A new knockdown resistance (*kdr*) mutation F1534L in *Aedes aegypti* associated with insecticide resistance

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ABSTRACT

The control of *Aedes aegypti* borne-infections mainly dengue, chikungunya, yellow fever and Zika virus relies mainly on vector control measures in the absence of specific drugs or vaccines available against these infections. Emergence of insecticide resistance in *Ae. aegypti* may pose serious threat to the success of insecticide-based vector control programme. Here, we report the presence of multiple knockdown resistance (*kdr*) mutations present in an Indian *Ae. aegypti* population including a new mutation F1534L (not reported earlier in *Ae. aegypti*) which is associated with DDT and pyrethroid resistance. DNA sequencing of partial domain II, III and IV of the voltage gated sodium channel (VGSC) performed on *Ae. aegypti* collected from Bengaluru, India, revealed the presence of four *kdr* mutations, i.e., V1016G and S989P in domain II and two alternative *kdr* mutations F1534C and F1534L in domain III. Allele specific PCR assays (ASPCR) were developed for the detection of *kdr* mutations V1016G and S989P while a PCR-RFLP based strategy was adopted for the genotyping of all three known mutations in domain III (F1534L, F1534C and T1520I). Genotyping of 572 *Ae. aegypti* samples collected in 2014 and 2015 revealed a moderate frequency of V1016G/S989P (18.27%) and F1534L (17.48%), a relatively high frequency of F1534C (50.61%) and absence of T1520I in the population. Mutations V1016G and S989P were in complete linkage disequilibrium while they were having negative linkage disequilibrium with *kdr* alleles F1534C and F1534L. The new mutation F1534L showed significant protection against permethrin, deltamethrin and DDT whereas F1534C showed protection against permethrin and DDT but not against deltamethrin.

Key words: *Aedes aegypti,* insecticide resistance, knockdown resistance, pyrethroid, voltage gated sodium channel.

Introduction:

Aedes aegypti is now a widely distributed mosquito species in tropical and subtropical regions and is responsible for the transmission of several arboviral infections in human mainly dengue, chikungunya, yellow fever and Zika virus. These arboviral infections are increasingly becoming a global health concern due to their rapid geographical spread and high disease burden [1]. The ancestral form of *Ae. aegypti* was found in Africa which used to

feed on nonhuman primates and after its domestication, *Ae. aegypti* has now expanded from Africa and colonized most of the pantropical world [2]. During last five decades, there have been an unprecedented emergence of epidemic arboviral diseases [2] and approximately half of the world population is under the threat of dengue [3,4]. In India, dengue and chikungunya are main arboviral infection with recent introduction of Zika virus (ZIKV). Recently there had been an outbreak of Zika infections in Jaipur city [5], where *Ae. aegypti* has been incriminated as Zika vector [6].

In absence of specific vaccines or drugs for the treatment of *Aedes*-borne infections, vector control and personal protection remain only measures to contain the spread of these arboviral infections. Use of insecticide-based control of this vector is a common approach used worldwide. Pyrethroid group of insecticides is of special interest which is being extensively used in long lasting insecticidal nets (LLIN), space spray as well as in household repellent and personal protection. Pyrethroids are preferred group of insecticides due to their low mammalian toxicity, degradability in nature and rapid knockdown effect on insect [7]. However, extensive use of pyrethroids in public health and also in agriculture sector has led to the emergence of resistance against these insecticides in many disease vectors including *Ae. aegypti*. Several reports of pyrethroid resistance in *Ae. aegypti* has been recorded from different parts of world mainly in Southeast Asia, Latin Americas and Africa, with such reports not available from India until the year 2015 [8].

Understanding the mechanisms of insecticide resistance in vector populations is crucial for an effective insecticide resistance management. One of the known mechanisms of insecticide resistance in mosquitoes against pyrethroids and DDT is knockdown resistance (*kdr*) which is conferred by alteration in the target site of action, i.e., the voltage gated sodium channel (VGSC) resulting from non-synonymous mutations. Several *kdr* mutations have been reported in *Ae. aegypti* in different parts of world, amongst which mutations at three loci i.e., Iso1011 (I→M/V) and Val1016 (V→G/I) in domain II and F1534 (F→C) in domain III are commonly associated with pyrethroid resistance [9-18]. Presence of such *kdr* mutations in the Indian subcontinent was screened in just one northern Indian population (Delhi) where we reported the presence of F1534C and a novel mutation T1520I [18]. This study reports a new mutation F1534L, co-occurring with mutations F1534C, S989P and

V1016G, in a southern Indian field population of *Ae. aegypti*. The new mutation F1534L showed positive association with DDT and pyrethroid resistance.

