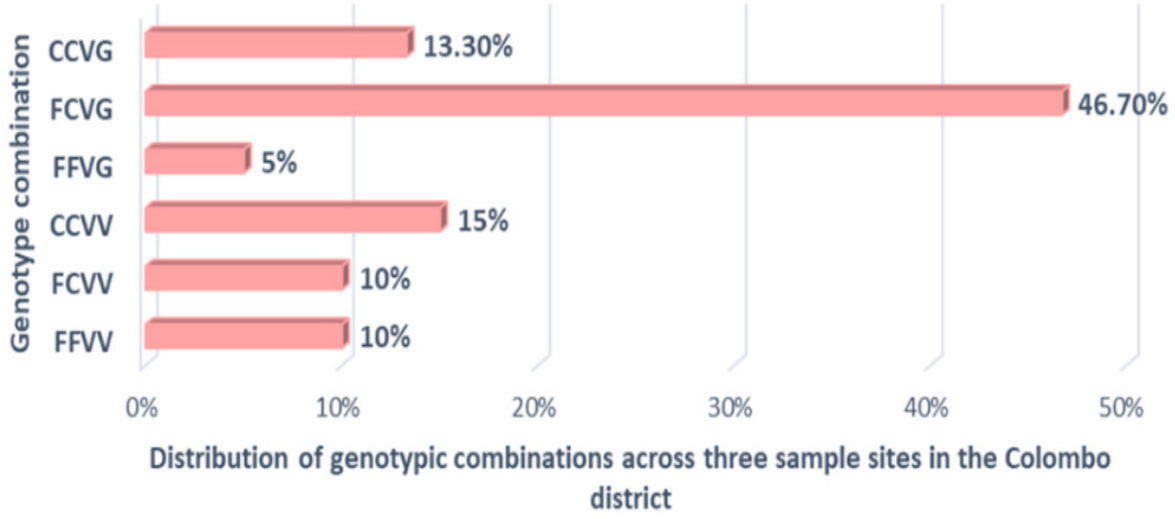

Fig. 2 Distribution of genotypic combinations across three sample sites in the Colombo district

Inbreeding coefficient and linkage disequilibrium:

Except for Battaramulla samples, the other two sites were in Hardy-Weinberg equilibrium for 1534 loci but only Wellawatte samples were in Hardy-Weinberg equilibrium for 1016 loci. When combined across sites, only the F1534C mutation was in Hardy-Weinberg equilibrium (P= 0.297) while the V1016G mutation showed a significant deviation from the equilibrium ($P< 10^{-4}$). Analysis of the Wright's inbreeding coefficient for F1534C and

V1016G *kdr* alleles revealed a negative value indicating the presence of higher heterozygotes than expected (*F*= -0.1547 and *F*= -0.4814 respectively). The results from Pairwise linkage disequilibrium analysis revealed that the linkage disequilibrium between F1534C and V1016G mutations is not consistently significant in the studied population (Table 3).

DISCUSSION

Identification of *kdr* mutations in natural populations of *Ae. aegypti* in the Colombo district can be useful in predicting pyrethroid resistance because it is routinely undergoing pyrethroid-adulticides spraying and also reporting the highest number of dengue cases every year. F1534C *kdr* mutation was first detected during 2016 (Fernando *et al.,* 2017) while V1016G was first reported in 2018 (Fernando *et al.,* 2018) in Sri Lankan *Ae. aegypti* populations. Following that F1534C and V1016G have been identified in pyrethroid-resistant *Ae. aegypti* as well as wild populations from numerous studies conducted in several areas across the island (Fernando *et al.,* 2017, 2018, 2020; Hegoda, 2017; Ranathunge *et al.,* 2019, 2021).

The present study documents the presence of two of the most prevalent *kdr* mutations F1534C and V1016G which have been established to confer pyrethroid resistance in the *vgsc* of *Ae. aegypti* (Du, 2013; Hirata *et al.,* 2014; Vera-Maloof *et al.,* 2015). In the current study, heterozygous mutant genotypes 1534FC and 1016VG predominated in the population with 56.6 and 65 per cent respectively, whereas only 1534CC was present as a homozygous mutant genotype with 28.33 per cent while 1016GG was absent in the population. These results are in line with a previous study finding where the 1534FC genotype was dominating the Colombo district samples with 43.7 and 56.8 per cent in 2018 and 2019 respectively (Ranathunge *et al.,* 2021). However, wild-type 1016VV was significantly higher in Colombo district populations with 79.1 and 74.5 per cent in 2018 and 2019 correspondingly (Ranathunge *et al.,* 2021).

