**Full title: *In vivo* functional validation of the V402L voltage gated sodium channel mutation in the malaria vector *An. gambiae*.**

**Short title: Characterization of a new knock down resistance mutation in the malaria vector *An. gambiae***

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**Abstract**

**BACKGROUND: Pyrethroids are the most widely used insecticides for the control of malaria transmitting *Anopheles gambiae* mosquitoes and rapid increase in resistance to this insecticide class is of major concern. Pyrethroids target the Voltage Gated Sodium Channels (VGSCs), that have a key role in the normal function of the mosquitoes’ nervous system. VGSC mutations L995F and L995S have long been associated with pyrethroid resistance and screening for their presence is routine in insecticide resistance management programs. Recently, a VGSC haplotype containing two amino acid substitutions associated with resistance in other species, V402L and I1527T, was identified. These two VGSC mutations are found in tight linkage and are mutually exclusive to the classical L995F/S mutations.**

**RESULTS: We identify the presence of the V402L-I1527T haplotype in resistant *An. coluzzii* colonized strains and in field populations from Burkina Faso at frequencies higher than previously reported; in some cases almost reaching fixation. Functional validation of V402L in insecticide resistance using a CRISPR/Cas9 genome modified line showed that it confers reduced mortality after exposure to all tested pyrethroids and DDT, but at lower levels compared to L995F. In contrast however to L995F, no fitness costs were identified for mosquitoes carrying V402L under laboratory conditions.**

**CONCLUSION: The V402L substitution confers pyrethroid resistance in *An. gambiae* in the absence of any other VGSC substitution and/or alternative resistance mechanisms. The lower fitness cost associated with this kdr mutation** **may provide a selective advantage over the classical kdr in some settings and genotyping at this locus should be added in the list of resistant alleles for routine screening.**

**Keywords: CRISPR/Cas9, LNA assays, target site resistance, functional validation, voltage gated sodium channel, mosquito.**

**Introduction**

**Pyrethroid insecticides have been, and remain, a central component of malaria control. The use of this insecticide class in Indoor Residual Spraying (IRS) and insecticide treated nets (ITNs)** has led to large reductions in the malaria burden in Africa (1). Over a billion ITNs, all impregnated with pyrethroids, have been distributed in Africa where it is estimated that at least 50% of the population at risk sleep under an ITN (2). However, as a consequence of the increasing use of pyrethroids, an immense selection pressure has been placed on malaria transmitting mosquitoes, and highly insecticide resistant populations have emerged threatening the efficacy of our most important tools (3). Whilst other chemistries have largely replaced pyrethroids for IRS, no insecticide class has yet been able to replicate the low toxicity, cost and fast mode of action of pyrethroids and, even newer ‘next generation’ nets, designed to control pyrethroid resistant mosquitoes still contain this chemistry in the presence of a second active ingredient (either another insecticide, sterilizing agent or synergist).

Pyrethroids target the Voltage Gated Sodium Channels (VGSCs) (4), that have a critical role in the function of the insect’s nervous system; VGSCs conduct sodium ions across the plasma membrane thereby initiating and propagating electrical signals needed for the insect’s movement, and reaction to external and internal stimuli with speed and coordination. Pyrethroid insecticides bind to sodium channels preventing their inactivation, which causes paralysis and eventually death (5). In *Anopheles gambiae s.s* and *Anopheles coluzzii* (two of the major malaria vectors in Africa belonging to the same species complex) two substitutions at codon 995 of the VGSC (within segment 6 of domain II), coding for the leucine to phenylalanine and serine substitutions (L995F/S), have been shown to reduce the VGSC’s sensitivity to pyrethroid insecticides and cause knock-down resistance (kdr) (6). Both mutations are found in high frequencies; L995F, also known as the classical *kdr* or 1014F, based on the *Musca domestica* codon numbering, approaches fixation in several parts of western and central Africa (7, 8) and L995S is widespread in central and eastern Africa (8). Thus, it is commonplace to screen for these two mutations as part of insecticide resistance monitoring and management programs.

Although VGSC is an essential protein, expected to be under strong purifying selection, examination of the phase I dataset of the *An. gambiae* 1000 Genomes Project (765 wild caught *An. gambiae s.l.* from 9 countries), identified 21 additional non-synonymous substitutions, at or above 5% frequency in one or more of the tested populations, indicating that target site resistance is likely more complex than thought (9). Thirteen of these substitutions were almost exclusively found on haplotypes carrying L995F. These include for example substitution N1570Y that has been shown to confer an additive protection to pyrethroids (7, 10) and two single-base pair substitutions at codon 1874, resulting in the P1874S and L variants, that have been hypothesized to compensate for fitness costs associated with the L995F mutation (11), but have also been associated with pyrethroid resistance in agricultural pests (12).