Results

Identification of *kdr* mutations in *Ae. aegypti* population

DNA sequencing of partial domain II, domain III and domain IV of the VGSC of *Ae. aegypti* population collected from Bengaluru, India (77° 56-57′ E, 12° 92-95′ N), revealed the presence of four mutations i.e., S989P and V1016G in domain II and F1534C and F1534L in domain III. No mutation was found in domain IV. The mutation F1534L is being reported for the first time in Indian *Ae. aegypti*. Mutations S989P and V1016G present in domain II were due to substitution on first codon (TCC→CCC) and second codon (GTA→GGA), respectively. In most of the cases, the identification of S989 and V1016 mutations were based on 1X sequencing data where forward sequence was used for identification of S989 alleles and reverse sequence was used for V1016 alleles. This was due to presence of ambiguous sequence in downstream sequence resulting from multiple indels present in intron between these two *kdr* locus. In course of study a total of 294 samples were sequenced for partial domain II of which 178 were homozygous wild for both residues (SS at residue S989 and VV at residue V1016), 92 were heterozygous (SP and VG) and 24 were mutant homozygous (PP and GG). Other two alternative mutations F1534C and F1534L present in domain III were due to T>C substitution on the first position of the codon, leading to Phe (TTC)→Leu (CTC) mutation, and T>G substitution on the second position of the codon leading to Phe (TTC)→Cys (TGC) mutations, respectively. Out of the 27 individuals sequenced for domain III, one was homozygous wild for FF (TTC) at residue F1534, seven were homozygotes for CC (TGC), four were homozygotes for LL (CTC), four samples were heterozygotes for each of FC and FL and seven were having mixed bases at first and second position of the codon, i.e., with YKC, which could be either heterozygote for LC (CTC+TGC) or FR (TTC+CGC). The latter combination was ruled out as sequencing of 15 cloned PCR products, from five such samples (having sequence YKC), revealed the presence of two haplotypes, one with CTC (L) and another with TGC (C). We also observed that haplotype with F1534L mutation had a restriction site for *Eco88*I (5'-C↓ YCGRG-3'). Therefore, all the seven heterozygote samples with the sequence YKC were subjected to PCR-RFLP with *Eco88*I and all were partially cleaved indicating the presence of LC (CTC+TGC) heterozygote. DNA sequencing

of 12 samples for partial domain IV revealed absence of any non-synonymous mutation including D1794Y reported elsewhere [19]. Additionally, 25 samples were checked for presence of D1794Y using PCR-RFLP following Chang *et al*., [19] and none were found positive.

Development of PCR-based assay for genotyping of *kdr* alleles

For genotyping of F1534-*kdr* alleles, we modified PCR-RFLP developed by Kushwah *et al*., (2015) [18] where an additional restriction enzyme *Eco88*I was used for identification of new allele F1534L. For genotyping of S989- and V1016-*kdr* alleles, we developed allele-specific PCRs (ASPCR) for each locus. Detailed methods are described in section Material and Methods.

These methods were validated through DNA sequencing. The genotyping result of PCR-RFLP based methods developed were well in agreement with DNA sequencing results of corresponding samples. The genotyping result of 27 samples that were sequenced for partial domain III of the VGSC (FF=1, CC=7, LL=4, FL=4, FC=4, LC=7) and 294 samples sequenced for domain II (SS/VV=178, PP/GG=24, SP/VG=92) matched with DNA sequencing results.

Genotyping of *kdr* alleles and their linkage association

Genotyping result of *kdr* alleles at loci F1534, S989 and V1016 carried out on 572 field collected F0 populations are shown in **Table** 1. The overall frequencies of F1534 (F), F1534C (C), F1534L (L), S989 (S), S989P (P), V1016 (V) and V1016G (G) were 0.32, 0.51, 0.17, 0.82, 0.18, 0.82 and 0.18, respectively, in collections carried out in year 2014 and 2015. The T1520I was absent in this population. It was observed that the frequency of observed F1534-*kdr* genotypes are not in Hardy-Weinberg equilibrium (HWE) while S989 and V1016-*kdr* genotypes were in HWE in pooled samples. Analysis of inbreeding coefficient for F1534-*kdr* alleles revealed positive value indicating presence of fewer of heterozygotes than expected (F=0.09). Additionally, 242 F1 individuals were also genotyped for *kdr* alleles. Thus, a total of 814 samples (F0 and F1 combined) were genotyped for *kdr* alleles present in domain II and III. The frequency of individuals with different genotype combination at three loci is shown in **Supplementary Table S1**. Estimation of gametic phase based on Gibbs sampling strategy implemented in Arlequin 3.536 revealed the presence of only 4 haplotypes

