

## Household and Structural Insects

# Multiple Mechanisms Conferring Broad-Spectrum Insecticide Resistance in the Tropical Bed Bug (Hemiptera: Cimicidae)

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### Abstract

The modern resurgence of the common (*Cimex lectularius* L.) and tropical bed bugs (*C. hemipterus* [F]) is thought to be primarily due to insecticide resistance. While there are many reports on insecticide resistance mechanisms in *C. lectularius*, such information in *C. hemipterus* is limited. We examined dichloro-diphenyl-trichloroethane (DDT), malathion, deltamethrin, permethrin, lambda-cyhalothrin resistance, and the underlying mechanisms in several *C. hemipterus* strains (Australia: Queensland [QLD-AU]; Malaysia: Kuala Lumpur [KL-MY], Tanjung Tokong [TT-MY], Christian [CH-MY], and Green Lane [GL-MY]). We used a surface contact method, synergism studies (utilizing piperonyl butoxide [PBO], *S,S,S*-tributyl phosphorotrithioate [DEF], and diethyl maleate [DEM]), and molecular detection of *kdr* mutations. Results demonstrated that all *C. hemipterus* strains possessed high resistance to DDT and the pyrethroids and moderate to high resistance to malathion. Synergism studies showed that deltamethrin resistance in all strains was significantly ( $P < 0.05$ ) inhibited by PBO. In contrast, deltamethrin resistance was not affected in DEF or DEM. Similar findings were found with lambda-cyhalothrin resistance. Malathion resistance was significantly ( $P < 0.05$ ) reduced by DEF in all strains. Resistance to DDT was not affected by DEM in all strains. Multiple *kdr* mutations (M918I, D953G, and L1014F) were detected by molecular analyses. TT-MY strain was found with individuals possessing three *kdr* mutation combinations; D953G + L1014F (homozygous susceptible: M918), M918I + D953G + L1014F (heterozygous resistant: I918), and M918I + D953G + L1014F (homozygous resistant: I918). Individuals with M918I + D953G + L1014F (homozygous resistant: I918) survived longer on deltamethrin (>12 h) than those ( $\leq 1$  h) with other combinations. M918I + L1014F mutations most likely conferred *super-kdr* characteristic toward pyrethroids and DDT in *C. hemipterus*.

**Key words:** synergism, cytochrome P450, esterase, *kdr*, *super-kdr*

Bed bugs, *Cimex lectularius* L. and *C. hemipterus* (F), are cryptic and nocturnal ectoparasites adapted to blood-feeding on humans (Usinger 1966, Doggett et al. 2018a). The common bed bug *C. lectularius* is most prevalent in temperate regions, whereas the tropical bed bug *C. hemipterus* is found mainly in tropical and subtropical areas (Dang et al. 2017a). However, there are overlaps in the regions where both species can be found, such as Africa (Fourie

and Crafford 2018), Australia (Doggett and Cains 2018, Geary et al. 2021), Central Europe (Balvín et al. 2021), China (Wang et al. 2013, Lee et al. 2018), France (Chebbah et al. 2021), Iran (Hosseini-Chegeni et al. 2019), Malaysia and Singapore (Lee et al. 2018), Russia (Capon 2016), and Thailand (Tawatsin et al. 2011). Both species have undergone a significant resurgence worldwide (Doggett et al. 2018a), and insecticide resistance is the most likely cause for

bed bug resurgence (Romero et al. 2007, Dang et al. 2017a, Romero 2018). Even though there is no evidence that bed bugs transmit human pathogens in their natural habitats (Doggett et al. 2012, Lai et al. 2016), the impact of insecticide resistance has resulted in the management of modern bed bugs being very challenging and expensive (Doggett et al. 2018b).

Insecticide resistance, particularly pyrethroid and dichlorodiphenyl-trichloroethane (DDT) resistance, has been well documented in *C. lectularius* and *C. hemipterus* worldwide (Dang et al. 2017a). More recently, resistance to the carbamates, organophosphates, neonicotinoids, and phenylpyrazoles have been reported in bed bugs during the modern resurgence too (Romero and Anderson 2016; Dang et al. 2017a, b; Romero 2018; Zulaikha and Majid 2019; Leong et al. 2020a, b; González-Morales et al. 2021; Soh and Veera Singham 2021). Three main resistance mechanisms are responsible for the development of insecticide resistance in bed bugs (Mamidala et al. 2011, Davies et al. 2012, Dang et al. 2017a), namely target site insensitivity, increased metabolic detoxication, and penetration resistance.

Point mutations in the voltage-gated sodium channel gene (VGSC) decrease the sensitivity of target sites for pyrethroids and DDT, which is known as knockdown resistance (*kdr*; Davies et al. 2007, Dong et al. 2014). Various mutations putatively conferring *kdr*-resistance to pyrethroids have been identified in *C. lectularius*, including V419L, L925I, and I936F (Yoon et al. 2008, Dang et al. 2015a) and in *C. hemipterus*, including A468T, L899V, M918I, D953G, Y/L995H, A1007S, V1010L, I1011F, I1011T, V1016E, L1014F, and L1017F/S (Dang et al. 2015b, Punchihewa et al. 2019, Ghavami et al. 2021, Soh and Veera Singham 2021).

Cytochrome P450 monooxygenases (P450s), esterases, and glutathione S-transferases (GSTs) are involved in the degradation, sequestration, or transformation of toxic compounds into nontoxic products before the compounds reach the target site of the insect (Li et al. 2007). Both P450s and esterases mediated resistance are common metabolic resistance mechanisms in *C. lectularius* (Adelman et al. 2011; Bai et al. 2011; Mamidala et al. 2011, 2012; Zhu et al. 2013; Lilly et al. 2016a; Gonzalez-Morales and Romero 2019; González-Morales et al. 2021). However, there are limited reports in *C. hemipterus* (Karunaratne et al. 2007, How and Lee 2011, Punchihewa et al. 2019, Soh and Veera Singham 2021). Lastly, penetration resistance due to increased thickening of cuticle leading to reduced insecticide penetration has been documented in *C. lectularius* (Koganemaru et al. 2013, Lilly et al. 2016b) and *C. hemipterus* (Soh and Veera Singham 2021).

Although efforts have been undertaken to study bed bugs' resistance status and mechanisms worldwide, the primary focus has

been on *C. lectularius*. The knowledge of the resistance mechanisms in *C. hemipterus* remains limited. This study aims to investigate insecticide resistance and the underlying mechanisms in several *C. hemipterus* populations from Malaysia and Australia. The susceptibility status of five *C. hemipterus* strains to DDT (organochlorines), malathion (organophosphates), and deltamethrin, permethrin, and lambda-cyhalothrin (pyrethroids) were assessed. Synergism assays with three common synergists, namely piperonyl butoxide (PBO), *S,S,S*-tributyl phosphotriothioate (DEF), and diethyl maleate (DEM), were used to investigate the involvement of cytochrome P450s, esterases, and glutathione S-transferases, respectively, in metabolic resistance. Molecular analyses were performed to assess *kdr* genotypes on the VGSC gene (Dang et al. 2015b).

## Materials and Methods

### Bed Bug Populations

Bed bug populations of *C. hemipterus* were collected from the field and maintained in the Urban Entomology Laboratory, Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia, at  $27 \pm 2^\circ\text{C}$ ,  $75 \pm 10\%$  relative humidity, and a 12-h photoperiod (Table 1). Colonies of bed bugs were blood-fed using Hemotek membrane feeding system (Discovery Workshops, Accrington, UK) with freshly drawn rabbit blood in lithium heparin tube (Vacutest Kima srl, Arzergrande [PD], Italy; Animal ethics approval: USM/Animal Ethics Approval/2016/[104] [819]). The Monheim *C. lectularius* strain was used as an insecticide-susceptible strain, as no insecticide-susceptible strain of *C. hemipterus* could be sourced worldwide (Dang et al. 2015b; Leong et al. 2020a, b). None of the strains underwent insecticide selection. All bed bug species were confirmed using Usinger (1966) before testing.

