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Insecticide susceptibility of *Aedes albopictus* from Sri Lanka: First report of the F1534C mutation in the country

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Abstract
Aedes albopictus, a vector of dengue, have increased concerns with its presence due to its rapid spread and the aggressive biting behavior. Resistance to commonly used insecticides is a major concern in vector mosquito control measures. The present study was carried out on local populations of *Ae. albopictus* in Western province, Sri Lanka. Adult and larval susceptibility tests were carried out for F_0 and F_1 populations of *Ae. albopictus* respectively against Deltamethrin, Permethrin and Temephos according to World Health Organization guidelines. The average mortality percentages recorded for permethrin and deltamethrin were 85.2% and 81.6% respectively. The mutant allele percentage varied 4%-5%. Larval bioassays showed 58.2% average mortality percentage. The study for the first time reveals the presence of F1534C mutation in pyrethroid resistant *Ae. albopictus* mosquitoes in Sri Lanka. The need of identifying the existing resistance mechanisms in local populations is important to carry out necessary implementations and reducing errors in vector control measures.

Keywords: *Aedes albopictus*, insecticide resistance, pyrethroids, Temephos, KDR, Sri Lanka

Introduction
Aedes albopictus, a major vector of foremost diseases, yellow fever, chikungunya, and zika [1], is the secondary vector of dengue fever (DF) in Sri Lanka. Dengue fever cases in Sri Lanka have been increasing dramatically over the past ten years and in the year 2017, 186,101 cumulative DF cases have been reported in the country with 34274 numbers of cases recording in the Colombo district [2].

Ae. albopictus is native to Southeast Asia, Western Pacific islands, and Islands of the Indian Ocean, nevertheless shows cosmopolitan distribution due to its invasive ability and ecological plasticity [3]. The larval breeding of *Ae. albopictus* occurs in fresh water in a broad range of natural sites (e.g., bamboo stumps, bromeliads and tree holes) to artificial containers. Additionally larvae have shown tolerance to salinity and have been found to breed in the brackish water systems [4]. The ability to withstand salinity helps invasive *Ae. albopictus* to increase the potential for transmission of dengue and chikungunya in temperate zone countries [5].

Vector control is an essential component of the vector borne disease control programs. Since studies for proper treatment or vaccination for DF is still ongoing, the current means of controlling of DF heavily relies on vector population reduction by use of chemical insecticides, due to its fast effectiveness and ease in use. Intense use of these insecticides has resulted in resistance in vector populations to almost all classes of insecticides available in the world. Among the four classes of synthetic insecticides, pyrethroids (PY) are the most widely used adulticide against *Aedes* mosquitoes, and recently the vector populations have shown resistance against PY. The target site insensitivity is common in pyrethroid resistant mosquito populations, and it can be occurred due to mutations in the amino acid sequences at voltage-gated sodium channels (VGSC) of nerve cell membranes. This will diminish the sensitivity of DDT and pyrethroid insecticides with the channels. The amendments at the target sites which leads to a resistance to insecticides are referred as knockdown resistance (*kdr*). This states the ability to withstand the prolonged exposure to insecticides [6].

The major vectors of dengue, *Ae. aegypti* and the vector *Ae. albopictus* shares the same ecological niche and exposes more frequently for chemical insecticidal activities at their larval stages [7]. Currently chemical treatments for the dengue vectors are carried out both indoors and outdoors in Sri Lanka and the vectors are exposed to chemical treatments frequently.

According to the previous studies done on the major vector, *Ae. aegypti* in Sri Lanka, it has shown resistance against PY insecticides with the presence of F1534C [8, 9] mutation due to heavy and frequent usage of PY against the vectors. The control of *Ae. albopictus* is far more difficult than *Ae. aegypti*, due to the fact that *Ae. albopictus* have a wider range of habitat. The heavy and frequent usage of temephos as a larval control measure is therefore practiced intensively which has led to a resistance to temephos [10, 11].

