Research Article

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Identification of putative *kdr* mutations in the tropical bed bug, *Cimex hemipterus* (Hemiptera: Cimicidae)

Kai Dang,[a,b] Cheryl S Toi,[a] David G Lilly,[a] Chow-Yang Lee,[c] Richard Naylor,[d] Apiwat Tawatsin,[e] Usavadee Thavara,[e] Wenun Bu[b] and Stephen L Doggett[a*]

Abstract

BACKGROUND: Bed bugs [both *Cimex hemipterus* (F.) and *Cimex lectularius* L.] are highly resistant to pyrethroids worldwide. An important resistance mechanism known as 'knockdown resistance' (*kdr*) is caused by genetic point mutations on the voltage-gated sodium channel (VGSC) gene. Previous studies have identified two point mutations (V419L and L925I) on the VGSC gene in *C. lectularius* that are responsible for *kdr*-type resistance. However, the *kdr* mutations in *C. hemipterus* have not been investigated.

RESULTS: Four novel mutations, L899V (*leucine* to *valine*), M918I (*methionine* to *isoleucine*), D953G (*aspartic acid* to *glycine*) and L1014F (*leucine* to *phenylalanine*), were identified in the domain II region of the *C. hemipterus* VGSC gene. This region has been widely investigated for the study of *kdr*-type resistance to pyrethroids in other insect pests. The V419L and L925I *kdr* mutations as previously identified in *C. lectularius* were not detected in *C. hemipterus*.

CONCLUSION: M918I and L1014F are considered to be probable *kdr* mutations and may play essential roles in *kdr*-type resistance to pyrethroids in *C. hemipterus*. Further studies are under way in the authors' laboratory to determine the non-*kdr*-type resistance mechanisms in *C. hemipterus*.

Keywords: *Cimex*; insecticide resistance; knockdown resistance; sodium channel

1 INTRODUCTION

Bed bugs [*Cimex hemipterus* (F.) and *C. lectularius* L.] have been undergoing a global resurgence over the last two decades.[1,2] An often integral part of the control strategy in bed bug control is insecticide application,[3] and pyrethroids have been widely employed owing to their low mammalian toxicity and fast knockdown activity. However, recent research indicates that bed bugs of both species are resistant to pyrethroids. Pyrethroid resistance in the tropical bed bug, *C. hemipterus*, has been documented in Africa, Sri Lanka and Thailand.[4–6]

An important resistance mechanism known as 'knockdown resistance' (*kdr*) has been identified in previous studies on other insect pests, caused by various point mutations on the voltage-gated sodium channel (VGSC) gene that reduce the target-site sensitivity of pyrethroids and DDT.[7–9] V419L and L925I *kdr* mutations have been identified in the common bed bug, *C. lectularius*, and are responsible for high resistance to pyrethroids.[10] Subsequently, these mutations have been detected widely across the United States.[11]

A modern population of *C. hemipterus* from Sri Lanka was suspected to have *kdr*-type resistance, with the suggestion that this mechanism was responsible for the observed high tolerance to both DDT and the pyrethroids; however, the assumption was not verified.[5] To date, *kdr*-mediated pyrethroid resistance in *C. hemipterus* remains unknown. Hence, the present study aimed to identify putative *kdr* mutations on the VGSC gene that could be associated with pyrethroid resistance in *C. hemipterus*.