### Chemicals

Five technical grade insecticides were used, namely DDT (98%, World Health Organization, Geneva, Switzerland), malathion (92.8%, PestAnal, Sigma Aldrich Laborachemikalien GmbH, Munich, Germany), permethrin (95.1%, Aventis Environmental Health, Malaysia), deltamethrin (97%, Bayer Environmental Science, Kuala Lumpur, Malaysia), and lambda-cyhalothrin (97%, Syngenta Crop Protection, Kuala Lumpur, Malaysia). Three technical grade synergists were used, namely *S,S,S*-tributyl phosphotriothioate (DEF; 98.3%, FMC Co., Agricultural Chemical Division, Middleport, NY), diethyl maleate (DEM; 97%, Sigma Aldrich, Saint Louis, MO), and piperonyl butoxide (PBO; 98%, FMC Co., Agricultural Chemical

**Table 1.** Susceptibility of the five *C. hemipterus* strains used in the study and the insecticide-susceptible Monheim *C. lectularius* strain

Species	Strain	Location	Year	Maximum generations <sup>a</sup>	Pyrethroids	DDT	Malathion
<i>C. lectularius</i>	Monheim	Monheim, Germany, laboratory colony	Late 1960s	NA	Susceptible	Susceptible	Susceptible
<i>C. hemipterus</i>	QLD-AU	North Queensland, AUSTRALIA, laboratory colony	2007	>100	High resistance	Moderate resistance	High resistance
	KL-MY	Kuala Lumpur, MALAYSIA, laboratory colony	2005	>100	High resistance	High resistance	High resistance
	TT-MY	Tanjung Tokong, Penang, MALAYSIA	2015	>10	High resistance	Moderate resistance	High resistance
	CH-MY	Christian, Penang, MALAYSIA	2015	>10	High resistance	High resistance	High resistance
	GL-MY	Green Lane, Penang, MALAYSIA	2015	>10	High resistance	High resistance	High resistance

<sup>a</sup>Based on minimum development period per generation of 31.3 d at 27°C (Omori 1941).

Division, Middleport, NY). Two types of oil, Risella oil (Shell, Malaysia) and olive oil (91.7%, Pomace Olive Oil Cosmetic Grade), were obtained from the Vector Control Research Unit in Universiti Sains Malaysia, Malaysia, and used as part of the diluent for DDT and malathion, respectively (WHO 1992, Dang et al. 2017b). Discriminating concentrations of each insecticide for the residual bioassays are shown in Table 2.

### Preparation of Insecticide-Impregnated Filter Papers

Filter papers (diam. 55 mm, Filtes Fioroni, Ingre, French) were treated with 0.3 ml of various insecticide solutions. For DDT and malathion, the solutions were prepared in a mixture of acetone-diluted insecticide and oil at a ratio of 1:2 (one part of acetone-diluted insecticide was added into two parts of oil; see Dang et al. 2017b for preparation details). Filter papers were treated with an equal volume of acetone/oil mixture at the same ratio (0.3 ml) as the control. For the three pyrethroids, the insecticide solution was prepared as an acetone-diluted insecticide only. Filter papers were treated with an equal volume of acetone (0.3 ml) as a control. The treated filter papers were allowed to air dry in a fume hood at room temperature for 24 h before bioassay.

### Residual Bioassays

Bed bugs were fed to repletion 5 d before testing. Ten adult bed bugs (randomly selected, with mixed-sex and age) were then transferred from rearing jars into a plastic Petri dish (60 mm dia. × 15 mm height, Citotest Labware Manufacturing Co. Ltd., Jiangsu, China) lined by the filter paper treated with an insecticide. For the three pyrethroids and malathion, knockdown of Monheim *C. lectularius* strain was recorded at intervals of 5 min for the first hour and subsequently at intervals of 10 min until all tested insects were knocked down. Knockdown of the five *C. hemipterus* strains was recorded at intervals of 12 h for up to 72 h. For DDT test on the Monheim strain, the recording was undertaken at intervals of 1 h until all tested insects were knocked down, while knockdown of all *C. hemipterus* strains was assessed at intervals of 24 h for up to 120 h. Control insects were exposed to the control filter papers in the plastic Petri dishes. An insect was considered knocked down if it could not move or right itself when gently touched with a pair of forceps. Three replicates were undertaken for each insecticide and each strain.

### Synergism Assays

Bed bugs (randomly selected adults, mixed-sex and age) were temporarily anesthetized with CO<sub>2</sub> for approximately 5 s before treatment. Twenty adults were treated topically with a synergist 2 h before being subjected to surface-contact exposure on the treated

filter papers. A 1 µl acetone solution of PBO (50 µg/µl), DEF (15 µg/µl), or DEM (50 µg/µl) was applied onto the ventral surface of the abdomen of the anesthetized bed bugs with a micro-applicator (Burkard Scientific PAX 100 Automatic Micro-Dispensing System) equipped with a 2.5 ml glass syringe (Eterna Matic, Sanitex, Switzerland; Chai and Lee 2010, Gonzalez-Morales and Romero 2019). After treatment, the insects were held in a plastic Petri dish (90 mm diam. × 15 mm height, Favorit, Malaysia) at room temperature (20–22°C).

Ten of the 20 treated insects were introduced into the Petri dish lined by filter paper treated with the insecticides as described above, 2 h after pretreatment with the synergist. Another 10 insects were transferred into a Petri dish lined with filter paper treated with acetone only or a mixture of acetone/oil as the control. The bed bugs were exposed for 72 h (malathion and the three pyrethroids) and 120 h for DDT, as DDT is slower acting (Davies et al. 2007). Knockdown was recorded at regular intervals as described above. Three replicates were carried out for each insecticide and each strain.

### Detection of the Putative *kdr* Mutation(s) on the VGSC Gene

Based on the manufacturer's protocol, total gDNA was extracted using the G-spin Total DNA Extraction Kit (Boca Scientific Incorporated, Dedham, MA). Polymerase chain reaction (PCR) amplification was done according to a previous study (Dang et al. 2015b). Forward primer, BBparaF3 (5'-GGAATTGAAGCTGCC ATGAAGTTG-3') and reverse primer, BBparaR3 (5'-TGCCTAT TCTGTTCGAAAGCCTCAG-3'), were used to amplify the region spanning the sites between L899 and L1017 of the voltage-gated sodium channel gene. The PCR reaction contained 12.5 µl of the KAPA HiFi Hotstart ReadyMix PCR Kit (2×) (Kapabiosystems, USA), 0.5 µl of both forward and reverse primers (10 µM), and 1 µl of the gDNA template in a total volume of 25 µl. Amplification was performed in a thermal cycler (Applied Biosystems Veriti™ 96-Well Thermal Cycler; Applied Biosystems, CA) under the following conditions: 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 45 s, and extended at 72°C for 10 min. PCR products were submitted to the Apical Scientific Sdn. Bhd., Selangor, Malaysia for both forward and reverse DNA sequencing. The sequences were aligned by ClustalW and analyzed using BioEdit and MEGA5 (Hall 1999, Tamura et al. 2011). DNA of five insects randomly from each of CH-MY and GL-MY strains were individually sequenced.