namely, FSV (wild), CSV, LSV and FPG (underlined are mutated alleles). The frequency of each haplotypes is shown in **Supplementary Data S1.** This indicate negative linkage disequilibrium of mutant *kdr* alleles (P and G) present in domain II with mutant *kdr* alleles in domain III (C and L). The two mutations in domain II, i.e., 989P and 1016G were in complete linkage disequilibrium.

Genetic association of *kdr* alleles with phenotype insecticide resistance

The distribution of individuals with different *kdr* allele genotypes in respect to F1534, S989 and V1016 loci in dead and alive mosquitoes (F0 and F1) after exposure to 0.75% permethrin (type I pyrethroid), 0.05% deltamethrin (type II pyrethroid) and 4% DDT is shown in **Supplementary Table S2.** Since mutations present in domain II (S989P and V1016G) were always found in association with wild form in domain III (F1534) and have been reported to have protection with insecticides, we removed data of individual having haplotype FPG from analysis of genetic association of F1534-*kdr* mutations with phenotypic insecticide resistance. Similarly, data of individual with CSV and LSV haplotypes were removed while analysing association of S989P/V1016G *kdr* mutations with phenotypic insecticide resistance. The analysis of genetic association of F1534-*kdr* alleles with phenotype insecticide resistance is shown in **Table 2**. Statistical analysis revealed that allele F1534L showed strong protection against permethrin (P <0.0001), moderate protection against deltamethrin (P< 0.01) and very low protection against DDT (P <0.05). Other allele 1534C showed strong protection against permethrin (P <0.0001), low protection against DDT (P <0.001) and no protection against deltamethrin. Mutations 989P and 1016G together have strong protection to permethrin and we could not establish their role against deltamethrin and DDT due to low frequency of alleles at S989 and V1016 locus.

Discussion

Aedes aegypti, a primary vector of dengue, yellow fever, chikungunya, Zika and other arboviral infections has attained global importance due to invasion of this species in different parts of the world from Africa. Currently, with no specific treatment or vaccine available to control transmission of these arboviral infections, vector control remains the mainstay in public health. One of the available potential methods for control of this vector is the use of

pyrethroid group of insecticides for space spray and personal protection measure. Development of resistance against these insecticides is major concern for success of such application. It is therefore desirable to have knowledge on underlying mechanism(s) of insecticide resistance for an effective insecticide resistance management.

Reduced sensitivity of VGSC—the target site for DDT and pyrethroids, is one of the mechanisms of resistance in insects due to conformational changes in protein resulting from one or more mutations leading to amino acid substitution, commonly referred to as knockdown resistance (*kdr*). In *Ae. aegypti,* several such mutations are documented, of which F1534C, S989P and V1016G are widely reported *kdr* mutation and is known to confer resistance against DDT and pyrethroids [20,21,22]. This study reports the presence of four *kdr* mutations viz. S989P, V1016G, F1534C and F1534L with the latter mutation is being reported for the first time in *Ae. aegypti*. although its presence in *Aedes albopictus* has been reported [23,24,25]. The new mutation F1534L has shown significantly higher protection against permethrin (type I pyrethroid), deltamethrin (type II pyrethroid) and DDT in *Ae. aegypti*. The discovery of new *kdr* mutations associated with insecticide resistance is of global concern as this may hamper pyrethroid-based vector control and personal protection measures.

Earlier we conducted a survey of *kdr* mutations on Delhi population in year 2014 [18], where we found F1534C mutation along with a novel mutation T1520I linked to F1534C but did not find the three mutations being reported in this study, i.e., S989P, V1016G and F1534L. Similarly, a novel mutation T1520I reported in Delhi was absent from Bengaluru. To ensure that we did not skipped detection of the other three mutations (S989P, V1016G and F1534L) in Delhi population during previous study, we genotyped 184 *Ae. aegypti* samples collected from Delhi in August 2018. We did not find any of these three mutations in Delhi population. Thus, there is a contrast difference in distribution of *kdr* alleles in two different geographical locations which are approximately 1700 km apart. In another part of India (West Bengal, eastern India), there is report of presence of three mutations, F1534C, T1520I and V1016G but the presence of S989P was not investigated in this study [26].

