INSECTICIDE RESISTANCE AND ITS UNDERLYING MECHANISMS, AND PRODUCT EVALUATIONS AGAINST TROPICAL BED BUGS, *Cimex hemipterus* (FABRICIUS) (HEMIPTERA: CIMICIDAE)

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF PLATES

Page

LIST OF SYMBOLS

P	P-value
ANOVA	Analysis of variance
χ^2	Chi-square

LIST OF ABBREVIATIONS

Abc	ATP-binding cassette
CCB	Centre for Chemical Biology USM
CS	Capsule suspension
DDT	Dichloro-diphenyl-trichloroethane
EC	Emulsifiable concentrate
GST	Glutathione-s-transferase
IPS	Institute of Postgraduate Studies USM
kdr	Knockdown resistance
KT	Knockdown time
LC	Lethal time
LD	Lethal dosage
LT	Lethal time
M	Microencapsulated
ME	Micro-emulsion
PCR	Polymerase Chain Reaction
RCMO	Research Creativity and Management Office USM
RH	Relative humidity
RR	Resistance Ratio
SBS	School of Biological Sciences USM
SC	Suspension concentrate
US EPA	United States Environmental Protection Agency
USM	Universiti Sains Malaysia
VGSC	Voltage-gated sodium channel
WHO	World Health Organization

KERINTANGAN INSEKTISID DAN MEKANISME KERINTANGAN, DAN PENGUJIAN PRODUK TERHADAP PEPIJAT TROPIKA, *Cimex hemipterus* (FABRICIUS) (HEMIPTERA: CIMICIDAE)

ABSTRAK

Kawalan kimia masih menjadi kaedah utama untuk mengawal serangga pepijat di Malaysia walaupun kerintangan pepijat terhadap insektisid telah banyak didokumentasikan. Sehingga kini, jumlah kajian yang telah dijalankan mengenai kerintangan *C.x hemipterus* adalah sedikit berbanding *C. lectularius*. Kajian ini bertujuan untuk mengkaji status kerintangan terhadap insektisid dan mekanismenya dalam lapan strain *C. hemipterus* yang dikumpul di Malaysia dan Australia. Strain *Cimex lectularius* (Monheim) yang rentan digunakan sebagai rujukan kerana tiada strain *C. hemipterus* yang rentan berjaya dijumpai. Tahap kerintangan strain *C. hemipterus* diuji dengan menggunakan produk komersial jenis sisa cair, pelapik tilam yang mengandungi permethrin (ActiveGuard), dan insektisid gred teknikal. Antara kelas-kelas insektisid yang dikaji ialah piretroid (deltamethrin, lambda-cyhalothrin, beta-cyfluthrin, d-tetramethrin, cyphenothrin, d-phenothrin), neonikotinoid (thiamethoxam, imidacloprid), organofosfat (fenitrothion, chlorpyrifos), pirol (chlorfenapyr), dan organoklorin (DDT). Parameter-parameter yang dinilai ialah kitaran hidup, tempoh pendedahan, dan tempoh pemerhatian kematian. Hasil kajian berjaya mengesan kerintangan dalam kesemua kumpulan dewasa strain *C. hemipterus* terhadap piretroid, neonikotinoid, organofosfat, dan pirol. Kerintangan yang tertinggi dikesan terhadap piretroid, diikuti dengan neonikotinoid, organofosfat, dan pirol. Instar pertama menunjukkan status kerintangan yang lebih rendah berbanding kumpulan dewasa. Kerintangan terhadap insektisid telah dikesan di kumpulan telur.

Tempoh pendedahan dan tempoh pemerhatian kematian berpotensi untuk memberikan kesan signifikan terhadap keputusan penilaian cerakinan masa-respons. Tempoh pendedahan yang lebih lama dan tempoh pemerhatian kematian yang lebih panjang dengan produk sisa cecair (kecuali Tandem) menunjukkan kadar kematian yang tinggi dan prestasi produk yang lebih baik. Pelapik tilam yang mengandungi permethrin menunjukkan prestasi yang lemah dalam membunuhi *C. hemipterus* terhadap piretroid. Pelapik tilam yang mengandungi permethrin didapati telah mengurangkan aktiviti pemakanan serangga pepijat. Sepanjang rawatan dengan insektisid gred teknikal, strain *C. hemipterus* mempamerkan kerentanan terendah terhadap deltamethrin (0-23.3% kematian), diikuti dengan chlorpyrifos (0-35.5% kematian) dan imidacloprid (51.7-100% kematian). Mekanisme kerintangan metabolisme *C. hemipterus* telah dikenalpasti melalui kajian sinergi dan ujian biokimia. Aktiviti metabolik tiga kumpulan enzim metabolisme utama (cytochrome P450s, esterases, glutathione-s-transferase) telah dipertingkatkan. Kerintangan terhadap DDT telah dikesan dalam strain *C. hemipterus*. Pengesanan molekul mutasi *kdr* dijalankan dan tiga tapak mutasi *kdr* (M918I, I1011T, L1014F) telah dikenalpasti dan berkorelasi dengan kerintangan terhadap piretroid. Kesimpulannya, kajian ini mendapati bahawa kesemua strain *C. hemipterus* mempunyai kerintangan terhadap insektisid seperti produk-produk komersial dan insektisid gred teknikal yang disebabkan oleh pelbagai mekanisme kerintangan. Kajian ini juga membuktikan bahawa kerintangan *C. hemipterus* bergantung kepada kitaran hidup (instar pertama, dewasa, dan telur). Selain itu, kajian ini mencadangkan reka bentuk eksperimen (tempoh pendedahan dan tempoh pemerhatian kematian) boleh mempengaruhi hasil penilaian kerintangan.