Bioassays results showed that some tested insects from the TT-MY strain were quickly knocked down within the first hour exposure to deltamethrin, while subsequent knockdown was observed after 12-h exposure. Therefore, for TT-MY strain, five

**Table 2.** Discriminating concentrations used in this study

Insecticide	Susceptibility baseline (LC <sub>90</sub> or LC <sub>99</sub> )	Discriminating concentrations <sup>d</sup>	References
Deltamethrin <sup>a</sup>	19.1 mg AI m <sup>-2</sup>	191 mg AI m <sup>-2</sup>	Barile et al. (2008)
Permethrin <sup>a</sup>	25.5 mg AI m <sup>-2</sup>	255 mg AI m <sup>-2</sup>	Fletcher and Axtell (1993)
lambda-cyhalothrin <sup>a</sup>	45.2 mg AI m <sup>-2</sup>	452 mg AI m <sup>-2</sup>	Fletcher and Axtell (1993)
DDT <sup>b</sup>	NA	2%	WHO (1992)
Malathion <sup>c</sup>	NA	5%	WHO (1992)

<sup>a</sup>Pyrethroids.

<sup>b</sup>Organochlorines.

<sup>c</sup>Organophosphates.

<sup>d</sup>The baselines for each pyrethroid insecticide was multiplied by ten and used as the discriminating concentration used for bioassays. For DDT and Malathion, the discriminating concentrations were from WHO (1992).

individuals were chosen randomly from those that were knocked down during the first hour by deltamethrin, and another two individuals from those that survived after 12-h exposure. These insects were sequenced for their *kdr*-mutations. The putative *kdr* mutations were confirmed by comparing sequence alignments with those from other studies (Dang et al. 2015b, Zhao et al. 2020, Soh and Veera Singham 2021), against the reference common bed bug *C. lectularius* (FJ031996) and other wild insect 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 (<https://www.ncbi.nlm.nih.gov/genbank/>).

### Statistical Analysis

Control knockdown was corrected using Abbott's (1925) formula. The knockdown times (KTs) for 50 and 95% of the test bed bugs (KT<sub>50</sub>s and KT<sub>95</sub>s) were generated using a natural log probit model performed on the IBM SPSS Statistic v22 for Windows (IBM Corp., 2013; Finney 1971). The goodness-of-fit test was used to confirm that the data set met the assumptions of the probit model. The cumulative knockdown (72 h) was examined for significance using one-way ANOVA with Tukey's test on the GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, <http://www.graphpad.com>). KT<sub>50</sub>s were considered significantly different if the 95% CI did not overlap (Payton et al. 2003, Wheeler et al. 2006). Inhibition levels of the synergists were determined by comparing knockdown between residual bioassays with and without the synergists. RR<sub>50</sub> (resistance ratio at KT<sub>50</sub>) = KT<sub>50</sub> of field strain/ KT<sub>50</sub> of Monheim strain; SR<sub>50</sub> (synergism ratio) = KT<sub>50</sub> in absence of PBO/ KT<sub>50</sub> in presence of PBO; RS (% resistance suppression) =  $[1 - (KT_{50} \text{ in presence of PBO} / KT_{50} \text{ in absence of PBO})] \times 100$  (Al-Sarar 2010). The classification of resistance is modified from that of Leong et al. (2020a): no resistance (RR<sub>50</sub> ≤ 1 fold), low resistance (1 fold < RR<sub>50</sub> ≤ 10 fold), moderate resistance (10 fold < RR<sub>50</sub> ≤ 25 fold), and high resistance (25 fold < RR<sub>50</sub>).

## Results

### Susceptibility of *C. hemipterus* to Insecticides

Residual bioassays demonstrated that all five *C. hemipterus* strains possessed high levels of resistance to the pyrethroids and DDT and moderate to high levels of resistance to malathion, compared with the susceptible Monheim *C. lectularius* strain (Tables 3 and 4).

For the pyrethroids (Table 3), the 72-h cumulative knockdown for all resistant strains was ≤40% (Table 3), while the susceptible Monheim strain achieved 100% knockdown within 1 h. Due to the high resistance levels, KT<sub>50</sub>s for all resistant strains to the pyrethroids could not be generated. The RR<sub>50</sub>s of all strains were >137-fold.

For DDT, all strains showed no knockdown after 12-h exposure. Even after 120-h exposure, the cumulative knockdown for each strain was ≤46.7% (Table 4). Similar to pyrethroids, the KT<sub>50</sub>s for DDT tests could not be generated. The Monheim strain achieved close to 100% knockdown after 12-h exposure. The RR<sub>50</sub>s of DDT were >29-fold (Table 4).

For malathion, compared with the Monheim strain, both QLD-AU and TT-MY strains showed moderate levels of resistance to malathion, with 100% of the test insects knocked down after 72 h, and RR<sub>50</sub>s ranging from 14.3- to 23-fold. The KL-MY, CH-MY, and GL-MY strains showed a high level of resistance to malathion with up to 76.7% of the insects knocked down (KL-MY: 76.7 ± 8.8%, CH-MY: 6.7 ± 3.3%, GL-MY: 73.3 ± 8.8%) after 72-h exposure, and RR<sub>50</sub>s at least 43.9- to >96.6-fold (Table 4).

### Synergism Assays

Pretreatment with PBO significantly (ANOVA, *P* < 0.05) increased the cumulative knockdown for all *C. hemipterus* strains to deltamethrin and lambda-cyhalothrin after 72-h exposure (Fig. 1A and C). For permethrin, PBO only significantly (ANOVA, *P* < 0.05) increased the cumulative knockdown of the GL-MY strain after 72-h exposure (Fig. 1B).

The addition of PBO to DDT significantly (*P* < 0.05) increased the susceptibility of four strains: QLD-AU (SR<sub>50</sub> > 10.8), TT-MY (SR<sub>50</sub> > 2.2), CH-MY (SR<sub>50</sub> > 4.1), and GL-MY (SR<sub>50</sub> > 6.4; Table 5). RS<sub>50</sub>s

**Table 3.** Susceptibility (knockdown time, KT) of the five *C. hemipterus* strains used in this study against various pyrethroids, with the insecticide-susceptible Monheim *C. lectularius* strain as the control

Insecticides	Strains	N	KT <sub>50</sub> (95%, CI) (min)	KT <sub>95</sub> (95% CI) (min)	χ <sup>2</sup> (df)	Slope ± SE	Knockdown (%) 72 h	RR <sub>50</sub>
Deltamethrin	Monheim	30	19.2 (18.0–20.1)	26.0 (24.3–29.2)	3.2 (3)	5.5 ± 0.9	100	1
	QLD-AU	30	>4,320	>4,320	—	—	10.0 ± 5.8	>224
	KL-MY	30	>4,320	>4,320	—	—	3.3 ± 3.3	>224
	TT-MY	30	>4,320	>4,320	—	—	40 ± 5.8	>224
	CH-MY	30	>4,320	>4,320	—	—	3.3 ± 3.3	>224
	GL-MY	30	>4,320	>4,320	—	—	3.3 ± 3.3	>224
Permethrin	Monheim	30	31.4 (30.3–32.4)	41.9 (39.0–44.3)	2.5 (6)	6.1 ± 0.7	100	1
	QLD-AU	30	>4,320	>4,320	—	—	13.3 ± 8.8	>137
	KL-MY	30	>4,320	>4,320	—	—	6.7 ± 3.3	>137
	TT-MY	30	>4,320	>4,320	—	—	30.0 ± 11.6	>137
	CH-MY	30	>4,320	>4,320	—	—	3.3 ± 3.3	>137
	GL-MY	30	>4,320	>4,320	—	—	6.7 ± 6.7	>137
Lambda-cyhalothrin	Monheim	30	21.0 (20.1–22.1)	30.0 (27.3–34.3)	6.3 (4)	4.7 ± 0.6	100	1
	QLD-AU	30	>4,320	>4,320	—	—	6.7 ± 6.7	>205
	KL-MY	30	>4,320	>4,320	—	—	3.3 ± 3.3	>205
	TT-MY	30	>4,320	>4,320	—	—	33.3 ± 8.8	>205
	CH-MY	30	>4,320	>4,320	—	—	0	>205
	GL-MY	30	>4,320	>4,320	—	—	6.7 ± 3.3	>205

RR<sub>50</sub> (resistance ratio at KT<sub>50</sub>) = KT<sub>50</sub> of resistant strain/ KT<sub>50</sub> of Monheim strain. 100% knockdown of Monheim strain was achieved within 1 h.

