INFESTATION LEVELS OF TROPICAL BED BUG (Cimex hemipterus F.) (HEMIPTERA: CIMICIDAE) IN PENINSULAR MALAYSIA AND THEIR RESISTANCE TO CONVENTIONAL INSECTICIDES AND ENTOMOPATHOGENIC FUNGI

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celcius
cm	Centimeter
mm	Millimeter
ml	Milliliter
%	Percent
/	Per
m^{-2}	per meter square
±	central range
km	Kilometer
χ^2	chi square
®	registered sign
$>$	more than
$<$	less than
µm	Micrometer
RH	relative humidity
LC_{50}	lethal concentration of 50% population
LT_{50}	lethal time of 50% population
ED_{50}	efficacy dose of 50% population
T_m	number of dead bugs in treated medium
C_m	number of dead bugs in control medium
TM1	*Trichoderma harzianum*
AM1	*Aspergillus tubingensis*

AR	Arau
IP	Ipoh
TI	Teluk Intan
HM	Hutan Melintang, Perak ()
PY	Puteri Jaya
KT	Kuala Terengganu
PC	Pantai Cenang
KG	Klang
PD	Port Dickson
SW	Senawang

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TAHAP SERANGAN PEPIJAT TROPIKA (Cimex hemipterus F.) (HEMIPTERA: CIMICIDAE) DI SEMENANJUNG MALAYSIA DAN KERINTANGAN TERHADAP RACUN SERANGGA LAZIM DAN KULAT ENTOMOPATOGENIK

ABSTRAK

Pada masa kini, majoriti industri kawalan makhluk perosak mengalami masalah pembasmian pepijat terutamanya di bilik asrama pekerja dan kawasan perumahan. Penggunaan pelbagai kelas racun serangga yang mengandungi bahan aktif kimia atau bukan kimia memerlukan sekurang-kurangnya 3 atau 4 kali untuk rawatan pembasmian sepenuhnya bagi jangka masa panjang. Oleh itu, tinjauan serangga pepijat di seluruh Semenanjung Malaysia dijalankan dengan mengumpul sampel dari kawasan perumahan, bilik asrama, hotel, motel dan pangsapuri. Tinjauan dilakukan di 11 buah negeri; Pulau Pinang, Perlis, Perak, Kedah, Terengganu, Kelantan, Pahang, Selangor, Negeri Sembilan, Melaka, Johor dan Wilayah Persekutuan Kuala Lumpur. Serangan pepijat yang aktif telah dikesan di 38 kawasan daripada 181 jumlah keseluruhan kawasan tinjauan. Namun, tiada perbezaan signifikan ditemui di antara status serangan di setiap negeri dan kawasan tinjauan. Kebanyakan pepijat didapati menduduki di sekeliling tilam, di celah rekahan dinding dan lantai, dan tempat duduk berkusyen di bilik asrama pekerja. Pepijat terkumpul kemudiannya dibiakkan di dalam makmal untuk tujuan kajian lain. Ini termasuklah kajian kerintangan dan kadar kematian apabila pepijat didedahkan kepada musuh semula jadi iaitu kulat, sebagai kaedah kawalan alternatif tanpa bahan kimia. Bagi kajian kerintangan racun, pepijat diuji dengan menggunakan kertas resap racun deltametrin dan propoxur yang disediakan oleh Pertubuhan Kesihatan Sedunia (WHO). Tiga replika mempunyai 10 ekor pepijat, setiap satu mengandungi kertas resap racun yang dibiarkan terdedah selama 14 hari. Kerintangan pepijat dikesan dalam populasi pepijat Arau (AR) (LT_{50} = 466.119 jam) selepas didedahkan kepada racun deltametrin. Propoxur sangat berkesan ke atas populasi pepijat Ipoh (IP) memandangkan nilai menyebabkan kematian adalah rendah, LT_{50} = 260.793 jam. Terdapat perbezaan signifikan antara kumpulan racun dan populasi pepijat. Interaksi antara populasi pepijat terdedah kepada deltametrin menunjukkan perbezaan signifikan, tetapi racun propoxur menunjukkan sebaliknya. Sebagai alternatif kepada kaedah menggunakan bahan kimia, satu formulasi spora *Aspergillus tubingensis* (AM2) dan *Trichoderma harzianum* (TM1) telah dibentuk untuk melihat kesannya ke atas pepijat. Tiga kepekatan spora konidia berbeza, 1 x 10^4, 1 x 10^5, 1 x 10^6 spora/ml disembur ke atas kain fabrik. Penilaian kebisaan kulat menunjukkan kadar kematian yang perlahan dengan masa kematian yang singkat bagi kesemua kepekatan kecuali kepekatan pengasingan tertinggi AM2, LT_{50} = 44.629j. Purata kelangsungan hidup (MST) bagaimanapun menunjukkan tiada perbezaan signifikan antara kesemua kepekatan. Oleh itu, pemeriksaan teliti terhadap tanda-tanda kehadiran pepijat semasa mengembara harus dilakukan untuk menghalang penyebaran pepijat ke seluruh negara. Pencegahan penggunaan racun piretroid terutamanya deltametrin boleh mengurangkan masalah kerintangan pepijat manakala kajian mengenai agen kawalan biologi perlu diterokai dengan mendalam untuk menentukan potensi kulat dalam kawalan pepijat.

