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Detecting of knock down resistance (*Kdr*) F1534C allele in the dengue vector *Aedes aegypti* in peri-urban areas of Colombo South, Sri Lanka

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Abstract
A *phenylalanine* (F) to *cysteine* (C) substitution at position 1534 within the third domain of the NaV is associated with resistance to type I pyrethroid, permethrin, among populations of *Aedes aegypti*. Mosquito eggs were collected from three dengue high risk MOH areas in the Colombo district. Samples were reared to adulthood and resistance status against permethrin was determined by standard WHO susceptibility bioassays. Permethrin-resistant insects were genotyped for the F1534C mutation. All the PCR products were separated on 3.5% agarose gel and visualized by UV trans-illumination. Overall susceptibility was 58.3%, with mortality rates varying widely from 12.0% to 61.0%. Samples obtained from all three sampling sites showed high degrees of incipient resistance (<80% mortality). There was no mortality among control mosquitoes. Amplicons of approximately 90 and 110 bp correspond to alleles 1534 Phe^{+} and 1534 Cys^{kdr}, were obtained from all the resistant genotypes. The mutant C allele frequency varied between 0.847 and 0.543. The 1534C mutation was significantly and positively associated with permethrin resistance.

Keywords: Knock-down resistance (*kdr*), voltage gated sodium channel (NaV), permethrin, *Aedes aegypti*, dengue, resistance, mutation, Sri Lanka

Introduction
Aedes aegypti is an important disease vector which is capable of transmitting dengue virus to humans [1]. At present, epidemic dengue is a major public health problem in Sri Lanka [2] and thousands of people are at continuous risk of this disease. During the last 12 months of the year 2016, 47834 suspected dengue fever cases have been reported to the Epidemiology Unit from all over the island. Approximately 49.48% of dengue fever cases were reported from the Western province. Out of other districts, Colombo has been identified as the highest risk area of dengue (Ministry of Health, Epidemiology Unit-Sri Lanka, 2016). In the absence of a licensed vaccine, the major focus in dengue disease control programs on the island is vector control through elimination of breeding sites and application of insecticides [3]. Reducing vector populations below thresholds capable of sustaining viral transmission requires the heavy use of space sprays of insecticides. Pyrethroid compounds are thus the primary insecticides used for the control of *Aedes* in Sri Lanka. Continuous exposure to insecticides leads to the development of insecticide resistance in vector populations [3]. A *phenylalanine* to *cysteine* substitution at position 1534 (F1534C) within the third domain of the NaV is associated with resistance to type I pyrethroid, permethrin, among populations of *Ae. Aegypti* [4]. The purpose of this study was to identify the pyrethroid resistance status and to detect the mutation and its frequency in *Ae. aegypti* in selected areas in Peri Urban areas of Colombo South Sri Lanka.

Materials and Methods
Study sites and sample collections
Mosquito eggs were collected from selected localities in Colombo district in Sri Lanka i.e. Dehiwala MOH area: Nedimala PHI area (6° 50′ 59″ N, 79° 52′ 44″ E), Maharagama MOH area: Jambugasmulla PHI area (6° 51′ 13″ N, 79° 54′ 19″ E) and Nugegoda MOH area: Gangodawila PHI area (6° 51′ 53″ N, 79° 54′ 7″ E) from July to October, 2016 (Figure 1). All the laboratory experiments were carried out in the Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka.

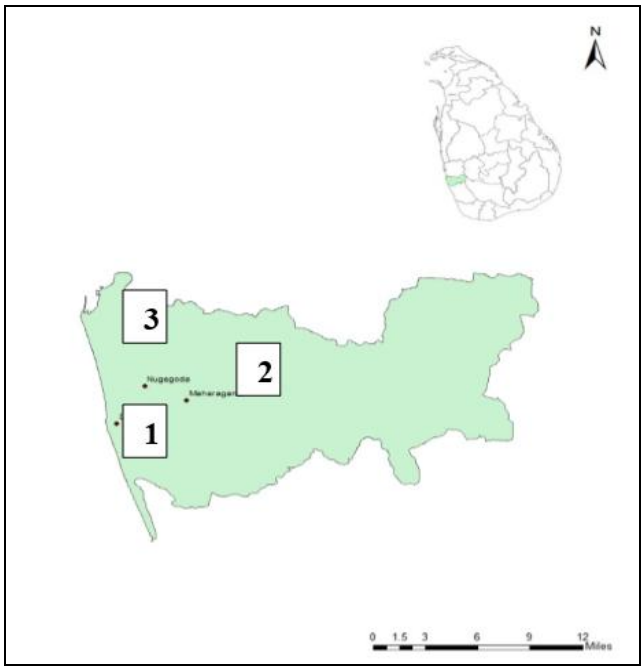

Fig 1: Collection sites of field samples of Ae. aegypti from Colombo South, Sri Lanka; 1 – Dehiwala, 2 – Maharagama; 3 – Nugegoda

