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Detection of *kdr* mutations in a laboratory-reared *Aedes aegypti* colony

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Abstract

Insecticide resistance poses a significant challenge to vector control efforts targeting *Aedes aegypti*. This mosquito is considered the primary vector for arboviruses such as dengue, Zika and yellow fever. This study investigates the resistance status and occurrence of knockdown resistance (*kdr*) mutations present in a laboratory-reared *Ae. aegypti* colony. At present two *kdr* mutations have been discovered with increasing frequency within Sri Lankan wild *Ae. aegypti* mosquito populations. These include F1534C, a mutation converting phenylalanine to cysteine at the position 1534 and V1016G, a mutation converting valine into glycine at the position 1016. To determine the presence of F1534C and V1016G mutations within the laboratory-reared mosquitoes, ten randomly selected mosquito samples from a colony reared in the laboratory for over forty generations were obtained. DNA extraction was performed according to the modified Ballinger - Crabtree (1992) protocol, followed by allele specific PCR (AS-PCR). Resulting PCR products were visualized using a 3% agarose gel. The AS–PCR technique effectively distinguished individual haplotypes of the two mutations. Wild type, mutant and heterozygous alleles were observed in the gel images. Results revealed the presence of both cysteine and glycine mutations. The findings suggest that the original mosquito population that was reared in the laboratory may have had the mutations but due to the lack of insecticide pressure the mosquitoes have tend to reverse their mutations and change back to their wild type thus making them susceptible to insecticides.

Keywords: *Aedes aegypti*, *kdr* mutation, Insecticide resistance, Laboratory-reared mosquitoes

1. Introduction

Viruses transmitted by mosquitoes are a primary health concern leading to the loss of human life on a global scale. Diseases commonly transmitted by mosquitoes are dengue and yellow fever which have made a major global impact during the last few years.[1] Dengue is considered an acute mosquito-borne viral disease transmitted by the principal vector *Aedes aegypti*. The *Ae. aegypti* mosquito is a species belonging to the genus that breeds in close association with humans and is often responsible for transmitting various arboviruses.

Ae. aegypti mosquitoes transmit dengue fever by biting humans infected with the dengue virus and then biting uninfected humans, thus transmitting the virus.[2] This mosquito was originated in Africa and later spread to tropical and sub-tropical areas particularly in urban and semi urban areas throughout the world. *Ae. aegypti* is a small, dark colored mosquito with white markings covering the abdomen and thorax area with alternating light and dark bands on its legs. Its life cycle consists of four stages including egg, larva, pupa and adult.[3]

According to the World Health Organization, the global incidence of dengue has increased a tenfold surge over the past two decades. Currently, dengue is reported in 129 countries and is influenced by climate, population density, mosquito vector abundance and urbanization. The key factors associated with the increased risk of dengue epidemic include the increasing change in distribution of the vectors including *Ae. aegypti* and *Ae.*

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albopictus in naïve countries and the lack of vaccines that help cure the disease.[4]

At present, many control methods are employed to minimize the population of *Ae. aegypti* mosquito vectors to reduce the spread of arboviruses among the human population. These methods mainly include source reduction and application of insecticides. Source reduction includes the elimination of *Ae. aegypti* mosquitoes by removing their potential breeding sites in domestic and peri–domestic areas. According to their lifestyle characteristics, these mosquitoes prefer to lay eggs in artificial water containers found around human habitation, such as discarded containers, flower pots, tires and any other water collecting materials that can serve as a breeding ground for mosquitoes[5]. Other sources include gutters and drains that can accumulate stagnant water due to improper waste management.[6] Insecticides belonging to the chemical classes of pyrethroids, carbamates, organophosphates and dichlorodiphenyltrichloroethane (DDT) are used for chemical control of mosquitoes.[7] Even though the use of insecticides helps eradicate and control vector populations, lack of planning and common usage of these insecticides has caused these mosquitoes (*Ae. aegypti*) to develop resistance towards them which is a major disadvantage.[8] Several mechanisms have been linked to the development of resistance such as altered behavior, enhanced detoxification, cuticular penetration and altered target sites.[9]

