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Characterization of Insecticide Resistance Mechanisms Through *kdr* Gene Analysis in Wild Mosquito *Anopheles Culicifacies*

M Bhaskar[1]*; S Sravani[1]; M Reddy Sri[2]; Arifullah Mohammed[3]

[1]Department of Zoology, Sri Venkateswara University, Tirupati-517502, AP, India.

[2]Sri Balaji Medical College and Hospital, Chromepet, Chennai-600044, TN, India.

[3]Faculty of Agrobased Industry, Universiti Malaysia Kelantan Jeli Campus.17600 Jeli, Kelantan, Malaysia.

***corresponding author:** **M Bhaskar**

Department of Zoology, Sri Venkateswara University, Tirupati-517502, AP, India.
Email: matchabhaskar@gmail.com

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Introduction

Malaria continues to be a life threatening public health problem. Over 40% of the world's population is at risk of malaria. Every year about 300-500 million cases of malaria are reported worldwide with one million deaths. worldwide. Malaria causes widespread premature deaths, imposes financial hardship on poor household, and reduces economic growth and improvement in standards. In some countries malaria accounts for up to 50% of all hospital admissions and outpatient visits. Indirect costs include lost income and productivity due to absence from work because of illness or caring for those suffering from malaria. Children's learning abilities similarly suffer due to poor school attendance for the same reasons. The dreaded disease is caused by a protozoan parasite of Genus *Plasmodium*. There are four species of *Plasmodium* that causes malaria in human beings namely *Plasmodium falciparum, P. vivax, P. malaria* and *P. ovale.* Among these four parasites, *P. falciparum* causes the most serious form of disease and majority of malarial deaths are attributable to this species.

The female Anopheles mosquitoes transmit disease from one person to other through?. A total of 424 Anopheline species are reported worldwide, of which 70 are major vectors of malaria. In India, 58 species of anophelines exist and nine are reported to be the vectors of malaria, of which six are primary importance [1,2]. These are namely, *Anopheles culicifacies* (transmits malaria in rural and peri-urban areas), *An. stephensi* (urban malaria), *An. fluviatilis* (hills and foothills), *An. minimus* and *An. dirus* (in northeastern states) and *An. culicifacies* alone transmits about 65% of the total cases of malaria in the country followed by *An. fluviatilis* (15%) and *An. stephensi* (12%) [2,3].

Now, wide range of vector strategies have been developed and used, and much emphasis has been laid on insecticide-treated nets for the past 20 years. Several formulations and fabrics were tested for impregnation. Child mortality was reduced from 14 to 63% in African trials, and trials in other countries by the use of bed nets, curtains and INT in reducing the malaria.

In India, malaria control is mainly focused on indoor residual spray and recently ITN and LLINs are being promoted from the control of rural malaria vectors, whereas for urban malaria control larviciding and thermal fogging are used. The control of *An. culicifacies* is treated as most appropriate as it is attributed for 65% of all malaria cases in India.

An. culicifacies is distributed in India and Bangladesh, Myanmar, Thailand, Laos and Vietnam in the east, Nepal and southern China, Combodia and Sri Lanka [1]. *An. culicifacies* is an important vector of malaria in India. Depending on the seasonal abundance, diurnal activity, man-biting behavior and vectorial potential significant differences were observed in the bionomics of *An. Culicifacies*. Due to the differences in biological characters this species might cover a range of "biological races" [4]. Now *An. culicifacies* has been recognized as a complex of five sibling species, provisionally designated as species A,

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B, C, D and E [5,6]. Vector control is very important for reducing malaria. The most successful methods for vector control are indoor residual spraying and usage of insecticide-treated nets (ITNs).

Knockdown resistance (*kdr*)-associated with the reduced sensitivity of the nervous system and involves modifications of sodium channel pharmacology [7]. The insensitivity may be due to reduced density of Na^+ channels or modified structure that reduces the amount of insecticides that binds [7]. *Kdr* is a type of target-site resistance arising from point mutations in sodium channel genes of the insect nervous system conferring resistance to pyrethroids, and can also confer cross-resistance to DDT [8]. Two *kdr* mutations have been described in *An. gambiae*. One is the substitution of a leucine amino acid (TTA: Wild-type allele - *kds*) by a phenylalanine (TTT: Termed as *kdr-w*) at position 1014 and is widely distributed in West Africa [9]. The second mutation involves the substitution of leucine (TTA) by a serine (TCA: Termed as *kdr-e*) at the same position, conferring high resistance to DDT [10] distributed in East Africa.

