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Advances in Molecular Research on Bed Bugs (Hemiptera: Cimicidae)

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Abstract With the resurgence and increase in infestations of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), across the world, there has been renewed interest in molecular research on this pest. In this paper, we present current information on the biology, medical importance, management practices, behavior and physiology, and molecular research conducted on bed bugs. The majority of molecular studies are focused towards understanding the molecular mechanism of insecticide resistance. Bed bugs are hematophagous insects with no prior record of vectoring any disease organisms. An improved understanding of how bed bugs lack vector competency may provide information to prevent disease transmission in other hematophagous insects. The genome of bed bugs has been sequenced, and genomic studies may provide a better understanding of bed bug behavior that might be utilized in developing effective management strategies. Recently, with the advancement of RNA interference (RNAi) as a tool to suppress insects, a few RNAi studies have been conducted in bed bugs. RNAi in bed bugs shows potential to suppress populations in laboratory conditions. However, delivery of double-stranded RNA (dsRNA) into bed bugs under field condition requires extensive research.

Key Words bed bugs, molecular research, insecticide, resistance, RNAi

Bed bugs, *Cimex lectularius* L., have followed humans all recorded history, contributing to human misery. Humans have been the primary host of bed bugs since ancient times when people were living in caves with bats. Bed bugs were nearly eradicated after World War II due to the excessive use of insecticides, such as organochlorines, organophosphates, and carbamates. However, restriction on the use of these insecticides along with the development of insecticide resistance and an increase in global travel have resulted in a resurgence of bed bugs, with a concomitant impact on human health and economy. Current bed bug management practices include insecticide application and cold and heat treatments. The use of molecular tools in insect identification, population genetics, and pest management has provided a new horizon in insect pest management. The RNA interference (RNAi) tool has been used to kill insects or reverse genetics of insects to increase the target's susceptibility to insecticides. Although funding in bed bug molecular research is limited, a few RNAi studies have been conducted to understand gene functions associated with behavior, reproduction, and physiology. Extraction of

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intact DNA from bed bugs is possible up to 1 yr if samples are kept in a sealed glass jar at room temperature (Basnet et al. 2017), and data from this study provided valuable information on the preservation techniques of bed bugs for molecular research. In this review herein, we present current information on molecular studies conducted on bed bugs and their importance in understanding basic biology, reproduction, behavior, physiology, and potential for vectoring disease organisms.

Biology and Life Cycle of Bed Bugs

The blood meal is obligatory for molting and growth in bed bugs. The life cycle of bed bugs consists of eggs and nymphs with five instars. Eggs are usually laid in clusters but can be laid singly, which are firmly attached to the surface by a white gelatinous substance to avoid dispersal. The eggs hatch usually within 2 weeks and develop into nymphs, which are creamy white in color. Each nymphal instar molts in approximately a week depending on ambient temperature and availability of blood meal. At least a single blood meal is required to complete each molt. Nymph survivorship is $>$90% in favorable conditions. A generation (from egg to adult) can be completed in 4 to 5 weeks at 28–30°C and a relative humidity of 75–80%. The life span of an adult depends on temperature, humidity, and food. A laboratory study revealed that adult bed bugs live for 3 mo without a blood meal (Polanco et al. 2011).

Male genitalia consists of an aedeagus and left clasper, and the female genitalia is functional and exclusively used for laying eggs. Mating occurs at a paragenital sinus called spermalege. An adult male expresses sexual interest to a recently fed female and has a peculiar method of copulation known as traumatic insemination. The sperm is directly ejaculated inside the body called Organ of Berlese (Usinger 1966). The injected sperm migrate to the ovaries to fertilize the eggs. The traumatic insemination causes physical injury to the female and she avoids aggregation and copulation until the previous injury is fully recovered (Stutt and Siva-Jothy 2001).