INFESTATION LEVELS OF TROPICAL BED BUG (Cimex hemipterus F.) (HEMIPTERA: CIMICIDAE) IN PENINSULAR MALAYSIA AND THEIR RESISTANCE TO CONVENTIONAL INSECTICIDES AND ENTOMOPATHOGENIC FUNGI

ABSTRACT

Nowadays majority of pest control industries are having difficulties in battling bed bugs, specifically in workers' dormitories and residential houses. Long-term treatment of many insecticides classes including chemical or non-chemical active ingredients are necessary at least three to four times for complete eradication. Here, a research is conducted to survey bed bug infestations across Peninsular Malaysia by collecting samples from residential houses, dormitories, hotels and motels and flats and apartments. The survey was carried out in 11 states; Pulau Pinang, Perlis, Perak, Kedah, Terengganu, Kelantan, Pahang, Selangor, Negeri Sembilan, Melaka, Johor and federal territory of Kuala Lumpur. Active bed bugs infestation was detected in 38 sites from the overall 181 surveyed sites. However, no significant difference was found between infestation status in each state and the surveyed premises. Most bed bugs were found harboring around mattresses, cracks and crevices of walls and floors as well as cushion seats in the workers' dormitories. Collected bed bugs were then colonized in the laboratory for other study purposes. This include resistance study and mortality testing on bed bugs using natural enemies of insect, fungi as an introduction to non-chemical method in managing the insect pest. In insecticide resistance bioassay, deltamethrin and propoxur were selected against live bed bugs, following protocol of World Health Organization (WHO) insecticide-impregnated papers. Triplicates of ten adult bed bugs were used, each replicate contained impregnated papers and they were left exposed for 14 days. Resistance in bed bugs were detected in Arau (AR) population (LT_{50} = 466.119h) after exposed to deltamethrin insecticides. Propoxur was highly effective against Ipoh (IP) population due to the low lethal value, LT_{50} = 260.793h. There was a significant difference between the insecticide groups and bed bugs populations. Interaction among the populations exposed to deltamethrin also showed significant difference but not in propoxur. As an alternative to chemical method, spore formulations of *Aspergillus tubingensis* (AM2) and *Trichoderma harzianum* (TM1) were made to observe their physical interaction against bed bugs. Three different concentrations of conidial spores, 1×10^4, 1×10^5, 1×10^6 spores/ml, were sprayed on fabric cloth substrate. Evaluation of both isolates in terms of virulence resulted in slower mortality rate due to low lethal hours in all concentrations except high concentration of AM2 isolate, LT_{50} = 44.629h. Mean survival times (MST) however showed no significant difference among all the concentrations. As such, vigilant inspections while travelling for any infestation signs should be made to prevent bed bugs from nationwide spreading. Prevention of pyrethroid insecticide especially deltamethrin may reduce resistance problems in bed bugs whereas studies on biological control agents should be further explored to determine the potential of fungi in controlling bed bugs population.