**Table 4.** Susceptibility of the five *C. hemipterus* strains used in this study to DDT and malathion, with the insecticide-susceptible Monheim *C. lectularius* strain used as the control

Insecticides	Strains	N	KT <sub>50</sub> (95%, CI) (min)	KT <sub>95</sub> (95% CI) (min)	χ <sup>2</sup> (df)	Slope ± SE	Knockdown (%) 120 h <sup>a</sup> /72 h <sup>b</sup>	RR <sub>50</sub>
2% DDT	Monheim	30	244.8 (220.6–266.6)	533.4 (451.1–710.8)	3.1 (6)	2.1 ± 0.3	100	1
	QLD-AU	30	>7,200	>7,200	—	—	26.7 ± 6.7	>29
	KL-MY	30	>7,200	>7,200	—	—	46.7 ± 12.0	>29
	TT-MY	30	>7,200	>7,200	—	—	26.7 ± 6.7	>29
	CH-MY	30	>7,200	>7,200	—	—	43.3 ± 6.7	>29
	GL-MY	30	>7,200	>7,200	—	—	30.0 ± 5.8	>29
5% Malathion	Monheim	30	44.7 (42.6–46.9)	65.6 (60.3–74.7)	2.4 (5)	4.3 ± 0.5	100	1
	QLD-AU	30	1,028.9 (912.0–1141.9)	1,921.5 (1631.4–2577.7)	1.4 (2)	2.6 ± 0.4	100	23
	KL-MY	30	1,982.9 (1521.3–2629.2)	>4,320	1.1 (2)	1.0 ± 0.2	76.7 ± 8.8	44.4
	TT-MY	30	640.4 (493.4–816.8)	5,804.5 (3732.9–11338.38)	8.7 (6)	0.6 ± 0.1	100	14.3
	CH-MY	30	>4,320	>4,320	—	—	6.7 ± 3.3	>96.6
	GL-MY	30	1,961.8 (1581.0–2496.2)	>4,320	0.5 (4)	0.9 ± 0.2	73.3 ± 8.8	43.9

<sup>a</sup>DDT, cumulated knockdown at 120 h.

<sup>b</sup>Malathion, cumulated knockdown at 72 h.

RR<sub>50</sub> (resistance ratio at KT<sub>50</sub>) = KT<sub>50</sub> of resistant strain/KT<sub>50</sub> of Monheim strain).

were at least 54.5–90.7%. PBO did not effectively suppress DDT resistance in the KL-MY strain.

PBO significantly suppressed malathion resistance in QLD-AU (SR<sub>50</sub> = 3.9, RS<sub>50</sub> = 74.4%,  $P < 0.05$ ; Table 5). PBO suppression in malathion resistance was recorded for the KL-MY, TT-MY, CH-MY, and GL-MY strains.

The addition of DEF did not effectively increase the knockdown for all strains to the pyrethroids. Surprisingly, DEF significantly suppressed resistance to DDT in TT-MY (SR<sub>50</sub> > 4.5, RS<sub>50</sub> > 77.8%,  $P < 0.05$ ) and GL-MY (SR<sub>50</sub> > 1.5, RS<sub>50</sub> > 33.3%,  $P < 0.05$ ) strains (Table 5). Pretreatment of DEF significantly increased the efficacy of malathion in all *C. hemipterus* strains (Table 5), with SR<sub>50</sub>s ranging from 1.7 to 3.7 and RS<sub>50</sub>s ranging from 41.2 to 83.1% (Table 5).

The addition of DEM did not increase the susceptibility of all *C. hemipterus* strains against pyrethroids and DDT (Fig. 1, Table 5). The pretreatment with DEM increased ( $P < 0.05$ ) the susceptibility of the QLD-AU strain (SR<sub>50</sub> = 1.9, RS<sub>50</sub> = 41.2%) to malathion (Table 5), but not on the other strains.

The SR<sub>50</sub>s and RS<sub>50</sub>s for all *C. hemipterus* strains against the pyrethroids were not calculated and compared in this study as all strains displayed high resistance levels, despite a significant increase in knockdown of *C. hemipterus* strains following PBO treatment (Fig. 1).

#### Detection of Putative *kdr* Mutations in *C. hemipterus*

DNA sequencing of domain II of the VGSC gene revealed three putative *kdr* mutations, M918I, D953G, and L1014F, in TT-MY, CH-MY, and GL-MY strains. In the CH-MY ( $N = 5$ ) and GL-MY ( $N = 5$ ) strains, all these individuals (100%) displayed M918I, D953G, and L1014F mutations. In the TT-MY strain ( $N = 7$ ), one individual displayed D953G and L1014F mutations, while six individuals displayed M918I, D953G, and L1014F mutations (refer to next section for further details of putative *kdr* mutations in TT-MY strain; Fig. 2). Both QLD-AU (M918I and L1014F) and KL-MY (L1014F) strains were previously reported (Dang et al. 2015b; Fig. 2).

#### Evidence of Mutations Associated with Pyrethroid Resistance in *C. hemipterus*

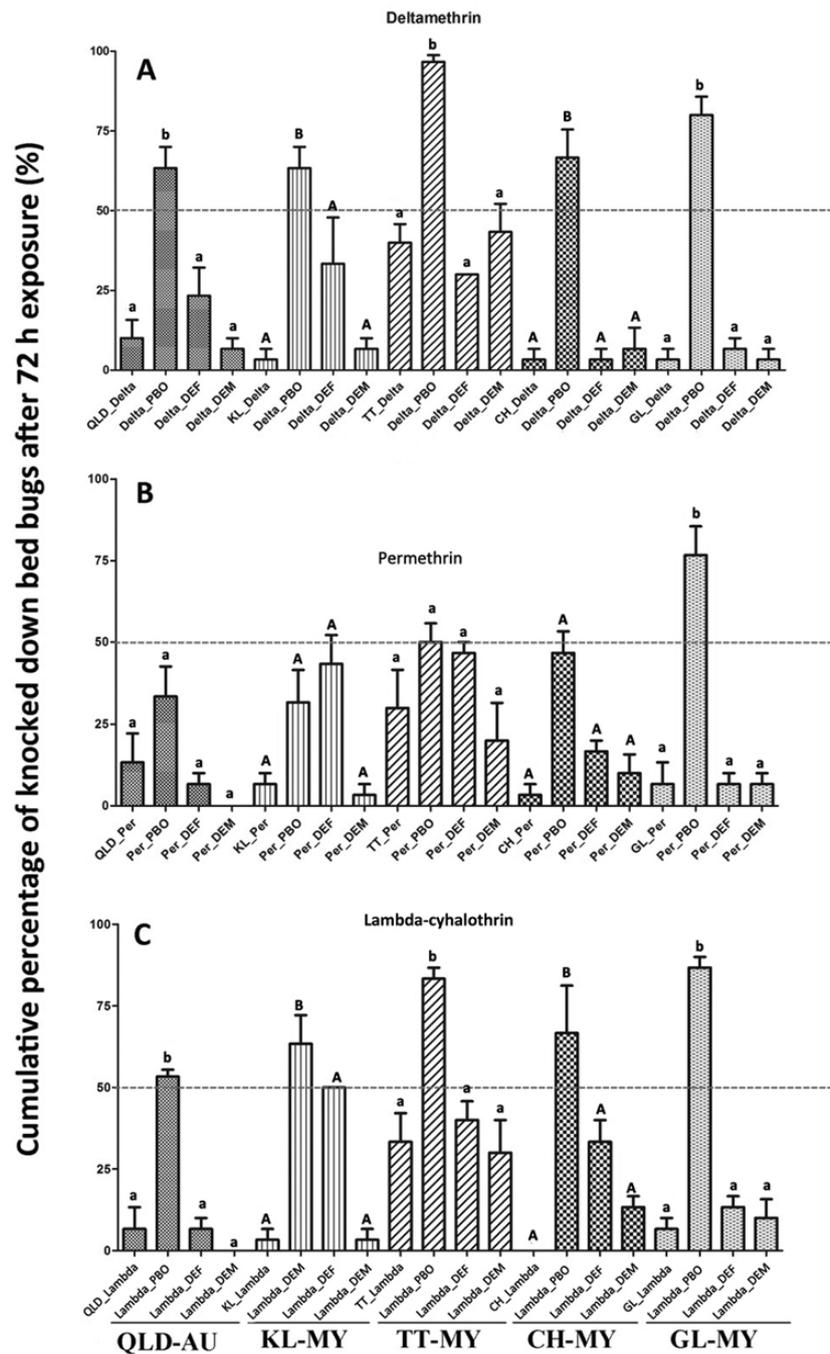
Bioassays results showed that 26.7 ± 6.7% of tested insects from the TT-MY strain were quickly knocked down within the first hour exposure to deltamethrin (Fig. 3A, shaded in green). Subsequent