In contrast, some of the newly identified non-synonymous substitutions were never found on the same haplotype with mutation L995F, indicating that these might be competing with the classical *kdr*. Among these is substitution V402L (at segment 6 of domain I) (Figure 1), that results from either of two nonsynonymous single nucleotide polymorphisms (9). The equivalent mutation in *Ae. aegypti*, V410L was recently identified and associated with resistance to both type I and type II pyrethroids (13). In *An. coluzzii* V402L also showed a very strong linkage with a second mutation, the I1527T (at segment 6 of domain III) (Figure 1); almost all individuals with the I1527T substitution, which is adjacent to a predicted pyrethroid binding site, had one of the two mutations resulting in the V402L substitution (9). The observation that the V402L-I1527T haplotype is under positive selection is supportive for a role in resistance in this species, but the levels of resistance conferred by these alternative mutations and their associated fitness costs are unknown. This information is crucial to evaluate the importance of the novel mutations in insecticide resistance in field populations.

In this study we explore the role of the V402L and I1527T mutations in *An. gambiae s.l* pyrethroid resistance. We report the presence of the V402L-I1527T haplotype in several highly resistant *An. coluzzii* colonized strains and in field populations from Burkina Faso. We observed changes in the frequency of this haplotype in several cases over time, occasionally reaching high levels, almost replacing the L995F haplotype. We functionally validated the contribution of the V402L mutation in insecticide resistance by generating a CRISPR/Cas9 genome modified *An. gambiae* strain carrying this mutation in an otherwise fully insecticide susceptible genetic background. We also designed LNA (Locked-Nucleic Acid) diagnostic assays for rapid monitoring of the novel V402L, I1527T and P1874S/L mutations.

**Materials and methods**

**Mosquito maintenance**

All mosquito colonies are maintained under standard insectary conditions: temperature of 26°C ± 2°C, 70% relative humidity ± 10% and L12:D12 hour light:dark photoperiod. The field colonized strains VK7 2014, Tiassalé, Banfora M and Tiefora are regularly selected for pyrethroid resistance at every 3rd to 5th generation by exposing them to standard WHO insecticide treated papers with either deltamethrin (0.05%) or permethrin (0.75%).

**Extraction of DNA from lab colonies and screening for VGSC mutations**

Genomic DNA was extracted from individual non-blood-fed females from each colony (Tiefora, Banfora M and VK7 2014) using the Qiagen blood and tissue DNA extraction kit according to manufacturer’s instructions. Initially, to determine if novel *vgsc* SNPs were present in resistant laboratory colonies, 16 individuals were screened in pools of four (combining 2 μl of DNA extract from each individual and using 2ul of the mixture as template). Three PCR reactions were performed to amplify fragments of the *vgsc* gene spanning the site of the three novel mutations (V402L, I1527T and P1874S/L). Each PCR reaction (total volume of 20μl) contained: 4 µl Buffer (HF), 0.2 µl Phusion Hot Start II High Fidelity Polymerase, 0.4 µl of 10 mM dNTPs, 0.5 µM of each primer (Supplementary Table 2), 11.4 µl H20, and 2 µl DNA template.

The PCR conditions were: an initial denaturation step of 98˚C for 30 seconds followed by 33 cycles of 98 ˚C for 10 seconds, 60 ˚C for 10 seconds, and 72 ˚C for 15 seconds, with a final extension of 5 minutes at 72 ˚C. 5 μl of the reaction were analysed by gel electrophoresis using a 1% agarose stained with peqGREEN (peqlab). The remaining 15 μl were purified using a PCR purification kit (Qiagen) in accordance with the manufacturer’s instructions. Amplicons were then sent for sequencing to Source BioScience, UK using either the forward or reverse primer (defined in Supplementary Table 2). The sequenced data were analysed with the sequence alignment software BioEdit and the presence of base pair substitutions evaluated. Screening for the L995F mutation was done using a previously established LNA based diagnostic assay (14). For strains in which the first 16 individuals were wild type at each of the three codons, 8 further pools of 4 mosquitoes were sent for sequencing.

Having established the presence of each of the VGSC mutants in the strains, PCR was conducted on individual mosquitoes to determine the frequency of *vgsc* mutations. DNA was extracted from individual non-blood fed females using either the Qiagen blood and tissue DNA extraction kit or by adding individual mosquitoes in 50 μl of STE buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) and incubating them at 95oC for 20-25 min. 1 μl of DNA extract was subsequently used in the LNA-based diagnostic assays described below for the novel mutations and the previously described LNA assay for the L995F mutation.

**Design of LNA based diagnostic assays for *kdr* mutations**

The design of the LNA-based diagnostic assays for the four novel mutations (I1527T, V402L and P1874S/L) was done as previously described by Lynd et al., 2018 (14). Briefly for each assay primers were designed to amplify a single region spanning the site of the mutation. PCR products were of the following sizes: 167 bp for V402L, 236 bp for I1527T and 117 bp for P1874S and L. Probes were designed using the IDT Biophysics software taking care to have an off-target Tm difference of at least 10 °C, whilst keeping the exact match target Tm within 3°C. This allows target binding, but prevents non-target binding. Reactions were set up using 1×Luna Universal qPCR Master Mix (NEB), 0.1 μM for each probe (IDT), 0.2 μM of primers and 1-2 μl of DNA extract in a total reaction volume of 10 μl. Reactions were run on an AriaMX qPCR cycler with the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Results were analysed using the AriaMX software V1.5. When the endpoint fluorescence value of a probe exceeded the threshold (background fluorescence) it was counted as a positive call for the respective allele (Supplementary Figures 1 ). Τhe 3D scatter plot for the V402L LNA assay (Figure 2) was done using the R package Plotly.