For monitoring of *kdr* mutations in field condition, we developed a highly specific PCR-RFLP-based assay for simultaneous detection of all the three mutations (total five alleles) reported in domain III-S6 of the VGSC at locus F1534 and T1520. The PCR-RFLP for the

identification of all five alleles is advantageous over other PCR-based methods being highly specific due to high sequence-specificity of restriction enzymes. Additionally, in this PCR-RFLP assay, unlike other assays, a single PCR is required for genotyping of all five alleles present at locus F1534 and T1520. For genotyping of domain II-S6 mutations, we developed two allele-specific PCR (ASPCR) assays, one each for S989P and V1016G mutations. Our ASPCRs for genotyping of S989P and V1016G alleles are advantageous over other available PCR assay for these mutations, as our PCR need single assay for each locus while method described by Stenhouse *et al*., (2013) [27] need two PCR assays for single locus. However, ASPCR being based on single base mismatch is prone to non-specific extension and high degree of optimization is required. ASPCR is sensitive to change of type of reagents and PCR thermal conditioning. Discrepancies were noticed with such PCR based genotyping of F1534 *kdr*-alleles in an earlier study [28].

Co-occurrence of F15134C with S989 and V1016 may be of serious concern in case all three mutations are present on same haplotype. It has been shown through the site directed mutagenesis that such combination (F1534C+S989P+V1016G) may result in a seriously high degree of resistance against permethrin and deltamethrin (1100- and 90-fold, respectively) [29]. In this study, we found that 989P, 1016G and 1534C/1534L were found together in a single mosquito but always in heterozygous condition. Phasing out of haplotypes revealed that there are just four haplotype FSV, FPG, LSV and CSV present in this population. Thus, 989P and 1016G are always found together but never with 1534C or 1534L. In such a population, a single recombination may lead to production of haplotype CPG or LPG which may have greater impact on insecticide resistance phenotype. Unlike our finding, a small proportion of mosquitoes in Myanmar have shown to have 1534C homozygotes with 1016G homozygotes (2.9%, double mutant) and with homozygous 989P+1016G+1534C (0.98%, triple mutant) suggesting presence of CPG haplotype [30]. Occurrence of such haplotype with three mutations may be selected in presence of insecticide pressure and may pose serious threat to insecticide-based vector control programme.

In this study we found 100% linkage association of 1016G with 989P [22,30,31,32]. Though, such association has been shown in several studies, the frequency of V1016G is reported higher than S989P where V1016G mutation may be found alone but S989P was always linked with V1016G [27,29,30,32]. Similar unidirectional linkage has been shown in domain III in VGSC of *Ae. aegypti* population, where a novel mutation T1520I (reported in Delhi,

India) was always found with F1534C and was suggested to be a compensatory mutation [18].

A disturbing fact we recorded in this study was the non-compliance of HWE for F1534-*kdr* mutations in Bengaluru. Similar departure from HWE for this locus was also noted in our previous study of Delhi-population [18] and in Grand Cayman Island [33]. It was interesting to note that another mutation T1520I that was linked to F1534C, was in compliance with HWE. While we don't have an explanation to this, probably remains this may be due to the presence of multiple VGSC gene or gene duplication as proposed by Martin *et al*., [34]. This will need a further investigation.

In conclusion, this study for the first time reports the presence of F1534L mutation in an *Ae. aegypti* population which is associated with pyrethroid resistance alongside presence of three other mutations F1534C, S989P and V1016G. Molecular methods were developed for monitoring of all these *kdr* mutations. Owing to the protection conferred by F1534L mutation, it is of paramount importance to screen other populations of *Ae. aegypti* for this mutation besides the *Ae. albopictus* which also plays important role in transmission of several arboviral infections.