knockdown was observed after 12-h exposure (Fig. 3A, shaded in yellow). Results showed these individuals had three genotype mutation combinations; D953G + L1014F (homozygous ss: M918), M918I + D953G + L1014F (heterozygous rs: I918), and M918I + D953G + L1014F (homozygous rr: I918). With the five individuals knocked down within the first hour, one individual had D953G + L1014F (homozygous ss: M918) mutations, while the other four individuals had M918I + D953G + L1014F (heterozygous rs: I918) mutations (Fig. 3C and D). However, the two individuals knocked down after 12 h have three mutations, M918I + D953G + L1014F (homozygous rr: I918; Fig. 3B). All seven individual TT-MY insects had homozygous D953G and L1014F (homozygous rr) mutations.

#### Discussion

Recent *C. hemipterus* infestations have been reported in the tropics, including Africa (Fourie and Crafford 2018), China (Guangxi and Guangdong provinces; Wang et al. 2013, Zhao et al. 2020), Australia (North Queensland; Doggett and Russell 2008), Indonesia (Soviana et al. 2019), Malaysia and Singapore (Zulaikha et al. 2016, Lee et al. 2018, Wan Mohammad et al. 2020), and Thailand (Tawatsin et al. 2011), subtropics including Australia (Sydney; Dang et al. 2015b, Doggett and Cains 2018), Iran (Hosseini-Chegeni et al. 2019) and United States (Florida; Campbell et al. 2016), and temperate regions including China (Wang et al. 2013), Central Europe (Balvin et al. 2021), France (Chebbah et al. 2021), Italy (Masini et al. 2020), Russia (Capon 2016), United States (Hawaii; Lewis et al. 2020). Accompanied by this resurgence, insecticide resistance has been widely reported in *C. hemipterus* (Dang et al. 2017a). Most insecticides used in the control of *C. hemipterus* today were initially developed and tested on *C. lectularius*. Different bed bug species may display varying resistance levels and other resistance mechanisms (e.g., *kdr* resistance; Dang et al. 2017a). In best practice, accurate identification is crucial, especially in those areas where both bed bug species are sympatric. For example, Dehghani et al. (2016) reported the common bed bug *C. lectularius* in Iran. However, based on the image that they provided in the publication, the species was *C. hemipterus*.

The DDT resistance may be an indirect outcome of the DDT usage in managing vector mosquitoes, such as malaria vector control programs (Myamba et al. 2002, Davies et al. 2012, Potter 2018). In Malaysia, the indoor residual spraying of DDT was carried out



**Fig. 1.** Cumulative percentage (mean  $\pm$  SE) of knocked down bed bugs for the five *C. hemipterus* strains used in this study after 72-h exposure to pyrethroids (A: Deltamethrin, B: Permethrin, C: Lambda-cyhalothrin) in synergism assays. Statistical differences among treatments within each strain were determined using one-way ANOVA and Tukey's test, with significant differences ( $P < 0.05$ ) indicated by different letters. Significant differences were considered when synergism knockdown was achieved above 50% in this study. Delta = Deltamethrin, Per = Permethrin, Lambda = lambda-cyhalothrin.

extensively since 1967 during the Malaria Eradication Program and was subsequently replaced with pyrethroids in the form of indoor spraying and impregnated bed nets in 1998 (Rohani et al. 2014). In this study, all *C. hemipterus* strains (QLD-AU [2007], KL-MY [2005], TT-MY [2015], CH-MY [2015], and GL-MY [2015]) displayed high levels of resistance to DDT, as well as the pyrethroids. The high levels of DDT resistance identified in the Malaysian strains in this study could also be due to the side-effect of the Malaria Eradication Program described above or the cross-resistance from pyrethroid to DDT since both classes of insecticides share a similar

mode of action. Compared to DDT and the pyrethroids, these five strains showed moderate to high levels of resistance to malathion. This resistance may be attributed to the repetitive use of organophosphates (e.g., malathion, diazinon, chlorpyrifos) and carbamates (e.g., bendiocarb, propoxur) in the past for bed bug management (Potter 2018). Despite serious resistance issues, pyrethroids remain a major class of insecticides widely used for bed bug control in Malaysia. Taken together, the sole use of pyrethroids should be avoided in the management of *C. hemipterus* infestations. Alternatively, newer formulations (e.g., neonicotinoid-pyrethroid mixtures), such as

**Table 5.** Effect of pretreatment with PBO, DEF, and DEM 2 h before application of DDT and malathion to the five *C. hemipterus* strains