2 MATERIALS AND METHODS

2.1 Bed bug populations

Test samples of *C. hemipterus* collected from multiple regions around the world, including Africa (AF), Australia (AU), India (IN), Malaysia (MA) and Thailand (TH), are listed in Table 1, as well as

* *Correspondence to: Stephen L Doggett, Department of Medical Entomology, Pathology West, ICPMR, Westmead Hospital, Locked Bag 9001, Westmead, NSW 2145, Australia. E-mail: Stephen.Doggett@health.nsw.gov.au*

a *Department of Medical Entomology, Institute for Clinical Pathology and Medical Research, Westmead Hospital, Westmead, NSW, Australia*

b *Institute of Entomology, College of Life Sciences, Nankai University, Tianjin, China*

c *Urban Entomology Laboratory, Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia*

d *Prior's Loft, Coleford Road, Tidenham, Monmouthshire, UK*

e *Department of Medical Sciences, National Institute of Health, Nonthaburi, Thailand*

Table 1. Susceptibility to d-allethrin and genotype within the Monheim *C. lectularius* strain and the international *C. hemipterus* strains

No.	Sample	Location	Date	*N*[a]	Resistance status	Mutations			
		Monheim, GERMANY, laboratory colony	Late 1960s	5	Susceptible	L899	M918	D953	L1014
1	DNT-AU	Darwin, Northern Territory, AUSTRALIA	1994	1	N/A[b]				L1014F
2	BQ-AU	Bundaberg, Queensland, AUSTRALIA	1998	1	N/A	L899V			L1014F
3	RBQ-AU	Rainbow Beach, Queensland, AUSTRALIA	~2004	1	N/A	L899V	M918I		L1014F
4	MQ-AU	Maroochydore, Queensland, AUSTRALIA	~2004	1	N/A	L899V	M918I		L1014F
5	NQ-AU	North Queensland, AUSTRALIA, laboratory colony	2007	8	Resistant[c]		M918I		L1014F
6	SP-AU	Surfers Paradise, Queensland, AUSTRALIA	2013	2	N/A		M918I		L1014F
7	M-AU	Melbourne, Victoria, AUSTRALIA	2009	3	N/A		M918I		L1014F
8	AS-AU	Auburn, Sydney, AUSTRALIA, laboratory colony	2013	7	Resistant			D953G	L1014F
9	B-IN	Bangalore, INDIA	1997	5	N/A		M918I		L1014F
10	KL-MA	Kuala Lumpur, MALAYSIA, laboratory colony	2005	3	Resistant				L1014F
11	K-AF	Kenya, AFRICA, laboratory colony in United Kingdom	2010	3	Resistant				L1014F
12	B-TH	Bangkok, THAILAND, laboratory colony	2011	4	Resistant		M918I	D953G	L1014F
13	C-TH	Chiang Mai, THAILAND, laboratory colony	2011	4	Resistant		M918I	D953G	L1014F
14	SHIP	Collected on ship; the origin of the bed bugs is not known, but suspected to be from Asia	2013	2	N/A		M918I	D953G	L1014F

[a] *N*: the tested number of bed bugs.
[b] N/A: only dead bed bugs were received, and thus the strains could not be tested via bioassay for resistance.
[c] The susceptibility of the NQ-AU strain was detected in a previous study by the present authors.[14]

the reference strain (Monheim) of the common bed bug, *C. lectularius*. The Monheim *C. lectularius* strain (from Bayer, Germany), maintained in the Department of Medical Entomology, Westmead Hospital, was used as an insecticide-susceptible control sample, as no susceptible strain of *C. hemipterus* could be sourced. Similar use of insecticide-susceptible mosquitoes as laboratory controls has been made in other mosquito resistance investigations.[12] Dead bed bug samples were stored in 95% alcohol at −20 °C. All bed bugs were identified according to Usinger[13] prior to testing.

2.2 Residual bioassay

The susceptibility of *C. hemipterus* to pyrethroids was established by the Dang mat assay.[14] Briefly, ten late nymphs (fourth or fifth instar) or mixed-sex adults from each international strain were confined to a d-allethrin impregnated mat, together with identical numbers of unexposed controls, and knockdown was recorded at 10 min intervals in the first hour, hourly to 6 h, and then again at 24 h exposure. Pyrethroid resistance status was tested for the following strains was: Auburn (AS-AU), Bangkok (B-TH), Chiang Mai (C-TH), Kenya (K-AF) and Kuala Lumpur (KL-MA) (Table 2). Except for the Auburn strain, three replicates were evaluated for each strain. However, owing to the small sample size, only one replicate was able to be evaluated for the Auburn strain. Bioassay data for the Monheim strain (adult) from a previous study[14] were used as a pyrethroid-susceptible reference.