Methods

Mosquito collection

Immatures (eggs, larvae and pupae) *Ae. aegypti* were collected from domestic and peri-domestic breeding sites from Basavangudi area of Bengaluru city (77° 56-57′ E, 12° 92-95′ N) during years 2014 and 2015. Oral informed consent was obtained from the owners of the houses for collection of immature at residential premises. Immature were reared in the laboratory till the emergence into adult (F0). In addition, F1 progenies were also obtained. To get F1 progenies, mosquitoes were fed on blood through artificial membrane (Parafilm®) and eggs were obtained after 72 hrs of blood feeding. Eggs were allowed to hatch in water and larvae were reared in enamel trays with a supplementation of fish-food till pupation. Pupae were removed from the tray and placed in a bowl containing water inside an insect cage (measuring 30 cm X30 cm X 30 cm) for their emergence into adult. Adults were fed on 10% glucose soaked on cotton pad. Insectary was maintained at a temperature of 27±°C, relative humidity (RH) 60-70% and photoperiod of 14h:10h (light:dark) ratio.

Exposure of insecticide to mosquitoes (Bio-assay)

Two-to four-days old sugar fed adult *Ae. aegypti* female mosquitoes (F0, and F1), were exposed to 0.05% deltamethrin-, 0.75% permethrin- or 4% DDT-impregnated papers (supplied by WHO collaborative centre, Vector Control Research, Universiti Sains, Malaysia) for one-hour following WHO's standard insecticide-susceptibility test guidelines [35]. Following exposure of insecticide, they were transferred to recovery tubes and mortalities were recorded after 24 hours of recovery. Individual dead and alive mosquitoes were kept in 1.5 mL micro centrifuge tubes with a piece of silica gel for DNA isolation and at -20 °C. All bioassays were carried out in a laboratory maintained at 25°C and RH 60-70%.

DNA isolation and sequencing

DNA from individual mosquitoes was isolated following the method described by Livak *et al*.,[36] after removing 1/3rd of the posterior abdomen that carries spermatheca (to avoid contamination of sperm from mating male partner) and stored at 4°C. Some of the mosquitoes were sequenced for domain II, III and IV of the VGSC. Primers used for amplification of domain II, III and IV are shown in **Supplementary Table 3**. A common PCR protocol and PCR conditions were used for amplification of partial domain II, III and IV of VGSC. The PCR reaction (25 µl) contained 1X buffer, 1.5 mM MgCl2, 200 μM of each dNTP, 0.2 μM of each primer and 0.625 units of taq polymerase (AmpliTaq Gold, Invitrogen corporation, USA). The PCR conditions were: an initial denaturation at 95 °C for 3 min followed by 35 cycles each of denaturation at 95 °C for 15s, annealing at 55°C for 15s and extension at 72°C for 30s with a final extension at 72°C for 7 min. PCR products were purified using ExoSAP-IT PCR Product Clean up (Exonuclease I - Shrimp Alkaline Phosphatase, Thermo Fisher Scientific Inc) and were sent to Macrogen Inc, South Korea, for sequencing. The sequence chromatograms were edited using Finch TV ver 1.5.0 (http://www.geospiza.com/).

Cloning and sequencing

It was not possible to identify the correct amino acid codon through DNA sequencing in samples which were found heterozygous for two nucleotide base positions i.e., first and second codon of the F1534 residue. We therefore cloned and sequenced five such heterozygous samples to identify the correct codon. PCR product was amplified using primers AekdrF and AekdrR and high fidelity taq DNA polymerase (Phusion® High-Fidelity DNA Polymerase) purified using QIAquick PCR Purification Kit (Qiagen Inc.) and cloned in

pGem-T Easy Vector Systems (Promega Corporation) as per vendor's protocol. Competent *E. coli* cells were transformed with the recombinant DNA and grown on LB-Agar plate. Positive clones were selected by blue/white screening and amplified using universal primers SP6 and T7. The PCR product of individual clone was sequenced at Macrogen, South Korea, using primers SP6 and T7. Sequences were aligned using Mega5 [37].