**Field sample collection and species identification**

Field mosquitoes were collected as larvae from rice fields in Tengrela village in the Cascades District Burkina Faso (GPS coordinates: 10° 37.447' N, 004° 33.201' E) in 2016, and 2019 and stored on silica gel before being shipped to the Liverpool School of Tropical Medicine. DNA was extracted by placing individual mosquitoes in 50 μl STE buffer (0.1M NaCl, 10mM Tris-HCl pH 8, 1 mM EDTA pH 8), followed by heating to 90 oC for 20-25 minutes in a thermo-cycler. 2 μl of the DNA extract were used for identifying the species ID and the molecular form of *An. gambiae s.s*. using the SINE PCR protocol described in (15).

**Generation of CRISPR/Cas9 modified Kisumu-402L/L line**

The CRISPR/Cas9 strategy described in (16) was followed to generate an *An. gambiae* line carrying the V402L mutation in a susceptible genetic background. Briefly, two plasmids were prepared:

Donor plasmid: a 1,600 bp region of the VGSC (AGAP004707-RA) gene having the 402 codon in the middle (homology arms extending 800 bp either direction) was PCR amplified using DNA extracted from the Kisumu insecticide susceptible *An. gambiae* colony and primers V402F\_Donor and V402R\_Donor (Supplementary Table 4) having overhangs for EcoRI and BamHI digestion respectively. The cycling conditions of the PCR were: 95 ˚C for 30 seconds followed by 35 cycles of 95 ˚C for 30 seconds, 55 ˚C for 30 seconds, and 72 ˚C for 80 seconds, with a final extension of 5 minutes at 72 ˚C. The PCR amplified region was purified with the QIAquick PCR Purification Kit (Qiagen) and cloned in the puc19 vector using restriction enzymes EcoRI and BamHI. Two mutations a G->C transversion generating the V402L mutation (codon alteration GTA->CTA) and a synonymous C->G transversion that abolishes the PAM site to avoid cleavage of the donor plasmid by Cas9 (Supplementary Figure 2) were introduced using NEB’s site directed mutagenesis kit and primers VGSC-V402L\_mutF and R (Supplementary table 4). The correct sequence of the donor plasmid was verified with sequencing (at GENEWIZ).

CRISPR plasmid: The p174 plasmid generated in (17), carrying: a human-codon-optimized Cas9 under the control of the germline specific zpg (zero population growth) promoter, a 3xP3::RFP marker and a U6::gRNA spacer cloning cassette was used. The gRNA target was identified and assessed for off targets using the ChopChop (https://chopchop.rc.fas.harvard.edu) website. Single stranded DNA oligos with complete homology to the target sequence (Supplementary table 4) and overhangs compatible with the BsaI digested p174 were annealed and cloned in p174 using Golden gated cloning. The correct cloning of the gRNA sequence was confirmed with sequencing (at GENEWIZ).

Kisumu embryo injections with the mixture of the two plasmids, identification of positive G1 transformants and establishment of the Kisumu-402L/L line were done as described in (16). The only modification we introduced was that the Kisumu females crossed with G0 injected males were left to lay *en masse* and not individually. The V402L LNA assay developed here was used to identify individuals carrying the mutation. The genome edited locus was verified by sequencing the PCR product of the VGSC-V402F\_Donor and VGSC-V402R\_Donor primers (Supplementary table 4) at GENEWIZ.

**Evaluating the insecticide resistance profile and levels of Kisumu-402L/L**

WHO tube bioassays (18) were performed to evaluate insecticide resistance of Kisumu-402L/L. Briefly, we exposed 2-5 day old female mosquitoes to insecticide impregnated papers of standard discriminating doses: 0.75% permethrin, 0.05% deltamethrin, 4% DDT and 0.05% a-cypermethrin (obtained from Universiti Sains Malaysia) for 60 min. Mortality was recorded after a 24h recovery period. Welch’s t test was performed to determine statistical differences between mortality rates in Kisumu-402L/L and Kisumu using GraphPad Prism 9.0.0. To determine the LT50 (exposure time resulting in 50% mortality) we varied the exposure time (Supporting Information dataset 1). Knock down was scored immediately after exposure and mortality after a 24h recovery period. LT50 values and Resistance Ratio were calculated by probit analysis (PoloPlus, LeOra Software). In all cases at least three replicates of 20 female mosquitoes (2-5 day old) were used per time point.