Mutant allele 1534C frequency was significantly higher (56.7%), while in 1016G mutant allele it was lower (32.5%). Nonetheless, the VG genotype was present in 65 per cent of the population suggesting the presence of higher heterozygotes than expected with a negative value achieved for Wright's inbreeding coefficient (F= -0.4814). This is the highest reported VG percentage so far by random sampling from a wild population of *Ae. aegypti* in Sri Lanka. Double heterozygous genotype combination, FC/VG was found to have the highest prevalence across the sample sites (46.7%). Furthermore, the frequency of an individual with FC having VG genotype was always significantly higher for all the sites; while for Battaramulla sample, it was 100 per cent. Taken together, these results suggest strong evidence of an increased *kdr* incidence considering random sampling from a wild population and the relatively small sample sizes being examined.

Table 3. Result of *kdr* genotyping of *Ae. aegypti* in field population (F_0): distribution of allelic frequencies and their compliance to Hardy–Weinberg equilibrium and distribution of mutation combinations

Collection Site	F1534C allele frequencies				V1016G allele frequencies				PHWE test		WiC	
	FF (WT)	FC (MT)	CC (MT)	C allelle	VV (WT)	VG (MT)	GG (MT)	G allele	F1534 C	V1016 G	F1534 C	V1016 G
Wellawatte (n=20)	0.4	0.45	0.15	0.375	0.6	0.4	0.0	0.2	1.000	0.538		
Borella (n=20)	0.05	0.5	0.45	0.7	0.3	0.7	0.0	0.35	0.613	0.045		
Battaramulla (n=20)	0.0	0.75	0.25	0.625	0.15	0.85	0.0	0.425	0.015	0.001		
Total(60)	0.15	0.567	0.283	0.567	0.35	0.65	0.0	0.325	0.297	<10-4	-0.1547	-0.4814

WT = Wild type; MT = Mutant type; PHWE = P value of Fisher's exact test; WiC = Wright's in breeding coefficient

The high frequency of a mutant allele for F1534C mutation is similar to the findings from previous studies however with different sample sizes from the Colombo district (Fernando *et al.,* 2020; Ranathunge *et al.,* 2021) as well as from the Gampaha district (Fernando *et al.,* 2018) and Galle (Fernando *et al.,* 2020). Similarly, a significantly high frequency for 1534C was reported in a bioassay study with 63.9 per cent in permethrin and 57.5 per cent in deltamethrin-resistant samples from the Colombo district (Fernando *et al.,* 2018). Also, several studies established that F1534C can alone elicit resistance towards permethrin (Du *et al.,* 2013; Hirata *et al.,* 2014). Meanwhile, a high level of pyrethroid has been confirmed when V1016G is present with F1534C mutation in combination (Kushwah *et al.,* 2020; Plernsub *et al.,* 2016). This scenario has also been confirmed in Sri Lankan populations (Fernando *et al.,* 2020; Hegoda, 2017).

However, all the previous local studies had a lower 1016G allele frequency in any district than the results of this study (Fernando *et al.,* 2018, 2020; Ranathunge *et al.,* 2021). Similarly, the homozygous mutant 1016GG genotype was not detected from any site in this study. This lower frequency of the 1016G allele is likely due to more exposure to type I pyrethroids (permethrin) compared to type II pyrethroids (deltamethrin) in these collection sites, and/or the V1016G mutation has not yet been established in the population and is still evolving. However, this account must be approached with some caution due to the smaller sample sizes tested here and for the previous studies, not all the analyses were done in wild populations and possible inbreeding might have occurred.

When considering the global distribution of *kdr* mutations, the 1534C mutant allele frequency was particularly high in India (Kushwah *et al.,* 2020; Saha *et al.,* 2019), Taiwan (Biduda *et al.,* 2019), and Malaysian (Akhir *et al.,* 2022; Ishak *et al.,* 2015) *Ae. aegypti* populations. However, studies in Myanmar (Kawada *et al.,* 2014; Naw *et al.,* 2020), Africa (Ayres *et al.,* 2020), and Indonesia (Wuliandari *et al.,* 2020) demonstrated a high prevalence of V1016G mutation. Findings of a 12-year linkage disequilibrium analysis in the Mexican population suggested the 1534C mutation is the one that was selected first with low fitness cost thus facilitating a combination of *kdr* mutation to establish and confer a high level of pyrethroid resistance over time (Du *et al.,* 2016; Vera-Maloof *et al.,* 2015). Similarly, F1534C happened to be the first *kdr* mutation reported in the Sri Lankan population, and only after that, other *kdr* mutations including S989P and V1016G were reported. Interestingly 71.79% of VG individuals possessed FC genotype in this study and also several other studies demonstrated a high proportion of FC genotype in individuals who were heterozygous for V1016G mutation from Sri Lankan (Fernando *et al.,* 2020; Ranathunge *et al.,* 2021) as well as global populations (Kushwah *et al.,* 2020; Wuliandari *et al.,* 2020) thus supporting the hypothesis from Vera-Maloof *et al.* (2015). Also, it has been suggested that in Asian populations, 989P+1016G and 1016G haplotypes are more likely to emerge from the F1534C haplotype (Cosme *et al.,* 2020; Du *et al.,* 2016). Taken together, these findings suggest the constant high frequency of F1534C mutation in Sri Lankan populations may likely establish the origin and dispersion of S989P and V1016G mutations thus resulting in strong resistance to both types I and II pyrethroids.

