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DETECTION OF KNOCKDOWN RESISTANCE (*KDR*) IN *CIMEX LECTULARIUS* AND *CIMEX HEMIPTERUS* (HEMIPTERA: CIMICIDAE)

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Abstract: Worldwide, there are many reports of pyrethroid resistance in bed bugs (*Cimex* spp.). Previous studies on the Common bed bug, *Cimex lectularius* L., have identified two mutations (V419L and L925I) in the voltage-gated sodium channel (VGSC) gene responsible for knockdown resistance (*kdr*). However, nothing is known on possible *kdr* mutations in Australian strains of *C. lectularius* or on the Tropical bed bug, *C. hemipterus* F. This study aimed to identify the status of *kdr* mutations in Australian *C. lectularius* strains, and *C. hemipterus* in Australia and international strains. Samples of *C. lectularius* were obtained from 24 sites across Australia, while the *C. hemipterus* were sourced from Australia, as well as Africa, India, Malaysia and Thailand. DNA was extracted, purified and examined for the *kdr*-related genes by PCR and Sanger sequencing. In *C. lectularius* field populations, the haplotypes A (neither V419L nor L925I), B (L925I only), and C (V419L and L925I) were found, with most (88%) of the field populations being haplotype B. A novel mutation, I936F, was identified in an 'Adelaide' strain (initially identified as haplotype A), which may be linked with *kdr*-type resistance. In *C. hemipterus*, the V419L and L925I mutations were not detected, however, three novel mutations, M918I (*Methionine* to *Isoleucine*), D953G (*Aspartic acid* to *Glycine*) and L1014F (*Leucine* to *Phenylalanine*), were identified. Samples from Thailand have the three mutations, while samples from Australia and India have both M918I and L1014F mutations. Only the L1014F mutation was evident in the samples from Malaysia and Africa. The M918I and L1014F were assumed to be *kdr* mutations and contribute to the high pyrethroid resistance in *C. hemipterus*. Further studies are in process to determine the non-*kdr* type resistance mechanisms.
Key words *Cimex*, insecticide resistance, pyrethroids, novel mutations.

INTRODUCTION

Over the last 15 years, as per other regions of the world, infestations of bed bugs (from the Common bed bug, *Cimex lectularius* L. and the Tropical bed bug, *Cimex hemipterus* F.) have undergone a dramatic reappearance in Australia (Doggett et al., 2003, 2004; Doggett and Russell, 2008; Doggett et al., 2011, 2012). An often integral part of the control strategy in bed bug eradication is insecticide application and the pyrethroids have been widely employed (Doggett and Russell, 2008). However resistance to this class of insecticides has been well documented, both in *C. lectularius* (Boase et al., 2006; Gangloff-Kaufmann et al., 2006; Moore and Miller, 2006; Romero et al. 2007; Kilpinen et al., 2008; Lilly et al., 2009), and in *C. hemipterus* (Myamba et al., 2002; Karunaratne et al., 2007; Tawatsin et al., 2011).

It is now known that multiple insecticide resistant mechanisms exist in *C. lectularius* against the pyrethroids, and other insecticide groups (Mamidala et al., 2011; Zhu et al., 2013; Dang et al., 2013). Resistance mechanisms documented in bed bugs against the pyrethroids included behavioural resistance (Romero et al. 2009), cuticular resistance (Koganemaru et al. 2013), metabolic resistance (Zhu et al. 2013), and target-site insensitivity resistance due to substitute mutations at the DNA level (Yoon et al. 2008).

Pyrethroid insecticides are widely used due to their low mammalian toxicity and high efficacy for killing insect pests. Like DDT, they target the Voltage-Gated Sodium Channel (VGSC) of insects, resulting in paralysis, an effect known as *knockdown,* which eventually leads to death. However, an important resistant mechanism evolved to combat the pyrethroids (and DDT) is known as knockdown resistance (*kdr*). This stems from various mutations on the VGSC gene that results in substitutions of the amino acid sequence of the VGSC protein. The result is to reduce the sensitivity of VGSC to pyrethroid insecticides. In previous studies, the mutations V419L and L925I were identified in *C. lectularius* and found to contribute to pyrethroid resistance (Yoon et al., 2008). Subsequently these mutations were detected widely across the USA (Zhu et al., 2010, 2013). Four mutation haplotypes were defined; haplotype A (no target site mutation), haplotype B (L925I mutation only), haplotype C (L925I and V419L mutations), and haplotype D (V419L mutation only).