**Assessing the impact of V402L on life history traits**

The impact of V402L on fertility, fecundity, larval development and female adult longevity was assessed following the same procedures as described in a previous publication (16), where we studied the impact of mutation L995F (using the same genetic background of Kisumu) on these life history traits

**Results**

**Identification of non-synonymous VGSC mutations in colonized *An. coluzzii* strains**.

Three insecticide resistant *An. coluzzii* strains from Burkina Faso: VK7 2014, Banfora M and Tiefora (origin and colonization date shown in Supplementary Table 1), that show mortality less than 13% after 1h exposure to standard WHO bioassays with the pyrethroids deltamethrin, permethrin and alpha cypermethrin and to the organochloride DDT (previously described in (19, 20)) , were tested for the presence of VGSC mutations L995F, I1527T, P1874S/L and V402L (Figure 1). A region spanning the codons for mutations I1527T, P1874S/L and V402L was PCR amplified (Supplementary Table 2) from 12-48 individuals per strain and sequenced in pools of four, while the presence of mutation L995F was tested using a previously established LNA assay (14). Mutations L995F and P1874S were present in all three strains, whilst mutations V402L and I1527T were present in Tiefora and Banfora M only. Mutation P1874L was not found in any of the strains.

**Design of LNA based molecular diagnostics.**

To enable rapid screening for mutations P1874S/L, V402L and I1527T we developed LNA based diagnostic assays. For the I1527T assay two probes were designed: a wild type specific, labelled with the HEX fluorophore and a mutant specific, labelled with the FAM fluorophore (Table 1). In the case of P1874S/L, a triplex assay was initially tested including two mutant probes (FAM labelled for P1874S and Cy5 labelled for P1874L) and a wild type probe (labelled HEX) (Supplementary table 3). However, in some reactions with template from wild type individuals, a low intensity background signal from the FAM and Cy5 probes was detected (Supplementary figure 1A). Thus, we developed two separate reactions (Table 1) to screen for the two mutations, as described in the methods, in which case we did not encounter issues with background signal (Supplementary Figure 1B and 1C). For V402L, a triplex assay was designed containing a wild type probe (labelled HEX) and two mutant probes, one for each of the two alleles reported to code for the leucine mutation (labelled FAM for the G>T transversion, and Cy5 for the G>C transversion) (Table 1). All three diagnostic assays were tested using specimens of known genotype. In the case of the V402L (C variant), in which we didn’t have access to a positive mosquito specimen, the CRISPR donor plasmid (described below) was used as control. After optimization all assays showed clear discrimination of the different genotypes, as can be seen from the clustering of samples in scatter plots (Figure 2).

**Changes in the frequency of mutations L995F and V402L-I1527T in colonized *An. coluzzii* strains.**

Using the above LNA assays we screened individuals from four insecticide resistant *An. coluzzii* strains using samples stored at different time points following their initial colonization (19). All 576 samples tested contained at least one *vgsc* mutation. The 402L and 1527T alleles were in complete linkage equilibrium, whereas the 402L-1527T haplotype was in disequilibrium, never being found with codons L995F/S . The frequencies of the two *vgsc* haplotypes fluctuated greatly in the Tiefora and Banfora M with the frequency of the 402L-1527T haplotype exceeding the frequency of the 995F at all sampling points. In contrast, in VK7 2014 and Tiassalé the initial frequency of the 402L-1527T haplotype was low (0.2 in VK7 2014 and 0.05 in Tiassalé) and remained always lower than the 995F, until it disappeared from the colonies and 995F became fixed (Figure 3). The disappearance of the 402L-1527T haplotype from these two colonies could be the result of genetic drift, given its low initial frequency and the limited mosquito population size that can be kept under laboratory conditions.

**Changes in the frequency of mutations L995F and V402L-I1527T in *An. coluzzii* field populations.**

*Anopheles* mosquitoes were collected in 2016 (N=35) and 2019 (N=49) from the rice fields of Tengrela in southwest Burkina Faso. All collected mosquitoes were identified as *An. coluzzii* using a PCR-RFLP assay (15) and screened for mutations L995F and V402L-I1527T using the established LNA assays. In 2016, the frequency of the 995F allele was 0.82 and the frequency of 402L-1527T 0.18. In 2019, the frequency of 995F dropped to 0.63, while the frequency of 402L-1527T increased to 0.37 (Figure 4).

**Genome modified mosquitoes carrying mutation V402L show increased levels of resistance to pyrethroids and DDT.**

CRISPR/Cas9 was used to introduce mutation V402L in the insecticide susceptible laboratory strain Kisumu (hereafter called Kisumu-402L/L). Approximately 650 eggs were injected, from which 170 larvae hatched. Seven of these larvae had transient RFP (Red Fluorescent Protein) expression (from the 3xP3:RFP present on the CRISPR plasmid) in their anal papillae. These RFP positive individuals were backcrossed with the Kisumu strain in sex specific cages. G0 females did not produce eggs, while Kisumu females crossed with G0 males produced progeny, in which positive transformants were identified at a frequency of 37%. A homozygous 402L/L line was established at the G2 generation.

