Insecticide resistance and molecular characterization of knockdown resistance (*kdr*) in *Culex quinquefasciatus* mosquitoes in Sri Lanka

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ABSTRACT: Resistance to pyrethroids (PY) and organophosphate (OP) insecticides is widespread among populations of *Culex quinquefasciatus,* the major vector of lymphatic filariasis (LF). The present study was designed to detect the L1014F *kdr* (knockdown resistant) mutation among *Cx. quinquefasciatus* populations in the filarial belt of Sri Lanka. Mosquitoes were reared from field-caught larvae from seven localities where LF is endemic. Susceptibility status of *Cx. quinquefasciatus* to adulticides, 0.05% deltamethrin, 0.75% permethrin, 5% malathion, and the larvicide temephos was determined using the standard WHO susceptibility tests. A fragment of *vgsc* gene was amplified and sequenced to identify the responsible *kdr* mutations. The susceptibility test results revealed less than 90% mortalities for 0.05% deltamethrin, and 0.75% permethrin and temephos. For 5% malathion, all study sites except Maharagama revealed greater than 90% mortality. The L1014F *kdr* mutation was observed in all studied populations. Although the overall microfilaria rate is less than 1% in the country, there is a high risk of re-emergence of LF in Sri Lanka due to abundant *Cx. quinquefasciatus* mosquitoes, increased resistant status to currently used insecticides, imported LF cases, higher rates of microfilaria among neighboring countries, and the advancement of tourism. ***Journal of Vector Ecology* 45 (2): 204-210. 2020.**

Keyword Index: Culex quinquefasciatus, insecticide resistance, filarial belt, knockdown resistance, L1014F mutation, Sri Lanka.

INTRODUCTION

The mosquito *Culex quinquefasciatus* Say 1832 is considered one of the most widespread mosquito species in the world. It is an important vector of a wide variety of pathogens and parasites of medical and veterinary diseases, including lymphatic filariasis, avian malaria, St. Louis encephalitis, western equine encephalitis, West Nile fever, and the newly emerged Zika (Goddard et al. 2002, Whiteman et al. 2005, Bhattacharya and Basu 2016, Guedes et al. 2017). *Culex quinquefasciatus* acts as an opportunistic feeder, and its host choice is regionally variable, feeding mainly on many species of mammals, birds, reptiles and amphibians (Mackay et al. 2010, Unlu et al. 2010). *Culex quinquefasciatus* is the predominant vector of the filarial worm *Wuchereria bancrofti,* the main agent of bancroftian filariasis in urban areas of Sri Lanka.

Filariasis has been known in Sri Lanka for many years. In the 1960s, bancroftian filariasis was widely distributed along the coastal belt of Sri Lanka starting from Negombo to Matara, covering less than 400 km^2 and including Colombo, Kalutara, Gampaha, Galle, Matara, Kurunegala, and Negombo. This area is also known by the Ministry of Health as the filarial belt (Dissanaike 1991). In studies carried out in 1990, the belt had extended to about 90 km north from Negombo to Puttalam and about 120 km to the southeast from Matara to Kataragama.

Due to implementation of various mass treatment programs and as a result of their success, in 2016 WHO validated that Sri Lanka has eliminated lymphatic filariasis as a public health problem. Although the overall microfilaria rate is less than 1% in the country, Sri Lanka is still recording filariasis cases and this has prompted public health concerns about the possibility of transmission of bancroftian filariasis again in Sri Lanka.

The control of vector mosquitoes using pyrethroid (PY) space-spraying for adults and applying temephos as a larvicide are the most extensively used measures as routine practices and as emergency measures in the event of an epidemic situation (Sirisena and Noordeen 2016). Pyrethroid-containing chemicals are also readily available in the market as numerous household anti-mosquito substances and are being extensively used in personnel protection.

The long-term use of insecticides in public health and agriculture has led to the development of resistance to commonly used insecticides (Karunaratne et al. 2013). Resistance to DDT and PYs has been documented in populations of *Cx. quinquefasciatus* in Sri Lanka and many other countries (Wondji et al. 2008, Roberts and Andre 1994).