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ABSTRACT

Chemical control remains as the principal mean of bed bug control in Malaysia despite insecticide resistance of bed bugs being widely documented. To date, there are fewer studies reported on insecticide resistance of *C.x hemipterus* as compared to *C. lectularius* . This study aims to investigate insecticide resistance status and its underlying mechanisms in eight field *C. hemipterus* strains collected in Malaysia and Australia. A *C. lectularius* (Monheim) susceptible strain was used as reference as no susceptible *C. hemipterus* strains were found. The *C. hemipterus* strains were evaluated for their resistance levels using commercialized residual liquid formulations, permethrin-impregnated (ActiveGuard) mattress liner, and technical grade insecticides. Insecticide classes investigated include pyrethroids (deltamethrin, lambda-cyhalothrin, beta-cyfluthrin, d-tetramethrin, cyphenothrin, d-phenothrin), neonicotinoids (thiamethoxam, imidacloprid), organophosphates (fenitrothion, chlorpyrifos), pyrrole (chlorfenapyr), and organochlorine (DDT). The parameters evaluated include developmental stages, exposure period, and mortality observation time. Results detected pyrethroid, neonicotinoid, organophosphate, and pyrrole resistance in adults of all *C. hemipterus* strains, exhibiting low to extremely high resistance levels. First instars demonstrated substantially lower resistance status than adults. Insecticide resistance was detected at egg stage. The outcomes of time-response assay could be affected significantly by the exposure period and mortality observation time. Longer treatment exposure periods and longer mortality observation time with

all residual liquid formulations (except Tandem) exhibited higher mortality and better product performance. Permethrin-impregnated mattress liner showed poor performance in killing the pyrethroid-resistant *C. hemipterus*. The permethrin-impregnated mattress liner was also found reduce feeding activity of bed bugs. . In treatment with technical grade insecticide, *C. hemipterus* strains exhibited the lowest susceptibility towards deltamethrin (0–23.3%), followed by chlorpyrifos (0–35.5%) and imidacloprid (51.7–100%). Underlying metabolic resistance mechanisms of the resistant *C. hemipterus* were characterized using synergism studies and biochemical assays. The activity of three important metabolic enzyme groups (cytochrome P450s, esterases, glutathione-s-transferase) was enhanced. Synergism study also suggested the involvement of metabolic resistance mechanisms (cytochrome-P450s, esterases, and GSTs). DDT resistance was detected in the *C. hemipterus* strains. Molecular detection of *kdr* mutations was conducted and found three putative *kdr* mutation sites (M918I, I1011T, L1014F) to correlate with pyrethroid resistance. In summary, the study detected insecticide resistance in all *C. hemipterus* strains towards commercialized products and technical grade insecticides, due to multiple resistance mechanisms. This study provides evidence of stage-dependent (first instars, adults, and eggs) resistance in *C. hemipterus* and suggested experiment design (exposure period and mortality observation time) could affect resistance assessment outcomes.

CHAPTER 1

INTRODUCTION

Infestations of bed bugs have been a long and persistent problem worldwide. Evidence of bed bugs co-existed with humans can be traced back to the times when humans were still inhabiting caves (Usinger, 1966; Potter, 2011). Bed bugs were found to initailly feed on bats but adapted to human blood when humans inhabited cave (Usinger, 1966). Researchers found first genetic evidence with bats are their origin blood host (Booth et al., 2015). As human moved out the cave, bed bugs hitch hike together with human and adapted to human dwellings. The hematophagous insects were classified as nuisance pests as there is no evidence of bed bugs being capable of transmitting disease. Bed bugs were adapted to pierce and suck through the host skins. Their bites were known to cause clinical consequences, the most common effects are dermatological reactions (Doggett et al., 2012).

Bed bug infestations were common globally before World War II (Potter et al., 2011; Davies et al., 2012; Doggett et al., 2012). The discovery of organochlorine dichloro-diphenyl trichloroethane (DDT) and organophosphates (malathion) as pesticides was a major success in insect pest control during World War II. Bed bug infestations became uncommon with the introduction of insecticides until their comeback since 15 to 20 years ago (Potter et al., 2011; Davies et al., 2012; Doggett et al., 2012).

Insecticide resistance was hypothesized as the major factor that leads to the modern resurgence of bed bugs (Dang et al., 2017b). Insecticide resistance in bed bugs was suggested as the consequence of the heavy reliance of insecticide in the earlier pest control practices. Pest control professionals ranked bed bugs as the most challenging urban pest to control because of insecticide resistance (Potter et al., 2015).

Despite the resistance, chemical control remains an important approach to bed bug control. Many studies evaluated the efficacy of insecticides (using technical grade insecticides and insecticide products) against bed bugs and detected insecticide resistance (How and Lee, 2011; Tawatsin et al., 2011; Campbell and Miller, 2015; Zahran and Ab Majid, 2019; Ashbrook et al., 2017; Vander Pan et al., 2019).

Bed bugs were frequently investigated for insecticide resistance, such as towards pyrethroids (Dang et al., 2015a,b,c; Lilly et al., 2016a,b; Dang et al., 2017a Gonzalez-Morales and Anderson, 2018; Lilly et al., 2018; Baraka et al., 2019; Berenji et al., 2019), neonicotinoids (Steelman et al., 2008; Romero and Anderson, 2016; Dang et al., 2017a; Lilly et al., 2018), organophosphates (Karunaratne et al., 2007; Kilpinen et al., 2011; Tawatsin et al., 2011; Punchihewa et al., 2019), organochlorine (DDT) (How and Lee, 2011; Dang et al., 2017a), and pyrroles (chlorfenapyr) (Wang et al., 2016; Ashbrook et al., 2017). Pyrethroids, neonicotinoids, and chlorfenapyr are commonly used in insecticide products designed for bed bug control, including in residual liquid formulations, insecticide dusts, pressurized aerosol spray, and insecticide-impregnated mattress liner. Performance evaluations of both technical grade insecticides and products are important to provide insights on the control failure of bed bugs in the field.

Unlike mosquitoes, there is no standard protocol or guidelines designed to evaluate and monitor insecticide resistance of bed bugs. Bed bug researchers often adhered to WHO guidelines(WHO, 2017), and the US EPA (2017) guidelines for the testing protocol for bed bug products, or other publications when developing the experimental protocol. Insecticide resistance assessment was found to be conducted using different developmental stages, treatment exposure time, experiment durations, or result interpretations. Outcomes may vary between resistance bioassays with

different experiment designs. Understanding these aspects could provide implications for future resistance detection and management.