 The resistance profile of the Kisumu-402L/L line was tested through standard WHO tube bioassays by exposing female mosquitoes to papers impregnated with a discriminating dose for permethrin (0.75%), deltamethrin (0.05%), α-cypermethrin (0.05%) and DDT (4%) for 1 h. Based on WHO criteria, (mortality of less than 90%, 24 h after exposure) Kisumu-402L/L showed resistance to permethrin, a-cypermethrin and DDT (Figure 5).

We also performed time response bioassays to obtain quantitative data on the resistance levels. We estimated the time required to obtain 50% mortality (LT50) in the Kisumu-402L/L line and compared it to the LT50 of the Kisumu susceptible line. The resistance ratio of Kisumu-402L/L was 5.1 fold for deltamethrin, 1.9 fold for permethrin, 7.1 fold for a-cypermethrin and 4 fold for DDT (Table 2).

**No fitness costs were observed for mosquitoes carrying the V402L mutation under lab conditions.**

We tested the effect of mutation V402L on several life history traits. No difference was observed in fecundity (12.2% of Kisumu-402L/L females and 12.9% of Kisumu females did not oviposit, P >0.99) (Figure 5A). In addition, no difference was observed in the mean number of eggs laid per female (62 (±24 SD) for Kisumu-402L/L and 61 (±31 SD) for Kisumu, P=0.96) (Figure 5B), nor in the mean number of larvae that hatched (34 (±28 SD) for Kisumu-402L/L and 44(±23 SD) for Kisumu, P=0.07) (Figure 5D). Similarly, no difference was observed in the development of L1 instar larvae to pupae (90% (±5 SD) of Kisumu-402L/L reached pupal stage vs 92% (±5 SD) for Kisumu). We also followed the longevity of females from the two strains and found no difference in lifespan (median survival 19 days for Kisumu-402L/L and 21 days for Kisumu, P=0.2).

**Discussion**

Pyrethroid resistance is a major threat for malaria control programs that largely rely on the use of this insecticide class to reduce the number of *Anopheles* mosquitoes, and thus the number of *Plasmodium* infectious bites. Managing the problem of insecticide resistance requires understanding its molecular basis. Target site resistance is one of the most commonly reported mechanisms in pyrethroid resistant *Anopheles* mosquitoes and its presence is predominantly identified through screening for the L995F and L995S mutations, as these are the most widely studied and well characterized VGSC mutations (21, 22). Here, we have functionally validated the role of the alternative V402L mutation, that based on *An. gambiae* whole genome sequencing data (9) appears to be mutually exclusive to L995F/S, and show that it confers reduced mortality to pyrethroids and DDT. This mutation has been previously reported in *An. coluzzii* from Ghana, Burkina Faso, Côte d'Ivoire and Guinea at low frequencies, not exceeding 0.127 (9). In this study we report the presence of V402L at much higher frequencies; in colonized *An. coluzzii* resistant populations, its frequency reached 0.99 and in field samples from Burkina Faso 0.37. More screening needs to be done in the future to see if the frequency of this mutation will remain at these levels or change.

By introducing V402L with CRISPR in the insecticide susceptible Kisumu strain, we were able to reveal its contribution to insecticide resistance when in isolation, and directly compare its effect size to that of the classical *kdr* mutation L995F, which we previously functionally characterized in the same way (16). Based on the standard WHO discriminating dose bioassay, the Kis-402L/L line would be characterized as resistant to permethrin, a-cypermethrin and DDT, while it would not be characterized resistant to deltamethrin, showing mortality of >90%. However, as has been previously reported (16) and shown here, the discriminating doses for the different insecticides are not directly comparable and quantitative data are required to compare resistance levels between insecticides and strains. The Kisumu-402L/L line showed resistance ratios for the three pyrethroids: deltamethrin, permethrin and a-cypermethrin of 5.1, 1.9 and 7.1 fold respectively, which were lower than the resistance ratios observed for Kis-995F/F (14.6 fold for deltamethrin, 9.9 fold for permethrin and 19.7 fold for α-cypermethrin). The same was shown for the organochloride DDT; resistance of Kis-402L/L was 4 fold, while resistance of Kis-995F/F was >24 fold. The equivalent to V402L mutation in *Ae. aegypti* (V410L) has also been associated with pyrethroid resistance and its effect has been characterized using the *Xenopus* oocyte system. *Ae. aegypti* sodium channels carrying the V410L mutation showed a 10 fold reduction in sensitivity to permethrin and deltamethrin (13) but the differences observed could also be related to the different functional validation methods used. Despite the relatively low levels of resistance conferred by V402L in isolation, it is important to note that it could have a combined effect (additive or even multiplicative) with mutation I1527T, with which it is found in strong linkage, or in the presence of other resistance mechanisms, like over-expression of detoxification enzymes, as has been previously shown for L995F (16, 23). Attempts to create a double mutant line carrying both V402L and I1527T were unsuccessful, but different gRNAs will be tested in the future.