Medical Importance of Bed Bugs

Vectoring capacity. Except for bed bugs, all other hematophagous insects are vectors of human diseases. *Triatoma* spp. and *Rhodnius prolixus* Stål are hemipteran insects that transmit Chagas disease. Mosquitoes transmit many human diseases including malaria, yellow fever, Zika fever, and Japanese encephalitis. The Oriental rat flea, *Xenopsylla cheopis* Roths, transmits bubonic plague, and the body louse, *Pediculus humanus humanus* L., is a vector of typhus fever. Other dipteran species such as black flies transmit onchocerciasis, and tsetse flies transmit human sleeping sickness. There is no factual evidence that bed bugs can transmit human diseases in natural conditions. However, speculation on bed bugs serving as a vector of human diseases has been reported in the older scientific literature (Doane 1910, Edwards 1892, Nelson 1963).

Adelman et al. (2013) speculated that three ecological circumstances can potentially cause bed bugs to become vectors of pathogens. Those are: (a) cimicids consisted of a diverse group of insects sharing common hosts and habitat; (b) bats are natural reservoir host for multiple arboviruses associated with human disease,

and bed bugs are also associated bats; (c) cimicids are repeatedly exposed to bats and viruses they harbor, and this interaction suggests the possibility of zoonotic transmission between these two groups. Salazar et al. (2015) disclosed that bed bugs can acquire *Trypanosoma cruzi* Chagas, a vector of Chagas disease, from infested mice and pass it to uninfected mice. Bed bugs can be a model insect to understand how it adapts itself to harbor pathogens but not transmit to other hosts. Studies also have revealed that bed bugs can harbor many human viruses, such as hepatitis B, C, and E, human immunodeficiency virus, and methacillin-resistant *Staphylococcus aureus* Rosenbach. These viruses have a remarkable capacity to mutate (RNA viruses can mutate faster than DNA viruses) to adapt a new host and environment. They can also have an adaptive mutation in response to specific selective pressures, and these mutations can cause viruses to live inside bed bugs and evolve as a causative agent of a specific disease.

Injury from bites. Bed bug bites can cause itchy welts or rash-like symptoms. However, these skin reactions are different among individuals, and some may not develop any symptoms at all. The common skin reactions are 2- to 5-mm-diameter itchy red spots at the bed bug feeding sites; usually one spot per bite. These disappear in about a week or so. The mental health issues associated with the bed bug infestation have been a subject of constant discussion. Infestation of bed bugs can cause distress, anxiety, and insomnia (Goddard and de Shazo 2012, Thomas et al. 2013). In a case study, a woman with a history of psychiatric morbidity committed suicide after she was heavily infested by bed bugs (Burrows et al. 2013).

Current Bed Bug Management Practices and Insecticides Resistance

Use of chemical insecticides have been the most effective means to control bed bugs and their use is likely to be the most desirable option until effective alternatives are discovered. The U.S. Environmental Protection Agency has registered $>$300 products for bed bug control that include different classes of insecticides such as pyrethrins, pyrethroids, desiccants, biochemicals, pyrroles, neonicotinoids, and insect growth regulators. Insecticide resistance is one of the most studied research areas in bed bugs (Mamidala et al. 2012; Moore and Miller 2009; Romero and Anderson 2016; Romero et al. 2007, 2009; Yoon et al. 2008). Pyrethroids are the most commonly used insecticides, but resistance, low residual activity, and avoidance of treated area by bed bugs are concerns for their uses (Romero et al. 2009). The field populations of bed bugs collected from different cities across the United States have shown medium to high levels of resistance to insecticides (Romero et al. 2007), making bed bug control more difficult. Use of insecticides with different modes of action has been implemented to address insecticide resistance, but very few classes of insecticides are registered for indoor treatment of bed bugs. The organophosphates are no longer registered, and a few labeled carbamates have too severe restrictions for indoor use. Chlorfenapyr is a pyrole insecticide registered for bed bug control, but it is slow acting and has limited residual activity. The neonicotinoid insecticides are also promising in bed bug control and have been used in rotation with pyrethroids to mitigate insecticide resistance. However, Romero and Anderson (2016) detected an elevated level of resistance to neonicotinoids in bed bugs.