After the detection of insecticide resistance status, it is also crucial to understand the role of several important resistance mechanisms in bed bugs such as target-site insensitivity (*kdr*) and metabolic resistance. Genetic studies, biochemical studies, and synergism studies were commonly used to characterize the underlying metabolic resistance mechanisms in insect pests. These studies are important to substantiate the findings from the insecticide efficacy test and monitor the resistance mechanisms involved.

As compared to *C. lectularius*, *C. hemipterus* was less studied. Both species of bed bugs dominated different geographical regions, *C. lectularius* are prevalent in the temperate region, while *C. hemipterus* are prevalent in the subtropical and tropical regions (Usinger, 1966; Koganemaru and Miller, 2013). Insecticide resistance assessment on *C. hemipterus* is critical to provide insights into bed bug management in Malaysia as only *C. hemipterus* strains were discovered in Malaysia.

The objectives of this study are described as below:

a. To evaluate the performance of several commercialized residual liquid formulations and the presence of stage dependent resistance in the field-collected *C. hemipterus* strains.
b. To determine the effect of exposure periods and mortality observation time on the outcomes of performance evaluations of residual liquid formulations.
c. To inspect the efficacy of permethrin-impregnated mattress liner and its effect of feeding success rate towards pyrethroid-resistant *C. hemipterus* strains.
d. To investigate the presence of deltamethrin, chlorpyrifos, and imidacloprid resistance in several field-collected *C. hemipterus* strains through topical

application method and the detection of underlying metabolic resistance mechanisms through synergism study and biochemical assay.

e. To evaluate the insecticide susceptibilities of several field-collected *C. hemipterus* strains when treated with deltamethrin and DDT through surface contact assay and detection of the presence of *kdr* mechanisms that were commonly linked to pyrethroid and DDT resistance through molecular study.

CHAPTER 2

LITERATURE REVIEW

2.1 Biology of bed bugs

Bed bugs are classified under Order Hemiptera and family Cimicidae. The insects are hematophagous and nocturnal ectoparasites. They have a pair of 4-segmented antenna that acts the main olfactory organ for odor and host detection (Haracca et al., 2010). Both nymphs and adults are hematophagous, they possess pierce-sucking mouthparts equipped with a proboscis for blood ingestion. When not fed, their bodies were flattened dorsal-ventrally, which enable them to hide in crack and crevices. The insects were flightless and possessed a pair of reduced wing pads on the thorax.

There are five nymphal instars in the life cycle of a bed bug. The immature stage of bed bugs required a full blood meal (fully engorged) to molt to the next nymphal stage until achieving adulthood. If the blood source is always available, the insect requires five to six weeks (at average) to develop from eggs to adults (Miller et al., 2019). The immature insects will be arrested at the same nymphal stage without a full blood meal. Late-instars and adult bed bugs were able to survive in the laboratory for more than six months without a blood meal, while the earlier instars were able to survive for about a month. During the first nymphal stage, the first instars appeared yellowish, the coloration gradually darkened as the insects molted into the later nymphal stage, and subsequently became reddish-brown at the adult stage.

Males and females can be differentiated by the abdomen, with females having a rounded abdominal end, while males having a pointed abdominal end. The adults, particularly the male insect will actively mate after every blood meal. The males would pierce the female's abdomen using its aedeagus (male reproductive organ) to transfer sperm into the female genitalia (Miller et al., 2019). The mating process is known as

traumatic insemination. Females with repeated mating experience may suffer injury to their abdominal region and would avoid mating (Miller et al., 2019). Multiple matings were found to reduce the fecundity and lifespan of the female bed bugs (Miller et al., 2019).

Mated females typically lay eggs two to five days after fed (Johnson et al., 1940; How and Lee, 2011; Polanco et al., 2011). Egg-depositing was observed in females aged between 30 to 200 days, with younger females having a maximum capacity in laying eggs (Johnson et al., 1940; How and Lee, 2011; Polanco et al., 2011). Egg production patterns may be different among different strains, but the reproductivity of female insects did not differ significantly (Polanco et al., 2011).

Cimex. lectularius and *C. hemipterus* can be differentiated by observing the thorax. *Cimex lectularius* has a wider thorax compared to the *C. hemipterus*. According to How and Lee (2011), *C. lectularius* showed better feeding efficiency than *C. hemipterus*. Through morphological evidence, *C. hemipterus* exhibited better vertical climbing ability compared to *C. lectularius* (Kim et al., 2017). Both species have different geographical distributions, *C. lectularius* is mostly distributed in the temperate region, while *C. hemipterus* were mostly distributed in the subtropical and tropical regions.

2.2 The history of bed bug

The two species of bed bugs, namely common bed bugs (*C. lectularius*) and tropical bed bugs (*C. hemipterus*) have been co-existed with humans for more than 3000 years (Usinger, 1966; Panagiotakopulu & Buckland, 1999). Bed bugs were known to first adapt to bat as their blood hosts and shifted to humans that were inhabiting the caves located in the Mediterranean and Middle Eastern regions (Usinger, 1966; Potter,

2011; Koganemaru and Miller, 2013). With the formation and expansion of civilization (villages and cities), bed bug infestations became established at the human settlements and were associated with humans since then (Potter, 2011).

Bed bugs became widespread globally as civilization and trade developed (Usinger, 1966). Despite lacking the ability to fly, the cryptic insects were able to hitchhike with travelers by hiding in their belongings or their transportations to other places around the world (Marlatt, 1916). Bed bug infestations became common in human dwellings regardless of economic status but affect mostly the poor due to crowded living space (Potter, 2011). During world war II, many people were suffering from the insects' irritating bites and obnoxious odor until the introduction of pesticides, such as DDT and Malathion (Potter, 2011; Dang et al., 2017b). With the widespread usage of DDT and malathion in the earlier pest control practices, bed bugs were successfully eradicated (Hirao, 2010; Potter, 2011; Cooper, 2011).

