Research Article Available online www.ijsrr.org ISSN: 2279–0543

International Journal of Scientific Research and Reviews

Molecular Analysis of *kdr* Mutation infield Populations of major filarial vector in Kerala

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ABSTRACT:

Knockdown resistance (*kdr)*, describes cases of resistance to insecticides like pyrethroids and DDT in insects and other arthropods. The point mutations in the *kdr* gene results reduced sensitivity of the nervous system towards the insecticides and confer resistance towards the specific insecticide. It has been shown to be a common resistance mechanism due to the continuous exposure towards the insecticides. The current study envisage to assess and analyze mutation in the knock down resistance gene of *Culex quinquefasciatus* of Kozhikode, Cochin, Malappuram, Thrissur, and Palakkad town areas of Kerala, India. Molecular assays using AS-PCR confirmed the presence of *kdr* mutations in the study areas with varying proportions. The investigations provide clear understanding of the status of insecticide resistance in some of the major cities in Kerala that can yield to revitalizing the mosquito control programmes in these areas.

KEYWORDS: *Culex quinquefasciatus,* insecticide resistance, *kdr* mutation

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INTRODUCTION:

The development of resistance in vector species, and emergence of new resistant genotypes among the vector population has been a major setback in the global efforts to control vector-borne diseases[1]. According to the World Health Organization, insecticide resistance is "the biggest single obstacle in the struggle against vector-borne diseases" [2]. Many studies have shown that multiple and complex resistance mechanisms, especially increased metabolic detoxification of insecticides and decreased sensitivity of the genes or target proteins are responsible for insecticide resistance. Gene over-expression and amplification, and mutations in protein-coding gene regions, has also frequently been implicated[3].

Alterations of amino acids responsible for insecticide binding at the site of action cause the insecticide to be less effective or even ineffective of its action. The target of organophosphorous (temephos, malathion, fenitrothion) and carbamate (propoxur, sevin) insecticides is acetylcholine esterase in nerve synapses, and target of organochlorines (DDT) and synthetic pyrethroids are the sodium channels of the nerve sheath.

Synthetic pyrethroids and DDT act as axonicexcitotoxins that prevent the closure of the voltage-gated sodium channels of the axonal membranes causing paralysis and thereby death of the insect[4]. Pyrethroids account for approximately 25% of the world insecticide market and used in coils, mats, aerosols, IRS and ISS. Pyrethroids are popular due to their very low toxicity in humans, and rapid killing effect on the insect[5]. Pyrethroids are the only class of insecticides approved for treating mosquito nets as they are safe for humans [6].

RESEARCH METHODS & MATERIALS:

Allele - specific PCR (AS-PCR) for detecting knock down resistance (*kdr*) mutation:

Primer selection: Four primers (Primer 1, 2, 3 and 4) were selected from the region II of para-type voltage gated sodium channel (vgsc) gene of *Cx. pipiens*[7, 8]. Two primers [Primer 1 (forward) 5´-GTGGAACTTCACCGACTTC - 3´and Primer 2 (reverse) 5´-GCAAGGCTAAGAAAAGGTTAAG- 3´] were used to amplify the fragment of sodium channel gene containing the *kdr* mutation site. The other two primers [Primer 3 (forward) 5´CCACCGTAGTGATAGGAAATTTA- 3´ and Primer 4 (forward) 5´CCACC GTAGTGATAGGAAATTTT- 3´] were allele-specific primers used in genotyping of knockdown susceptible (Primer 3) and knockdown resistant (Primer 4) alleles by allele-specific PCR assay. The

allele-specific primers were identical except at the 3´-OH end where 'A' in Primer 3 was replaced by 'T' in Primer 4. Both primers 3 and 4 could amplify a 380bp corresponding region.

AS-PCR Assay: The PCR was performed according to Martin-Torres *et al.,* protocol [7] with modifications to detect *kdr* mutation in the mosquito population. Two PCR reactions were run in parallel. One reaction contained the primers 1, 2 and 3 (10 pmol each). In the other reaction primer 3 was replaced by primer 4. 10 ng of mosquito DNA was added as template in each reaction.