Knockdown resistance *(kdr)* is a form of target site insensitivity and is a major mechanism responsible for reduced susceptibility to pyrethroid insecticides in *Ae. aegypti*. The primary target of pyrethroid insecticides is the voltage gated sodium channel *(vgsc)*. Single or multiple mutations appearing in the *vgsc* are responsible for the decreased target site insensitivity for pyrethroid insecticide leading to the knockdown resistance (*kdr*).[10] In *Ae. aegypti*, eleven *kdr* mutations responsible for pyrethroid resistance have been identified and these mutations vary depending on the geographical spread of the mosquito, frequency of the insecticide application, and effect it has on the resistance phenotype. However, among the eleven mutations, only five have been linked to functional resistance in pyrethroids, namely, F1534C, V1016G, S989P, I1011M and V410L. At present in Sri Lanka, three *kdr* mutations have been discovered in increased frequency within *Ae. aegypti* mosquito populations including F1534C which is the conversion of phenylalanine to cysteine at the position 1534, V1016G which is the conversion of valine into glycine at the position 1016 and S989P which is the conversion of serine to proline.[11] However, it has been suggested that once the application of insecticides ceases, mosquitoes tend to reverse their mutation. The objective of the current study was to determine the insecticide resistance status and the presence of two major *kdr* mutations F1534C and V1016G in *Ae. aegypti* mosquito colonies that were reared in the laboratory for over forty generations without any insecticide pressure. Monitoring resistance to commonly used insecticides and understanding their underlying mechanisms within the *Ae. aegypti* mosquito population are critical in the management of disease transmission, resource optimization, and development of effective control methods and management strategies worldwide.[12]

2. Methodology

2.1 Mosquito samples. Ten samples were collected from Ae. aegypti colonies which were reared in the Insectary, Centre for Biotechnology, University of Sri Jayewardenepura, Sri Lanka for over forty generations without any insecticide pressure.

2.2 DNA extraction. DNA extraction was carried out according to the modified Ballinger Crabtree (1992) protocol with an additional Phenol-Chloroform step. The obtained DNA pellets were dissolved in 100µl of TE buffer.[13]

2.3 F1534C mutation. To detect F1534C mutation, allele specific PCR (AS-PCR) was carried out.[14] Each PCR reaction was performed in a total volume of 25 µl consisting of 12.5 µl Promega master mix, 2.0 µl DNA, 0.625 µl of common reverse primer (5'-TCTGCTCGTTGAAGTTGTCGAT-3'), and two forward primers; 0.625 µl F1534F (5'-GCGGGCTCTACTTTGTGTTCTTCATCAT ATT-3') and 0.2 µl of C1534F (5'-GCGGGCAGGGCGGCGGGGGCGGGGCC TCTACTTTGTGTTCTTCATCATGTG-3').

The thermal cycling conditions followed were initial denaturation for 2 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C, extension for 30 seconds at 72°C with a final extension step for 2 minutes at 72°C.

2.4 V1016G mutation. To detect V1016G mutation, AS-PCR was conducted. Each PCR reaction contained a 25 µl total volume consisting of 12.5µl Promega master mix, 2.0 µl DNA, 0.625 µl common forward primer (5'-ACCGACAAATTGTTTCCC-3'), two reverse primers; 0.3125 µl G1016R (5'-GCGGGCAGGGCGGCGGGGGCGGGGCC AGCAAGGCTAAGAAAAGGTTAACTC-3') and 0.3125 µl V1016R (5'-GCGGGCAGCAAGGCTAAGAAAAGGTT AATTA-3'). The thermal cycling conditions followed for the above AS–PCR process was initial denaturation for 2 minutes at 94°C followed by 35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, extension for 30 seconds at 72°C with final extension for 2 minutes at 72°C.