Nevertheless, the resurgence of bed bugs was observed worldwide in the 1990s. Bed bug infestations were mostly reported in heavily travel places (hospitality sector) during the earlier stage of the modern resurgence (Doggett et al., 2018a), which suggested the high turnover rate of local/international travelers likely contribute to the bed bug infestations. The report on the bed bug infestations then expanded to other places including offices, movie theaters, elder-living facilities, healthcare facilities, houses, retail stores, aircraft cabins, and many more (Davies et al., 2012; Doggett et al., 2012; Bandyopadhyay et al., 2015). Other postulated contributing factors to the modern resurgence include insecticide resistance, changes in pest control practices and sale of second-hand items.

2.3 The importance of bed bugs

The two species of bed bugs, namely common bed bugs (*C. lectularius*) and tropical bed bugs (*C. hemipterus*) have been co-existed with humans for more than 3000 years (Usinger, 1966; Panagiotakopulu & Buckland, 1999). Bed bugs were known to first adapt to bat as their blood hosts and shifted to humans that were inhabiting the caves located in the Mediterranean and Middle Eastern regions (Usinger, 1966; Potter, 2011; Koganemaru and Miller, 2013). With the formation and expansion of civilization (villages and cities), bed bug infestations became established at the human settlements and were associated with humans since then (Potter, 2011).

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Bed bug infestations have brought negative economical and financial consequences to individuals, businesses, and organizations (Doggett et al., 2018c). Hospitality, housing, and retail sectors may suffer from revenue loss or having to afford treatment costs for the infested rooms or houses/apartments (Doggett et al., 2018c). The fear of encountering accommodations with bed bugs among travelers could also cause devaluations of hospitality sectors.

Treatment services on bed bugs are challenging due to their cryptic behavior. There are several available treatment options to get rid of bed bug infestations, such as chemical control, heat treatment, cold treatment, and fumigation. However, the equipment used for the treatments can be costly and may not be affordable for small- or medium-scale pest control businesses (Doggett et al., 2018c). Residual insecticide formulation is the least expensive treatment option and is still widely used by pest control operators in Malaysia. Nonetheless, this treatment approach often required follow-up services. Insecticide formulation could not act on bed bug eggs effectively during initial treatment, follow-up treatments are usually needed to kill the newly hatched first instars to completely eradicate the infestation (Wang et al., 2013; Cooper et al., 2015).

Bed bugs were not known as a disease vector probably due to lacking a forest cycle (Koganemaru and Miller, 2013). There was still no evidence reported yet on the bed bug's ability to transmit human disease. According to a few laboratory studies, bed bugs were able to transmit Chagas disease (pathogen: *Trypanosoma cruzi*) and trench

fever (*Bartonella quintana*) (Leulmi et al., 2015; Sentana-Lledo et al., 2016). A previous study also found that the human hepatitis B virus (HBV) was transmitted transtadially in bed bugs and excreted in the fecal matter (Blow, 2001).

The hematophagous behavior of bed bugs was frequently reported to cause health impacts on humans. The insect bites could cause a varying dermatological effect in the human hosts, ranging from no reaction or mild itchiness to severe allergic reaction (Doggett et al., 2012; Doggett et al., 2018b). Redness, rashes, and papules were previously reported in humans suffering from bed bug bites (Goddard, 2008; Pritchard and Hwang, 2009; Doggett et al., 2012; Doggett et al., 2018b). As humans could react to bed bug bites differently, the diagnosis of bed bug bites should not rely solely on the dermatological symptoms (Leverkus et al., 2006). Repeated feeding of bed bugs could lead to other health complications such as blood loss. Humans with compromised health, older people, and younger children were likely to be affected by bed bugs (Pritchard and Hwang, 2009; Paul-Korinek et al., 2012; Sabou et al., 2013). There were cases reported people were suffering from anemia, iron deficiency, and low hemoglobin level after associated with bed bug infestations for some time (Pritchard and Hwang, 2009; Paul-Korinek et al., 2012; Sabou et al., 2013).

Bed bugs are nocturnal insects and typically forage at night. The biting of bed bugs could lead to sleep loss due to the irritation caused by the bites. Bed bugs injected their proboscis into human skin repeatedly until they located the capillary space for feeding (Koganemaru and Miller, 2013). The repeated probing tends to irritate humans, inducing humans to scratch the bitten skin areas all night, which subsequently leads to sleep deprivation. According to a survey, about 30% of people who are suffering from bed bug infestations were unable to sleep at night (Potter et al., 2010).

2.4 Insecticide resistance in bed bugs

Amongst the aforementioned speculating factors of the modern resurgence of bed bugs, insecticide resistance could be the major contributing factor. According to Insecticide Resistance Action Committee (IRAC), insecticide resistance is defined as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species'. The introduction of organochlorine and organophosphate in the earlier pest control practice could have subjected the bed bugs to insecticide selection pressure, resulting in the increasing frequency of resistant individuals in the field population. The resistant individuals can resist insecticide action due to the presence of resistance mechanisms. Reports on insecticide resistance in the modern bed bugs began in the 21st century, starts with the first resistant report on modern *C. lectularius* in the UK (Birchard, 1998). Myamba et al. (2002) then identified insecticide resistance in Tanzania *C. hemipterus* population (Myamba et al., 2002). More resistance reports were then documented on both *C. lectularius* and *C. hemipterus* strains. Insecticide resistance assessments on the field *C. hemipterus* strains often used a susceptible *C. lectularius* strain for comparisons as no susceptible *C. hemipterus* strain exists (Dang et al., 2017a; Dang et al., 2021). According to Dang et al., (2017), molecular analysis of the voltage-gated sodium channel (VGSC) (at domain IS6 to part of domain I -II linker and parts of domains IIS4 to IIS6) detected ~98% similarity between both bed bug species.