The PCR conditions were 5 min at 94 °C for the first cycle, followed by 1 min at 94 °C, 2 min at 49 °C and 2 min for 72 °C for 29 cycles, and 10 min at 72 °C for the final extension.The DNA fragments were separated by electrophoresis on 1.5% agarose gel and were visualized by ethidium bromide staining under UV light. The presence of 380bp band corresponding to resistant and susceptible specific primers revealed the genotype of the mosquitoes.

Detection of site specific mutation in *kdr* gene: The DNA isolated from the different locations were amplified the fragment of sodium channel containing *kdr* gene. Allele specific primers used in genotyping of knock down resistant (*kdr*) and knock down susceptible (*kds*) alleles by allele-specific PCR assay (AS-PCR). The AS-PCR assay revealed the presence of leucine – phenylalanine *kdr* mutation in the field strains of *Cx. quinquefasciatus*. PCR assay showed three genotypes, identified by the characteristic 380bp band corresponding to resistant and susceptible specific primers. The 380bp PCR product with both the knock down specific [*kds* (primer3) and *kdr* (primer4)] primers in an individual mosquito indicates heterozygous condition (SR). The appearance of this band only in susceptible specific primer (*kds* - primer3) indicates homozygous susceptible (SS) and in resistant specific primer (*kdr* - primer4) indicates homozygous resistant (RR).

RESULTS & DISCUSSION:

The result shows (Table 1 and plate A, B&C) that out of 20 samples taken CLT and EKM samples have 5 homozygous resistant mosquitoes. From MPM out of 20 samples evaluated 1 homozygous resistant, 4 heterozygous resistant and 15 homozygous susceptible genotypes obtained. The homozygous resistant genotypes obtained from PKD and TCR were 4 and 3 mosquitoes respectively. The number of homozygous susceptible genotypes obtained in the mosquitoes found in MPM, PKD, TCR, CLT and EKM area were 15, 11, 10, 7 and 6 respectively. The CLT population having 7 homozygous susceptible, 8 heterozygous resistant and 5 homozygous resistant mosquitoes out of the 20 mosquitoes taken for the experiment.The EKM population having a higher number of heterozygous resistant mosquitoes and MPM having the least number of resistant mosquitoes.

Knock-down resistance due to a point mutation (designated the L to F *kdr* mutation) in the voltage gated sodium channel is a common mechanism of resistance to pyrethroids. Simple and reliable techniques are in great need to detect and monitor pyrethroid resistance among mosquito populations in the field. Allele specific polymerase chain reaction (AS-PCR) method is used to detect the L to F *kdr* mutation in the mosquito *Cx. quinquefasciatus*.

After optimizing experimental conditions, AS-PCR could effectively distinguish individual mosquitoes that were homozygous or heterozygous for the mutations. The results indicate that all the mosquito populations showing resistance towards pyrethroids. As a conclusion of the present study, the, target site mutations in *kdr* gene were observed in *Cx. Quinquefasciatus* from the study populations. This is the first description showing evidence of the existence pyrethroid insecticide resistance mechanisms in *Cx. Quinquefasciatus* in Kerala. The pattern of resistance and the mechanisms involved can be expected to have a number of implications on resistance management strategies.

The observation made on the incidence of *kdr* mutation may also be considered seriously as pyrethroids are used for IRS and ISS for the immediate control of mosquito populations in areas reporting high incidence of lymphatic filariasis cases. The *kdr* mutation renders the *Cx. quinquefasciatus* populations resistant to the common household control measures used, as pyrethroids are the common constituent of mosquito mats, coils and repellents.