Understanding insecticide resistance mechanisms is an essential component in vector control strategies. Resistance mechanisms can be divided into two major groups as decreased sensitivity of the target site and increased metabolic detoxification of insecticides (Hemingway et al. 2004). The target site insensitivity appears due to mutations in the amino acid sequences at voltage-gated sodium channels (*vgsc*) of nerve cell membranes. Pyrethroid group insecticides act as an agent that binds to voltage-gated sodium channels (*vgsc*) of the neurons. Pyrethroids bind preferentially to open channels and this bound form then remains at open or activated status which leads to repetitive nerve firing. This results in the uncontrolled status of nerve activity, which is

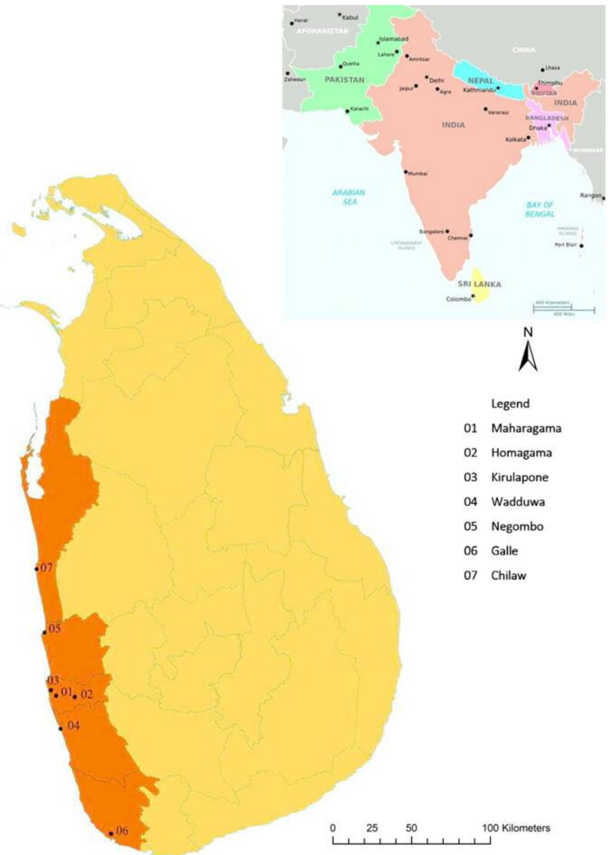

Figure 1. Collection sites of immature stages and adults of *Cx. quinquefasciatus* during the study period within the filarial belt from September, 2016 to December, 2017.

Table 1. Geographic description of study sites.

Study Site No.	District	Locality	Geographical Description
01	Colombo	Maharagama	6° .8524 N 79° .9031 E
02	Colombo	Homagama	6° .8447 N 80° .0101 E
03	Colombo	Kirulapone	6° .8840 N 79° .8737 E
04	Kalutara	Wadduwa	6° .6627 N 79° .9285 E
05	Gampaha	Negombo	7° .2142 N 79° .8373 E
06	Galle	Galle city	6° .0334 N 80° .2176 E
07	Puttalam	Chilaw	7° .5755 N 79° .7911 E

referred as 'knockdown.' Mutations at the binding site of the voltage-gated sodium channel induce knockdown resistance (*kdr*) in vectors. (Vijverberg and Bercken 1990, Dong et al. 2014). In *Cx. quinquefasciatus,* the most common target site insensitivity is the L1014F *kdr* mutation in the *vgsc* gene, conferring resistance to DDT and PYs (Soderlund and Knipple 2003).

Though there are no targeted vector control programs against the main lymphatic filariasis vector *Cx. quinquefasciatus* in Sri Lanka at the moment, PY insecticides are widely used all over the country to control dengue vectors during epidemics. Although control measures are ongoing, the number of reported dengue cases increases continuously. The increase in reported dengue cases is suspected to have been due to the development of resistance to commonly used insecticides, making the insecticide application less effective. As a result of long-term intensive use of PY insecticides, non-targeted mosquito species like *Cx. quinquefasciatus* tend to develop resistance.