Table 2. Knockdown response and percentage mortality of the five international strains of *Cimex hemipterus* and the Monheim strain of *C. lectularius* following exposure to the d-allethrin-impregnated mats

Strains	*n*	KT_{50} (95% CL) (min)	KT_{95} (95% CL) (min)	χ^2	df	Slope ± SE	Percentage mortality at 24 h exposure	RR_{50}
Monheim[a]	30	11 (8–13)	26 (21–39)	2.725	2	4.4 ± 0.8	100[d]	1
Kenya (K-AF)[b]	30	337 (244–539)	>1440	10.857	7	2.0 ± 0.2	100	30
Auburn (AS-AU)[a]	10	408 (310–752)	1190 (680–9508)	1.919	3	3.5 ± 1.1	100	37
Bangkok (B-TH)[a]	30	>1440	NA	NA	NA	–	20	>130
Chiang Mai (C-TH)[a]	30	>1440	NA	NA	NA	–	10	>130
Kuala Lumpur (KL-MA)[c]	30	>1440	NA	NA	NA	–	13	>130

[a] All adults.
[b] Fifth-instar nymphs.
[c] Mixed fourth- and fifth-instar nymphs.
[d] 100% knockdown at 30 min exposure.

2.3 DNA isolation

DNA from individual bed bugs was extracted using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA).[15] Briefly, fresh and stored bed bugs were ground individually in a 5 mL tube (LabServ, Cat. No. LBSSP2006) containing five glass beads and 800 μL of phosphate buffered saline (PBS) via a mechanical grinder that was held in a cold room set at 4 °C for 1 h.[16] With the ground bed bug, 180 μL of the grind was treated with 20 μL of proteinase K at 55 °C for 1 h and then extracted following the manufacturer's protocol [QIAGEN Supplementary Protocol: Purification of total DNA from insects using the DNeasy Blood and Tissue kit (DY14 Aug-06)]. DNA was eluted twice in 100 μL of elution buffer.

2.4 Detection of the putative *kdr* mutation site(s) on the VGSC gene targets

Polymerase chain reaction (PCR) amplification followed by Sanger sequencing was used to identify the putative *kdr* mutations at the below-mentioned domains of the VGSC gene. Sense and antisense primers BBparaF1 (5-AACCTGGATATACATGCCTTCAAGG-3), BBparaR1 (5-TGATGGAGATTTTGCCACTGATG-3) and BBparaF3 (5-GGAATTGAAGCTGCCATGAAGTTG-3), BBparaR3 (5-TGCCTATTCT GTCGAAAGCCTCAG-3) were used to amplify the regions spanning the V419 and L925 sites respectively.[11] The PCR reaction contained 12.5 μL of MyTaq HS Mix (Bioline, London, UK), 0.5 μL of both forward and reverse primers (20 μM) and 2 μL of the DNA template in a total volume of 25 μL. Amplification was performed in a thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) under the following conditions: 95 °C for 2 min, followed by 40 cycles of 94 °C for 20 s, 58 °C for 30 s and 72 °C for 40 s, and extended at 72 °C for 10 min. The amplified fragments of the expected size were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) at 37 °C for 30 min, followed by 80 °C for 30 min, and held at 4 °C. The purified product was sequenced by AGRF (Australian Genome Research Facility Ltd, Sydney, Australia) using the reverse primer as a sequencing primer for both regions. The sequences were aligned by ClustalW and analysed by means of BioEdit, MEGA5 and Sequence Scanner v.1.0. The *kdr* mutation was confirmed by comparing sequence alignments of the tested bed bugs against the Monheim strain, the reference *C. lectularius* (FJ031996) and other insect wild species, including the housefly *Musca domestica* L. (U38813), mosquito *Anopheles gambiae* Giles (AM422833), German cockroach *Blattella germanica* (L.) (U71083) and head lice *Pediculus humanus capitis* De Geer (AY191156) from the NCBI GenBank.