Mosquitoes have created protection from essentially every insecticide that has been utilized, all over the world. The strategies for vector control have turned out to be progressively troublesome as the quantity of new insecticides in the health sector is low. Therefore discovering systems for postponing the advancement of development of resistance by vector mosquitoes to the couple of accessible insecticides is captious. Subsequently it is fundamental to identify the mutations responsible for the resistance in order to improve precise monitoring programs, to comprehend the population genetics and the evolution of resistance and to plan viable measures to moderate or to a slowdown the improvement of resistance.

The present study was carried out to determine the susceptibility of Sri Lankan *Ae. albopictus* mosquitoes to the most commonly used adulticides permethrin and deltamethrin and the most commonly used larvicide, temephos. The resistant *Ae. albopictus* mosquitoes were genotyped to determine the presence of F1534C mutation in the target site.

Materials and Methods

Mosquito collection

The study was carried out in five divisional secretariats (DS) in the Colombo district, Nugegoda (6^0 52' 22" N, 79^0 53' 27" E), Maharagama (6^0 50' 59" N, 79^0 55' 59"E), Homagama (6^0 50' 26" N, 80^0 00' 50"), Moratuwa (6^0 46' 22" N, 79^0 52' 53" E), Ratmalana (6^0 49' 11" N, 79^0 53' 6" E). The collection sites were selected with an emphasis on areas with previous reports of dengue incidences (Figure 1).

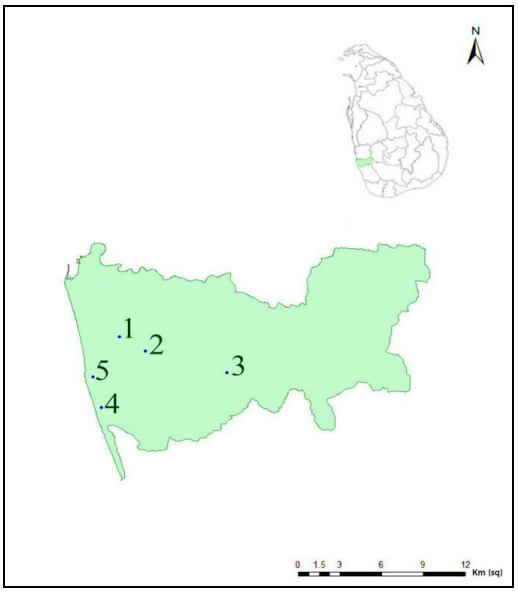

Fig 1: Map of Western province Sri Lanka with areas of mosquito collection; 1- Nugegoda, 2-Maharagama, 3-Homagama, 4-Moratuwa, 5-Ratmalana

Eggs, larvae and pupae of *Ae. albopictus* mosquitoes were collected from May-August 2017 using 100 ovitraps per site. Ovitraps were made from black stained plastic cups with a capacity of 250 ml and a wooden paddle sized 3cm*10 cm was placed inside the cup resting on the upper rim. Ovitraps were placed at least 15 m distance from each other and within 5 m distance from each house in outdoor habitats at investigated sites. The ovitraps were inspected after five days and the paddles and water containing the larvae and pupae were transported to the laboratory. Egg-containing paddles were dried at room temperature and stored at 28^0C. New paddles and water were placed in the cups for next round collections.

Eggs were hatched in the insectary and reared to adults. The emerged F_0 adult mosquitoes were identified morphologically using standard identification keys [13]. As adult feed, 10% sucrose-soaked cotton balls were provided on top of the cage and kept at 27 ± 2 °C temperature and relative humidity of 80 ± 10% at the insectary.

About 100-125 none blood-fed female mosquitoes were separated in to the cages from each location for adult susceptibility bioassays and the remaining adult female and male mosquitoes were separated in to another cage to induce mating. After mating has occurred between male and female mosquitoes, the female mosquitoes were fed with chicken blood to induce egg laying using artificial membrane feeder to obtain F_1 progeny. Larvae obtained from F_1 progeny were used for the larval susceptibility bioassays.