This implies that VGSC gene has multiple binding sites for different and/or same class of insecticides [11,12]. Probably, the number of mutations that are positively implicated in causing IR is just a tip of iceberg. Discovery of many new *kdr*- like mutations still on [12] from the other regions of the VGSC need special attention to study their role in development of IR.

Close phenotypic and genotypic association of *Kdr* to DDT and pyrethroid-resistance has been studied in various insect species, for example, *An. Gambiae* [10,13-14], *An. Stephensi* [15], *An. Arabiensis* [16], housefly [17], German cockroach [18], *Drosophila* [19], peach-potato aphid [20] etc. Several gene expression studies has been reported earlier in various organisms in our laboratory, such as simple sequence repeats distribution in genomes of *Brucella* species [21], binding mode of ATP in the kinase domains of Hexokinase family [22], molecular dynamics simulations of MODY2 mutated glucokinase structures [23], structural variations of human glucokinase Glu256Lys in MODY2 [24], isolation and characterization of ethanol tolerant yeast strains [25], polymorphism in Kir 6.2 gene in Type 2 diabetic patients [26], screening and subtractive genomic approach exhibited new virulence factors, potential drug targets against bio-war pathogen, *Brucella melitensis* 16M [27], and expression of anti and pro apoptotic genes in the *in vivo* grown and cultured ovarian follicles in sheep [28].

Development of insecticide resistance depends on genetics and intensive application of insecticide. Insect's resistance to a particular insecticide was inherited to later generations. Insects are known to exhibit resistance at alarming rates among all the different categories of pests. More than 500 species of insects and related arthropods are resistant to insecticides worldwide.

Resistance may develop to only a single insecticide which is more common for insects that exhibit resistance to one insecticide to be resistant (or develop resistance more rapidly) to other insecticides with the same moment of alwaysness (MOA). For example Housefly became resistant to DDT in the 1950's, also exhibited resistance, with no previous exposure, to pyrethroid insecticides used decades later. Same MOA was for DDT and pyrethroids which is known as cross-resistance.

The genotyping of DDT and pyrethroid resistance using *kdr* assays were found misleading in some cases, because of random association of *kdr* mutations and pesticide resistance. Evidence from Cˆote d'Ivoire gave powerful indication that *kdr* may not always produce significant resistance phenotype to stall control interventions [29]. Darriet *et al.,* [30] and Ansari *et al.,* [31-32] showed that pyrethroid treated bed nets retained a measurable degree of toxicity against *An. gambiae* populations with high frequencies of *kdr.* Hay *et al.,* [33] showed that sufficient use of pyrethroid treated bed nets were able to reduce asymptomatic malaria infection in areas where the frequency of *kdr* is high in the vector *An. gambiae*. Moreover, Yu and Caterwall [34] proposed that *kdr* variation at the genomic DNA level may not be sufficient to produce a resistance phenotype unless it is transcribed into RNA variation at the RNA editing stage.

Kdr like mutations other than the described *kdr* locus are responsible for same kind of action, i.e. increasing of insensitivity of sodium ion channel to the target insecticides. Recently, such mutations have been identified in *An. culicifaciess.* and *An. stephensi* from Indian subcontinent [35-37]. Recently, Singh *et al.,* [36] has identified the *kdr* mutations in classical sodium channel *loci*. However, to confirm their association with the insect resistance needs results from more field studies to validate the suggested role. The discrepancies observed in case of role of *kdr* in causing insect resistance can be explained in different angles. The polymerase chain reactions standardized for genotyping of this locus itself has some inherent errors. The perfect association between the PCR results and the SNP present the genome is lacking. Some studies have performed to improve the situation. During the comparative analysis as much as 50% errors have been reported. Hence, the cause for the non-association findings reported with the phenotypic and genotypic association need to be considered with caution.

Above all, large number of studies have found and concluded that the genotyping and monitoring of *kdr* and *kdr*- like mutations have large impact on the management of insect resistance, thereby keeping vector borne diseases under control.

Hence, the present study was undertaken to characterize the insecticide susceptibility of adult mosquitoes and characterize insecticide resistance mechanisms in *Anopheles culicifacies* mosquitoes.