It has recently been reported that in Sri Lanka, there have been F1534C, V1016G, and S989P mutations in the *vgsc* gene of *Aedes aegypti* and F1534C in Aedes albopictus, which are the major mechanisms of pyrethroid resistance in Sri Lanka and neighboring countries. *Aedes albopictus*, which are the major mechanisms of pyrethroid resistance in Sri Lanka and neighboring countries (Hegoda et al. 2017, Fernando et al. 2018, Abeyrathna et al. 2019). Thus, the purpose of the study was to analyze the current status of resistance development of *Cx. quinquefasciatus* populations in the filarial belt to currently used insecticides in Sri Lanka and to analyze the mutant *kdr* alleles in the resistant populations.

MATERIALS AND METHODS

Mosquito collection

Larvae and pupae of *Cx. quinquefasciatus* were collected from polluted drains and wastewater tanks/containers using standard dipping and scooping techniques. The study was carried out from September, 2016 to December, 2017, in urban and semi-urban areas from seven selected locations. In each location, 25 study sites were selected for sample collection. These sites included previously highly reported LF areas, located in residential areas of Colombo, Gampaha, Kalutara, Galle, and Puttalam districts. Three localities from Colombo district and one locality from each above-mentioned district were selected (Figure 1) (Table 1). Collected larvae were reared to adulthood in the insectary at the Department of Zoology, University of Sri Jayewardenepura, Sri Lanka.

Insecticide susceptibility tests

Adult and larval susceptibility tests were conducted according to WHO standard procedures. In the adult susceptibility test, batches of 25 non-blood-fed 1-3 day-old adult females and males (4:1 ratio) were exposed to 0.05% deltamethrin, 0.75% permethrin and 5% malathion impregnated papers for 60 min in a standard WHO test kit. In the control experiment, adult mosquitoes were exposed to papers without insecticide. The mosquitoes and the control were held for a 24 h recovery period and mortality was recorded.

The percentage mortality of the larvae was tested for the WHO recommended dose 0.012 µg/ml for temephos. For a negative control, 1 ml of absolute alcohol was taken. Batches of 20 larvae were placed into each plastic cup containing temephos. The cups were kept for 24 h at 28° C, during the experiment. Mortality was recorded after 24 h. The experiment was carried out in five replicates.

Percent mortality was corrected using Abbott's formula (Abbott 1925) when the percent mortality from the control was greater than 5%. The susceptibility test results were interpreted according to WHO criteria as susceptible (>97% mortality), possible resistant (90-97% mortality), and resistant (<90% mortality).

Genomic DNA extraction and *kdr* mutation analysis

Genomic DNA was extracted from resistant and susceptible adult mosquitoes using a modified phenol chloroform extraction method (Ballinger-Crabtree et al. 1992). Extracted DNA was stored in 100 µl of Tris Ethylenediaminetetraacetic Acid (TE) buffer.

A fragment of the *vgsc* gene, containing the IIP- IIS6 region, was amplified by PCR, carried out in a 25 µl reaction volume containing 0.2 U *Taq* DNA polymerase, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1 X PCR buffer, and 0.5 µM of IIP_F and IIIS_R primers. The amplification consists of an initial heat activation step 95° C for 2 min, followed by 35 cycles of 95° C for 30 s, 63° C for 30 s and 72° C for 30 s with a final extension step at 72° C for 2 min. The amplified fragments were analyzed by electrophoresis on 1.5% agarose gel and visualized under UV light by ethidium bromide staining.

Successfully amplified fragments were sent to Macrogen Inc. (Macrogen Inc., Republic of Korea) in order to obtain the nucleotide sequences. DNA Baser v4.36.0 (DNA Sequence Assembler v4 (2013), Heracle BioSoft, www.DnaBaser.com), Multiple Sequence Alignment by CLUSTALW (*https://www.genome.jp*), and BioEdit version 5.0.6 software was used to assemble, align, and analyze sequence DNA data.