Larval susceptibility bioassays

Standard WHO larval bioassays were conducted to detect the level of susceptibility to temephos using F_1 larvae [14]. For the bio assays, 2nd or 3rd instar larvae were used. The larval mortality was tested at the diagnostic dosage, 0.012 mg/L with 100 ml of water and 100% ethanol as the control with 5 replicates per each site. After 24 hours numbers of dead (susceptible) and alive (resistant) larvae were counted and the mortality percentage was calculated.

Adult susceptibility bioassays

Adult bioassays were conducted using one to three days old non-blood fed adult female mosquitoes (n=125) from each location according to WHO standard guidelines [15, 7]. Two pyrethroid insecticides deltamethrin 0.05% and 0.75% permethrin were tested. For every test a control assay was also carried out using untreated control papers. After 24 hours mortality percentages was calculated separately for the two insecticides.

DNA extraction and KDR genotyping

Total DNA was extracted from adult mosquitoes which showed resistance to each insecticide separately, along with the susceptible strains in each location, using a phenol-chloroform extraction method [16]. Allele specific PCR (AS-PCR) was carried out to observe the *phenylalanine* to *cysteine* substitution at position 1534 (F1534C) within the third domain of the Na_V [17]. Each 25 µl PCR reaction contained, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1U of *Taq* DNA Polymerase (Promega), 0.2 mM of Phe Forward primer, 0.2 mM of Cys Forward primer, 0.25 mM of common reverse primer [18], and 10 ng of template DNA. The cyclic conditions were 95 °C for 2 minutes, 35 cycles of 95 °C for 30 seconds, 60°C for 30 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 2 minutes. The amplified PCR products were subjected to agarose gel electrophoresis at 80V for one hour and were trans-illuminated under a UV light.

Statistical analysis

The bioassay results were interpreted according to WHO criteria, as susceptible (>97% mortality), possible resistant (90-97% mortality), resistant (< 90% mortality).

The PCR results were analyzed using Hardy-Weinberg Equilibrium (HWE). The data obtained were statistically analyzed using paired t-test.

Results

Figure 2 shows the percentage mortality for temephos. According to the larval susceptibility tests, samples collected from all five DS indicated resistance to temephos. The highest percentage of larval mortality was recorded in Homagama DS (68%) while the lowest was recorded from Moratuwa (43%). There was no mortality among control mosquitoes.