Most studies evaluated bed bug resistance towards pyrethroids. Pyrethroids were commonly used in indoor pest control due to the low mammalian toxicity and fast action mechanism (Davies et al., 2007). Pyrethroid was also the most popular choice amongst all insecticide classes to be employed in insecticide formulations (Lee et al.,

2018). Many over-the-counter insecticide formulations contained pyrethroids as active ingredients, such as aerosol spray, mosquito mat, and insecticide-impregnated bed nets. Besides, pyrethroids were also incorporated in several popularly used residual formulations by pest control professionals, for instance, Temprid SC (imidacloprid + beta-cyfluthrin) and Tandem (thiamethoxam + lambda-cyhalothrin). Pyrethroids were synthetic insecticides that were structurally similar to pyrethrin. Pyrethrin was an organic insecticide and was used in controlling insect pests for thousands of years. Both pyrethroid and pyrethrin were neurotoxic and shared similar action mechanisms. Pyrethroid targets the voltage-gated sodium channel (VGSC) in insects, changing the permeability of the nerve membrane to sodium and potassium ions, thereby leading to neuronal hyperexcitation. Despite pyrethroids possessed efficient insecticide properties, bed bugs were found to show pyrethroid resistance. Pyrethroid-resistant bed bugs were detected worldwide including Asia, Africa, North and South America, Europe, and Oceania (Dang et al., 2017b). Both species of bed bugs were frequently reported to show pyrethroid resistance (Karunaratne et al., 2007; Yoon et al., 2008; Zhu et al., 2010; Adelman et all., 2011; Dang et al., 2015a,b,c; Punchihewa et al., 2019; Zahran and Ab Majid, 2019; Dang et al., 2021).

DDT and pyrethroid were frequently linked together due to their same target sites in insects. DDT was initially discovered for its insecticidal properties by Paul Hermann Müller, a Swiss chemist, and was first introduced in pest management during the 1940s to tackle insect vectors (WHO, 1979). The usage of DDT was then extended to control bed bug infestations (Potter, 2011). After a few years since the introduction of DDT, control failure was first reported in Pearl Harbour, Hawaii in managing bed bug infestations (Johnson and Hill, 1948). Since then, DDT resistance in bed bugs was widely reported (Busvine, 1958; WHO, 1963, 1970; Brown and Pal, 1971),

organophosphates (malathion, chlorpyrifos, and diazinon) and lindane were used as alternatives. Despite DDT resistance were frequently documented, DDT showed remarkable performance (long residual effect and no repellency) in controlling bed bug infestations around 50 to 70 years ago. In the 1980s, due to the health impact and persistence of DDT in the environment, many countries banned the usage of DDT (WHO, 1979). The efficiency of DDT in killing bed bugs was still frequently assessed and detected DDT resistance in both *C. lectularius* (Steelman et al., 2008; Tawatsin et al., 2011) and *C. hemipterus* (How and Lee, 2011; Tawatsin et al., 2011; Dang et al., 2017a).

Imidacloprid (neonicotinoid) was initially introduced in 1991, followed by several other compounds from the same class including acetamiprid, nitenpyram, thiamethoxam, thiacloprid, clothianidin, and dinotefuran (Nauen and Denholm, 2005; Bass et al., 2015). This insecticide class was widely used in controlling various sucking pests from different insect orders, such as Diptera, Coleoptera, and Lepidoptera (Elbert et al., 2008). Several neonicotinoid insecticides were incorporated in bed bug residual formulations together with pyrethroids. These pyrethroid-neonicotinoid formulated products (Temprid SC and Tandem) were widely used in controlling bed bug infestations in Malaysia. Neonicotinoids were neurotoxic insecticides. It acts by binding to the insect nicotinic acetylcholine receptors (nAChRs) at the post-synaptic neurons, leading to neural hyperstimulation and eventually death. Neonicotinoids were first used in crop protection and expanded to controlling urban insect pests (Elbert et al., 2008). Treatment with imidacloprid alone was found less efficient in controlling *C. hemipterus* alone when compared to pyrethroids and phenyl pyrazole (How and Lee, 2011; Lilly et al., 2018). Neonicotinoid resistance was detected in *C. lectularius* field strains (Romero and Anderson, 2016).

Organophosphates were frequently used as an alternative to DDT in the earlier pest control practices including bed bug management. However, organophosphates were already banned for indoor usage in many countries now due to their chronic toxicity to humans and strong odor (Lee et al., 2018). Organophosphates acted on insects by inhibiting acetylcholinesterase (involved in degrading acetylcholine (AChE)) in the nervous system. The accumulation of AChE disrupts the function of the acetylcholine post-synapse and eventually death. Several organophosphate compounds were investigated for their effectiveness against bed bugs, such as malathion, fenitrothion, diazinon, dichlorvos, and so on (Lee et al., 2018). Organophosphates showed delayed toxic action when compared to other neurotoxic insecticides (pyrethroids and neonicotinoids) as the conversion of organophosphate to the toxic organophosphate-oxon form requires a longer period (Chai and Lee, 2010). However, as compared to pyrethroids, organophosphate performed better in killing bed bugs (Potter et al., 2011; Dang et al., 2017a; Dang et al., 2021). Evidence of organophosphate-resistant bed bugs has been documented in India (Sen, 1958), around 15 years since the introduction of organophosphate as insecticides. Both species of bed bugs were reported for organophosphate resistance in many countries including Israel (Barkai, 1964), Sri Lanka (Karunaratne et al., 2007; Punchihewa et al., 2019), Denmark (Kilpinen et al., 2011), Thailand (Tawatsin et al., 2011), Iran (Berenji et al., 2019) and Malaysia (Dang et al., 2017a; Dang et al., 2021).

Chlorfenapyr-formulated products, such as Phantom have been investigated for the performance against bed bugs in several studies (Wang et al;., 2016; Ashbrook et al., 2017;). Chlorfenapyr belongs to a slow-acting and newer insecticide class, pyrrole. This insecticide class targets the mitochondria and disrupts the production of ATP from ADP. Romero et al. (2010), Tawatsin et al., (2011), and Wang et al. (2016a) suggested

chlorfenapyr performed well in bed bug control. However, Ashbrook et al. (2017) detected chlorfenapyr resistance in *C. lectularius*. On the other hand, chlorfenapyr was also evaluated in repellency assay towards *C. lectularius* and was found non-repellent against bed bugs (Romero et al., 2009a).