2.5 Statistical analysis

Data were corrected for control knockdown mortality using Abbott's formula.[17] Knockdown data were pooled and subjected to probit analysis using the POLO-PC program (LeOra Software, Berkely, CA, 1997) to estimate the KT_{50} and KT_{95} values.[18] A non-overlap of 95% confidence interval was used to determine the significant differences between the values. The resistance ratio at KT_{50} (RR_{50}) was calculated by dividing the KT_{50} values of the field strains with the corresponding knockdown time for the Monheim strain of *C. lectularius*.

3 RESULTS AND DISCUSSION

3.1 Susceptibility of tropical bed bug strains to d-allethrin

In previous studies using the Dang mat assay method, a high level of d-allethrin resistance was shown in the NQ-AU strain of *C. hemipterus* compared with the susceptible Monheim strain of *C. lectularius*.[14] A 100% knockdown within 30 min exposure was produced in adults of the Monheim strain, whereas none of the NQ-AU strain was knocked down at 6 h post-exposure. Knockdown was evident in only 17% of bed bugs at 24 h post-exposure in the NQ-AU strain. In the present study, the susceptibility of five strains of *C. hemipterus* (Table 2) around the world was examined by the Dang mat assay (Fig. 1). The results showed that these five strains have a moderate to high resistance to d-allethrin, in which the B-TH and C-TH strains from Thailand (adults) and the KL-MA strain from Malaysia (fourth- and fifth-instar nymphs) show high resistance with ≤20% knockdown after 24 h exposure ($RR_{50} > 130$), while the K-AF strain from Africa (fifth-instar nymphs) and the AS-AU strain (adults) showed moderate resistance with 23.3% knockdown (K-AF, $RR_{50} = 30$) and 20% knockdown (AS-AU, $RR_{50} = 37$) within the first 4 h, and both produced 100% knockdown after 24 h.

3.2 Regions of interest of the VGSC gene

Two regions of the VGSC gene, containing most key putative *kdr* mutation sites associated with *kdr*-type resistance to pyrethroids in a range of insect pests, were initially investigated. The first region (~500 bp) included four putative *kdr* mutation sites, namely V410, V419, V421 and E435, encoding for the domain IS6 and part of the domain I–II linker region of the VGSC gene. The second region (~800 bp) encompasses five putative *kdr* mutation sites, namely M918, L925, T929, L932 and L1014, encoding for the domain IIS4–IIS6 region.[9,11] The *kdr* mutations, V419L and L925I, of its related species (*C. lectularius*) are also located in these two regions.[11,15]

3.3 Identification of mutations in *C. hemipterus*

A 510 bp region and a 513 bp region encompassing the V419 mutation site (domain IS6 and part of the domain I–II linker) of *C. lectularius* (Monheim strain) and *C. hemipterus* (NQ-AU) respectively were examined. DNA sequence alignments showed that the fragment included two intron regions and three exon regions (Figs 2A and B). Comparison of exon 1 (125 bp), exon 2 (162 bp) and exon 3 (99 bp) of *C. hemipterus* against the reference Monheim strain of *C. lectularius* showed a similarity of 98.2%, differing only by seven nucleotides. To identify mutations within these exon regions, the exon nucleotide sequences were translated into protein sequences and compared. No amino acid change was evident at the site V419 (*valine*/V: GTC) in *C. hemipterus* (NQ-AU) (Fig. 2B). The alignment results showed that both protein sequences of *C. lectularius* and *C. hemipterus* were the same (335–482 aa).