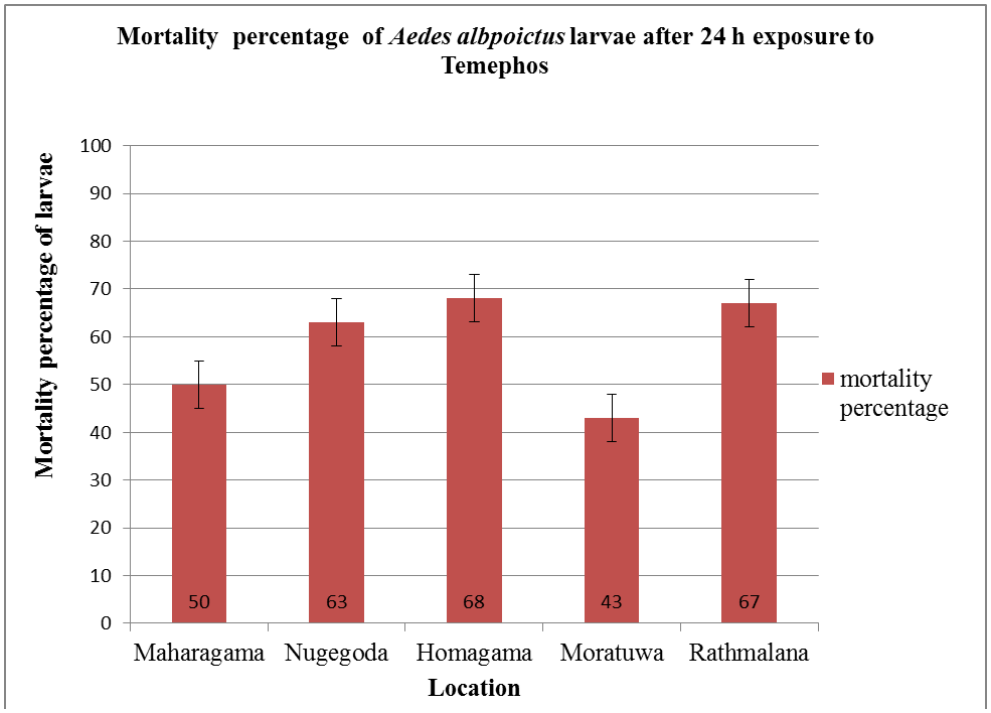

Fig 2: Susceptibility status of *Ae. albopictus* larvae for Temephos in the studied locations.

According to (figure 3 and figure 4) the adult susceptibility tests, the highest mortality percentage for permethrin (93%) and deltamethrin (89%) were recorded in Homagama DS, and the lowest was recorded from Maharagama DS with 79% and 67% mortalities respectively. Mosquito populations from Maharagama, Moratuwa and Rathmalana DS were in 'confirmed resistance' as the mortality percentages were less than 90%. Mortality percentages of Nugegoda and Homagama DS were between 90-97% and categorized as in the 'possible resistance' category. The average mortality percentage for permethrin was 85.2% while the average percentage mortality for deltamethrin was 81.6%.

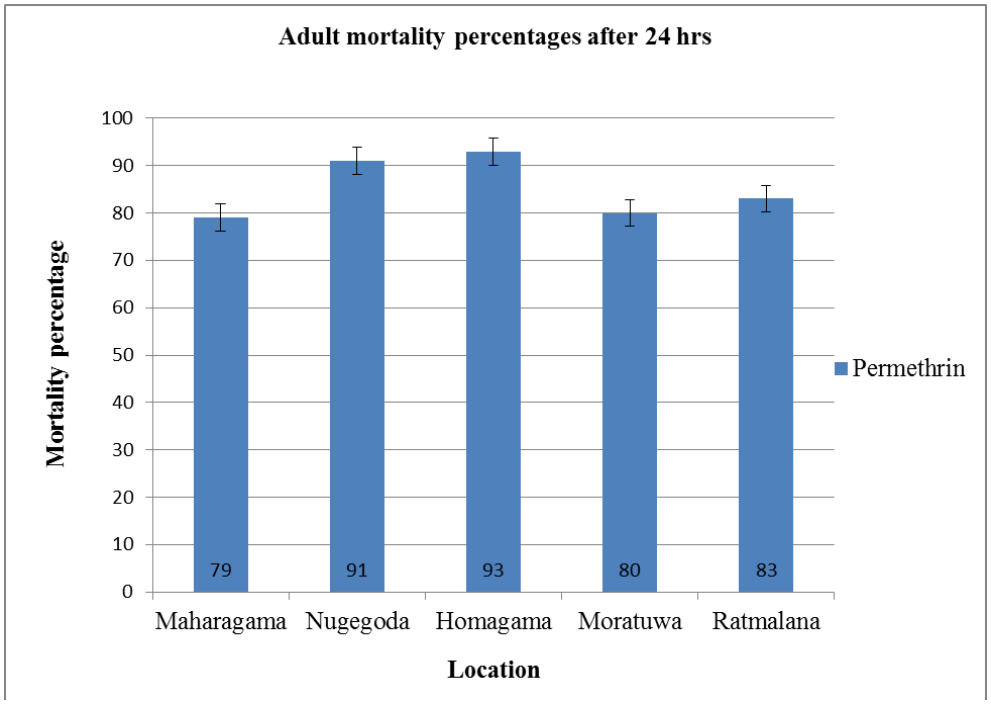

Fig 3: Susceptibility status of adult female *Ae. albopictus* for permethrin in the studied locations.