Exposure to extreme high and low temperatures can kill bed bugs and their eggs. Heat treatment has been an effective method to treat bed bugs in private homes and hotels. Bed bugs are killed if they are exposed at 39–40°C for >90 min. If the temperature is increased to 48°C, bed bugs can be killed in 20 min. Exposure time and temperature to kill eggs are, respectively, 90 min at 48°C (Miller 2014).

Bed bugs are also susceptible to low temperatures. Both adults and nymphs can be killed when exposed continuously to −12°C for 1 week, while the eggs should be exposed to −31.2°C for 1 week (Olson et al. 2013). Use of cold temperature is only feasible for small samples that can be placed in freezers.

Different fungal pathogens have been evaluated against bed bugs. A study by Zahran et al. (2017) determined rapid mortality (as high as 90%) in bed bugs following exposure to *Aspergillus tubingensis* Mosseray. Barbarin et al. (2017) reported >94% mortality in 6 d when insecticide-resistant bed bugs were exposed to *Beauveria bassiana* (Balsamo) Vuillemin. Ulrich et al. (2014) reported that *Metarhizium anisopliae* Sorokin is a poor pathogen for bed bug control. These data suggest some fungal pathogens can be effective for indoor control of bed bugs. However, the bed bugs must come in direct contact with the spores for infection to occur.

Bed Bug Behavior and Physiology

Otti et al. (2017) studied the microbiome abundance in bed bugs and reported differences in their abundance during feeding and mating. In addition, these microbiomes communicate during mating and feeding and possibly have a role in healing wounds caused during traumatic insemination. The most abundant microbial symbionts in bed bugs are *Wolbachia*, followed by *Clostridium* (Rosenfeld et al. 2016). This study also identified that *Wolbachia* is essential for bed bug growth and reproduction by supplying vitamin B. Talbot et al. (2017) identified two salivary protein genes that enhance the efficiency of blood feeding in bed bugs by inhibiting platelet aggregations and vasoconstriction. Liu et al. (2017) identified aldehydes/ketones as the most efficient stimuli, while carboxylic acids and aliphatics/aromatics were comparatively less effective in eliciting responses from bed bug olfactory receptors. Weeks et al. (2011) identified potential kairomones, which have been shown to have an additive effect when used in a heated bed bug trap with carbon dioxide and is the only available trap with proven efficacy in the field. Liu et al. (2017) elucidated the molecular basis of DEET repellent in bed bugs. In this research, DEET-sensitive receptors were functionally deciphered in bed bugs. DEET produced a blocking effect on the neuronal responses of bed bugs to specific human odors and showed inhibitory effect on the function of odorant receptors in responding to certain human odor. Bed bugs could be exposed to ethanol, caffeine, and ibuprofen because of the intensive use of these produced across the world. Narain and Kamble (2015) determined that ibuprofen negatively impacts oviposition, while caffeine adversely impacts feeding, fecundity, and egg hatch. In addition, ethanol consumption in bed bugs reduced fecundity (Narain 2015). Siljander et al. (2008) and Gries et al. (2015) isolated two compounds, (*E*)-2-hexenal and (*E*)-2-octenal, from the pheromones of bed bugs, which opens the

possibility of the commercial development of these compounds as a pest management tool for an attract-and-kill strategy.