RESULTS

Insecticide susceptibility bioassay

Adult susceptibility test results showed percentage mortalities varied from 16% to 87%, from 8% to 79%, and from 89% to 100% for 0.75% permethrin, 0.05% deltamethrin (PY), and 5% malathion (OP), respectively. Samples obtained from all localities of the filarial belt showed high degree of resistance (<80% mortality) for PY adulticides, whereas OP adulticide showed susceptible for most of the study sites. Interestingly, mosquito populations of Maharagama showed resistance to the OP adulticide, malathion, with a mortality percentage of 89±0.9. Samples collected from Homagama (93±1.3), Galle (96±1.4), and Kirulapone (93±2.1) also exhibited possible resistance. No mortalities were observed among control experiments. Study site Maharagama showed significantly lower mortality rates of all tested insecticides except for adulticide 5% malathion when compared with other localities.

The larval susceptibility test indicated resistance to temephos in samples collected from all localities of filarial belt. The highest percentage of larval mortality was recorded in Negombo, which was 76.2% while the lowest was recorded from Maharagama which was 16.2% (Table 2). No mortality was recorded among control experiments.

***kdr* allele frequencies**

Fifty-six pyrethroid-exposed mosquitoes, consisting of four resistant (two permethrin resistant and two deltamethrin resistant) and four susceptible individuals per location, were randomly selected and genotyped for the L1014F mutation (Table 3). A 668-bp fragment of IIP-IIS6 region of the *vgsc* gene was amplified and sequenced. Analysis of the *vgsc* sequences showed a substitution of A to T at the third position of codon 1014, which results in the substitution of leucine (L) with phenylalanine (F), known as the L1014F mutation.

Among sequenced *Cx. quinquefasciatus* populations, homozygote-susceptible genotype (SS) (TTA) and homozygote-resistant genotypes (TTT and TTC) were

Table 2. Percentage mortalities with their respective standard deviation (SD) values for each locality for each tested insecticide.

Location	Percentage Mortality ± SD			
	Larvicide	Adulticides		
	Temephos	0.75% Permethrin	0.05% Deltamethrin	5% Malathion
Maharagama	16.2±2.5	16±3.3	8±3.3	89±0.9
Homagama	65±4.1	87±2.1	69±8.9	93±1.3
Kirulapone	17.5±3.5	41±0.9	62±1.3	96±1.4
Negombo	76.3±4.8	50±1.3	53±1.7	99±1.7
Wadduwa	35±4.1	57±2.5	36±1.6	97±0.9
Galle	45±4.7	71±1.7	79±1.3	93±2.1
Chilaw	51.3±2.5	41±0.9	49±1.7	100±0.7

Table 3. Genotype distribution of *kdr* mutations in samples from the filarial belt.

Location	Susceptible/ Resistant	Number tested	Genotypes					
			TTA (SS)	TTA/C (RS)	TTC (RR)	TTA/T (RS)	TTC/T (RR)	TTT (RR)
Maharagama	Susceptible	4	3	-	-	1		
	Resistant -D	2						2
	Resistant -P	2						2
Homagama	Susceptible	4	4	-				
	Resistant -D	2				1		1
	Resistant -P	2			1		1	
Kirulapone	Susceptible	4	3	-	-			1
	Resistant -D	2					1	1
	Resistant -P	2					2	
Negombo	Susceptible	4	4	-	-			
	Resistant -D	2						2
	Resistant -P	2				1		1
Wadduwa	Susceptible	4	4	-	-			
	Resistant -D	2						2
	Resistant -P	2					1	1
Galle	Susceptible	4	4	-				
	Resistant -D	2			1	1		
	Resistant -P	2					2	
Chilaw	Susceptible	4	4	-	-	-		
	Resistant -D	2						2
	Resistant -P	2				1		1

SS, homozygote-susceptible genotype; RS, heterozygote-susceptible / resistant genotype; RR, homozygote-resistant genotype; D, 0.05% deltamethrin; P, 0.75% permethrin.

recorded at all study sites (Table 3). Considering the two types of homozygote-resistant genotype, *Cx. quinquefasciatus* populations belong to Homagama and Kirulapone, and Wadduwa represents both of them (TTT and TTC/T). *Culex quinquefasciatus* belongs to Negombo, and the Chilaw study sites shows only the TTT type homozygote-resistant genotype. Only one type of heterozygote-susceptible genotype (TTA/T) was recorded and it was recorded among *Cx. quinquefasciatus* populations of Maharagama, Homagama, Negombo, Chilaw, and Galle.