Strains	Tests	N	KT <sub>50</sub> (95% CI) (h)	KT <sub>95</sub> (95% CI) (h)	Slope ± SE	χ <sup>2</sup> (df)	Knockdown <sup>d</sup> (%)	SR <sub>50</sub>	RS <sub>50</sub>
QLD-AU	2% DDT	30	>120	>120	—	—	26.7 ± 6.7	1	—
	2% DDT + PBO	30	11.1 (9.7–12.6)*	52.4 (42.3–69.3)	1.1 ± 0.1	1.8 (5)	100	>10.8	>90.7%
	2% DDT + DEF	30	>120	>120	—	—	23.3 ± 12	1	—
	2% DDT + DEM	30	>120	>120	—	—	10	1	—
	5% Mala	30	17.1 (15.4–18.8)	32.7 (27.6–44.5)	2.5 ± 0.4	1.9 (3)	100	1	—
	5% Mala + PBO	30	4.4 (3.7–5.1)*	10.5 (8.7–14.1)	1.9 ± 0.3	0.3 (3)	100	3.9	74.4%
	5% Mala + DEF	30	6.1 (4.9–7.3)*	16.0 (12.9–22.2)	1.7 ± 0.2	0.8 (2)	100	2.8	64.3%
KL-MY	5% Mala + DEM	30	10.1 (8.8–11.7)*	25.1 (20.0–35.7)	1.8 ± 0.2	1.1 (4)	100	1.9	41.2%
	2% DDT	30	>120	>120	—	—	46.7 ± 12	1	—
	2% DDT + PBO	30	114.0 (94.1–158.4)	>120	1.2 ± 0.2	1.5 (3)	51.3 ± 6.8	>1.1	>9.1%
	2% DDT + DEF	30	>120	>120	—	—	33.3 ± 14.5	1	—
	2% DDT + DEM	30	>120	>120	—	—	16.7 ± 8.8	1	—
	5% Mala	30	31.0 (24.0–39.5)	>72	0.9 ± 0.2	2.3 (3)	76.7 ± 8.8	1	—
	5% Mala + PBO	30	30.2 (24.6–37.7)	>72	1.1 ± 0.2	1.1 (3)	78.2 ± 2.8	1	—
TT-MY	5% Mala + DEF	30	12.0 (9.2–14.0)*	32.9 (25.1–62.3)	1.6 ± 0.4	1.2 (2)	96.7 ± 3.3	2.6	61.5%
	5% Mala + DEM	30	17.9 (12.2–26.3)	>72	0.5 ± 0.1	1.14 (5)	80 ± 5.8	1	—
	2% DDT	30	>120	>120	—	—	26.7 ± 6.7	1	—
	2% DDT + PBO	30	55.8 (45.1–69.7)*	>120	0.9 ± 0.1	2.4 (4)	78.8 ± 13.2	>2.2	>54.5%
	2% DDT + DEF	30	26.9 (20.3–35.1)*	>120	0.7 ± 0.1	3.2 (6)	93.3 ± 3.3	>4.5	>77.8%
	2% DDT + DEM	30	>120	>120	—	—	13.3 ± 3.3	1	—
	5% Mala	30	10.7 (8.2–13.6)	96.7 (62.2–189.0)	0.8 ± 0.1	8.7 (6)	100	1	—
CH-MY	5% Mala + PBO	30	9.7 (6.9–12.7)	102.9 (62.6–238.3)	0.7 ± 0.1	0.5 (5)	93.3 ± 6.7	1	—
	5% Mala + DEF	30	2.9 (2.1–3.7)*	12.4 (8.6–22.3)	1.1 ± 0.2	3.1 (2)	100	3.7	72.9%
	5% Mala + DEM	30	10.3 (8.2–12.8)	71.3 (47.3–133.7)	0.9 ± 0.1	1.0 (6)	96.7 ± 3.3	1	—
	2% DDT	30	>120	>120	—	—	43.3 ± 6.7	1	—
	2% DDT + PBO	30	29.4 (22.9–37.4)*	>120	1.0 ± 0.2	3.9 (3)	90 ± 5.8	>4.1	>75.6%
	2% DDT + DEF	30	>120	>120	—	—	13.3 ± 3.3	1	—
	2% DDT + DEM	30	>120	>120	—	—	13.3 ± 8.8	1	—
GL-MY	5% Mala	30	>72	>72	—	—	6.7 ± 3.3	1	—
	5% Mala + PBO	30	>72	>72	—	—	23.3 ± 6.7	1	—
	5% Mala + DEF	30	26.3 (22.9–30.3)*	68.4 (52.8–108.4)	1.7 ± 0.3	0.8 (3)	89.6 ± 5.8	>2.7	>63%
	5% Mala + DEM	30	>72	>72	—	—	6.7 ± 3.3	1	—
	2% DDT	30	>120	>120	—	—	30 ± 5.8	1	—
	2% DDT + PBO	30	18.7 (16.0–22.0)*	132.3 (94.8–210.0)	0.8 ± 0.1	6.3 (5)	97.1 ± 1.8	>6.4	>84.4%
	2% DDT + DEF	30	80.1 (63.1–111.7)*	>120	0.9 ± 0.2	0.6 (3)	66.7 ± 8.8	>1.5	>33.3%
	2% DDT + DEM	30	>120	>120	—	—	30 ± 5.8	1	—
	5% Mala	30	32.7 (26.4–41.6)	>72	0.9 ± 0.2	0.5 (4)	73.3 ± 8.8	1	—
	5% Mala + PBO	30	32.1 (26.7–39.6)	>72	1.2 ± 0.2	1.0 (3)	86.7 ± 8.8	1	—
	5% Mala + DEF	30	19.8 (17.1–22.4)*	47.6 (39.3–64.8)	1.9 ± 0.3	2.3 (4)	100	1.7	41.2%
	5% Mala + DEM	30	45.6 (39.5–53.6)	>72	1.7 ± 0.3	0.8 (2)	76.7 ± 8.8	1	—

\*KT<sub>50</sub>s were considered significantly different ( $P < 0.05$ ) if their 95% CI did not overlap.

<sup>d</sup>Cumulated knockdown to DDT at 120 h, and Malathion at 72 h.

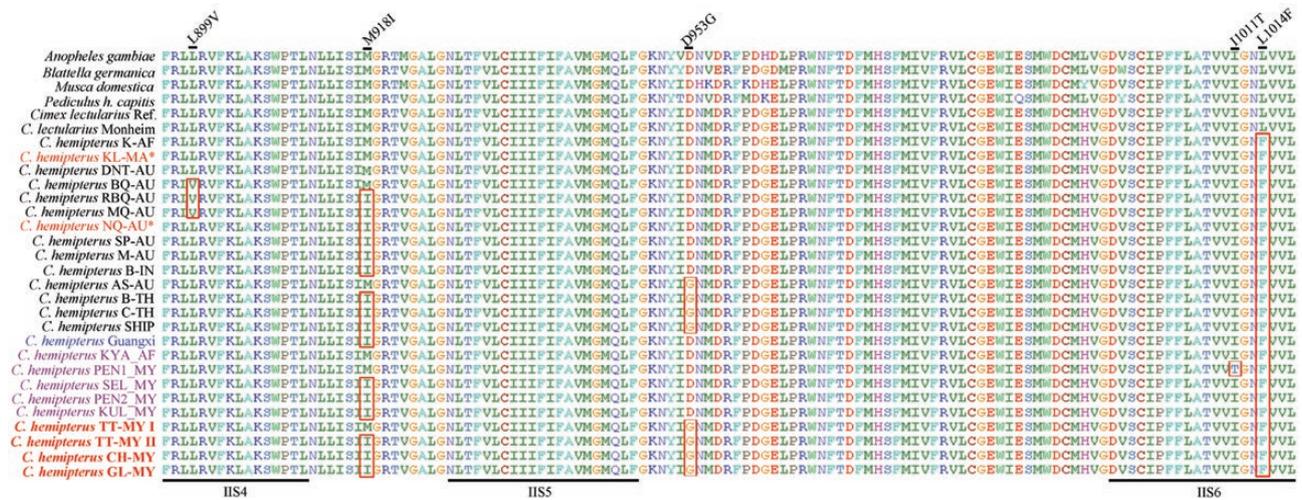
SR<sub>50</sub> (synergism ratio at KT<sub>50</sub>) = KT<sub>50</sub> in absence of PBO/KT<sub>50</sub> in the presence of PBO). RS<sub>50</sub> (% resistance suppression) = [1 - (KT<sub>50</sub> in presence of PBO / KT<sub>50</sub> in absence of PBO)] × 100, same to DEF and DEM.

Temprid (21% imidacloprid, 10.5% beta-cyfluthrin) and Triloa® ZC (also known as Tandem, 11.6% thiamethoxam, 3.5% lambda-cyhalothrin), and nonchemical methods such as heat treatment should be considered in best practice.

P450s are the primary enzyme family associated with resistance to most insecticides, especially pyrethroids (e.g., permethrin, deltamethrin; David et al. 2013). Previous studies have identified that P450s-mediated metabolic resistance to pyrethroids occurs in *C. lectularius* (Dang et al. 2017a, Gonzalez-Morales and Romero 2019, Khalid et al. 2019). In contrast, this resistance mechanism remains the least understood in *C. hemipterus*. Although recent biochemical assays identified varied P450s activities in different *C. hemipterus* strains (Punchihewa et al. 2019, Soh and Veera Singham 2021), the role of P450s in insecticide resistance warrants more investigation. In this study, the addition of PBO effectively increased the susceptibility of all five *C. hemipterus* strains to

deltamethrin and lambda-cyhalothrin (Fig. 1), suggesting P450s involvement in pyrethroid resistance in *C. hemipterus*. P450s can metabolize a broad range of substrates and lead to cross-resistance with different classes of insecticides (Mamidala et al. 2012, David et al. 2013). For example, two synergism studies suggested that P450s likely metabolize fipronil in bed bugs due to cross-resistance, even though fipronil was not registered for bed bug control and not used in bed bug management (How and Lee 2011, González-Morales et al. 2021). In this study, PBO effectively increased the susceptibility of *C. hemipterus* to DDT in four strains and malathion in the QLD-AU strain. This study suggests that P450s are conferring metabolic resistance to pyrethroids and other classes of insecticides in *C. hemipterus*.