Table 1: Genotypes of *Cx. quinquefasciatus* collected from the laboratory and field populations predicted by AS-PCR

Area	Total no of samples	AS PCR		
		SS (500 bp, 380bp cq3)	**SR (500bp, 380bp cq3 & cq4)**	**RR (500bp, 380 bp cq4)**
LAB	20	20	-	-
CLT	20	7	8	5
EKM	20	6	9	5
MPM	20	15	4	1
PKD	20	10	6	4
TCR	20	11	7	3

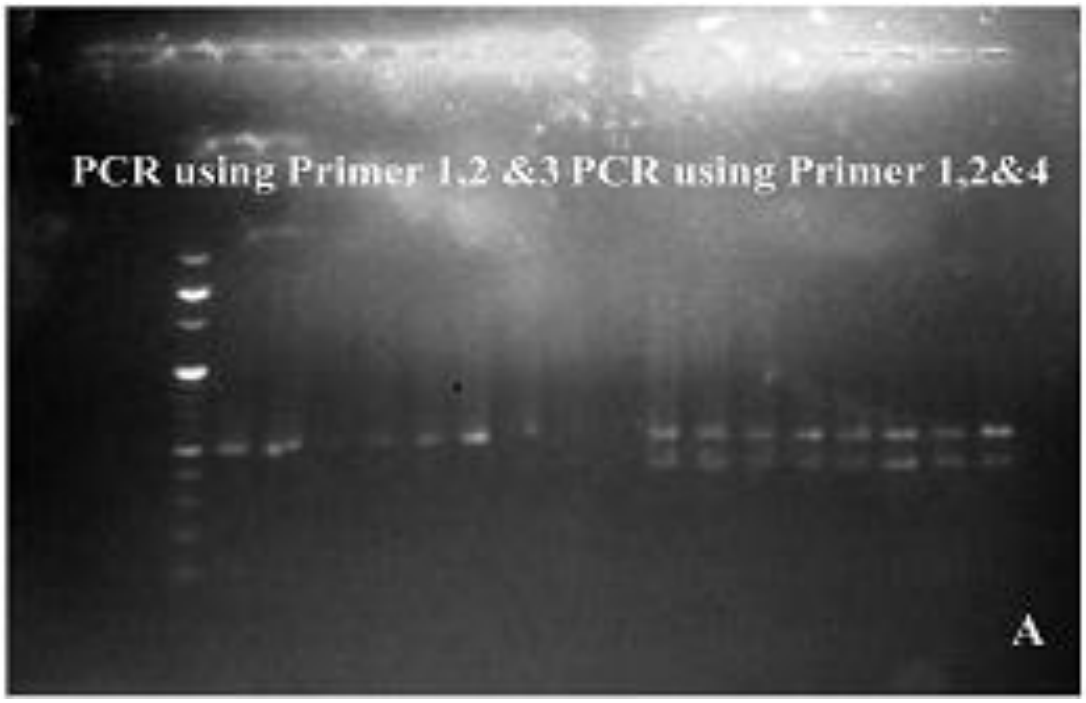

Figure 1: Agarose gel showing the *kdr* genotypes of *Cx. quinquefasciatus* after Allele specific PCR: Homozygous susceptible genotype