Mosquito eggs were collected using ovitraps prepared using black plastic cups and ovipaddles made from hardboard were used as oviposition substrates. Ten ovitraps were placed randomly in each area. Ovitraps were placed inside the houses as in the living room in close proximity to shelves or racks or hanging cloths.

Collection of ovipaddles with eggs from the ovitraps placed in field sites was done on a weekly basis. Carefully removed ovipaddles were brought to the laboratory and immersed in plastic basins containing dechlorinated tap water, allowing the eggs to hatch and reared in separate basins. Powdered fish meal pellets were given as larval food. Once the adults were emerged, they were transferred to adult mosquito cages and fed on cotton pads soaked in 10% glucose solution (25 ± 2 ^{0}C, 80% RH). No blood meals were provided.

Adults mosquitoes were morphologically identified using Standard Mosquito Identification keys [5]. Adult male and female *Ae. aegypti* mosquitoes were separated into two different cages. This was performed in all three areas.

Permethrin bioassays

For each population, insecticide bioassays were carried out using the WHO standard bioassay kits [6]. Two to five day old unfed female mosquitoes were used for permethrin susceptibility testing. At least 100 females obtained from each location were used for testing. This provided four replicates of 25 mosquitoes. Following the procedure, each replicate group was placed into a holding tube and initially observed for injured or otherwise unsuitable mosquitoes were present. Thereafter they were transferred into four exposure tubes, each lined with 0.75% permethrin-impregnated papers (WHO, Malaysia) and a control group was transferred to another holding tube, which was lined with oil-impregnated papers. Insecticide exposure lasted one hour. Thereafter, knockdown individuals were scored. Following exposure, the mosquitoes were reintroduced into their respective holding tubes and again provided a 10% w/v sucrose solution. After 24 hours dead mosquitoes, as well as those alive but incapable of coordinated movement, were scored as susceptible (S). Remaining survivors were scored as resistant (R). All samples were subsequently put in to separate Eppendorf tubes with small holes on their tips for removal of moisture. After separating mosquitoes in to tubes, tubes were stored in sealed bags with dehydrated silica gel until they were taken to the DNA extraction. Data were considered only if the control mortalities were less than 20%. If there were mortalities in controls, actual mortalities were calculated using Abbott's formula.

F1534C AS-PCR genotyping

For each test of 100 mosquitoes, total resistant mosquitoes were processed for genotyping. Genomic DNA was obtained by using modified Ballinger-Crabtree, 1992 [7] method.

Stock solutions were prepared and used for AS-PCR genotyping. For our study, we sought an AS-PCR assay which would utilize a standard PCR thermal cycler and the products of which could be visualized by gel electrophoresis. The AS-PCR assay follows the protocol of one of the previous studies [8].

Each reaction was performed in a 25 µl volume with final concentrations of 1.5 mM $MgCl_2$, 5x PCR buffer, 1.25 µM Phe forward primer (5′-GCGGGCTCTACTTTGTGTT CTTCATCATATT-3′), 1.25 µM Cys forward primer (5′-GCGGGCAGGGCGGCGGG GGCGGGGCCTCTACTTTG TGTTCTTCATCATGTG-3′), 0.5 µM common reverse primer (5′-TCTGCTCGTTGAAGTT GTCGAT-3′), 200 µM dNTP mix (Promega, USA), 0.2 units Taq DNA polymerase (Promega, USA), and 25 ng template DNA. Amplification was done at 95 °C for 2 min initial activation stage and followed by 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, in turn followed by a final extension at 72 °C for 2 min. PCR products were loaded onto 3.5% agarose gels and electrophoresis was conducted at 100 V for 45 min.