Results for both F1534C and V1016G were visualized by agarose gel electrophoresis. PCR products were loaded into 3% agarose gel containing TBE buffer solution and the electrophoresis was conducted for 45 minutes at 100V with a 50 bp DNA ladder.

3. Results and Discussion

Figure 1 and Figure 2 show the visualization of the amplicons in a 3% agarose gel electrophoresis, run under 100V for 45 minutes and stained with ethidium bromide. Figure 1 shows the banding pattern obtained for F1534C mutation. Heterozygotes are presented by the presence of two bands corresponding to both the wild type and mutant alleles. Amplicons approximately close to 93bp were considered to be the wild type whereas the amplicons closer to 113bp were considered to be the mutant allele. Additionally, a 50 bp DNA ladder was included as a reference for size estimation. According to Figure 1 starting from the left contains the 50bp DNA ladder followed by L1 containing the mutant allele (C/C) and the rest of the lanes L2, L3 L4 containing heterozygous alleles (F/C), and no band was visible in the L5 lane since it is the negative control. Figure 2 Shows the banding pattern obtained for V1016G mutation. Amplicons approximately close to 60bp were considered to be the wild type, while amplicons closer to 80bp were considered to be the mutant alleles. The 50bp DNA ladder was placed in the left corner followed by four lanes L1, L2, L3 and L5 containing wild type homozygous alleles (V/V) and lane L4 containing heterozygous allele (V/G).

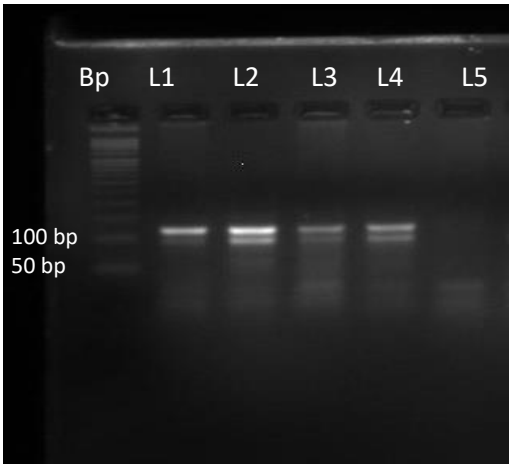

Figure 1. Gel electrophoresis results of F1534C. Two of the three genotypes are shown from left to right: mutant homozygous (C/C) in L1, heterozygous (F/C) in L2, L3, L4 and negative control on L5. The lane to the far left contains DNA ladder (bp).

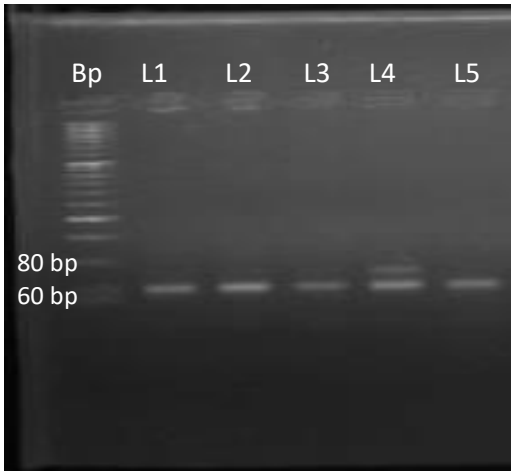

Figure 2. Gel electrophoresis results of V1016G. Two of the three genotypes are shown from left to right: wild type homozygous (V/V) in L1, L2, L3, L5, heterozygous (V/G) in L4. The lane to the far left contains DNA ladder (bp).