Similarly, a 746 bp region and a 752 bp gene region encompassing the L925 mutation site that forms part of the IIS4–IIS6 domain in *C. lectularius* (Monheim) and *C. hemipterus* (NQ-AU) respectively were sequenced and analysed. The structure of the genomic fragments included three short intron regions and four exon regions, indicated in Figs 2C and D. The exon sequences of *C. hemipterus* were 98.1% similar to *C. lectularius*, with a difference of ten nucleotides. A comparison between the aligned protein sequences of both bed bug species in exons 2 and 3 showed base-pair substitutions. Two transitions respectively at sites I918 (*isoleucine*/I: ATA) and F1014 (*phenylalnine*/F: TTC) were evident in *C. hemipterus* (NQ-AU) compared with site M918 (*methioine*/M: ATG) and L1014 (*leucine*/L: CTG) in *C. lectularius* (Monheim) and the *C. lectularius* reference gene (Figs 2C to D). A CTC (CTT in *C. lectularius*) transition was apparent at site L925 in *C. hemipterus*, with no change in the encoded amino acid, *leucine*/L (Fig. 2D).

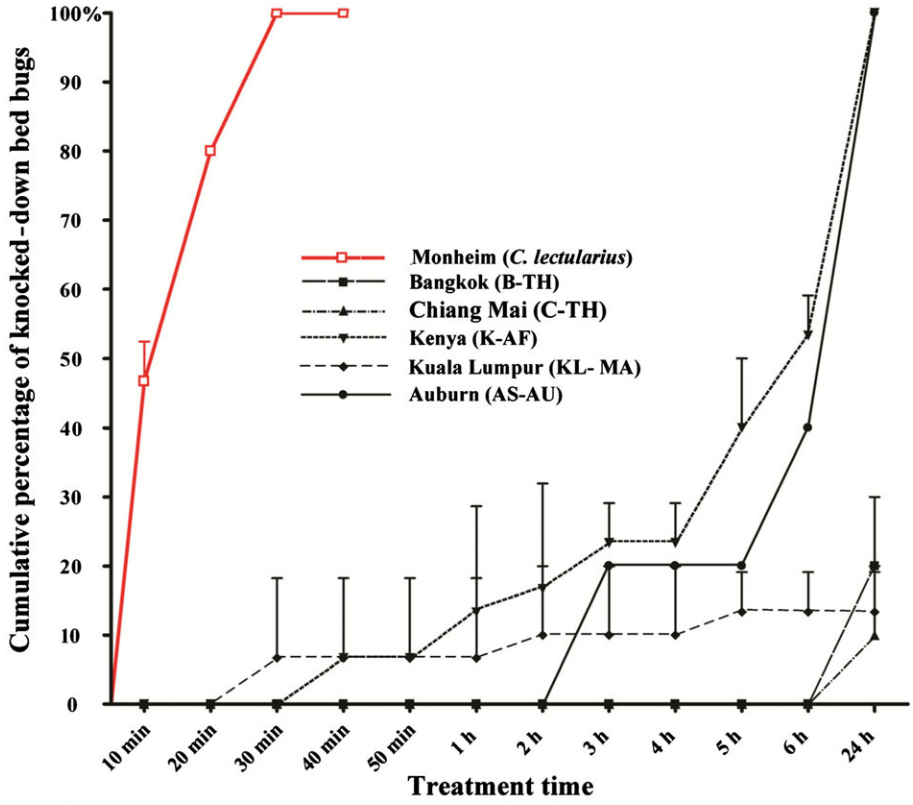

Figure 1. Comparison of cumulative percentage of knockdown (mean ± SD) between the Monheim strain (*C. lectularius*, susceptible) and the five strains of *C. hemipterus* following exposure to the d-allethrin impregnated mats. Stages of bed bugs tested: Bangkok and Chiang Mai – adults, three replicates; Kenya – fifth-instar nymphs, three replicates; Kuala Lumpur – fourth- and fifth-instar nymphs, three replicates; Auburn adults because of the small sample size, then one replicate, no error bar). The data of the Monheim strain are from a previous study by the present authors.[14]