CHAPTER ONE

INTRODUCTION

1.1 Background Study

Bed bugs are known as little ‘vampire’ as they feed mainly on human blood to maintain their survivals. In physical, bed bugs have a brownish flattened oval-shaped body which is almost similar to the apple seed size (Miller 2008). They are notoriously active at night in order to hunt their victims for blood supply. Once bed bugs are fully consumed, they flee back to their hiding places which are not too far from the host (How and Lee 2010a). Since bed bugs prefer small, damp and narrow spaces to aggregate, cluttering activities may create new harborage sites for them to hide (Wang and Wen 2011). The list of favored harborages could be as follows; seams of mattresses as well as pillows, headboards, wooden furniture, in cracks and crevices along walls and floors or even electrical fuse boxes (How and Lee 2010b).

Before World War II, these Old World pests have been tagging along with the immigrants to access in well-developed countries to establish infestations that later causes nuisance among humans (Usinger 1966). Several possible factors such as open international migration activities and increase level of resistance of the pest against many insecticides have been linked to their reemergence up to this date (Emmanuel et al. 2014). According to the study of Zorilla-Vaca et al. (2015), two species of bed bugs, *Cimex hemipterus*, have been severely affecting human in Southeast Asia, China, Japan, Korea whereas *Cimex lectularius* seems to be dominant in Europe countries and America based on the number of reported cases and publications. Even though they are not capable of transmitting pathogens and diseases in the field, however this pest is affirmed to be public health importance due to clinical effects including skin reactions and psychological distress on human daily lives (Ashcroft et al. 2015). Although bed bugs are less likely to become vectors of human disease but they are considered to be significant treated pest due to their blood-sucking behavior (Haynes et al. 2010).

1.2 Statement of Problem

Recent reports showed most pest control industries found it difficult to eliminate bed bug populations unless minimum infestation level was detected in an area. Long-term treatment of various types of insecticides including chemical or non-chemical active ingredients was essential with about three to four times application for complete eradication. Hotels, dormitories, apartments, homeless shelters and residential houses were often heavily infested with these opportunist insects (Hwang et al. 2005, Quarles 2007). Unaware immigrants and travelers were the quick access for the bugs to crawl alongside their belongings and initiate a new infestation in much favorable place (Kaufmann et al. 2006, Haynes et al. 2010, Davies et al. 2012, Doggett et al. 2012). The fastest and easiest solution for the pest control company in handling an established infestation was exposing them to insecticides (Moore and Miller 2006).

In Thailand, excessive residual spray of pyrethroid insecticides on mattresses in hotels had caused resistance in bed bugs (Tawatsin et al. 2011). However, even though the whole places in a house have been filled with pesticides, traces of bed bugs still can be detected since their flattened body-shaped helped them to flee in tiny corners such as inside electrical appliances or even cracks on the ceiling. Resistance in the bugs itself also become one of the major causes to this problem. Dichloro-diphenyl-trichloroethane (DDT) was popular in usage back then before 20th century in controlling insect vectors to non-insect vectors like mosquitoes, cockroaches and bed bugs (Quarles 2007). Bed bugs showed resistance towards pyrethroid compared to other major classes of insecticides and combined pesticides with insect growth regulator (IGR) (Busvine 1958, Zhu et al. 2013). Still, there are no detailed researches regarding insecticide resistance in tropical bed bug populations obtained across Peninsular Malaysia. Therefore, this study is aimed to find out whether tropical bed bug populations in Malaysia have developed resistance against insecticides and finding an alternative solution via application of entomopathogenic fungus to control them.

1.3 Significance of Study

This research is conducted to study bed bug infestations in selected areas of Malaysia. Resurgence in other foreign countries causes a need of this research to be carried out to investigate infestation levels of bed bug especially in states of Malaysia. It is also to find out the possible factors of the resurgence of bed bugs that previously reported that migration of people around the world caused wider distribution. Other factors include insecticide resistance which will be tested on the bugs by exposing them to certain classes of insecticides (Busvine 1958). Common bed bugs are known to be resistant to pyrethroid groups, however tropical bed bugs may have different metabolism in their body due to climatic change and environmental condition in tropic countries (Tawatsin 2011). Despite of using chemical control, biological control is a component of integrated pest management (IPM) to control this bed bug population. In this study, fungi, *Aspergillus tubingensis* and *Trichoderma harzianum* are chosen as biological agent against bed bugs.