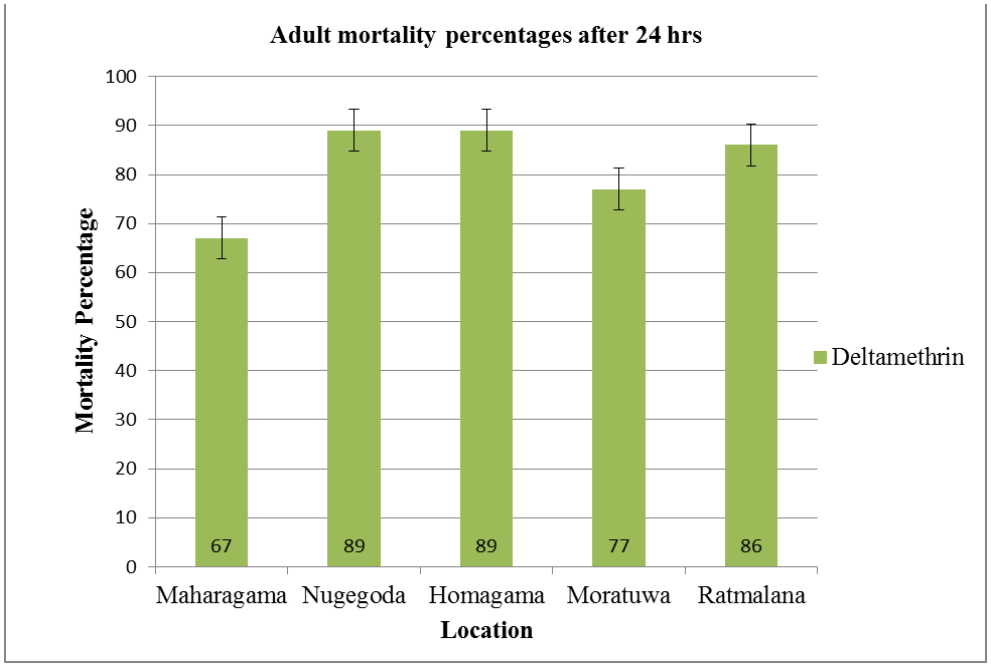

Fig 4: Susceptibility status of adult female *Ae. albopictus* for deltamethrin in the studied locations.

AS-PCR and *kdr* genotyping

Presence of mutations at alleles of 1534 Phe^{+} and 1534 Cys^{kdr} were revealed by the amplified PCR bands at 93 bp and 113 bp respectively (Figure 5).