Bed bugs were also found to show resistance towards several other insecticide classes such as organochlorines (dieldrin, gamma-HCH, methoxychlor, and aldrin) and carbamates. Despite being banned for usage after many years, Tawatsin et al. (2011) still detected dieldrin resistance in the field bed bug strains. Carbamates shared a similar mode of action with the organophosphates (Lee et al., 2018) by binding to acetylcholinesterase to inhibit the breakdown of acetylcholine, causing the accumulation of acetylcholine. Nonetheless, the inhibition process for organophosphate is non-competitive, with the organophosphate bound permanently to the acetylcholinesterase, while carbamates performed a competitive inhibition (Mwila et al., 2013). Like organophosphates, despite being effective against pyrethroid-resistant bed bugs, the usage of carbamates as insecticides were withdrawn from many countries but remained accepted in some Asian countries due to their effectiveness against pyrethroid-resistant insects (Lee et al., 2018). Technical grade carbamate (Karunaratne, 2007; Steelman et al., 2008; Tawatsin et al., 2011) and carbamate-formulated products such as liquid spray (Newberry, 1991), wettable powder (Barile et al., 2008; Turner and Brigham, 2008), emulsifiable concentrate (Tawatsin et al., 2008) were tested against bed bugs. Carbamate (propoxur and bendiocarb) resistance was reported in bed bugs (Boase et al., 2006; Karunaratne et al., 2007; Tawatsin et al., 2011).

2.5 Insecticide resistance mechanism

The development of insecticide resistance in bed bugs occurred due to the continuous changes in the environment (insecticide exposure) that created a selection pressure against the insect populations. Survival of the fittest is the evolutionary response of the insect populations towards insecticide-selection pressure. When exposed to insecticides, the susceptible insects towards insecticide will be eliminated, leaving the resistant insects (individuals with inherited ability to survive insecticide action) in the populations. The resistant insects that survived will continue to reproduce, passing on the resistant traits in the next generation and more. Eventually, the resistant traits became more and more abundant in the population, reducing the susceptibility of the whole population towards insecticide.

Physiological resistance and behavioral resistance were both documented as the major means of insecticide resistance in insects. For physiological resistance in insects, insects could reduce their susceptibility towards insecticide action through physiological modification, for instance, reduced target-site insensitivity, enhanced metabolic resistance, and reduced cuticular penetration resistance. Physiological resistance was widely documented in many insect pests, including bed bugs. The study on physiological resistance is a very important aspect of chemical control in bed bugs. Cross-resistance between insecticides of different classes due to the similar insecticide-detoxification pathway or similar target site were previously discussed in several bed bug studies (Gordon et al., 2014; Dang et al., 2017b). On the other hand, several studies also found that bed bugs could possess multiple resistance mechanisms that could associate with extremely high resistance levels (Adelman et al., 2011; Zhu et al., 2013; Punchihewa et al., 2019; Vander Pan, 2020). Further monitoring and studying on

insecticide resistance of bed bugs is warranted to develop management strategies against resistant bed bugs.

2.5.1 Metabolic resistance mechanism

Resistant bed bugs were frequently found to show increased enzymatic activities that potentially enhanced the detoxification pathway of the toxins. Previously published reviews have discussed the roles of various important detoxification enzyme groups (cytochrome P450s, esterase, glutathione-s-transferase (GST), and ATP-binding cassette (Abc) transporters) in the metabolic resistance of resistant bed bugs (Mamidala et al. 2011, Dang et al., 2017b). Enzymes played an important role in metabolic detoxification by breaking down the toxins (drugs, insecticides) into their non-toxic forms. The enzyme groups were reported to have a wide spectrum of catalyzing activity towards insecticide from different classes (Mamidala et al., 2011; Dang et al., 2017b).

Cytochrome P450s comprised the largest superfamilies of protein amongst all living organisms (Agosin, 1976; Scott, 1999). The cytochrome P450-mediated system is one of the most important metabolic systems involved in the catabolism and anabolism of toxins that lead to insecticide resistance. Evidence of enhanced cytochrome P450s activity was frequently documented in various insect pests including mosquitoes (Perera et al., 2008; Low et al., 2013;), German cockroaches (Scharf et al., 1999; Chai and Lee, 2010), houseflies (Markussen and Kristensen, 2010; Gao et al., 2012), bed bugs (Karunaratne et al., 2007; Adelman et al., 2011; How and Lee, 2011; Gonzales-Morales and Romero, 2018; Punchihewa et al., 2019; Vander Pan et al., 2020), and so on.

Cytochrome P450s have varying substrate specificity, for example, CYP1A1 was able to metabolize at least 20 substrates, while CYP7A1 was found to metabolize one substrate so far (Rendic and Di Carlo, 1997; Mansuy, 1998). During the reaction,

the cytochrome P450s system reacted with oxygen molecules and acquired an electron from nicotinamide adenine dinucleotide phosphate (NADPH), eventually forming water molecules (Berge et al., 1998). The reaction involved the electron transport system in the cell mitochondrion (Hanukoglu, 1996; Hubbard et al., 2001; Zhu et al., 2012).

Overexpression of cytochrome P450 genes was reported to be caused by constitutive transcriptional overexpression at mRNA levels or induced transcriptional overexpression (Liu, 2012). To date, only constitutive transcriptional overexpression was detected in *C. lectularius* (Adelman et al., 2011; Mamidala et al., 2012; Zhu et al., 2013), there has been a lack of related studies in *C. hemipterus*. Through synergism study, How and Lee (2011) detected the role of cytochrome P450s in pyrethroid resistance of *C. hemipterus*. Similarly, pyrethroid-resistant *C. lectularius* showed reduced pyrethroid susceptibility after pretreatment with PBO (inhibitor of cytochrome P450s) (Lilly et al., 2016a; Gonzalez-Morales and Romero, 2018). Cáceres et al. (2019) also reported a synergistic effect when using PBO with deltamethrin towards three field-collected *C. lectularius* strains. According to Adelman et al. (2011), the resistant Richmond strain showed significantly higher cytochrome P450 activities compared to the susceptible Harlan strain and detected overexpression of CYP397A1, CYP6DM2, and CYP400A1 genes. Several other members of cytochrome P450s including CYP9, CYP397A1V2, CYP6A2, CYP6A13, CYP398A1, CYP6DN1, and CYP4CM1 were previously reported to be overexpressed in pyrethroid-resistant *C. lectularius* through transcriptomic analysis and dsRNA-mediated interference (RNAi) method (Bai et al., 2011; Mamidala et al., 2012; Zhu et al., 2013).