Molecular Research in Bed Bugs

Bed bug genome. The genome of bed bugs has been sequenced, and approximately 15,000 protein-coded genes have been predicted (Benoit et al. 2016, Rosenfeld et al. 2016**)**. The phylogenetic relationship showed the *R. prolixus*, a vector of the Chagas parasite, is the closest relative of bed bugs (Rosenfeld et al. 2016), and a sister species of another hemipteran, *Acyrthosiphon pisum* (Harris) (Benoit et al. 2016). Genes related to traumatic insemination, insecticide resistance, host–symbiont interactions, and chemosensors have been identified, but their specific functions have yet to be confirmed in laboratory settings (Benoit et al. 2016). Further, Benoit et al. (2016) identified genes associated with the olfactory receptors, an odorant-binding protein, which will provide a better understanding of how bed bugs are attracted to hosts for feeding. Another species of bed bugs, the tropical bed bugs *Cimex hemipterus* F., have also been established in Florida and Australia, and their establishment could further aggravate the infestation of bed bugs (Campbell et al. 2017, Doggett et al. 2004). Whole-genome sequencing of all bed bug species and their comparative analysis can provide us a better understanding of the behavior and physiology of different bed bug species. Besides, there are also several blood-feeding cimicids, and genomic studies on all these insects may provide us with details on how these insects have different host preferences and vary in vector competence.

Population genetics research. Population genetics is a study of genetic variations among different populations of a species. In bed bugs, few studies have been conducted to understand the divergence among different bed bug populations. Balvín et al. (2012) reported no genetic differences between bed bug populations collected from humans and bats based on the mitochondrial DNA sequence analysis. Molecular markers have been used to understand the diversity of bed bugs found within an apartment complex. Booth et al. (2012) have disclosed the relatively low level of divergence of bed bugs collected from different rooms within an apartment complex, suggesting dispersal of the same population. The study in tropical bed bugs collected from various locations in Malaysia indicated that the populations were homogenous (Seri Masran and Ab Majid 2017). Surprisingly, the only study that showed great diversity in bed bug populations was by Narain et al. (2015) in the populations collected from various locations of the midwestern United States. The population found in the eastern United States did not resemble populations from any other geographical location, suggesting the multiple introductions of bed bugs likely occurred in the United States (Saenz et al. 2012). They also found that the populations in the eastern United States are developed from a single mated female and descendants, further suggesting that bed bug reproduction is highly prolific and a single population can spread very rapidly. Resurgence of bed bugs also has been reported from Canada (Hwang et al. 2005), Australia (Doggett et al. 2004), the United Kingdom and Germany (Boase 2001), Italy (Masetti and Bruschi 2007), Denmark (Kilpinen et al. 2011), and Thailand (Tawatsin et al. 2011). With the introduction of tropical bed bugs in new areas, there

may be confusion in the identification of bed bugs at the species level, and DNA-based identification of the species can be developed.

Molecular mechanism of insecticide resistance. The multiple mechanisms of insecticide resistances identified in bed bugs are target site mutations, differential gene expression, thickening of the bed bug cuticle, and behavior modifications. Zhu et al. (2013) identified five mechanisms involved in insecticide resistance that includes knockdown resistance mutation, enhanced metabolic detoxification, cuticular proteins, and ATP-binding cassette (ABC) transporters. Yoon et al. (2008) identified the mutations V419L and L925I in voltage-gated sodium channel of an α-subunit gene that led to deltamethrin resistance in bed bugs. The majority of bed bug populations across the United States and Europe had this mechanism of insecticide resistance when tested by Zhu et al. (2010) and Booth et al. (2015) in their individual experiments. Palenchar et al. (2015) reported that the majority of the bed bugs collected in Israel had L925I mutation and no V419L mutation.

The major detoxifying enzymes identified for insecticide resistance are cytochrome P450s, carboxylesterases, and glutathione-*S*-esterase. These enzymes are responsible for expression of cuticular proteins and decrease in cuticular penetration of insecticides. Benoit et al. (2016) have identified multiple ABC transporters, carboxylesterases, and cuticle proteins that could have caused insecticide resistance in bed bugs. Adelman et al. (2011) reported that deep sequencing analysis of pyrethroid-resistant bed bugs yielded high expression of cytochrome P450 and carboxylesterase genes, which were significantly overexpressed in the resistant Richmond strain when compared to susceptible Harlan strain. Furthermore, Koganemaru et al. (2013) disclosed that thickening or mutated chemical composition of cuticle may have contributed to decreased insecticide penetration into bed bugs. Lilly et al. (2016) demonstrated the relation between the cuticle thickness and time-to-knockdown the bed bugs upon exposure to an insecticide. Behavior resistance also has been reported in bed bugs in terms of avoiding areas sprayed with pyrethroids (Romero et al. 2009), but the molecular mechanism involved in the behavior resistance is poorly understood.