1.4 Limitations of Study

The survey was restricted within the states of Peninsular Malaysia; Pulau Pinang, Perlis, Kedah, Perak, Kelantan, Terengganu, Pahang, Selangor, Negeri Sembilan, Melaka, Johor and main federal territory, Kuala Lumpur. The study was mainly focused on urban areas, since no signs of infestation were observed in rural areas. Major sites targeted were residential houses, flats and apartments, dormitories for workers, hostels in schools and public transportations like airports and bus stations. Collection of bed bug samples were limited to visual inspection in particular harborage sites. Thus, the total number of samples was differed for each location. Since the samples collection differed according to their locations, several strains were chosen for laboratory testing while the remaining were colonized at room condition. Adult bed bugs were used in both insecticide resistance bioassay and fungi testing due to insufficient number of bed bug at nymph stages.

1.5 Objectives

The objectives in this study have been divided into three parts;

Objective 1 (Chapter 3): Survey on infestation levels of tropical bed bug *(Cimex hemipterus)* F. in Peninsular Malaysia.

1. To investigate the infestation levels of bed bugs across Peninsular Malaysia within the target premises.
2. To determine the harborage preferences of bed bugs within each harborage site.

Objective 2 (Chapter 4): Insecticide resistance bioassay in tropical bed bug *(Cimex hemipterus)* F. using two major classes of insecticide.

1. To evaluate insecticide resistance level in tropical bed bugs by using impregnated papers of two major classes of insecticide group; pyrethroid and carbamate.
2. To compare difference in the efficacy between two insecticide groups on ten populations of bed bugs.

Objective 3 (Chapter 5): Exposure of entomopathogenic fungi towards tropical bed bug *(Cimex hemipterus)* F.

1. To investigate the effectiveness of fungi as a biological control agent on bed bugs.
2. To evaluate virulence of two fungal species, *Aspergillus* and *Trichoderma* sp. in killing bed bugs.

CHAPTER TWO

LITERATURE REVIEW

2.1 Bed bugs Origin and Biology

Bed bugs are worldwide pests with various names such as *bugge* in Middle English, *buks* in Middle East region while Swedish called *Cimex*, which is also the largest pest control company in Sweden (Quarles 2007). Originally, bed bugs are from the order of Hemiptera due to their features with small hemelytral pads based on Miller (2008), and later is classified under Cimicidae family. They are considered as pest since their blood-feeding activities causes discomfort to humans that may end with skin reactions which in turn becoming serious cases, involving lawsuit against a hotel and airplane company (Doggett et al. 2012). Over 74 species of cimicids were identifiable such as bed bugs, bat bugs, chicken bugs, swallow bugs, and pigeon bugs (Miller 2013).

There are three identified species which are closely related to human, common bed bug *(Cimex lectularius)* Linnaeus, tropical bed bug *(Cimex hemipterus)* Fabricius and bat bugs (*Leptocimex boueti)* (Khan and Rahman 2012, Tawatsin et al. 2013). These bugs initially made human as their primary blood source except *Leptocimex boueti*, bat bug which are dominantly found in West Africa depend on bat's blood to survive but they may also feed on humans as an alternative nutrient supply (Benoit 2011). While most of common bed bugs were widely distributed in United States and some parts of other country such as Thailand, specifically in Chiangmai whereas *C. hemipterus* prefer to live in tropical habitats especially in Southeast Asia regions including Singapore, Vietnam, Indonesia, Taiwan, Malaysia (Koehler et al. 1993, Miller 2008).

Bed bugs undergo incomplete metamorphosis which includes three stages: egg, nymph and adult. Its life cycle begins with a unique copulation between male and female, called as traumatic insemination (Kamimura et al. 2014). Interestingly, the male will puncture the body wall of female and insert his genitalia to inject sperms into the spermalege that causes wound. The process lasts for a few minutes up to half an hour in some cases. After mating, female is able to produce eggs within 3 days with 1-12 eggs per day (Khan and Rahman 2012). Mating bed bugs have shorter lifespan compared to the virgins.

The eggs are usually laid individually on rough surfaces or in crack and crevices. A sticky substance or a glue-like substance held the eggs either singly or in cluster forms to the surface (Khan and Rahman 2012, Doggett 2013). Within 10 days, the eggs started to hatch and the emerged nymph are able to feed right away. Nymphs have five instars or molting stages. After the long nymphal stage, bed bug reaches its maturity (Plate 2.1). In order to develop and molt to the next stage, blood meal is required in each stage. It takes about 4-5 weeks for the developmental stages from egg to adult under optimal conditions (Reinhardt and Siva-Jothy 2007).