Objectives

- To examine the insecticide susceptibility status of *An. culicifacies* populations collected from Almatti dam Catchment area, Karnataka, India. To determine the insecticide susceptibility of the *An. culicifacies* populations to diagnostic concentrations of DDT (4%) an organochlorine, Deltamethrin (0.05%), a synthetic pyrethroid.

- To analyse the frequency of knock-down resistance (kdr) alleles, and to correlate its role (kdr mutation) in conferring the insecticide resistance either to DDT or pyrethroid or both of the insecticides, if any in *An. culicifacies* populations.

Material and methods

Anopheles culicifacies mosquitoes were collected from field through hand catch collection and identified using standard keys [38-40]. Larvae were reared in enamel trays. Finely ground dog biscuit and yeast powder in the ratio of 40:60% was provided as larval food during rearing. Every two days the water in the larval trays was changed. Boiled and cooled dechlorinated water was used for rearing of larvae. Enamel trays of 12 inch were used for rearing of larvae. About 200-300 larvae were reared in a tray. Mosquito species were identified and *An. culicifacies* mosquitoes emerged from the field collected larvae were housed in separate cages. All the mosquitoes were reared in an insectarium at National Institute of Malaria Research Field Unit, Bengaluru. The insectarium is maintained at 27 ±2°C and relative humidity of 70-80%. Adults were caged in 2x2x2 feet mosquito cages. Adult mosquitoes were provided with 10% glucose solution during maintenance and blood source was given through membrane feeding for oviposition. Eggs were collected three days post feeding and kept for hatching in enamel trays and reared to adults as per standard rearing techniques. F_1 generation of the colonised mosquitoes was used for different tests.

Determination of insecticide susceptibility

Susceptibility of adult mosquitoes to different diagnostic concentration of DDT (4%) (Organochlorine) and deltamethrin (0.05%) was determined using tube test [41]. Insecticide impregnated papers were procured from Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia. In brief, the following procedure was used for tube test. Green dot, red dot tubes and stages were cleaned with ethanol soaked cotton pads and allowed them to dry. Necessary entries in the data sheet, such as temperature, humidity, test species, age, no. of use of impregnated papers, date, etc. were recorded. For each insecticide four test replicates and two control replicates were run simultaneously. The test was conducted at 27 ±2°C and relative humidity of 70-80%. Nylon mesh was inserted into the tubes and was properly screwed. The stages were prepared to ensure proper movement of stage plate, and fastened with rubber band if required and locked with a cotton plug. Four green dot tubes were taken for the test and one tube for control and labelled them with replicate number with marker pen. Control filter paper (without insecticide) was inserted into the green dot tubes, held by two silver rings. One end of the tube was closed with screw cap with mesh and other end by inserting the stage. Mosquitoes were collected directly by means of a suction tube and were released gently into the test tubes @ 25 females/ tube through small hole and the stage of the holding green dot tube was closed. Mosquitoes were allowed to acclimatize for some time (1 h) and were observed for any dead and damaged mosquitoes. Number of dead mosquitoes was deducted from the total number of mosquitoes per replicate and recorded the same in the data sheet. Insecticide impregnated paper was inserted into the red dot tubes by placing impregnated surface inside so that inscription readable through the tube. The insecticide impregnated paper was held with2 copper rings, and then the tube was closed with a mesh and screwed. Green dot and red tubes were joined and screwed tightly to the stage.

The mosquitoes were then gently blown through the big hole by slowly sliding the stage of the plate and then closed the stage by pushing back the plate. Mosquitoes were exposed for 1 hour in exposure tube under moderate diffuse lighting. Knock down mosquitoes were scored after 1 hour post exposure. At the end of exposure period, mosquitoes were transferred from exposure tubes into the holding tubes in the same way as previously done. Insecticide impregnated papers from the red dot tubes were removed and marked with pencil about the number of times of usage and stored at 4°C for further use. Green dot tubes (holding tubes) were kept for 24 hours in a chamber/room with controlled temperature of 26 to 28°C and RH of 65-75% in dark to record the mortalities. During holding, 10% glucose/honey solution in a swab was provided. After

24 hours of holding, the number of dead/live mosquitoes were scored and noted on data sheets. Mortality was calculated for each replicate and recorded. Abbott's [42] formula as mentioned below was used to correct the test mortality when the mortality in control replicates was between <5% and 20%. When the mortality in control replicates was more than 20% the test was discarded and repeated.