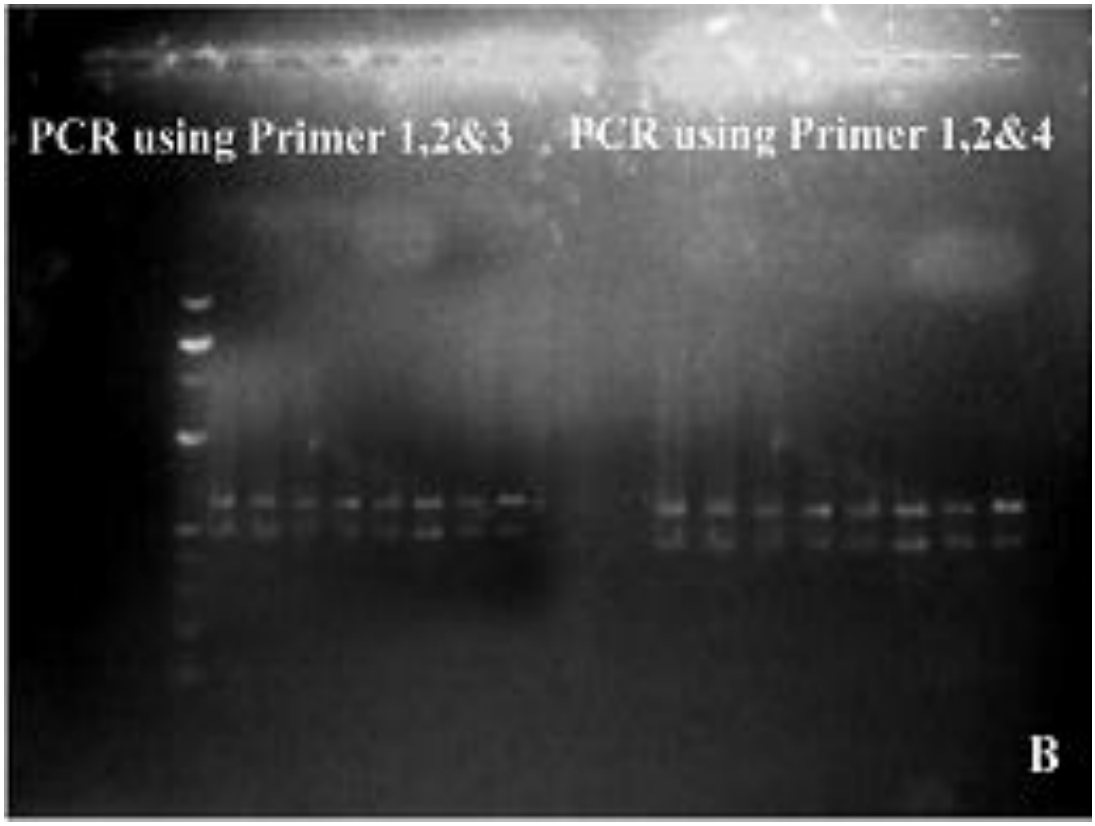

Figure 2: Agarose gel showing the *kdr* genotypes of *Cx. quinquefasciatus* after Allele specific PCR: Heterozygous resistant genotype