RNAi research in bed bugs. Recently, RNAi has developed as a novel method of insect management because of its potential to kill insect pests. RNAi technology is highly specific in targeting the genes of interest, versatile in that any genes of interest can be targeted, and efficient with $>$90% of the genes can be silenced (Zhang et al. 2013). Therefore, a concept of RNAi-based pest suppression was developed by silencing the genes involved in the vital functioning and arresting the growth, fecundity, or the survival of insects. In numerous studies, the RNAi tool is used to suppress critical pathway genes for insect control (Huvenne and Smagghe 2010, Whyard et al. 2009).

RNAi research on bed bugs was first initiated by Zhu et al. (2012). These authors silenced the cytochrome P450 genes in bed bugs and increased the susceptibility of bed bugs to pyrethroids. Mamidala et al. (2012) characterized the knockdown potassium (Kir) channels in bed bugs by RNAi. Zhu et al. (2013) also identified 14 molecular markers associated with pyrethroid resistance and tested the relative contribution of each gene by knocking down different genes involved in the resistance. Gujar and Palli (2016a) identified three genes coding for vitellogenin and reported their hormonal regulation by RNAi. Gujar and Palli (2016b) further revealed the interactions among Krüppel homolog 1, early ecdysone response gene, and

juvenile hormone in the regulation of metamorphosis in bed bugs. Moriyama et al. (2016) knocked down vitellogenin in bed bugs and found reduced fecundity and survival in bed bugs. Tsujimoto et al. (2017) characterized the water channel protein aquaporin, silenced the gene by RNAi, and demonstrated its critical function in excretion, water homeostasis, and reproduction in bed bugs. Knockdown of critical genes in bed bugs by RNAi has the potential to cause >90% mortality in bed bugs, along with disruption of the reproductive system (Basnet and Kamble 2017, 2018). These studies showed that RNAi has the potential to suppress bed bug populations through injection of double-stranded RNA (dsRNA). However, the field application RNAi for bed bug control seems very difficult due to the constraints of dsRNA delivery. Our laboratory studies showed that dsRNA is quickly degraded in the blood, which delimits the use of blood products for dsRNA delivery into bed bugs. Whitten et al. (2016) demonstrated significant knockdown of vitellogenin and reduced fecundity in *R. prolixus* through a symbiont-mediated dsRNA expression. *Rhodnius prolixus* is a close relative of the bed bug. Genetically engineered endosymbionts have the potential to suppress the insect population in a laboratory setting, but the delivery technique limits use in the field. A proof of concept that can be evaluated in future is the identification of a virus specific to bed bugs, and genetically manipulate these viruses for dsRNA delivery into bed bugs.

Conclusions and Future Perspectives

Bed bug infestation is likely to increase due to the globalization, the rapid increase in urban areas, as well as limited use of chemical insecticides indoors. Insecticide resistance has aggravated the infestation levels, and cross resistance of insecticides has also been reported, which could further complicate the use of chemical insecticides. Fungal pathogens have also shown the promising results against bed bugs. RNAi has provided a better understanding of bed bug behavior and physiology, and this tool also has shown promising results for development in insect control. However, delivery of RNAi-based molecules seems feasible in phytophagous insects only as of now. Effective delivery of dsRNA to hematophagous insects is a major challenge of using RNAi for control of hematophagous insects. dsRNA is not stable in the blood, and using blood as a component of a pest management product is not practical. A single tactic that can completely control bed bugs is not possible. Therefore, the feasibility of different control tactics including RNAi, fungal pathogens, and pheromones should be studied or used together with insecticides for successful suppression of bed bug populations. In addition, bed bugs have the potential to transmit diseases, requiring further research to understand its potential or the mechanism underlying vector incompetence.

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