Genotyping of domain II *kdr* alleles (V1016G and S989P)

For genotyping of *kdr* alleles at residue S989 and V1016, separate allele-specific PCR assays were designed. The list of primers used for ASPCR are shown in **Supplementary Table 3**. The PCR conditions for both PCR were identical. The PCR was carried out using Thermo Scientific DreamTaq Hot Start DNA Polymerase ready reaction mixture. The thermal cycling conditions of PCR were: initial denaturation at 95 °C for 3 min followed by 20 cycles, each of denaturation at 95 °C for 15 s, annealing for 30 °C at temperature starting from 65 °C with an increment of -1 °C each cycle and extension at 72 °C for 60 s. In remaining 15 cycles, the cycling conditions were similar except annealing temperature was kept constant at 45 °C. The primers used for S989-ASPCR were 1.0 μM of Aed2F, 1.5 μM of Aed2R, 0.6 μM of SSR and 0.45 μM of PPF. For V1016-ASPCR, primers used were 1.2 μM of Aed2F, 2.25 μM of Aed2R, 0.45 μM of VVF and 1.2 μM of GGR. The sizes of universal amplicon and allele-specific amplicons are as follows: universal amplicons: 620/636 bp, S989: 129 bp, 989P: 525/549 bp, V1016: 209 bp and 1016G: 446/462 bp. PCR products were electrophoresed on 2.0% agarose gel containing ethidium bromide and visualized under UV in gel documentation system.

Genotyping of domain III *kdr* alleles (T1520I, F1534C and F1534L)

Earlier we reported a PCR-RFLP for the genotyping T1520I and F1534C, where a single PCR product amplified using primers AekdrF and AekdrR were subjected to RFLP using two restriction enzymes *BsaB*I and *Ssi*I respectively [9]. In order to include RFLP for identification of new mutation F1534L, we searched for F1534L-specific restriction enzyme site using an online tool available at http: //insilico.ehu.es/restriction/two_seq. We found a unique restriction site *Eco88*I in the DNA sequence with F1534L and therefore for genotyping of all *kdr* alleles present in domain III, PCR-RFLP method was modified. In modified procedure additional restriction-digestion was performed in a separate tube with *Eco88*I in addition to *BsaB*I and *Ssi*I. Additional restriction-digestion reaction mixture (20 µl) contained 5 µl of PCR-amplified product, 2 units of *Eco88*I enzyme and 1X buffer

(Thermo Fisher scientific). This was incubated for 4 hours or overnight at 37°C following electrophoretic run on 2.5% agarose gel. The RFLP product and visualized in gel documentation system. The criteria for scoring of F1534 alleles were modified which are presented in Table 3, while the criterion for scoring of T1520 alleles remain unchanged.

Statistical analysis:

Hardy-Weinberg equilibrium (HWE) of *kdr*-alleles in a population was tested using Fisher's exact test. Phasing of haplotypes and estimation of their frequencies were performed using Arlequin 3.5 software [38]. Association of *kdr* genotypes with phenotype resistance was tested using co-dominant model and test of significance was estimated using Fisher's exact test and odds ratio (OR).

The Wright's inbreeding coefficient was calculated using formula F = (He-Ho)/He, where 'He' is expected heterozygosity and 'Ho' is observed heterozygosity.

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Author contributions statement

R. B. S. K. and T. K. performed DNA isolation, PCR and genotyping of samples; R. H. K. collected mosquitoes; C. L. D. performed cloning experiments; N. K. and T. K. contributed to the manuscript; O. P. S. designed study, sequencing and genotyping strategies, analysed data and wrote draft manuscript. All authors reviewed and approved the manuscript.

Competing Interests

The authors declare no competing interests.