Analysis of results

The results of the permethrin bioassays were interpreted as susceptible (98–100% mortality), possibly resistant (80–97% mortality) and resistant (<80% mortality) according to WHO recommendations [6].

In the bioassay test, according to WHO test procedure [6], the mortality of test sample was calculated by summing the number of dead mosquitoes across all four exposure replicates and it was expressed as a percentage of the total number of exposed mosquitoes. (As control mortalities were not recorded, mortality was not corrected using Abbots formula.)

Recorded mutant C allele frequency was calculated according to Hardy-Weinberg law.

Results

Permethrin bioassays and adult genotyping

A total of 300 female *Ae. aegypti* mosquitoes collected from 3 peri urban areas in Colombo South were tested for permethrin susceptibility and resistance (Table 1).Overall susceptibility was 58.3%, with mortality rates varying widely from 12.0% to 61.0%. Samples obtained from all three sampling sites show high degrees of incipient resistance (<80% mortality). There was no mortality among control mosquitoes. Genotype and allele frequencies were determined from 175 resistant mosquitoes selected at random.

Amplicons of approximately 90 and 110 bp correspond to alleles 1534 Phe^+ and 1534 Cys^{kdr}, were obtained from all the resistant genotypes as in Figure 2. The highest C allele frequency (0.847) was obtained from Nugegoda, Gangodawila PHI area and the lowest number of C allele frequency (0.543) was obtained from Dehiwala, Nedimala PHI area (Table 1).

Table 1: F1534C allele specific PCR results for testing mosquito samples in each area.

Area	Location	Mortality %	Status	n	Total PCR	Resistance Genotype			C Allele Frequency
						F/F	F/C	C/C	
Dehiwala	Nedimala	46	R	49	46	5	32	9	0.543
Maharagama	Jambugasmulla	61	R	38	35	3	18	14	0.657
Nugegoda	Gangodawila	12	R	88	75	1	21	53	0.847
Total		58.3	R	175	156	9	71	76	0.715

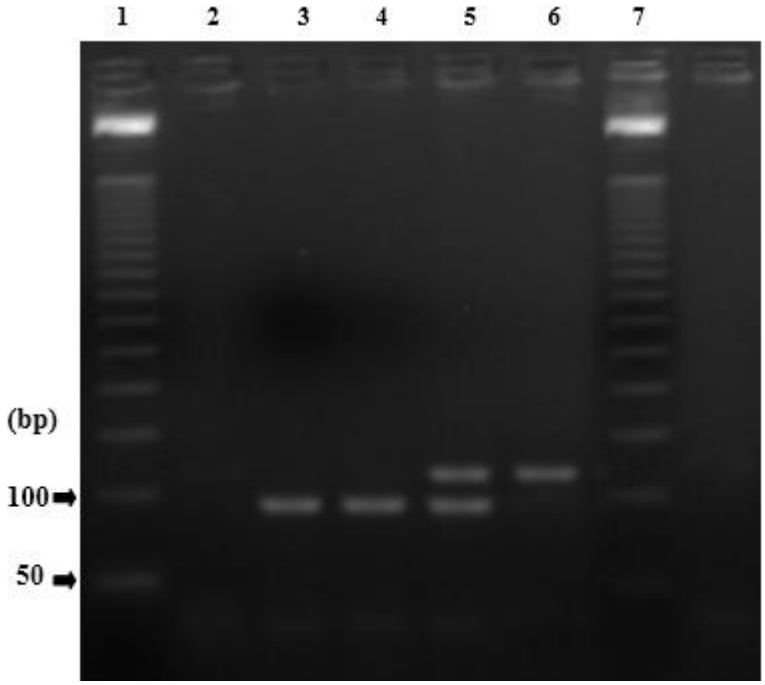

Fig 2: Gel photograph showing the genotypes of F1534C alleles

Each of the three genotypes is shown from left to right: wild-type homozygous (F/F), heterozygous (F/C) and mutant homozygous (C/C). The lane to the far left contains low-molecular weight DNA ladder (L). (1: 50bp Ladder; 2: Negative control without DNA; 3. Positive control with DNA of wild type homozygous susceptible strain of lab colony; 4: 1534 Phe^{+}/Phe^{+}: wild type homozygous; 5: 1534 Phe^{+}/Cys^{kdr}: heterozygous; 6: 1534 Cys^{kdr}/ Cys^{kdr}: mutant homozygous; 7: 50bp Ladder)