The wide catalyzing ability of cytochrome P450s could be the key reason of cytochrome P450-mediated resistance was associated with many insecticide classes

such as pyrethroids, neonicotinoids, and organochlorines. Due to the similar metabolic detoxification pathway and the broad-spectrum enzyme activity, the cytochrome P450-mediated system could confer cross-resistance. Cross-resistance between the pyrethroid, neonicotinoids, and organochlorines was confirmed by molecular docking studies due to the upregulation of cytochrome P450 genes (Mamidala et al., 2012).

The esterase-mediated system was also an important metabolic resistance mechanism that was frequently reported in many insect pests. Esterase constitutes many enzymes including phosphorotriester hydrolases and carboxylesterases (Yan et al., 2009). Esterases catalyzed hydrolysis of carboxyl ester compounds through water addition (Yan et al., 2009). The reaction resulted in acid and alcohol compounds (Yan et al., 2009). The classification of the esterase group is challenging due to the overlapping spectrum of substrate specificity. The detoxification capability of esterases enables it to confer resistance to several insecticide classes, with organophosphates and carbamates, as well as pyrethroids in some cases were found as the substrates (Liu et al., 2006; Yan et al., 2009).

Esterase confers resistance through two major mechanisms which are the elevated esterase-based mechanism (quantitative) and non-elevated esterase based mechanism (qualitative) at the esterase coding sequences (Hemingway et al., 2004). Quantitative changes of esterase in insects are caused by the overexpression of the esterase genes. The amplification of enzyme genes produced large quantities of esterases in the insects, lead to elevated enzyme activity in the insects when the insects were exposed to insecticides (Liu et al., 2006). Enhanced esterase activities were previously documented in many resistant insect pests such as cockroaches (Lee et al., 2000; Enayati and Motevalli, 2007), mosquitoes (Low et al., 2013; Bharati et al., 2016), and bed bugs (Karunaratne et al., 2007; Adelman et al., 2011; Zhu et al., 2013;

Gonzalez-Morales and Romero, 2018). Through transcriptome analysis, two carboxylesterase-encoding genes (CE3959 and CE21331) were found overexpressed in the pyrethroid-resistant Richmond (*C. lectularius*) strain (Adelman et al., 2011). Similarly, another study by Zhu et al. (2013) identified CE21331 (known as ClCE21331 in the article) to be overexpressed in the resistant strains. Several synergism studies also detected increased pyrethroid susceptibility in resistant *C. lectularius* after pretreatment with esterase inhibitors (DEF (Gonzalez and Morales, 2018), TPP (Gonzalez-Morales, 2018), and EN16/5–1 (Lilly et al., 2016a). The studies suggested the importance of the esterase-mediated system in resistant bed bugs. There has been a lack of molecular evidence on the overexpression of esterase genes in *C. hemipterus*. However, Karunaratne et al. (2007) and Punchihewa et al. (2019) detected elevated esterase activities in Sri Lanka strains of *C. hemipterus* that could be associated with organophosphate and carbamate resistance via biochemical analysis.

Non-elevated esterase-based mechanism is when an insect showed increased enzymatic activity without overproduction of esterases (Liu et al., 2006). The mechanism was first revealed in organophosphate-resistant house flies (Van Asperen and Oppenoorth, 1959). It was speculated to be ‘mutant aliphatic esterase hypothesis’, a mutation (point mutation or substitution) at the aliphatic esterase (carboxylesterase) gene encoding sequences that affect the normal function and reduce the hydrolyzing activity for carboxylesterase substrates but gained the ability to hydrolyze toxins such as organophosphate (Liu et al., 2006). Amino acid substitution at the LcaE7 gene was detected in organophosphate-resistant sheep blowflies (Newcomb et al., 1997) and house flies (Claudianos et al., 1999). Nonetheless, it was not reported yet in both species of bed bugs.

Like cytochrome P450s and esterases, GSTs were multi-functional enzymes that were commonly associated with insecticide resistance. GST-mediated system was frequently reported in various insect pests, for instance, fruit flies (Sun et al., 2011), cockroaches (Lee et al., 2000; Enayati and Motevali, 2007), mosquitoes (Lumjuan et al., 2005; Xu et al., 2005), and bed bugs (Karunaratne et al., 2007; Punchihewa et al., 2019).

GST-mediated system was known to associate with organochlorine-, pyrethroid-, and organophosphate-resistance. The role of the GST-mediated system in organophosphate resistance was initially reported by Fukami and Shishido (1966). Reduced glutathione (GSH) derived from GST involved in the catabolism of conjugated electrophilic compounds (Enayati et al., 2005; Li et al., 2007). GST played an important role in the metabolic detoxification process through sequestrations, binding to xenobiotics, transporting endogenous compounds intracellularly (Li et al., 2007). Besides, GSTs could also demonstrate DDTase ability by involving in dehydrochlorination of DDT with the cofactor, GSH (Li et al., 2007).

In a synergism study on field *C. lectularius* strains, after pretreatment with DEM (GST inhibitor), the insects were found less resistant towards deltamethrin (Gonzalez-Morales and Romero, 2018). The study suggested pyrethroid resistance in bed bugs could be associated with GSTs. Mamidala et al. (2012) and Adelman et al. (2013) revealed the upregulated GST genes were present in resistant *C. lectularius* strains through molecular analysis. There has been a lack of molecular analysis and synergism study on the role of GST in resistant *C. hemipterus*. However, two studies (Karunaratne et al., 2007; Punchihewa et al., 2019) in Sri Lanka revealed that GST could potentially confer resistance to DDT as high GST activity was detected in the DDT-resistant *C.*

hemipterus field strains. More study is warranted to further confirmed the involvement of the GST-mediated system in insecticide resistance of bed bugs.