The association of insecticide resistance mechanisms with fitness costs is a critical prerequisite for resistance management strategies that rotate the use of insecticides with different mode of action and rely on the presence of fitness costs to reduce the frequency of resistant alleles. The availability of the Kisumu and Kisumu-402L/L lines that share the same genetic background provided the opportunity to test the impact of mutation V402L on life history traits, with the least possible confounding effects. No difference between the two strains was observed in terms of fertility, fecundity, development of larvae to pupae and longevity. On the other hand, mutation L995F has previously been shown to have pleiotropic effects resulting in reduced fecundity and longevity, alongside an effect on larval development. Thus, although V402L confers lower levels of resistance compared to L995F, at least in isolation, we hypothesize that it could persist in field populations due to the lower (or absent) fitness costs. Furthermore, it could compete with the L995F mutation and increase in frequency in cases where insecticide selection pressure decreases or if its combined effect with other mechanisms equals (or exceeds) the resistance levels provided by haplotypes carrying the L995F. Further screening needs to be done in the future, at different locations and over time, to reveal how the frequency of these two mutations changes and if there are patterns that could be related to the selection pressure applied.

Insecticide resistance management strategies are aided by the availability of DNA-based diagnostics that can easily and reliably detect the presence (even at low frequencies), increase and spread of resistance in field populations (24). Here we have developed LNA-based diagnostics for four VGSC mutations: the V402L and I1527T, as well as the two: P1874S and P1874L, that are almost exclusively found in combination with L995F. The developed assays can reliably distinguish the wild type, heterozygote and mutant homozygote haplotypes in a rapid assay.

 In conclusion, our study provides an enriched toolbox to screen for knock down resistance in the malaria vector *An. gambiae* and demonstrates the role of additional *vgsc* mutations in conferring pyrethroid/DDT resistance. We have shown that the frequency of the 402L-1527T haplotype is increasing in southwest Burkina Faso, and monitoring for the emergence of this haplotype in other locations along with further studies on the implication for pyrethroid resistance, are important priorities for resistance management. Our data also caution against interpreting reductions in the frequency of DNA based makers, such as the classical 995F kdr marker, as signs that resistance is declining; resistance monitoring programs need to be ever vigilant for the emergence of new resistance mechanisms.

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**Conflict of Interest Statement**

The authors declare that they have no conflicts of interests.

**References**

1. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature. 2015;526(7572):207-11.

2. WHO. World malaria report 2019. Geneva: World Health Organization.

3. Churcher TS, Lissenden N, Griffin JT, Worrall E, Ranson H. The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. Elife. 2016;5.

4. Narahashi T. Neuroreceptors and ion channels as the basis for drug action: past, present, and future. J Pharmacol Exp Ther. 2000;294(1):1-26.

5. Dong K, Du Y, Rinkevich F, Nomura Y, Xu P, Wang L, et al. Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem Mol Biol. 2014;50:1-17.

6. Burton MJ, Mellor IR, Duce IR, Davies TG, Field LM, Williamson MS. Differential resistance of insect sodium channels with kdr mutations to deltamethrin, permethrin and DDT. Insect Biochem Mol Biol. 2011;41(9):723-32.

7. Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, Donnelly MJ, et al. Footprints of positive selection associated with a mutation (N1575Y) in the voltage-gated sodium channel of Anopheles gambiae. Proc Natl Acad Sci U S A. 2012;109(17):6614-9.

8. Silva AP, Santos JM, Martins AJ. Mutations in the voltage-gated sodium channel gene of anophelines and their association with resistance to pyrethroids - a review. Parasit Vectors. 2014;7:450.

9. Clarkson CS, Miles A, Harding NJ, O'Reilly AO, Weetman D, Kwiatkowski D, et al. The genetic architecture of target-site resistance to pyrethroid insecticides in the African malaria vectors Anopheles gambiae and Anopheles coluzzii. Mol Ecol. 2021.

10. Wang L, Nomura Y, Du Y, Liu N, Zhorov BS, Dong K. A mutation in the intracellular loop III/IV of mosquito sodium channel synergizes the effect of mutations in helix IIS6 on pyrethroid resistance. Mol Pharmacol. 2015;87(3):421-9.

11. Lucas ER, Rockett KA, Lynd A, Essandoh J, Grisales N, Kemei B, et al. A high throughput multi-locus insecticide resistance marker panel for tracking resistance emergence and spread in Anopheles gambiae. Sci Rep. 2019;9(1):13335.

12. Sonoda S, Tsukahara Y, Ashfaq M, Tsumuki H. Genomic organization of the para-sodium channel alpha-subunit genes from the pyrethroid-resistant and -susceptible strains of the diamondback moth. Arch Insect Biochem Physiol. 2008;69(1):1-12.