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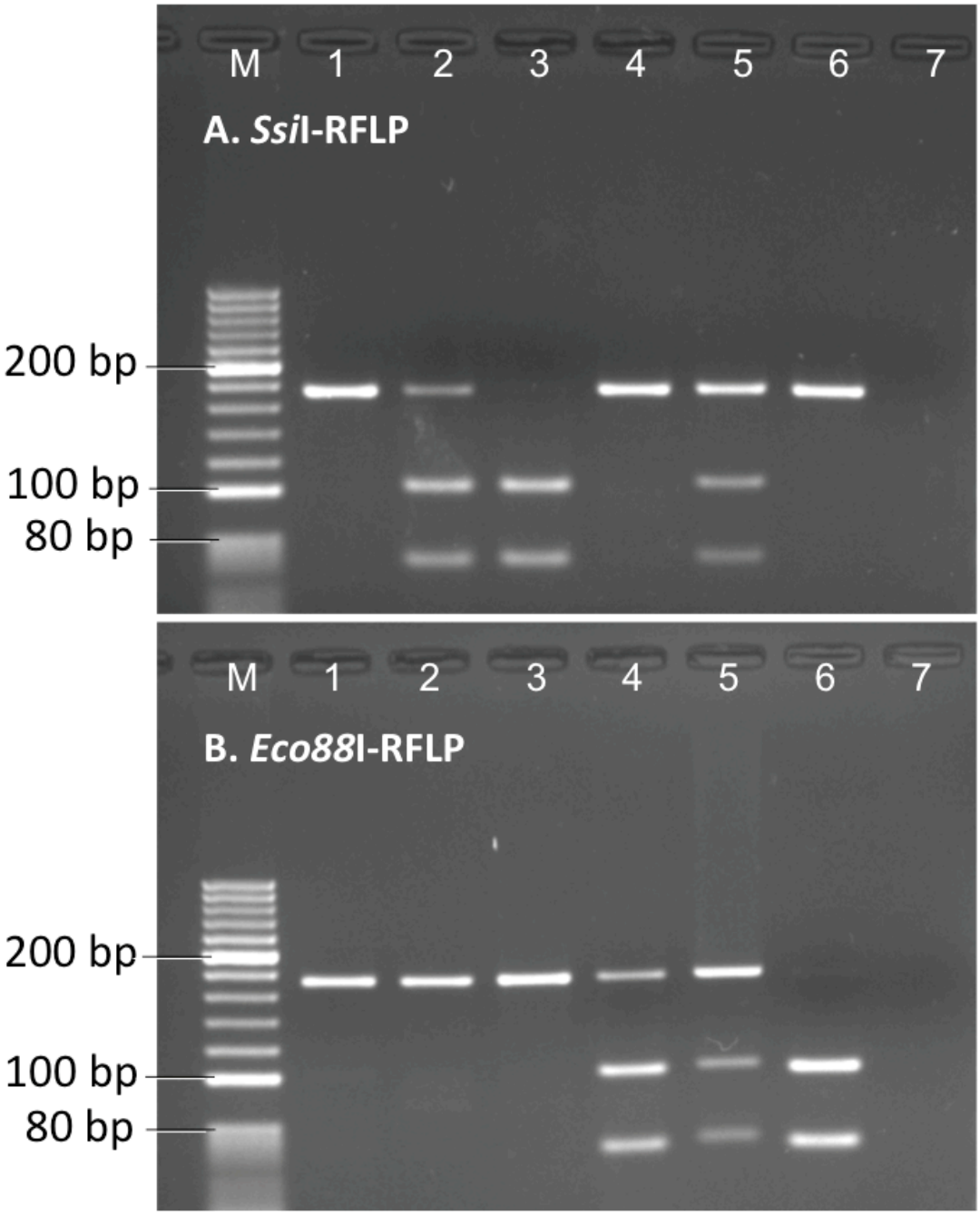

Figure 1: Gel photographs of (A) *Ssi*I- and (B) *Eco88*I-PCR-RFLP digests showing banding pattern of the different F1534-*kdr* genotypes. Lane M represents 20 bp ladder, lanes 1 to 6 represents genotypes FF, FC, CC, FL, LC and LL respectively; lane 7 represents negative control. Full-length gels are presented in Supplementary Figure 1 and 2.