Co-occurrence of S989P and V1016G in domain II of the *vgsc* gene was prevalent in Asian *Ae. aegypti* populations (Cosme *et al.,* 2020; Wuliandari *et al.,* 2020) while obtaining a perfect linkage disequilibrium between two mutations (Kushwah *et al.,* 2020; Wuliandari *et al.,* 2020). Also, this observation was later recorded in some Arabian populations as well (Al Nazawi *et al.,* 2017). Further, the combination of S989P and V1016G has exhibited a synergistic effect to confer resistance to deltamethrin (Hirata *et al.,* 2014; Srisawat *et al.,* 2010). However, most of the Asian populations have not shown a significant linkage disequilibrium between F1534C and V1016 (Cosme *et al.,* 2020; Du *et al.,* 2016; Kushwah *et al.,* 2020;). Likewise, this study reported, no significant linkage disequilibrium between F1534C and V1016G in both pooled samples. Therefore, it is likely that in Asian populations S989P+V1016G combination is more

prevalent than the F1534C+V1016G combination (Cosme *et al.,* 2020). More *kdr* genotyping analyses in the future will confirm the association between F1534C with V1016G and S989P with 1016G in Sri Lankan *Ae. aegypti* populations. Intriguingly, the F1534C mutation has always been in a high frequency while the 1016G mutant allele has been under 50% in all the local studies done up to date. However, in the Sri Lankan population, 1534C is more prevalent than 989P (Fernando *et al.,* 2018; Ranathunge *et al.,* 2021) and field identifications with larger sample sizes will explain this further. Even though previously published *kdr* occurrence in Sri Lankan *Ae. aegypti* populations are limited to a few studies, there has been a notable increase in *kdr* mutant allele frequencies through the years. Especially, a rapid increase was visible in 1534C allele frequency in 2017 compared to the 2015 collection from 17.5 (n=39) to 80.2 per cent (n=48) in the Colombo district (Fernando *et al.,* 2020). Another early study demonstrated a 71.5 per cent (n=156) frequency for the 1534C allele when combined across three sample sites from the Colombo district which had an extreme level of resistance to permethrin (Hegoda, 2017). In a recent study, F1534C and S989P *kdr* mutations were found from randomly selected *Ae. aegypti* samples from the Colombo district showed resistance towards Cyfluthrin, Permethrin, ë-Cyhalothrin, and DDT (Nugapola *et al.,* 2021).

The Colombo district has been reporting dramatic *kdr* incidence as opposed to other areas on the island through the years (Fernando *et al.,*2018,2020; Ranathunge *et al.,* 2019, 2021) and the highest recorded pyrethroid resistance in *Ae. aegypti* populations (Fernando *et al.,* 2018, 2020; Nugapola *et al.,* 2021) while being accounted for the area with the highest number of reported dengue cases every year (Epidemiology Unit, 2022; National Dengue Control Unit-Disease, 2022) Moreover, a lot of studies have explained that compared to *Ae. albopictus,* pyrethroid resistance in *Ae. aegypti* occur mainly due to *kdr* mutations rather than metabolic resistance (Leong *et al.,* 2019; Nugapola *et al.,* 2021). These collective pieces of evidence suggest the rapid increase in F1534C is not likely because of genetic drift or founder effect but caused by the convergent selection for resistance to DDT and/or pyrethroids. Therefore, on-field diagnosis of *kdr* alleles is vital in predicting pyrethroid resistance ideally before every spray season.

The findings suggest a possibility of high pyrethroid resistance. An excess of heterozygosity reported for both F1534C and V1016G mutations likely indicates an emerging selection of *kdr* alleles due to the constant exposure to the pyrethroids.

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