13. Haddi K, Tome HVV, Du Y, Valbon WR, Nomura Y, Martins GF, et al. Detection of a new pyrethroid resistance mutation (V410L) in the sodium channel of Aedes aegypti: a potential challenge for mosquito control. Sci Rep. 2017;7:46549.

14. Lynd A, Oruni A, Van't Hof AE, Morgan JC, Naego LB, Pipini D, et al. Insecticide resistance in Anopheles gambiae from the northern Democratic Republic of Congo, with extreme knockdown resistance (kdr) mutation frequencies revealed by a new diagnostic assay. Malar J. 2018;17(1):412.

15. Fanello C, Santolamazza F, della Torre A. Simultaneous identification of species and molecular forms of the Anopheles gambiae complex by PCR-RFLP. Med Vet Entomol. 2002;16(4):461-4.

16. Grigoraki L, Cowlishaw R, Nolan T, Donnelly M, Lycett G, Ranson H. CRISPR/Cas9 modified An. gambiae carrying kdr mutation L1014F functionally validate its contribution in insecticide resistance and combined effect with metabolic enzymes. PLoS Genet. 2021;17(7):e1009556.

17. Hammond A, Karlsson X, Morianou I, Kyrou K, Beaghton A, Gribble M, et al. Regulating the expression of gene drives is key to increasing their invasive potential and the mitigation of resistance. PLoS Genet. 2021;17(1):e1009321.

18. Organization WH. Test Procedures for Insecticide Resistance Monitoring in Malaria Vector Mosquitoes (World Health Organization, Geneva, Switzerland, ed. 2, 2016).

19. Williams J, Flood L, Praulins G, Ingham VA, Morgan J, Lees RS, et al. Characterisation of Anopheles strains used for laboratory screening of new vector control products. Parasit Vectors. 2019;12(1):522.

20. Williams J, Ingham V, Morris M, Aristide H, Hyacinthe T, Dabire R, et al. Sympatric populations of the An gambiae complex in southwest Burkina Faso evolve multiple diverse resistance mechanisms in response to intense selection pressure with pyrethroids. Submitted. 2021.

21. Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004;34(7):653-65.

22. Donnelly MJ, Isaacs AT, Weetman D. Identification, Validation, and Application of Molecular Diagnostics for Insecticide Resistance in Malaria Vectors. Trends Parasitol. 2016;32(3):197-206.

23. Samantsidis GR, Panteleri R, Denecke S, Kounadi S, Christou I, Nauen R, et al. 'What I cannot create, I do not understand': functionally validated synergism of metabolic and target site insecticide resistance. Proc Biol Sci. 2020;287(1927):20200838.

24. Weetman D, Donnelly MJ. Evolution of insecticide resistance diagnostics in malaria vectors. Trans R Soc Trop Med Hyg. 2015;109(5):291-3.

**Tables**

**Table 1**: **Primers and probes used in the LNA molecular diagnostics.** + preceding a base indicates it is an LNA nucleotide. IBFQ (Iowa Black Fluorescent Quencher).

|  |  |  |  |
| --- | --- | --- | --- |
| Name | 5’ Fluorescence modification  | Sequence (5’-3’) | 3’ Quencher modification  |
| **I1527T** |
| 1527-F primer |  | GTCGGTAAACAGCCTATACGGG |  |
| 1527-R primer |  | TTCTAGCGATCCACCAGC |  |
| I1527 Wildtype Iso | HEX | ACC+CAAA+GA+T+A+A+TAAAG | IBFQ |
| 1527T Mutant Thr | FAM | C+CA AA+G A+T+A +G+TA AAG | IBFQ |
| **V402L** |
| 402-F primer |  | GTGTTACGATCAGCTGGACCG |  |
| 402-R primer |  | CCGAAGTGCTTCTTCCTCGG |  |
| V402 Wildtype Val | HEX | TT+A+C+AA+G+GTAAAA+CGA | IBFQ |
| 402L(T) Mutant Leu | FAM | AATT+A+A+AA+G+GTAAAA+C+GA | IBFQ |
| 402L(C) Mutant Leu  | CY5 | TT+A+G+AA+G+GTAAAA+CG | IBRQ |
| **P1874S** |
| 1874-F primer |  | AAGGCTTAACTGATGACGATTATG |  |
| 1874-R primer |  | GGTTCCAGCACATCCAAA |  |
| 1874 Wildtype Pro | HEX | TC+GA+T+C+CTGACG | IBFQ |
| 1874S Mutant Ser | FAM  | C+C+G T+C+A +G+AA T | IBFQ |
| **P1874L** |
| 1874-F primer |  | AAGGCTTAACTGATGACGATTATG |  |
| 1874-R primer |  | GGTTCCAGCACATCCAAA |  |
| 1874 Wildtype Pro | HEX | TC+GA+T+C+CTGACG | IBFQ |
| 1874L Mutant Leu | CY5  | TC+GAT+C+T+TGA+CGG | IBRQ |