Esterases have been implicated in carbamates and organophosphate resistance in numerous insect species, including *C. lectularius* (Adelman et al. 2011, Mamidala et al. 2012, Zhu et al. 2013, Dang



**Fig. 2.** Amino acid sequence alignments of partial VGSC genes of the *C. hemipterus* strains from the study (TT-MY, CH-MY, and GL-MY strains: red letter) and those of previous studies (Dang et al. 2015b: black letter, Zhao et al. 2020: Guangxi strain, Soh and Veera Singham 2021: purple letter), against the reference common bed bug *C. lectularius* (FJ031996) and other wild insect 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. \*NQ-AU is the QLD-AU strain, KL-MA is the KL-MY strain in this study. CH-MY (N = 5): M918I, D953G, L1014F. GL-MY (N = 5): M918I, D953G, L1014F. TT-MY (N = 7; refer to Fig. 3): I (N = 1), D953G, L1014F; II (N = 6), M918I, D953G, L1014F.

et al. 2017a) and *C. hemipterus* (Karunaratne et al. 2007, Punchihewa et al. 2019). Our investigations demonstrated that esterases conferred resistance to malathion, as pretreatment of DEF effectively reduced malathion resistance in all five strains (Table 5). However, altered acetylcholinesterases (AChEs) might also be a resistance mechanism against the organophosphates in *C. hemipterus* (Karunaratne et al. 2007, Punchihewa et al. 2019, Komagata et al. 2021). Recently, a novel mutation, F348Y (phenylalanine 348 to tyrosine), on the AChE gene was identified and was thought to contribute organophosphate and carbamate resistance in both *C. lectularius* and *C. hemipterus* (Komagata et al. 2021). Esterases have also been shown to confer cross-resistance to the pyrethroids and fipronil in *C. lectularius* (Lilly et al. 2016a, Dang et al. 2017a, Gonzalez-Morales and Romero 2019, Gonzalez-Morales et al. 2021).

In this study, DDT resistance was suppressed by DEF in the TT-MY and GL-MY strains (Table 5). This observation must be treated with caution as DEF is not a completely specific inhibitor of esterases. DEF could also inhibit microsomal oxidases at concentrations of  $>10^{-4}$  M (Scott 1990). Compared with *C. lectularius*, the role of esterases in conferring pyrethroid resistance still requires further investigation in *C. hemipterus*, as all synergism assays with DEF pretreatment showed negative results (Fig. 1).

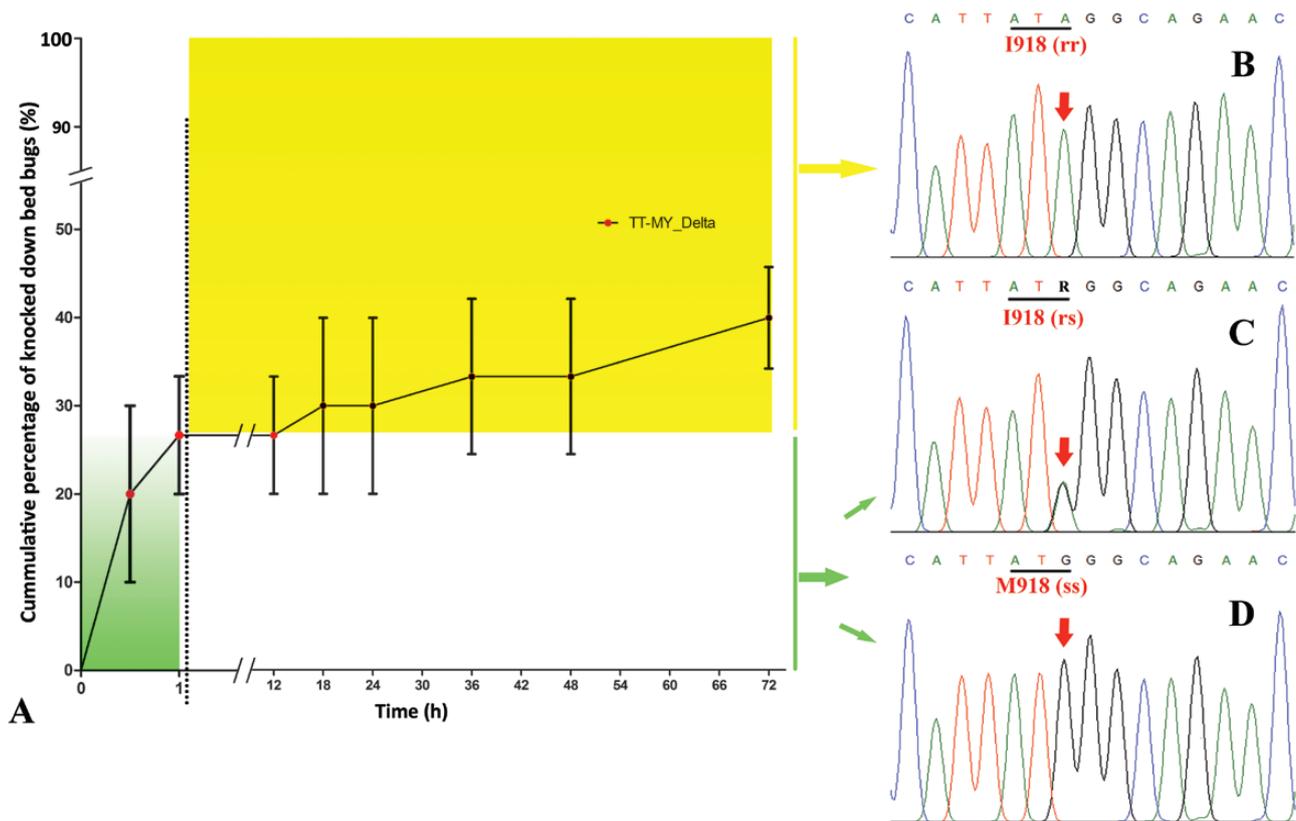
DDT dehydrochlorination by GSTs is a major route of detoxification in insects (Enayati et al. 2005). In this study, the pretreatment of DEM did not effectively increase the susceptibility of all *C. hemipterus* strains to DDT, as well as the pyrethroids. DEM only suppressed malathion resistance in the QLD-AU strain. Despite GSTs-mediated resistance, other metabolic mechanisms such as P450s could instead contribute towards DDT resistance in the five strains. In addition, DDT shares the same VGSC target site with the pyrethroids. *Kdr* mutations in the VGSC gene may be responsible for high levels of DDT resistance in the five strains (Karunaratne et al. 2007). On the contrary, DDT resistance in Sri Lankan *C. hemipterus* populations was due to their high GSTs levels rather than *kdr* mutations (Punchihewa et al. 2019).

Results from the synergism studies provide insights on the involvement of metabolic enzymes in insecticide resistance and provide

important information about the potential use of insecticides with synergists in the formulation for the management of resistant bed bug infestations. For example, PBO and 3-phenoxybenzyl hexanoate (PBH) were suggested for use as synergists with pyrethroids against resistant *C. lectularius* (Romero et al. 2009, Hardstone et al. 2015), and *C. hemipterus* (How and Lee 2011).