% Corrected mortality = (Test mortality–Control mortality)/(100–control mortality)x 100

The following WHO criterion was used to ascertain susceptibility or resistance to a particular insecticide as per the percent corrected mortality obtained in test replicates: >98% mortality– susceptible; 90-98%– verification required; <90% mortality – resistant.

Determination of kdr gene frequency

Regarding the second section of the project, i.e. molecular characterization of the knock down resistance (kdr) gene mutations in different *An. stephensi* populations, the responsive locus and the related region (pl. see red circle in the below figure) primers were designed and assay was developed. For this purpose, different voltage gated sodium channel gene sequences of different populations available from public domain were used.

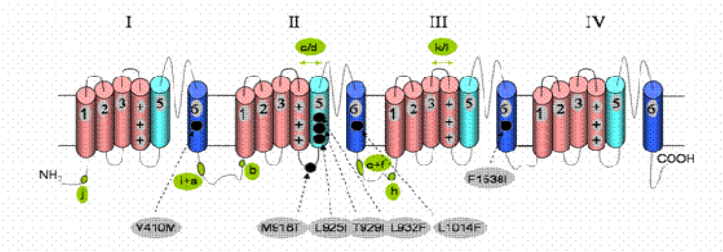

Figure 1: Showing different mutations (both kdr and kdr-like) in the voltage-gated sodium ion channel that are implicated in knock-down resistance in broad range of insect species. The red circle in the figure denotes the classical targeted kdr mutation for monitoring the DDT or pyrethroid resistance in different anopheline vectors.

The primer 3 software was employed to design the primers to amplify the region of interest. The designed primers are tested in the laboratory reared *An. stephensi* mosquitoes. The primer sequences that are designed for this purpose are as follows:

S1	CTTCGAGTTTTTAAGCTCGCTA
S2	CGACATAGTTCTTTCCGAACA
S3	ATAATGTGGATAGATTCCCCG
S4	CTGTCGGTTGAACGGATGCTA
s5	ATGTCAACCTCGGCTGG
s6	TCAGACATCTGCCGTTC

Isolation of genomic DNA

Isolation of good quality genomic DNA (gDNA) is a prerequisite for any PCR-based assays. The commercially available kits are having an adequate amount of DNA from a single individual mosquito sufficient for PCR-based assays. The whole adult mosquitoes collected from the field are individually preserved by storing either in 100µl isopropanol for the DNA extraction. DNazol solution was procured from Invitrogen (10503-027). Ethanol and Nuclease free water were of standard grade.

Procedure

From adult female mosquitoes at the half-gravid stage body parts were isolated and preserved in isopropanol (100%) for DNA extraction. In the case of blood-fed females care was taken to dissect out the abdomen before DNA extraction to avoid mosquito genomic contamination with vertebrate DNA from the blood. Mosquito sample was taken out from isopropanol and washed twice with distilled water and kept in sterile 1.5 ml centrifuge tube. Exactly 100 µl of DNazol into the tube and the mosquito sample ground with the help of pestle until it is completely homogenized. Then centrifuged the homogenate for 10 min at 10,000 g at 4ºC or room temperature and transferred viscous supernatant to fresh sterile 1.5 ml centrifuge tube. To the supernatant 50 µl of 100% ethanol was added and mixed by inverting the tube 5-8 times, and incubated at room temperature for 1-3 min. (DNA shall be visible at this stage as cloudy precipitate). This was centrifuged at 4,000 × g for 1-2 min at room temperature or 4°C and the supernatant was discarded.

DNA pellet was washed twice with 1 ml of 75% ethanol. At each wash, the DNA pellet was suspended in ethanol by inverting the tubes 3-6 times. The tubes were stored vertically for 0.5-1 min to allow the DNA to settle to the bottom of the tubes and excessive ethanol was removed by pipetting or decanting. Residual ethanol was discarded from the tube by air drying the tube at room temperature about strictly for 5-15 sec (if the DNA is exposed to air for more than few seconds it will be difficult to dissolve in water). Then, resuspended the pellet with 50-100 µl of nuclease free water and kept the vials over night at 4^0C to maximize pellet rehydration. After that DNA samples were stored at -20^0C.