**Table 2**: **Time response bioassay results**. The LT50 (time required to obtain 50% mortality) values are given for each strain. Resistant Ratios (LT50 resistant strain/ LT50 control strain) are given in comparison to Kisumu. Upper and Lower limits represent the 95% fiducial limits of the LT50. For each time point at least 3 replicates of 20 female mosquitoes 2–5 day old were used per strain. Raw data provided in Supplementary dataset 1.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Insecticide** | **Strain** | **LT50** | **Upper and Lower Limits (0.95)** | **Resistance Ratio (0.95)** |
| **Deltamethrin** | Kisumu-402L | 17.5 min | 20.4-14.7 | **5.1** (6.5-3.9) |
|  | Kisumu | 3.4 min | 4.4-2.5 |  |
|  |  |  |  |  |
| **Permethrin** | Kisumu-402L | 34.3 min | 48.8-20.7 | **1.9** (2.3-1.6) |
|  | Kisumu | 17.6 min | 21.8-13.9 |  |
|  |  |  |  |  |
| **α-cypermethrin** | Kisumu-402L | 36.8 min | 45.2-29.1 | **7.1** (8.6-5.8) |
|  | Kisumu | 5.1 min | 6.2-4.1 |  |
|  |  |  |  |  |
| **DDT** | Kisumu-402L | 129.4 min | 151.3-108.6 | **4.0** (4.6-3.5) |
|  | Kisumu | 31.8 min | 37.7-26.1 |  |

**Figure legends**

**Figure 1: Schematic representation of the *An. gambiae* Voltage Gated Sodium Channel.** Mutations referred to in this work are represented with red dots.

**Figure 2:** **Scatter plots showing distinct genotype groupings for VGSC mutations, as determined by the endpoint fluorescence measurement for the different probes used in the LNA assays.** A) 3D scatter plot for the V402L triplex assay with: V402 wild type probe labelled Hex, 402L (G->T base change) mutant probe labelled Fam and 402L (G->C base change) mutant probe labelled Cy5. B) Bi-directional scatter plot for the I1527T assay: I1527 wild type probe labelled Hex and 1527T mutant probe labelled Fam. C) Bi-directional scatter plot for the P1874S assay: P1874 wild type probe labelled Hex and 1874S mutant probe labelled Fam. D) Bi-directional scatter plot for the P1874L assay: P1874 wild type probe labelled Hex and 1874L mutant probe labelled Cy5.

**Figure 3:** **Graphs showing the change in the frequency of the 995F and 402L-1527T haplotypes in four insecticide resistant strains at or close to the time of colonization and thereafter.** Tiefora 2018 N=42, Tiefora 2019 N=77, Tiefora 2020 N=46, Banfora 2018 N=46, Banfora 2019 N=63, Banfora 2021 N= 48, VK7-2014 Feb2015 N= 25, VK7-2014 Oct2015 N=44, VK7-2014 2016 N=48, Tiassale 2013 N=44, Tiassale 2015 N=44, Tiassale 2018 N=48.

**Figure 4**. **Graph showing the change in the frequency of the 995F and 402L-1527T haplotypes in field collected *An. coluzzii* mosquitoes from Tengrela (Burkina Faso) collected in 2016 and 2019.** Samples analysed: N=35 from 2016 and N=49 from 2019.

**Figure 5**. **WHO discriminating dose bioassays**. 2–5-day old females were exposed to standard WHO discriminating doses for 1h and mortality recorded 24h later. Error bars represent the SD (at least three replicates of 20 female mosquitoes each were used per strain). The dotted line marks the WHO 90% mortality threshold for defining resistance. Welch’s t test with P\* < 0.05 significance cutoff. \*\*P <0.01; \*\*\*P <0.001; \*\*\*\*P <0.0001.

**Figure 6: Assessing the effect of VGSC mutation V402L on life history traits**. A) Pie charts showing no difference in female fecundity (percentage of females that laid at least one egg vs females that did not lay any eggs) between the Kisumu and Kisumu-402L/L strains; Fisher’s exact test, P >0.9; number of females tested: 54 Kisumu and 41 Kisumu-402L/L. B) The mean number of eggs laid per Kisumu and Kisumu-402L female is depicted. Standard errors represent the standard deviation (SD). No significant difference was observed; Mann Whitney test, P =0.96; number of females tested: 47 Kisumu and 36 Kisumu-402L/L ). C) The percentage of L1 larvae reaching the pupae stage is shown for the two strains. Error bars represent the SD, seven replicates of 70 larvae each were tested. No significant difference was observed; Unpaired t-test, P =0.5). D) The mean number of larvae that hatched from each female’s egg batch is shown. No difference is observed between Kisumu and Kisumu-402L strains; Mann Whitney test, P=0.07; number of females tested: 47 Kisumu and 36 Kisumu-402L/L). E) The lifespan of thirty Kisumu and Kisumu-402L females was followed. No significant difference was observed between the strains; Mantel-Cox test, P = 0.2). Raw data are provided in Supplementary dataset 1.