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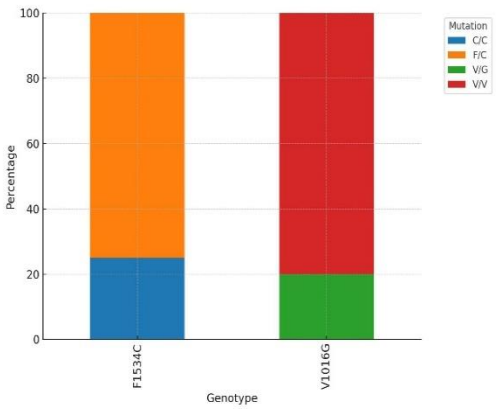

Figure 3. Percentages of haplotypes recorded in F1534C and V1016G. Two of the three genotypes are shown in F1534C; C/C mutant homozygous 25%; F/C heterozygous 75%. Two of the three genotypes are shown in V1016G; (V/V) wild type homozygous 80%; (V/G) heterozygous 20%.

According to Figure 3, among 10 samples 25% of the population had homozygous mutant allele (C/C) and 75% of the population had heterozygous allele (F/C) in F1534C mutation and in V1016G mutation, 80% of the population had wild type homozygous allele (V/V) and 20% of the population had heterozygous allele (V/G).

The resistant allele frequency was calculated using the Hardy-Weinberg equilibrium. The frequency obtained for F1534C mutation was 0.5 while frequency value obtained by V1016G mutation was 0.1. Since *kdr* mutations usually result from an evolutionary response to selective pressure from insecticide exposure, their incidence of mutations is expected to be low or absent in laboratory-reared mosquitoes that have not been exposed to any insecticides and would tend to reverse their mutations thus becoming susceptible to the insecticides.[15]

The current study was performed to identify the presence of *kdr* mutation and the occurrence of insecticide resistance in *Ae. aegypti* laboratory-reared mosquito colonies. *Ae. aegypti* is considered as the vector of many arboviral diseases such as dengue, Zika and yellow fever. Even though use of insecticides has been found to be effective in eliminating the presence of these viral mosquitoes lack of planning and common usage have caused the mosquitoes to develop resistance, making them no longer effective.

In this study ten samples of *Ae. aegypti* mosquitoes were obtained to determine the insecticide resistance status and occurrence of two *kdr* mutations such as F1534C and V1016G in laboratory-reared mosquitoes. *Kdr* mutations in *Ae. aegypti* are primarily associated with resistance to pyrethroid insecticides. Since *kdr* mutations usually result from an evolutionary response to selective pressure from insecticide exposure, their incidence of mutations is low to absent in laboratory reared mosquitoes that have not been subjected to any insecticides. However, it is not impossible for *kdr* mutations to occur spontaneously due to genetic variation or other factors but nonetheless it would occur at a very low frequency compared to mosquito populations under insecticide pressure.[17] Resistant allele frequencies obtained for F1534C and V1016G showed very low frequency levels such as 0.5 and 0.1. It could be assumed that the original mosquito population which was reared in the laboratory may have contained these two mutations, however, overtime due to zero insecticide exposure the mosquitoes have tend to reverse back to their wild type making them more susceptible for insecticides. Nevertheless, even though genetic mutations in mosquitoes may provide an advantage in surviving environments with high insecticide pressure they can also impose fitness costs on the mosquitoes.[18] Fitness cost is the adaptive process in which it leads to the survival and reproductive success of individuals exposed to a natural or induced adverse conditions such as exposure to insecticides which eventually leads to loss of biotic potential. Such changes that can be observed within *Ae. aegypti* mosquito populations are vulnerability to predation, reduced competitive potential among male mosquitoes, increased development time, decrease size of individuals, reduced flight range, reduce reproductive success including lower mating efficiency and egg production and overall reduced survival rates. Thus, in the absence of insecticide pressure, mosquitoes will reverse their resistance mechanisms.[19]

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4. Conclusion

The study revealed the presence of *kdr* mutations at low frequencies in *Ae. aegypti* mosquitos that were reared in the laboratory for over forty generations. Long term rearing of the mosquitoes without any insecticide pressure has caused the mosquitoes to reverse their mutations and revert back to their wild type thus making them susceptible for insecticides.[20] Nevertheless, regular monitoring and management strategies such as use of different insecticides must be implemented to detect and prevent widespread of insecticide resistance within the laboratory-reared mosquito population.[21]

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