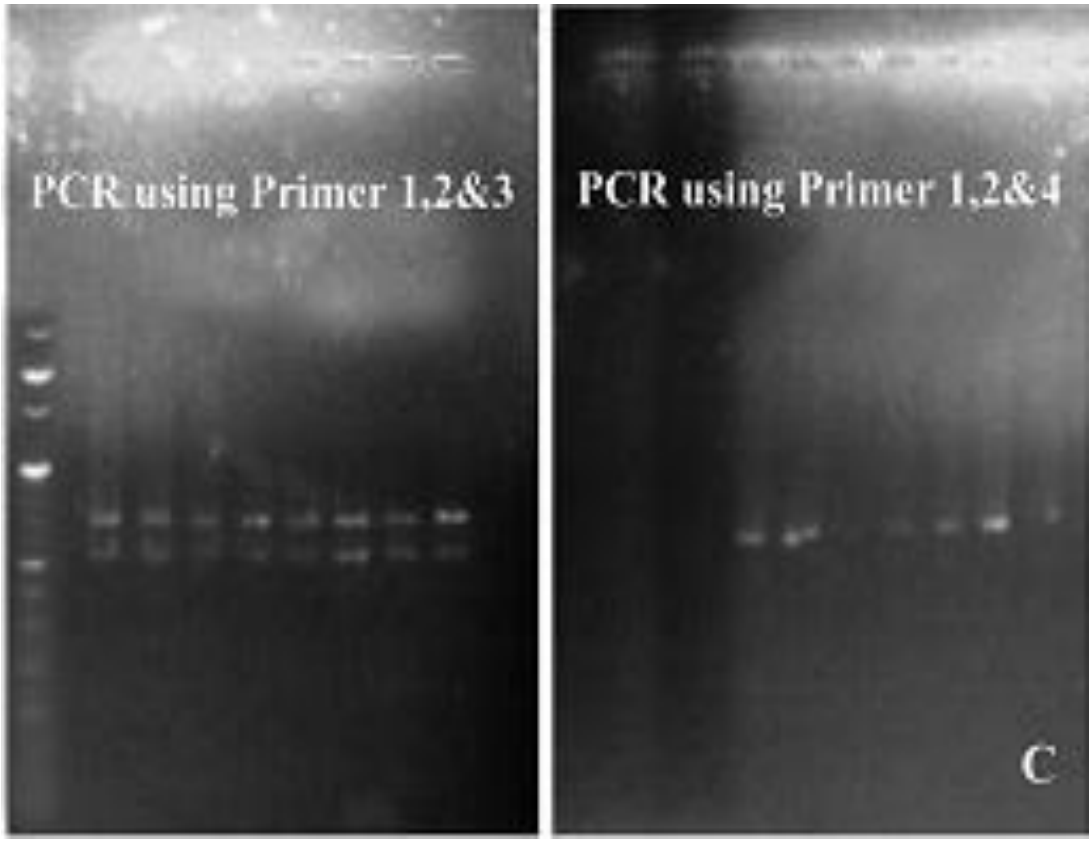

Figure 3: Agarose gel showing the *kdr* genotypes of *Cx. quinquefasciatus* after Allele specific PCR: Homozygous resistant genotype

The observation made on the incidence of *kdr* mutation may also be considered seriously as pyrethroids are used for IRS and ISS for the immediate control of mosquito populations in areas reporting high incidence of lymphatic filariasis cases. The *kdr* mutation renders the *Cx. quinquefasciatus* populations resistant to the common household control measures used, as pyrethroids are the common constituent of mosquito mats, coils and repellents.

Various insecticide selection pressures particularly from agriculture and domestic use of insecticides were suspected to be the cause of resistance to the insecticides. It also appears that the presence of urban pollutants in mosquito breeding sites probably has a direct or indirect impact on mosquito resistance. For this reason, proper management of waste, particularly in urban areas, and effective regulation of use of pesticides appear to be critical in resistance management programs. One way is to reduce the mosquito population, especially in urban areas where most of the important larval habitats have been shown to be anthropogenic[8] and such habitats can easily be managed through proper waste management, proper construction of drains and the change of the inhabitants' behaviour through proper education. Hence proper management and control measures are necessary for the control of vector mosquitoes.

CONCLUSION:

The observation made on the incidence of *kdr* mutation may also be considered seriously as pyrethroids are used for IRS and ISS for the immediate control of mosquito populations in areas reporting high incidence of lymphatic filariasis cases. The *kdr* mutation renders the *Cx. quinquefasciatus* populations resistant to the common household control measures used, as pyrethroids are the common constituent of mosquito mats, coils and repellents.

The most suitable strategy for controlling disease vectors especially mosquitoes, the rotational use of insecticides of different modes of action altogether, rather than merely alternating members of any one chemical class or different chemical classes that address the same target site. For example, the presence of *kdr* resistance renders DDT and pyrethroids less effective, whereas carbamates such as bendiocarb, or organophosphates can still be used, but the presence of modified *ace* warns the regular usage of organophosphates in the field[9].

The evidence of development of resistance to synthetic insecticides in mosquitoes observed in the present study points to the need of employing new phytochemicals in the field as an alternative to synthetic chemical pesticides which would be easily degradable and have less harmful effect on other organisms.

ACKNOWLEDGMENT:

Authors are thankful to UGC BSR for providing financial support and UGC SAP Laboratory, Department of Zoology, University of Calicut for providing instrumentation facilities to carry out this work.

CONFLICTS OF INTEREST:

The authors have no conflicts of interest.

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