Resuspending the PCR primers

The oligos were reconstituted in the laminar flow hood, in nuclease free water (NFW) to make a 100 µM master stock solution. The original primer tubes were used for making 100 µM master stock. Number of µl of NFW to be added to make the 100 µM stock was calculated as per manufacturer's instructions. After adding the required volume of NFW mixes the tube well and allows sitting at room temperature for 10 minutes. From the 100 µM master stock 10µM working primer stock solution was prepared. Then diluted the master stock in a sterile micro centrifuge tube 1:10 with NFW inside the laminar flow hood (10 µl of primer master stock + 90µl of NFW will give 100µl of 10µM working stock).

Analysis of kdr through PCR

Paralysis and death of the insects was caused by the neurotoxins DDT and pyrethroids .These act on the voltage-gated sodium channels by modifying their gating kinetics. Finally they result in the prolonged opening of individual channels. Reduced target site sensitivity is caused by pyrethroid resistance in insects. This is referred to as knock-down resistance (*kdr*) . The phenotype is commonly conferred by a single point mutation (L1014F) in the IIS6 segment of voltage gated sodium channel. For testing DDT/pyrethroid resistant mosquitoes PCR based assay called Amplification Refractory Mutation System (ARMS) was used.

Primer sequence

α) KdrF- 5'-GGA CCA YGA TTT GCC AAG ATG-3'

β) KdrR- 5'-CGA AAT TGG ACA AAA GCA AAG-3'

χ) KdrA1- 5'-GGG TTA CTGCTA GGT TAC TTA CGG CT -3'

δ) KdrT1- 5'- CTG GCT ACA GTA GTG ATA GGA AAT CTT-3'

Procedure

The PCR reaction condition consists of initial denaturation at 95°C for 5 min, followed by 35 cycles each of 95°C for 30 S, 48°C for 45 S and 72°C for 60 S, and a final extension step of 72°C for 7 min. The product was run on 2% agarose gel containing ethidium bromide and visualized under UV and 100 bp DNA ladder was used for analyzing the band size. Genotypes were scored based on the presence of 271, 191 and 132 bp bands. The presence of 271 and 191bp band represents SS, 271 and 132bp represents RR and the presence of all the three bands (271, 191 and 132 bp) represents RS genotype.

PCR reaction mixture composition

S. NO	Reagents	Working volume (µl)
1	10x Taq buffer	2.0
2	25mM $MgCl_2$	0.9
2	10mM dNTP	0.3
3	kdrF	1.5
4	kdrR	0.6
5	kdrA1	0.6
6	kdrT1	3.0
7	Taq polymerase	0.5
8	gDNA	2.0
9	Nuclease free water	3.6
	Reaction volume	**15** µl

Where, SS: Homozygous Susceptible; RS: Heterozygous Resistance; RR: Homozygous Resistance.

Results and discussion

The present study was conducted to analyse the insecticide susceptibility status of *An. culicifacies* populations collected from Almatti Dam Catchment area, Bagalkot district of Karnataka state. Mortality of exposed mosquitoes to diagnostic dosages of DDT and deltamethrin are shown in Table-1. The results revealed that the *An. culicifacies* wild mosquitoes collected from Almatti Dam catchment area showed resistance to DDT 4% impregnated papers with a corrected % mortality of 27.5% and knock down percent in 1 h of 40%. The percent mortalities in mosquitoes exposed to diagnostic dosages of deltamethrin 0.05% was 86% and the knockdown percent was 89% indicating pyrethroid resistance in this population.

Table 1: Susceptibility status of *An. culicifacies* populations from Almatti, Karnataka to DDT (4%) and deltamethrin 0.05%.

S. No.	Insecticide and Concentration	No. of mosquitoes exposed in test	No. knockdown in 60 min	% Knockdown in 60 min	No. of dead in test 24 h	% Corrected Mortality	Resistant Status
1	Deltamethrin 0.05	100	89	89	86	86%	Resistant
2	DDT (4%)	80	32	40	22	27.5	Resistant

S: Susceptible> 98% mortality; VR: Verification requited (91-98% mortality) and - Resistant <90% mortalite.