2.5.2 ABC transporter mediated resistance mechanism

Abc transporters played an important role by importing or exporting compounds across cell membranes. Due to their ability to transport xenobiotics, Abc transporters could remove toxins, such as insecticides away from the target sites. Abc transporters could confer resistance to several insecticide classes, such as pyrethroids, organophosphates, carbamates, neonicotinoids, and organochlorines (Dang et al., 2017b). The involvement of Abc transporters in metabolic resistance was previously suggested in resistant *C. lectularius* through RNAi studies (Zhu et al., 2013). Mamidala et al. (2012) also detected upregulated Abc transporter genes in bed bugs and suggested Abc transporters could involve in insecticide resistance. Nevertheless, more studies are warranted to better understand the role of Abc transporters in insecticide resistance.

2.5.3 Target site resistance mechanism

Insecticide targets different sites according to their mode of action. For instance, DDT and pyrethroids target VGSC at neurons, neonicotinoid targets nicotinic acetylcholine receptors (nAChRs) at post-synapse, while organophosphates and carbamates target acetylcholinesterase at post-synapse. Insects were known to develop resistance by reducing the sensitivity of the target sites, reducing the binding of insecticides. Modifications at the target sites avoid the neurotoxic action to take place by inhibiting the insecticides from reacting with the target sites. Target site insensitivity was previously documented in various insect pests, namely knockdown resistance (*kdr*), altered nAChRs, altered AchEs, and GABA receptors insensitivity (*rdl*).

Knockdown resistance (*kdr*) is very important in conferring pyrethroid and DDT resistance in many insect pests (Williamson et al., 1996; Reimer et al., 2008; Kawada

et al., 2009; Smith et al., 2019; Yoshimizu et al., 2019; Brownell et al., 2020; Villanueva-Segura et al., 2020). Pyrethroid and DDT bind at VGSC of insects, causing the constant influx of sodium ions, leading to the repeated firing of nerve impulses in insects. Genetic alterations at the gene encoding VGSC sequences allow the insects to resist the insecticide action of pyrethroids and DDTs by reducing the affinity of the target sites (Soderlund, 2008). Several mutations (amino acid substitution and point mutation) were identified at the VGSC genes and were known to be associated with pyrethroid and DDT resistance in insects. *kdr* resistance was first suggested by Busvine (1951) on insecticide-resistant house flies. Milani (1954) as well as Milani and Travaglino (1957) then conducted genetic studies and confirmed the presence of *kdr* mechanisms as a recessive factor in house flies.

kdr mutations (V419L and L925I) were identified in a pyrethroid-resistant *C. lectularius* strain (Yoon et al., 2008). In the same study, the same resistant *C. lectularius* strain was investigated for metabolic resistance through biochemical assay but show no significant difference in enzyme (7-ethoxycoumarin O-deethylases, esterases, and GSTs) activities compared to that of susceptible strain. The findings suggested pyrethroid resistance of bed bugs was associated with *kdr* mutations. Zhu et al. (2010) reported *kdr* mutations were widespread amongst 110 *C. lectularius* strains collected in the USA. Different *kdr* haplotypes were identified among all tested strains: haplotype A (without both V419L and L925I mutations, haplotype B (with L925I only), haplotype C (with both V419L and L925I), and haplotype D (with V419L only). Besides mutations V419L and L925I, I936F was also detected in an Australian *C. lectularius* strain being correlated to low resistance level towards d-allethrin (Dang et al., 2015b). Other studies also reported the presence of *kdr* mutations in pyrethroid-resistant *C. lectularius* (Adelman et al., 2011; Durand et al., 2012; Zhu et al., 2013; Dang et al.,

2015b,c; Palenchar et al., 2015; Raab et al., 2016; Gaire et al., 2020; Vander Pan et al., 2020; Akhoundi et al., 2021).

kdr mechanism in *C. hemipterus* was first described by Dang et al. (2015b) and identified four mutations at domain II region VGSC gene, including L899V, M918I, D953G, and L1014F. Mutations located at residues M918 and L1014 were suggested to link with high pyrethroid resistance in *C. hemipterus* (Dang et al. 2015b). Residue sit 918 was previously described as *super-kdr* mutation (M918T). The mutation at residue 1014 was reported to have different amino acid substitutions in various insect pests (L1014C/H/S/W) (Rinkevich et al., 2013). Bed bugs were found to demonstrate higher resistance towards d-allethrin when having both M918I and L1014F mutations compared to individuals with L1014F only (Dang et al., 2015c). M918I mutations were detected with L1014F in all individuals tested in the study. Similarly, another study in China also detected the double M918I + L1014F mutation in the field *C. hemipterus* strains (Zhao et al., 2020). However, the authors did not assess the pyrethroid resistance level of the *C. hemipterus* strains tested.

In Sri Lanka, Punchihewa et al. (2019) did not detect mutation M918I in their *C. hemipterus* field strains. Their findings showed seven mutations (Y/L995H, A1007S, V1010L, I1011F, L1014F, V1016E, L1017F/S) at the VGSC gene. All mutation sites were previously described as *kdr*-associated mutations (some with different amino acid replacements) in arthropods (Dinparast Djadid et al., 2007; Kawada et al., 2009; Singh et al., 2009; Singh et al., 2010; Ilias et al., 2014; Kawada et al., 2014; Silva et al., 2014), except for A1007S (Menze et al., 2016). Nonetheless, to confirm the association of these *kdr* mutation sites with pyrethroid resistance in *C. hemipterus*, more studies are required. Lewis et al. (2020) detected the presence of M918I, D953G, Y/L995H, and L1014F in *C. hemipterus* strains collected at Honolulu, Hawaii. Samiei et al. (2020a)