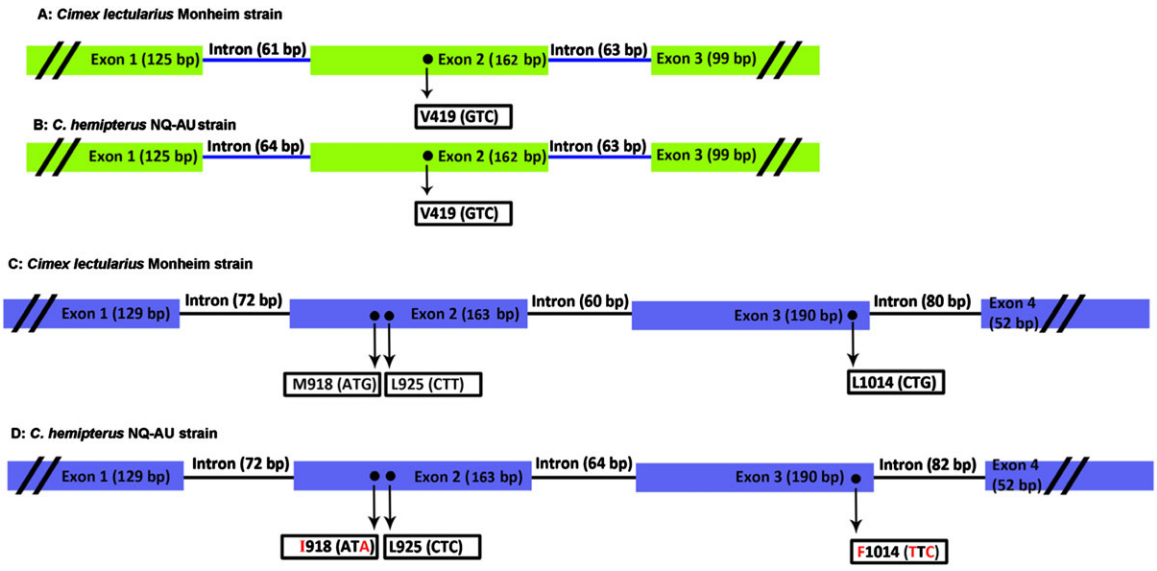

Figure 2. Exon–intron structure of genomic fragments of the VGSC gene respectively encompassing V419 (A and B) and L925 (C and D).

Comparison of protein sequences of *C. hemipterus* and *C. lectularius* aligned with the reference VGSC genes of the other insect wild species mentioned above showed that the area encompassing the M918 and L1014 mutation sites forms part of a conserved region (Fig. 3). This suggests that the M918 and L1014 mutation sites are also conserved in *C. hemipterus* and *C. lectularius*, and implies that within the VGSC gene the 918 and 1014 sites were originally *methionine* (M) and *leucine* (L) respectively. However, the study herein showed a synonymous, missense mutation respectively at 918(I) and 1014(F) sites in *C. hemipterus* (NQ-AU). Hence, the M918I and L1014F transitions in *C. hemipterus* (NQ-AU) can be considered to be probable mutations. Unlike the pyrethroid-resistant strains of C. *lectularius*, the V419L and L925I *kdr* mutations were not found in *C. hemipterus*. No other putative *kdr* mutations in