The frequency of the genotypes is presented in Table 4. A high proportion of individuals with susceptible phenotype was scored as SS genotype (TTA). A high proportion of individuals with resistant phenotype was also scored as homozygote-resistant genotype (TTT).

DISCUSSION

Culex quinquefasciatus is an important vector of filariasis in Sri Lanka. We investigated the status of resistance of *Cx. quinquefasciatus* to the most commonly used insecticides for vector control and understanding the underlying resistance mechanisms in order to guide the use of these chemicals and their efficacy as vector control tools.

The susceptibility test results of *Cx. quinquefasciatus* larvae in all seven mosquito populations showed mortality percentages well below 80%. Considering WHO resistance levels according to the mortality percentages, *Cx. quinquefasciatus* larvae in the filarial belt express the existence of resistance to temephos larvicide. Larval mortality percentages less than 90% confirm the existence of resistant genes for temephos larvicide (OP). Different mortality percentages for different study sites could be attributed to the variations in larvicide application among different areas.

Table 4. Genotype frequencies at the L1014F in the voltage-gated sodium channel gene of the sampled *Cx. quinquefasciatus.*

	TTA	TTA/T	TTC	TTC/T	TTT
From total population	0.464	0.089	-	0.161	0.285
Maharagama	0.375	0.125	-	-	0.5
Homagama	0.5	0.125	0.125	0.125	0.125
Kirulapone	0.375	-	-	0.375	0.25
Negombo	0.5	0.125	-	-	0.375
Wadduwa	0.5	-	-	0.125	0.375
Galle	0.5	0.125	0.125	0.25	-
Chilaw	0.5	0.125	-	-	0.375

Extremely low mortality percentages were recorded from Maharagama (16.25±2.50) and Kirulapone (17.5±3.45) areas. These observations are consistent with those reported in the world by several researchers, who reported that a high level of resistance was acquired in urban larval populations of *Culex* sp. after continuous treatment with organophosphates (Tmimi et al. 2018).

All selected localities expressed mortalities below 90% for both 0.05% deltamethrin and 0.75% permethrin in mosquito susceptibility tests. This confirms the existence of resistant genes in the test populations against PYs insecticides. Dissimilarities among localities for deltamethrin and permethrin may be due to variations in exposure to different insecticides. Highly urbanized areas are exposed to the households as well as to control measures taken by urban councils and Ministry of Health (MOH) offices. Household insecticidal applications such as aerosols, mosquito coils, mosquito vaporizers, and various types of natural as well as artificial repellents contain permethrin, *d*-tetramethrin, and esbiothrin-like PY compounds. Control measures taken by urban councils and MOH offices include space application of synthetic insecticides, especially PYs. Due to high mosquito densities and current epidemics of dengue, dense urban areas such as Maharagama are exposed to both types of above-mentioned control measures that lead to the development of resistance compared to the less insecticide-exposed semi-urban and rural areas, such as Homagama.

Considering the three types of insecticides, a significant difference between PY and OP groups of insecticides was observed at the 95% confidence level. This is due to differences in modes of action and resistance mechanisms of two different insecticide groups. Pyrethroids act on the insect nervous system, targeting the *vgsc* and disrupting the activation and inactivation of *vgsc* channels leading to repetitive firing in motor and sensory axons (Li et al. 2012). Insecticides of the OP group are responsible for the inhibition of the acetylcholinesterase (AChE) enzyme that is needed to degrade acetylcholine (ACh), the excitatory neurotransmitter. Due to inhibition of AChE nerve impulses, transmission through cholinergic synapses gets terminated, leading to overexcitation and death (Siegfried and Scharf 2001). Susceptibility test results suggest that permethrin and deltamethrin adulticides have comparatively lower efficacy in controlling *Cx. quinquefasciatus* mosquitoes in the filarial belt. In similar studies, it was shown that permethrin and deltamethrin no longer effectively control the major dengue vectors *Ae. aegypti* and *Ae. albopictus* in Sri Lanka (Fernando et al. 2018, Abeyrathna et al. 2019, and Hegoda et al. 2017).