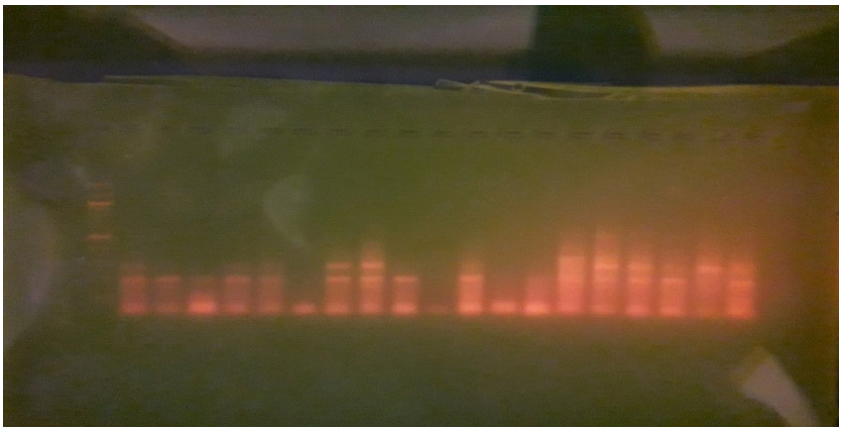

Figure 2: Genotype frequencies of Kdr mutations in *An. culicifacies* wild populations collected.

Table 2: Genotype frequencies of *kdr* like mutations in *An. culicifacies* wild populations collected.

Sample No.	Presence of bands			kdr Genotype status	Allelle Status
	132bp	191bp	271bp		
1	+	-	+	RR	Homozygous Resistance
2	+	+	+	RS	Heterozygous Resistance
3	+	+	+	RS	Heterozygous Resistance
4	-	+	+	SS	Homozygous Susceptible
5	-	+	+	SS	Homozygous Susceptible
6	-	+	+	SS	Homozygous Susceptible
7	-	+	+	SS	Homozygous Susceptible
8	-	+	+	SS	Homozygous Susceptible
9	-	+	+	SS	Homozygous Susceptible
10	-	+	+	SS	Homozygous Susceptible
11	-	+	+	SS	Homozygous Susceptible
12	-	+	+	SS	Homozygous Susceptible
13	-	+	+	SS	Homozygous Susceptible
14	+	-	+	RR	Heterozygous Resistance

15	+	+	+	RS	Heterozygous Resistance
16	-	+	+	SS	Homozygous Resistance
17	+	+	+	RS	Heterozygous Resistance
18	+	+	+	RS	Heterozygous Resistance
19	+	+	+	RS	Heterozygous Resistance
20	-	+	+	SS	Homozygous susceptible

RR- 2; RS= 6; SS=12

Anopheles culicifacies a major malaria vector in India. This has developed widespread resistance to DDT and pyrethroids recommended for the impregnation of bed nets. Knock-down resistance due to a point mutation in the voltage gated sodium channel at L1014 residue (kdr) is a common mechanism of resistance to DDT and pyrethroids. The selection of this resistance may pose a serious threat to the success of the pyrethroid-impregnated bed net programme. This study reports the presence of *kdr* mutation (L1014F) in a field population of *An. culicifacies*.

The point mutation at residue L1014 leading to Leu-to-Phe change in voltage gated sodium channel is a most common mechanism associated with knock-down resistance in insects against DDT and pyrethroids. DDT and pyrethroids are neurotoxins that act on the voltage- gated sodium channels by modifying their gating kinetics, resulting in the prolonged opening of individual channels leading to paralysis and death of the insect. One of the mechanisms of pyrethroid resistance in insects is referred to as knock-down resistance (*kdr*) caused by reduced target site sensitivity. The phenotype is commonly conferred by a single point mutation (L1014F/S/H) in the IIS6 segment of voltage gated sodium channel. Other mutations in different regions of the gene also confer knock-down resistance in some insects, but among anophelines this is the only locus where point mutations have been reported to date conferring resistance.

Only two point's mutations have been reported in anophelines at this locus–L1014F in *Anopheles gambiae* (West African *kdr*), *Anopheles arabiensis* and *Anopheles stephensi*, and L1014S in *An. gambiae* (East African *kdr*).

The study showed that due to presence of many heterozygous alleles there is a possibility of development of resistance in the population. The results indicate that there are other resistance mechanisms may also be involved in the population.

Conclusion

Insecticide susceptibility tests on *An. culicifacies* mosquitoes collected in wild region showed resistance to DDT 4% and deltamethrin 0.05%. Knock down resistance (kdr) gene mutations as assessed through PCR and gel electrophoresis for the presence of mutations of L1014S did not reveal significant evidence for involvement of kdr gene like mutations responsible for development of resistance in the population. Only two homozygous resistant alleles could be found and could not be attributable for the resistance mechanisms in the population.

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