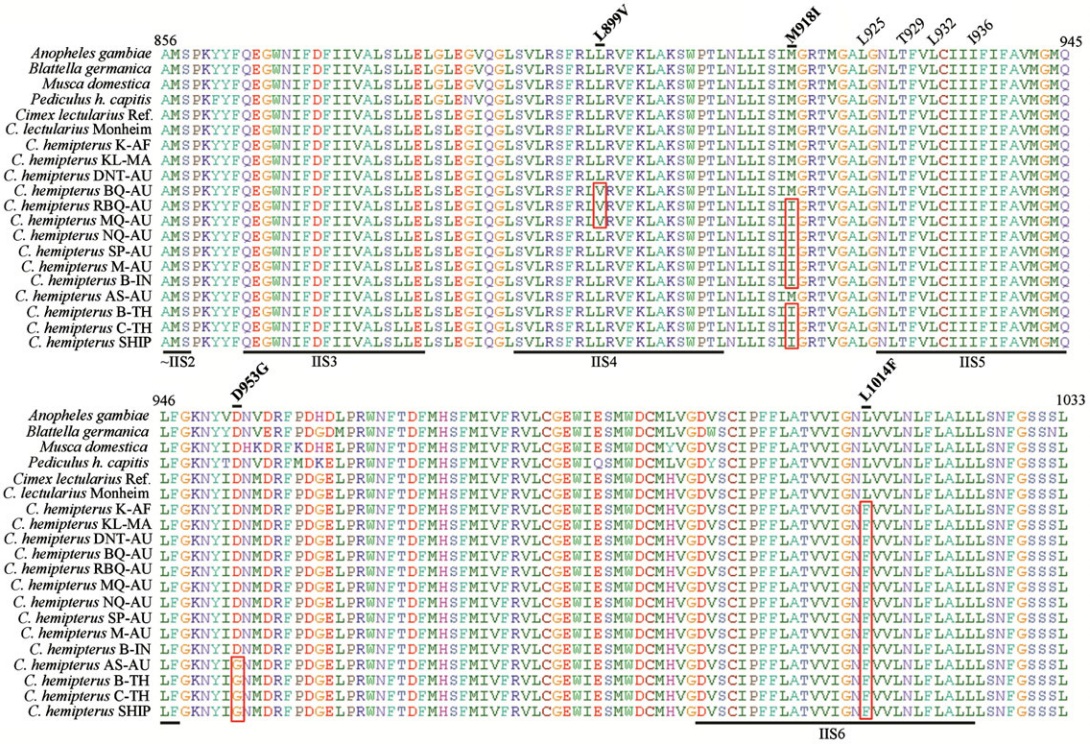

Figure 3. Protein sequence alignments of the VGSC genes from *C. hemipterus* [Australia (AU), Africa (AF), Malaysia (MA) and Thailand (TH), and samples collected from a ship (SHIP)] and *C. lectularius* (Monheim) against the NCBI reference VGSC genes of wild-type insect sequences [common bed bug *C. lectularius* L. (FJ031996), housefly *Musca domestica* L. (U38813), mosquito *Anopheles gambiae* Giles (AM422833), German cockroach *Blattella germanica* (L.) (U71083) and head lice *Pediculus humanus capitis* De Geer (AY191156)].

the IS6 and part of the DI–II linker and the IIS4–IIS6 regions were detected.

DNA sequence analysis was also performed on other international strains of *C. hemipterus* (Table 1). Samples from RBQ-AU (collected in 2004), MQ-AU (2004), M-AU (2009), SP-AU (2013), B-IN (1997), B-TH (2011), C-TH (2011) and a ship (SHIP, 2013) had both the M918I and L1014F mutations. However, only the L1014F mutation was evident in the bed bugs from DNT-AU (1994), BQ-AU (1998), AS-AU (2013), KL-MA (2005) and K-AF (2010).

Another two novel transitions were subsequently identified at sites L899 and D953 (Fig. 3). The first transition, CTC to GTC at the first base of the L899 site located on DIIS4 residue, was identified in the field samples of the BQ-AU, RBQ-AU and MQ-AU strains. The other mutation, GAT to GGT at the second base of the D953 site located on the DIIS5-S6 linker of the VGSC gene, was found in the ship-originated strain (SHIP), AS-AU, and the two Thailand strains (B-TH and C-TH). Both L899 and D953 are considered to be conserved sites within the VGSC gene. Hence, both transitions are indicative of two respective novel mutations [L899V: *leucine* (CTC) to *valine* (GTC) and D953G: *aspartic acid* (GAT) to *glycine* (GGT)].