According to genotype distribution of *kdr* in samples from the filarial belt, all *Cx. quinquefasciatus* mosquitoes that were susceptible to PY expressed the homozygote-susceptible genotype and those not susceptible to PY expressed resistant genotypes. During our study, the TTA- homozygote-susceptible genotype, TTA/T- heterozygote-resistant genotype, and TTC/T, TTC, and TTT types of homozygote-resistant genotypes were identified.

The L1014F mutation occurring at the IIS6 in the *vgsc* was the first *kdr* mutation to be discovered and associated with insecticide resistance (Rinkevich et al. 2013). The mutation was first discovered in *Musca domestica*. Pyrethroid-resistant populations have revealed several substitutions (C, H, S, or W) at position 1014 (Ponce et al. 2016). It has been known that L1014F provides variable levels of resistance to Type I and Type II pyrethroids and DDT. Evidence for resistance associated with permethrin (Type I) has been found in Alabama, U.S.A. (Xu et al. 2005) and Mexico (Ponce et al. 2016), whereas resistance to deltamethrin (Type II) has been verified in China (Chen et al. 2010).

A study carried out with 43 colonies of *Cx. quinquefasciatus,* including susceptible (S-Lab) and resistant ($HAmCg^{G9}$) strains, has revealed a clear correlation between the prevalence of the allelic expression of A or T in the L to F *kdr* at the RNA level and their susceptibility level to permethrin. The prevalence of substitutions at the codon 1014 has been previously analyzed in Sri Lankan *Cx. quinquefasciatus* populations (Roberts and Andre 1994). The study revealed the presence of both A-to-C and A-to-T mutations. However, A-to-C mutation revealed a high predominance over A-to-T mutation in the particular study (Roberts and Andre 1994). It is of interest to observe that in the present study, the predominance levels have changed with A-to-T mutation being the predominant.

Considering the lymphatic filariasis statuses in Sri Lanka, WHO validates Sri Lanka as a country that has eliminated lymphatic filariasis as a public health problem due to a less than 1% microfilaria rate. The neighboring countries of Sri Lanka, such as India, have become a major concern of filariasis, with around 250 endemic districts and around 600 million affected people. Since a reasonable amount of migration occurs every year between Sri Lanka and India, Sri Lanka is at a high risk for re-emerging lymphatic filariasis due to parasite re-establishment from India. If the country has to face such a situation, it will be difficult to control vector populations due to their development of resistance.

The results of the present study suggest that there are serious problems in the vector control activities which are currently undertaken in the filarial belt of Sri Lanka. Due to the absence or very low rate (less than 1%) of microfilaria among filarial vector populations in Sri Lanka, vector population reduction programs have been neglected. Rapid unplanned developments, large-scale agriculture, poorly managed wastewater, blocked drainage systems, and less attention to vector control programs contribute to an increasing abundance of the main lymphatic filariasis vector, *Cx. quinquefasciatus,* in many areas of Sri Lanka. Abundant *Cx. quinquefasciatus* mosquitoes showed increased resistance to currently used insecticides, and spreading microfilaria from migrant workers traveling to and from neighboring countries where microfilaria levels are high increases the risk of reemergence of LF in Sri Lanka.

This study that was conducted in the filarial belt provides further evidence that control programs have not been successful in maintaining mosquito populations at low densities, since *Cx. quinquefasciatus* was predominant. The continued use of PY insecticides in such areas has decreased the efficacy of the insecticide. It is expected that the results of the present study will help health authorities to use appropriate insecticides to delay the onset of resistance and to get the maximum effect through space-spraying. Also, an assessment of the resistant status of *Cx. quinquefasciatus* populations is important, considering reemerging conditions of filariasis and possible invasion of the newly emerging Zika virus.

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