Discussion

The current study was performed to identify the presence of *kdr* mutation and its frequency in *Ae. aegypti* the primary vector of dengue in selected areas of Dehiwala, Nugegoda and Maharagama. Dengue vector *Ae.aegypti* is considered as the vectors of three important viral diseases—dengue, yellow fever, and chikungunya and is capable of transmitting a number of others [9]. Vector surveillance is an essential component in the implementation of an optimum vector control program to combat dengue. Under that, vector control through insecticide application is considered as one of the main steps to control dengue. All over the world, chemical insecticides are still the most widely used vector control strategy. Hence, monitoring of insecticide resistance is crucial to the long-term maintenance of this strategic tool [10].

In this study, to collect *Aedes* mosquitoes, an ovi traps based study was used and all the traps were placed inside of houses in each sampling area. The present study reveals a high prevalence of *Ae. aegypti*, the reported principal vector of dengue in Sri Lanka in the residential areas of Dehiwala, Nugegoda and Maharagama, in Colombo District. When comparing with Maharagama, Jambugasmulla sampling area this is in contrast to a previous study that reported a continuous appearance of *Ae. albopictus* and the absence of *Ae. Aegypti* [11]. Increase of the development and the urbanization, with time might make that chance to *Ae. aegypti* to maximize its number in indoor environments.

In all these three areas, to control the dengue vector different control measures have been used by their MOH offices and by the Urban Councils. Among these control measures insecticide application for dengue vector control plays a major role. So the study of insecticide resistance of the dengue vector in all these three areas is essential in order to modify their control measures to control high dengue cases.

According to Surendran *et al*., 2012 [12], in Sri Lanka Dichloro-diphenyl-trichloroethane (DDT), a synthetic organochloride, was used until mid-1970s, when due to the development of DDT resistance it was replaced by the synthetic organophosphate (OP) Malathion. The use of Malathion in IRS was discontinued in 1993 in most parts of the country as a result of the development of resistance, and replaced with l-cyhalothrin, a synthetic pyrethroid. At present insecticides such as fenitrothion, l-cyhalothrin, cyfluthrin, deltamethrin and etofenprox are used in different districts on a rotational basis to delay the development of resistance in mosquitoes.

As described by WHO, 1998 [3], space spraying of insecticides and application of larvicides are the major methods of chemical control used in rapid control of vector populations, especially during the outbreaks. Malathion, pesguard FG161 and deltacide are commonly used for space spraying in Sri Lanka while temephos are used as the main larvicide. It is important to assess the efficacy of these insecticide based control methods in order to strengthen the

present vector control programmes.

According to the bioassay results, unfortunately all three mosquito populations showed a mortality less than 80%, confirming the existence of resistant genes in the test population (Table 1). It suggests that Permethrin, a PY insecticide will have low efficacy for *Aedes* control. This resistance level is considered to be an underestimate due to our use of 0.75% permethrin paper which is at a higher concentration than the discriminating dose (0.25%) for adult *Ae. aegypti* recommended by WHO [13].

The mortality that was recorded in Maharagama, Jambugasmulla PHI area and Nugegoda, Gangodawila PHI area, abled to indicate that they have high resistance to the Permethrin, a PY insecticide. Although both have been applying IRS and outdoor fogging regularly, the presence of the resistance to insecticides of these mosquitoes might be a reason for the increase of dengue patients.

In this study, we have successfully applied a simple AS-PCR technique to detect the F1534C mutation in *Ae. aegypti* in selected sampling areas. Although the mutant allele was found in all three areas, it is evidently widespread in Nugegoda compared with the other two areas.

The 1534 Cys^{kdr} significantly diminished the NaV sensibility to type I PY. From the AS-PCR results, these genotypes of resistance checked *Ae. aegypti* mosquitoes of all three sampling areas were observed as in Figure 2. So the evidence was found for target-site (NaV) resistance. Homozygous mutant females would be resistant and most wild-type homozygous would be susceptible, whereas intermediate resistance would be displayed by heterozygous mosquitoes to permethrin. According to the results all the resistance mosquitoes were not showing the homozygous mutant 1534 Cys^{kdr}/ Cys^{kdr} allele. The survival of heterozygous mosquitoes and wild type homozygous mosquitoes indicates that the F1534C mutation is not the only mechanism involved and that either, other *kdr* or enzymatic mechanisms may confer cross resistance or enhance resistance.