3.4 Inference of *kdr* mutations in *C. hemipterus*

Several point mutations associated with pyrethroid resistance clustered on domains IIS4–IIS6 in the VGSC gene have been widely observed in a range of important agricultural pests and disease vectors, indicating that the region is a major site of pyrethroid interaction.[8] The L1014F mutation, originally found in housefly *M. domestica*, is now confirmed as a critical *kdr* mutation and has been reported from several insect pests.[8,19] Variant replacements at this site, L1014C/H/S/W, have also been recorded.[9]

The *kdr* mutation is commonly associated with resistance to DDT and pyrethroids. In particular, the M918 site is strongly associated with *kdr*- or *super-kdr*-type resistance to pyrethroids in many insect pests, with variant replacements of M918I/L/T/V/ reported.[9] Expression studies have also shown that incorporation of the M918T mutation into the *Drosophila* VGSC gene and expressed in the oocytes of the African clawed frog, *Xenopus laevis* Daudin, conferred strong insensitivity to pyrethroids.[20] The I874M substitution, which is equivalent to M918 residue of *M. domestica* or *C. hemipterus*, greatly increases the sensitivity of the rat brain IIA sodium channel to deltamethrin by more than 100-fold.[21] In addition, the M918I mutation, reported previously in the diamondback moth, *Plutella xylostella* (L.), is associated with *kdr*-type resistance to pyrethroids.[22–24]

By inference with the research on other insect species, the residue M918 and L1014 are likely sites for binding of pyrethroids. Hence, it is probable that both the M918I and L1014F mutations contribute towards *kdr* resistance to pyrethroids in *C. hemipterus.* However, this needs to be experimentally verified, such as has been undertaken in the common fruit fly, *Drosophila melanogaster* Meigen.[20] The M918I mutation was always found with the L1014F mutation in the present study. In addition, the bioassay results showed that the strains (NQ-AU,[14] B-TH and C-TH, with ≤20% knockdown after 24 h exposure, $RR_{50} > 130$) with both M918I and L1014F mutations showed higher resistance to d-allethrin (pyrethroid) than the strains just with L1014F mutation (K-AF and AS-A, with 100% knockdown after 24 h exposure, $RR_{50} < 40$). Therefore, this suggests that the M918I mutation, like the previously described M918T mutation (as *super-kdr*),[8] probably plays an important role in enhancing pyrethroid resistance in *C. hemipterus*. A similar inference was demonstrated in *P. xylostella*.[23]

The L899V and D953G mutations were identified in this study. So far there have been no published reports of these two mutations in other insects. The L899V and D953G mutations are possibly linked by way of resistance to pyrethroids. However, further validation studies, such as functional expression of each mutation individually and in combination using the *Xenopus* oocyte expression system with two-electrode voltage-clamp electrophysiology,[20] are required.

4 CONCLUSIONS

This is first attempt to investigate possible *kdr*-type resistance to pyrethroids in the tropical bed bug, *C. hemipterus*. Two point mutations, M918I and L1014F, were identified and implicated as *kdr* mutations contributing resistance to pyrethroids in *C. hemipterus*, based on evidence found in other pyrethroid-resistant insects. Two other mutations, L899V and D953G, were also found; however, their function requires further investigations. This study confirms the suggestion that high tolerance to both DDT and pyrethroids in *C. hemipterus* is inferred by the *kdr*-type resistance mechanism, based on bioassay and biochemical assays.[5] The KL-MA strain, like the K-AF strain, has only the L1014F mutation; however, it demonstrated high resistance, with the resistance responding similarly to the NQ-AU strain. In light of the multiple resistance mechanisms identified in *C. lectularius*,[25–29] it is probable that other non-*kdr*-type resistance mechanisms are also present in *C. hemipterus*, and these are currently under investigation.

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