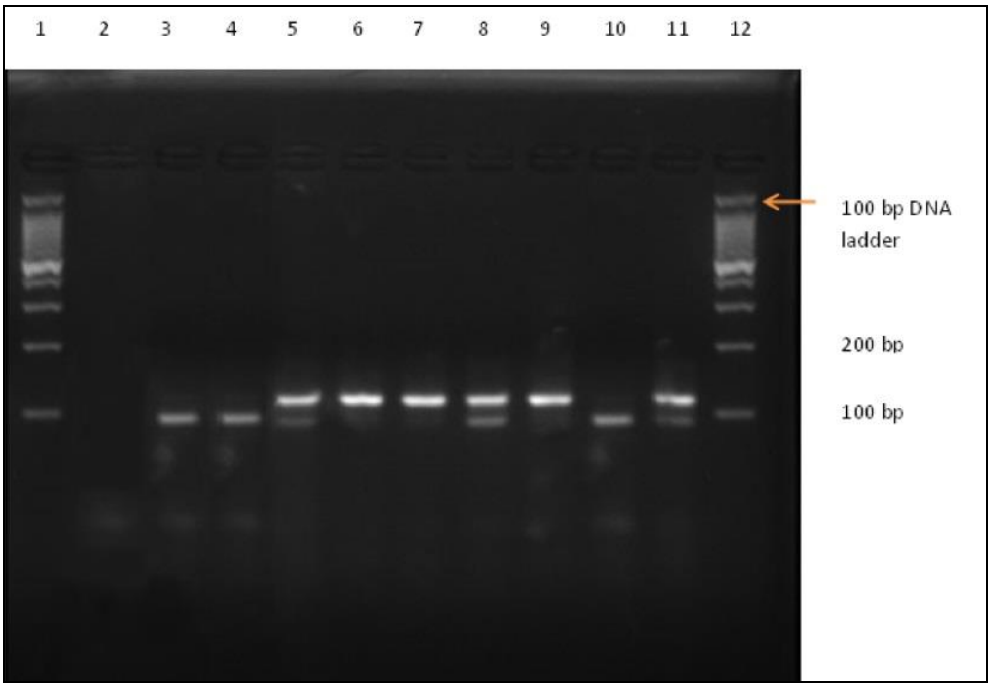

Fig 5: Gel electrophoresis results for the AS-PCR to detect F1534C mutation in pyrethroid resistant *Ae. albopictus*. Lane 1- 12 shows, 1- 100 bp DNA Ladder, 2- Negetive control without DNA, 3- Positive control with DNA of wild-type homozygous, susceptible strain, 4-1534 Phe^{+}/Phe^{+}: wild type homozygous, 5-1534 Phe^{+}/Cys^{kdr}: heterozygous, 6- 1534Cys^{kdr}/Cys^{kdr}: mutant homozygous, 7-1534Cys^{kdr}/Cys^{kdr}: mutant homozygous, 8- 1534 Phe^{+}/Cys^{kdr}: heterozygous, 9- 1534Cys^{kdr}/Cys^{kdr}: mutant homozygous, 10- 1534 Phe^{+}/Phe^{+}: wild-type homozygous, 11- 1534 Phe^{+}/Cys^{kdr}: heterozygous and 12- 100bp DNA Ladder respectively.

Among the genotyping of the permethrin resistant mosquitoes the highest C allele frequency was observed in Nugegoda DS and the lowest in the Moratuwa DS (Table 1). In deltamethrin resistant mosquitoes, highest mutant allele frequency was observed in Maharagama and Ratmalana DS and the lowest was from Moratuwa DS (Table 1). According to HWE: Chi square test with 1 degree freedom (Table 1). The populations are in Hardy-Weinberg Equilibrium. According to the Paired t- test of the mutant allele frequency regarding the two insecticides, there is no significant difference between mutant allele frequencies for both insecticides for the same location.

Table 1: AS-PCR results for tested deltamethrin and permethrin resistant mosquito samples in each area.

Location	Status	N	Total PCR	Resistance Genotype			P	χ^2	C Allele frequency
				F/F	F/C	C/C			
Permethrin									
Maharagama	Resistant	21	20	0.3	0.55	0.15	0.4	0.1	0.6
Nugegoda	Resistant	9	8	0.25	0.5	0.13	0.36	0.3	0.64
Homagama	Resistant	7	7	0.43	0.43	0.14	0.45	0.3	0.55
Moratuwa	Resistant	20	20	0.35	0.6	0.1	0.65	0.5	0.35
Ratmalana	Resistant	17	15	0.27	0.53	0.13	0.62	0.7	0.38
Deltamethrin									
Maharagama	Resistant	33	30	0.27	0.6	0.13	0.57	0.5	0.43
Nugegoda	Resistant	11	11	0.27	0.64	0.09	0.59	0.6	0.41
Homagama	Resistant	11	11	0.36	0.45	0.18	0.59	1	0.41
Moratuwa	Resistant	23	20	0.3	0.6	0.1	0.6	0.5	0.4
Ratmalana	Resistant	14	14	0.36	0.43	0.21	0.57	0.9	0.43

N: Total number of resistant mosquitoes to permethrin/deltamethrin, F/F- homozygous dominant, F/C-heterozygous, C/C –homozygous recessive, p: frequency for homozygous dominant, and χ^2:chi square value.

Discussion

The present study revealed the presence of F1534C mutation in the pyrethroid resistant *Ae. albopictus* mosquitoes for the first time in Sri Lanka. The study also revealed resistance to the most commonly used adulticides, permethrin and deltamethrin and the larvicide Temephos in *Ae. albopictus* populations in Sri Lanka. In the five study sites mosquitoes were more resistant to deltamethrin than permethrin and *Ae. albopictus* from all five study sites were resistant to Temephos.