The *kdr* mutation is commonly associated with DDT and pyrethroid resistance in a range of insect pests, including bed bugs (Yoon et al. 2008; Davies et al. 2007, 2012; Dang et al. 2015b). Dang et al. (2015b) identified four mutations, namely L899V, M918I, D953G, and L1014F, in *C. hemipterus* from various localities. Subsequently, another eight mutations (A468TY/L995H, A1007S, V1010L, I1011E, I1011T, V1016E, and L1017F/S) were identified but not widely reported (Punchihewa et al. 2019, Ghavami et al. 2021, Soh and Veera Singham 2021). Both M918I and L1014F mutations were commonly identified to occur together in *C. hemipterus* populations from various locations, including Australia (Dang et al. 2015b), Central Europe (Balvin et al. 2021), China (Zhao et al. 2020), India (Dang et al. 2015b), Iran (Ghavami et al. 2021), Malaysia (Dang et al. 2015b, Soh and Veera Singham 2021), and United States (Hawaii; Lewis et al. 2020). Similarly, the *C. hemipterus* strains that were highly resistant to both pyrethroids and DDT in this study were also found to possess both M918I and L1014F. On this basis, M918I and L1014F mutations presumably play a significant role in *kdr* resistance and are widespread among the *C. hemipterus* populations worldwide. Specific molecular markers based on M918I and L1014F mutations could be developed to detect *kdr* resistance in *C. hemipterus*.

Our previous study (Dang et al. 2015b) identified that when L1014F in *C. hemipterus* accompanies M918I, it may be acting as a ‘super-*kdr* mutation’ in pyrethroid resistance (much as the combined M918T + L1014F mutations that has been reported in the house fly [Davies et al. 2007]). Similar findings were reported in later studies (Zhao et al. 2020, Lewis et al. 2020, Balvin et al. 2021, Ghavami et al. 2021, Soh and Veera Singham 2021). Irrespective of the presence of point mutation I1011T, Soh and Veera Singham (2021) reported that PEN1\_MY and KYA\_AF strains, which possessed only



**Fig. 3.** A. Cumulative percentage (Mean  $\pm$  SE) of knocked down *C. hemipterus* of the TT-MY strain within 72-h exposure (knockdown was recorded at 30 min, 1 h, 12 h, 18 h, 24 h, 36 h, 48 h, and 72 h). B–D. Chromatograms showing mutation genotypes of *C. hemipterus*; B. genotype I918 (rr), C. genotype I918 (rs), D. genotype M918 (ss). rr = homozygous resistant, rs = heterozygous resistant, ss = homozygous susceptible. R(ATR)=A/G. DNA sequencing was carried out on seven individual TT-MY insects. In these seven insects, two individuals were knocked down after 12 h by deltamethrin (yellow area of Fig. 3A). These two showed homozygous rr mutation of M918I, refer to Fig. 3B. Five individuals were knocked down during 1 h by deltamethrin (green area of Fig. 3A); one individual showed homozygous ss of M918 (Fig. 3D), while four individuals showed heterozygous rs of M918T (Fig. 3C).

*kdr*-like mutation L1014F, were significantly more susceptible to deltamethrin than that of three strains with both M918I and L1014F mutations. In the present study, the results obtained from both bioassays and molecular analyses of the TT-MY strain provided further evidence to support this hypothesis. Individuals ( $N = 5$ ) that were knocked down during the first hour by deltamethrin had either D953G + L1014F (homozygous ss: M918, in one insect) or M918I + D953G + L1014F (heterozygous rs: I918, in four insects; Fig. 3A, C, and D). However, the remaining two individuals that were knocked down after 12-h exposure displayed M918I + D953G + L1014F (homozygous rr: I918) mutations (Note: both D953G and L1014F mutations were homozygous rr in these individual insects; Fig. 3A and B). Taken together, the results suggested M918I + L1014F mutations confer *super-kdr* resistance, which enhanced deltamethrin resistance in *C. hemipterus*. Compared to P450s, the *super-kdr* may play a significant role in pyrethroid resistance in *C. hemipterus*, such as shown in the TT-MY strain. 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, are required (Usherwood et al. 2007).

In the KL-MY strain, bed bugs possessed the L1014F mutation only but displayed high pyrethroid and DDT resistance levels. In comparison to the four strains (QLD-AU, TT-MY, CH-MY, and GL-MY; Table 6), other resistance mechanisms, such as cuticle thickening (Lilly et al. 2016b, Soh and Veera Singham 2021), may also

be present in the KL-MY strain. Further investigations of biochemical, morphological, and molecular assays (especially whole genomic sequencing) on potential resistance mechanisms are required for *C. hemipterus*.

Unlike *C. lectularius*, no insecticide-susceptible *C. hemipterus* strain exists worldwide, as all modern strains are known to display a certain degree of resistance to different classes of insecticides (Karunaratne et al. 2007; How and Lee 2011; Tawatsin et al. 2011; Dang et al. 2015b, 2017b; Leong et al. 2020a, b; Punchihewa et al. 2019; Soh and Veera Singham 2021; Komagata et al. 2021). Therefore, in this study, we used the susceptible *C. lectularius* Monheim strain for comparison. It is common to select an insecticide-susceptible sympatric or similar species as a reference when no such strain exists for the species under study. Previously, the Monheim strain had been used as a reference in tropical bed bug studies (Dang et al. 2015b, 2017b; Lilly et al. 2018; Leong et al. 2020a, b). In a recent study, Komagata et al. (2021) also selected one insecticide-susceptible strain of *C. lectularius*, TKD as a reference for both *C. lectularius* and *C. hemipterus*. Similarly, in the mosquito *Anopheles funestus* resistance studies, the insecticide-susceptible *Anopheles gambiae* Kisumu strain was used as a reference as no susceptible strain of *A. funestus* was available (Morgan et al. 2010).

In conclusion, the present study showed that both P450s and esterases were involved in metabolic resistance and played significant but varied roles in *C. hemipterus* resistance between different strains (Table 6). Molecular analyses of the VGSC gene identified

**Table 6.** *kdr* resistance and metabolic resistance identified in this study

Insecticides	Resistance mechanisms	QLD-AU <sup>a</sup>	KL-MY <sup>a</sup>	TT-MY <sup>b</sup>	CH-MY	GL-MY
DDT/pyrethroids	<i>kdr</i> -like mutations	M918I, L1014F	L1014F	D953G/L1014F M918I/D953G/ L1014F	M918I, D953G, L1014F	M918I, D953G, L1014F
Deltamethrin	Metabolic	P450s	P450s	P450s	P450s	P450s
Permethrin	Metabolic	ND	ND	ND	ND	P450s
Lambda-cyhalothrin	Metabolic	P450s	P450s	P450s	P450s	P450s
DDT	Metabolic	P450s	ND	P450s	P450s	P450s
Malathion	Metabolic	P450s, Esterases, GST	Esterases	Esterases	Esterases	Esterases

<sup>a</sup>As determined by Dang et al. (2015b).

<sup>b</sup>Further detailed investigation on the Tanjung Tokong (TT-MY) strain found individuals with three types of *kdr*-like mutation combination; D953G + L1014F (homozygous ss: M918), M918I + D953G + L1014F (heterozygous rs: I918), and M918I + D953G + L1014F (homozygous rr: I918), refer to Fig. 3.

ND = not detected, the synergism result is negative.

that *kdr* mutations (M918I, D953G, and L1014F) were present in the TT-MY, CH-MY, and GL-MY *C. hemipterus* strains. The *kdr* mutations in QLD-AU (M918I and L1014F) and KL-MY (L1014F) strains have been identified in our previous study (Dang et al. 2015b). The high levels of resistance to the pyrethroids and DDT may be due to these mutations. Both bioassays and molecular assays provided evidence that M918I + L1014F mutation most likely conferred *super-kdr* characteristic towards pyrethroids and DDT in *C. hemipterus*. Compared with *C. lectularius*, published information on insecticide resistance mechanisms in *C. hemipterus* is relatively limited. In the light of the recent advances into resistance mechanisms within *C. lectularius*, more studies are warranted to investigate other underlying resistance mechanisms present in *C. hemipterus*.

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