According to Karunaratne *et al*., 2013 [3], *Ae. aegypti* populations from Kandy, Kurunegala, Ratnapura, Gampaha and Puttalam have also shown the resistance to the Permethrin, a PY insecticide. Screening of Sri Lankan *Ae. aegypti* populations for the presence of these mutations is important for effective dengue vector control strategies.

This study which was conducted in these cities provide further evidence that the control program has not been successful in maintaining mosquito populations at low densities, since *Ae. aegypti* was detected in all three areas prevalently. The continued use of PY insecticides in such areas will certainly decrease the efficacy of the insecticide.

It is expected that the results of the present study would help health authorities to use appropriate insecticides to delay the onset of resistance and to use appropriate insecticides to get the maximum effect through space spraying.

References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, *et al*. The global distribution and burden of dengue. Nature. 2013; 496(7446):504-507.
2. Abeyewickreme W, Wickremasinghe AR, Karunatilake K, Sommerfeld J, Axel K. Community mobilization and household level waste management for dengue vector control in Gampaha district of Sri Lanka; an intervention study. Pathogens and global health. 2012; 106(8):479-487.
3. Karunaratne SHPP, Weeraratne TC, Perera MDB, Surendran SN. Insecticide resistance and, efficacy of space spraying and larviciding in the control of dengue vectors Aedes aegypti and Aedes albopictus in Sri Lanka. Pesticide biochemistry and physiology. 2013; 107(1):98-105.
4. Stenhouse SA, Plernsub S, Yanola J, Lumjuan N, Dantrakool A, Choochote W, *et al*., Detection of the V1016G mutation in the voltage-gated sodium channel gene of Aedes aegypti (Diptera: Culicidae) by allele-specific PCR assay, and its distribution and effect on deltamethrin resistance in Thailand. Parasites & vectors. 2013; 6(1):253.
5. Amerasinghe FP. Illustrated keys to the genera of mosquitoes (Diptera: Culicidae) in Sri Lanka. Journal of the National Science Foundation of Sri Lanka. 23(4).
6. World Health Organization. The Global plan for insecticide resistance management in malaria vectors (GPIRM). Geneva: World Health Organization, 2012.
7. Ballinger-Crabtree ME, Black WC, Miller BR. Use of genetic polymorphisms detected by the random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for differentiation and identification of *Aedes aegypti* subspecies and populations. The American journal of tropical medicine and hygiene. 1992; 47(6):893-901.
8. Yanola J, Somboon P, Walton C, Nachaiwieng W, Somwang P, Prapanthadara LA. High-throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in permethrin-resistant *Aedes aegypti* and the distribution of this mutation throughout Thailand. Tropical Medicine & International Health. 2011; 16(4):501-509.
9. Morrison AC, Zielinski-Gutierrez E, Scott TW, Rosenberg R. Defining challenges and proposing solutions for control of the virus vector Aedes aegypti. PLoS medicine. 2008; *5*(3):e68.
10. Martins AJ, de Andrade Lins RMM, Linss JGB, Peixoto AA, Valle D. Voltage-gated sodium channel polymorphism and metabolic resistance in pyrethroid-resistant *Aedes aegypti* from Brazil. The American journal of tropical medicine and hygiene. 2009; 81(1):108-115.
11. Gunathilake KVK, De Silva BGDNK. *Aedes* mosquito survey within the premises of University of Sri Jayewardenepura to determine its population density and seasonal shift in relative abundance. Vidyodaya Journal of Science. 2012; 15.
12. Surendran SN, Jude PJ, Weerarathne TC, Karunaratne SP, Ramasamy R. Variations in susceptibility to common insecticides and resistance mechanisms among morphologically identified sibling species of the malaria vector Anopheles subpictus in Sri Lanka. Parasites & vectors. 2012; 5(1):34.
13. World Health Organization. Test procedures for insecticide resistance monitoring, bio-efficacy and persistence of insecticides on treated surfaces. Geneva: World Health Organization, 1998.
14. Surendran SN, Kajatheepan A, Sanjeefkumar KF, Jude PJ. Seasonality and insecticide susceptibility of dengue vectors: an ovitrap based survey in a residential area of northern Sri Lanka, 2007.