Ae. albopictus is a container breeding mosquito. As WHO recommendation the most suitable and only approved larvicide for container breeding mosquitoes is Temephos [19]. However, in the present study, in all five divisional secretariats, the mortality percentages recorded were less than hundred for the WHO recommended discriminating dosage for mosquito larvae. Based on the bioassay results, it could be stated that the larval populations are having an incipient resistance towards the insecticide Temephos in urban areas like Moratuwa and Maharagama where the number of dengue cases are increasing and the application of the larvicides are more frequent. Thus, the present study raises the need for regulating the larval control measures and also to improve the knowledge and the practice of the personals which are engaged in applying the larval control measures at epidemic states. Also, the sole reliance on the chemical insecticides for larval control must be regulated and should be more oriented towards integrated vector control with more biological methods of applications.

According to adult bioassay results, all populations of the vector *Ae. albopictus* showed resistance towards deltamethrin and permethrin. As for deltamethrin, the mortality percentage is less than permethrin indicating *Ae. albopictus* population is more resistance towards deltamethrin than permethrin. In Sri Lanka mostly space spraying is done with Pesgaurd FG161 and deltacide whereas in the latter deltamethrin is the main ingredient [20].

These results suggest that permethrin and deltamethrin have a low efficacy in controlling the vector mosquitoes in the country. According to previous studies done on the resistance state of the vector *Ae. albopictus* it has shown resistance to malathion and DDT in Sri Lanka [4]. The ultimate results of both larval and adult bioassays have shown resistance in the areas of Maharagama and Moratuwa. These areas are more urbanized with more potential breeding sites for the vector mosquitoes and frequent and unplanned fogging activities with the use of pyrethoids in this area may have given rise to a resistance.

The allele-specific PCR has suggested the existence of *kdr* mutations in the vector population of *Ae. albopictus* in Sri Lanka of which the mutant allele frequency is varying between 4%-5%. Based on AS-PCR results, the occurrence of F1534C mutation has mostly shown heterozygosity. And the levels of the mutant allele frequency were lower. It is clear that the emergence of the *kdr* mutations of these mosquitoes has just started in the country. This was also observered recently in other parts of the world [18]. Previous studies carried out on *Ae. aegypti* in Sri Lanka have shown lower mortality rates and high frequency of mutant alleles which suggests higher resistance levels to pyrethroids in the country [8, 9]. As pyrethroids being the frequent applicant to all dengue vectors it can also cause high level of resistance in *Ae. albopictus* too.

The statistical analysis results from the paired t-test of the *kdr* genotyping have suggested as *kdr* mutant allele distribution for two different pyrethroids of deltamethrin and permethrin in the same location is significantly not different to each other. Also, the average mutant allele frequency for permethrin is higher than deltamethrin. According to HWE, the population is in Hardy–Weinberg proportions. The resistance levels at the bioassays are considered to be an underestimate due to the usage of insecticide papers with a higher concentration than the discriminating dose recommended by WHO.

The results of this study concern the authorities on arising resistance of the vector *Ae. albopictus* to be a threat in the country. This suggests that resistance has arisen to the commonly used insecticides and the application of chemical control methods need to be revised in the country accordingly. This is due to improper applications with heavy quantities and frequent application of the same insecticide throughout a long period of time. This implies a strong implication on the government to be more careful with the application of the insecticides bring more alternative vector control methods like biological control strategies specially release of *Wolbachia* infected male mosquitoes which become more and more important in the current world situation.

Conclusion

Insecticide resistance is a global scenario which turns the authorities to be more concern in the application of the insecticides and manage their system to be more centered

towards an integrated vector control system. This study would help the health authorities to use appropriate and well-managed vector control system against the vector *Ae. albopictus* while maximizing the effect of pyrethroids and temephos as chemical control strategies.

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