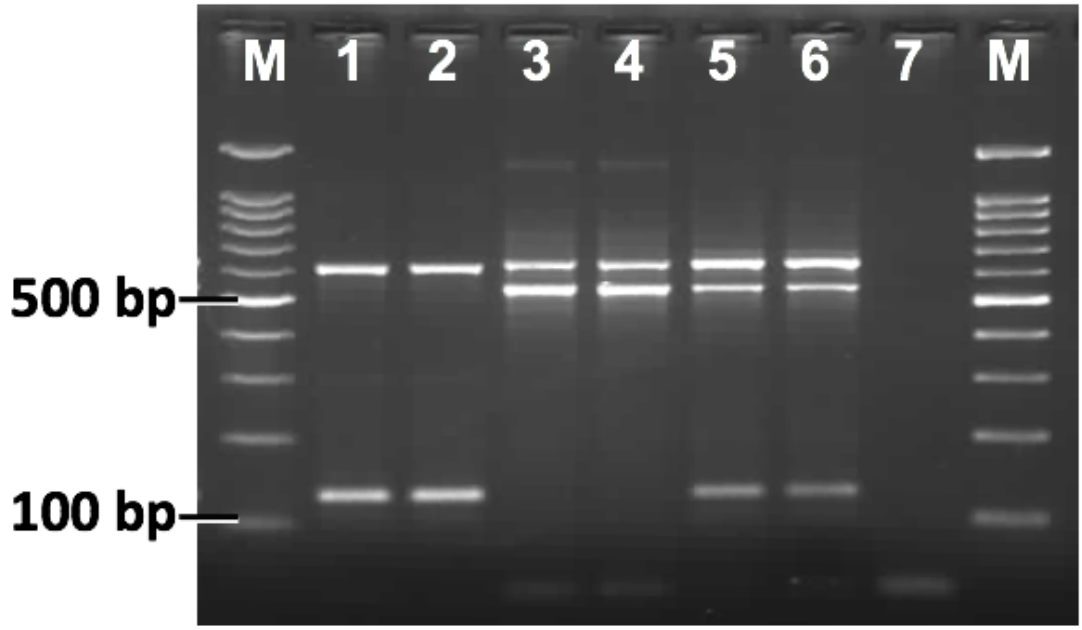

Figure 2: Gel photographs of allele-specific PCR for identification of S989-*kdr* alleles. Lane M represents 100 bp ladder, lanes 1 and 2 represents genotype SS, 3 and 4 PP, 5 and 6 SP and lane 7 represents negative control. Full-length gel is presented in Supplementary Figure 3.

Figure 3: Gel photographs of allele-specific PCR for identification of V1016-*kdr* alleles. Lane M represents 100 bp ladder, lanes 1 and 2 represents genotype VV, 3 and 4 GG, 5 and 6 VG and lane 7 represents negative control. Full-length gel is presented in Supplementary Figure 4.

Table 1: Genotyping result of different *kdr* alleles

Collection date	N	*kdr* genotypes												Allele frequencies							P_{HWE}		
		F1534						S989			V1016			F1534			S989		V1016		F1534	S989	V1016
		FF	FC	CC	FL	LL	LC	SS	SP	PP	VV	VG	GG	F	C	L	S	P	V	G			
Oct-Nov 2014	148	12	26	20	20	8	62	120	20	8	120	20	8	0.24	0.43	0.33	0.88	0.12	0.88	0.12	0.00	0.00	0.00
Mar-Apr 2015	424	61	151	110	22	0	80	270	135	19	270	135	19	0.35	0.53	0.120	0.80	0.20	0.80	0.20	0.00	0.92	0.92
Total	572	73	177	130	42	8	142	390	155	27	390	155	27	0.32	0.51	0.17	0.82	0.18	0.82	0.18	0.00	0.09	0.09

P_{HWE}= test of significance for deviation from Hardy-Weinberg equilibrium

Table 2: Distribution of *kdr* alleles in dead and alive mosquitoes (F_0 and F_1) following one-hour exposure to the insecticide impregnated papers and test of genetic association of *kdr*–alleles with insecticide resistance

Insecticide		Alleles at locus					Test of genetic association					
		F1534*			S989/V1016#		Fisher's exact test			OR (fiducial limit at 95% CI)		
		F	C	L	S/V	P/G	F vs C	F vs L	S/V vs P/G	F vs C	F vs L	S/V vs P/G
DDT 4%	Alive	44	149	23	22	16						
	Dead	12	8	0	5	2	p <0.001	p <0.05	NS	5.08 (1.95-13.21)	Infinity	1.82 (0.31-10.59)
PER 0.75%	Alive	25	236	89	5	22						
	Dead	38	74	24	16	6	p <0.0001	p <0.0001	p <0.0001	4.85 (2.75-8.56)	5.64 (2.86-11.09)	11.73 (3.04-45.27)
DEL 0.05%	Alive	77	190	75	31	10						
	Dead	46	79	17	24	2	NS	p <0.01	NS	1.44 (0.92-2.25)	2.64 (1.39-5.00)	3.87 (0.77-19.35)

*Excluding individuals having mutant alleles 989P/1016G; #excluding individuals having mutant alleles 1534C/L

OR= Odd Ratio; CI= confidence interval; DDT=dichloro diphenyl trichloroethane; PER=permethrin; DEL= deltamethrin

Table 3. Criteria for scoring of F1534 alleles in PCR-RFLP assays

F1534 genotypes	Size of PCR-RFLP bands in	
	*Eco88*I-RFLP	*Ssi*I-RFLP
FF	171	171
FC	171	171, 103 and 68
FL	171, 103 and 68	171
CC	171	103 and 68
CL	171, 103 and 68	171, 103 and 68
LL	103 and 68	171