To date, insecticide resistance profiling in Australian *Cimex* has been undertaken on one laboratory colony strain; the 'Sydney' *C. lectularius* strain (Lilly et al., 2009), which has been shown to be highly resistant to the pyrethroids and moderately resistant to the carbamates. However, no studies have been undertaken at the molecular level examining the basis of the resistant mechanisms in Australia. Therefore, the purpose of this study was to examine for such resistance, beginning with the status of *kdr* mutations in Australian *C. lectularius*, and *C. hemipterus* collected from around the world.

METHODS AND MATERIALS

Samples of *C. lectularius* were obtained from 24 sites across Australia, while the *C. hemipterus* were sourced from Australia, as well as Africa, India, Malaysia and Thailand (Table 1). The pyrethroid-susceptible 'Monheim' *C. lectularius* strain was used as a control. No insecticide susceptible strain of *C. hemipterus* was available. All bed bug samples were identified to species according to the key of Usinger (1966) prior to any testing.

DNA was extracted, purified and examined for the *kdr*-related genes by PCR and Sanger sequencing. Two gene regions were amplified and sequenced (Zhu et al., 2010); the fragment (~800bp) encodes the domain IIS4–IIS6 region of the VGSC gene, which contains six putative mutation sites previously associated with *kdr* pyrethroid resistance in a large range of insect pests, namely M918, L925, T929, L932, I936 and L1014. Another region (~500bp) encodes the domain IS6 and front part of domain I–II linker region which contains four putative mutation sites, namely V410, V419, V421 and E435. All sequences were aligned by ClustalW using BioEdit and MEGA5.

RESULTS AND DISCUSSION

kdr mutations in *Cimex lectularius*

In *C. lectularius* field populations (Table 1), the haplotypes A (no V419L or L925I mutation), B (L925I only), and C (V419L and L925I) were identified, with most (88%) of the field populations showing haplotype B. No haplotype D (V419L only) was detected. A novel mutation, I936F, was identified in an Adelaide strain, which was otherwise haplotype A. Interestingly, a mutation (I936V) was previously identified in *Drosophila melanogaster* Meigen, which had been found to contribute to significant

pyrethroid resistance (Usherwood et al., 2007). This is circumstantial evidence that I936F is potentially linked to pyrethroid resistance in *C. lectularius*, however further work is required to confirm this.

For locations within the United States that exhibited high proportion of *kdr* haplotypes, the haplotypes B and C were more prevalent than the others (Zhu et al., 2010). In comparison, a study from France reported a high incidence of *kdr* with all being haplotype B (Durand et al., 2012). The variable frequencies of *kdr* haplotypes in disparate countries would probably relate to the types that were initially introduced and subsequently spread throughout the respective nation. Although studies are limited, to date all investigations examining *kdr* have revealed a high proportion of the mutations suggesting that they are probably widespread in bed bugs across the world. The continuous use of pyrethroid insecticides (despite not being very efficacious) may be contributing to the maintenance of high *kdr* mutation frequencies.

kdr* mutations in *Cimex hemipterusIn initial testing of the 'North Queensland' laboratory strain of *C. hemipterus*, the V419L and L925I *kdr* mutations were not present. Except for synonymous substitutions, however, two novel mutations were found; M918I and L1014F.

Table 1. *kdr* mutations found in *Cimex lectularius* and *C. hemipterus* used in this study.

Species	Strain	Collection Year	Mutation					
			V419L	L925I	I936F	M918I	D953G	L1014F
C. lectuarius	Monheim colony	1960's						
	Sydney colony	2004		*				
	St Ives, SYDNEY	2007		*				
	Redfern I, SYDNEY	2007		*				
	Glebe, SYDNEY	2007		*				
	Byron Bay I	2007		*				
	Byron Bay II	2007		*				
	Darlinghust I, SYDNEY	2008		*				
	Redfern II, SYDNEY	2011		*				
	Abbotsford, SYDNEY	2011		*				
	Darlinghust II, SYDNEY	2011	*	*				
	Auburn, SYDNEY	2012		*				
	Parramatta, SYDNEY	2012		*				
	Northbridge, SYDNEY	2013		*				
	Maryland, NEWCASTLE	2013		*				
	Northbridge, PERTH	2007		*				
	Nedlands, PERTH	2007		*				
	Cottesloe, PERTH	2013		*				
	Southbank, MELBOURNE	2007		*				
	Ripponlea. MELBOURNE	2013		*				
	Moonee Ponds, MELBOURNE	2013	*	*				
	South Yarra, MELBOURNE	2013		*				
	West Melbourne	2013		*				
	Alice Springs, NT	2013		*				
	Narangba, BRISBANE	2007		*				
	Semaphore Park, ADELAIDE	2013			*			
C. hemipterus	Australia	2007				*		*
	India	1997				*		*
	Malaysia	2005						*
	Africa	2010						*
	Thailand	2011				*	*	*

Analysis of the other strains showed that samples from India, like the Australian strains, have both M918I and L1014F mutations, but only the L1014F mutation was present in colonies from Malaysia and Africa (Table 1). No other *kdr* mutation in either the domain IS6 and part of domain I–II linker, and the domain IIS4–IIS6 regions were detected in *C. hemipterus*. However, another novel mutation, D953G (GAT-to-GGT) located on DIIS5-S6 linker of the VGSC gene, was subsequently identified in the samples from Thailand. So far, there have been no published reports of the D953G mutation in other insects.

The L1014F mutation is reported as a critical *kdr* mutation in several insect species (Davies and Williamson, 2009). Additionally, the mutation at M918 site has been confirmed to be associated with *kdr* or *super-kdr* type resistance to the pyrethroids (Rinkevich et al., 2013). The M918I+L1014F *kdr* mutations were identified in the diamondback moth, *Plutella xylostella* (L.) and are responsible for resistance to the pyrethroids (Sonoda et al., 2008). Hence, both the M918I and L1014F mutations in *C. hemipterus* are assumed to be *kdr* mutations, contributing towards pyrethroid resistance. The study herein appears to confirm the suggestion of Karunaratne et al. (2007) that high tolerance to both DDT and the pyrethroids was due to a '*kdr*'-type resistance mechanism. The D953G mutation is probably associated with the resistance to the pyrethroids, however further studies are required to verify this. Studies are ongoing to determinate non-*kdr* type insecticide resistance mechanisms in *C. hemipterus* (and *C. lectularius*) in our laboratory.

CONCLUSIONS

It is noteworthy that different *kdr* mutation types (V419L and L925I in *C. lectularius*, and M918I and L1014F in *C. hemipterus*) have evolved independently in the two bed bug species, which are both closely related. In contrast, similar *kdr* mutation types have evolved in other insects that are related (Rinkevich et al., 2013). The evolutionary mechanisms of the respective *kdr* mutation types between the bed bug species are unknown and further research is required to elucidate these mechanisms.

In light of the widespread resistance and resistance mechanisms (including *kdr*), the control of bed bug infestations should follow an integrated management approach encompassing non-chemical means of control and a reduced reliance on the pyrethroid insecticides. It is important that pest managers follow 'best practice' as defined in the various bed bug management industry standards to reduce the risk of treatment failure.

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The 'Monheim' *C. lectularius* strain was imported (Permit IP060247782) from Bayer CropScience, Monheim, Germany and continued to be stored in accordance with Australian Quarantine and Inspection Service (AQIS) requirements. Storage and culturing of the bed bug strains was conducted as approved by the Westmead Hospital Animal Ethics Committee (WHAEC Protocol N$^{o.}$ 1008) and in accordance with NSW Animal Research Review Panel (ARRP) *Guidelines for the Housing of Rats in Scientific Institutions*. This study was partly supported by the Chinese Scholarship Council (No. 201206200048).

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