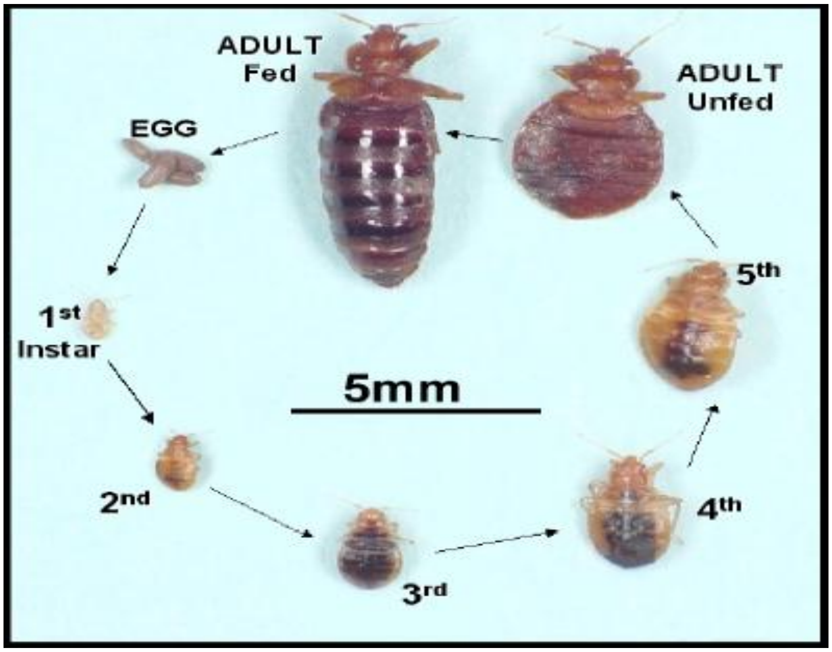

Plate 2.1. Life cycle of bed bugs (Doggett 2006).

Adult bed bugs are easier to spot due to their reddish-brown colour with flat, oval appearance when unfed but elongated as they are fully engorged (Cohen 2010). Their flat body structure allow them to hide in thin and narrow places like cracks inside wooden furniture, floors and even in electrical wiring on the walls aside from the bedding perimeter (Reinhardt and Siva-Jothy 2007, Miller 2008). The body length is broad, typically 5-8 mm and width about 4 mm. The four-segmented antennae projects forward on the head while other features include the extended labrum and rostrum. Rostrum consists of 3-segmented labium, mandibular and maxillary stylets and salivary canals on the head part. It has reduced wings form which exposed its 11 dorsal abdominal segmented body (Miller 2008). The abdomen may expand or grow larger as it has intersegmental membranes within the second and fifth abdominal segments. Adults also bears 3-segmented tarsus (How and Lee 2010b).

2.2 Identification and Behavior of Bed Bugs

Distinguishing sexes between male and female bed bugs can be done by observing size and shape the tip of their abdomen. Male bed bug has abdomen with much narrower and pointed asymmetrical shape which bears genitalia on the ventral side near the tip (How and Lee 2010a). Meanwhile, in female bed bug, the abdomen is broader and has no open reproductive system but has a notch-like spermalege which is located on the left ventral side (Thomas et al. 2004). The immature stages of bed bugs (nymph) are almost similar to the adults except the size is much smaller. The other comparative features include the nymph has a translucent white colour, abdominal segments lack of sclerotize and hemelytral pads, 2-segmented tarsus and reproductive organ is not well-developed (Miller 2008).

Minor comparison between common and tropical bed bugs requires experts and entomologist to specify the details using light microscope to observe the bed bug structure (Usinger 1966). *C. lectularius* species had more rounded body appearance with expanded pronotum and hairy on the sides of the pronotum (Figure 2.1). In contrast, *C. hemipterus* species had also rounded body-shaped but slightly smaller and darker than common bed bugs. Its prothorax is less excavated and the hairs on each side are distanced apart and fewer (Khan and Rahman 2012). Although they shared most of their morphological characteristics, other method also can be used to differentiate between them aside from requirements of experts and that is via phylogenetic analysis (Tawatsin et al. 2013). Usage of molecular techniques such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) can easily compare the species in a short amount of time (Hemingway and Ranson 2000).

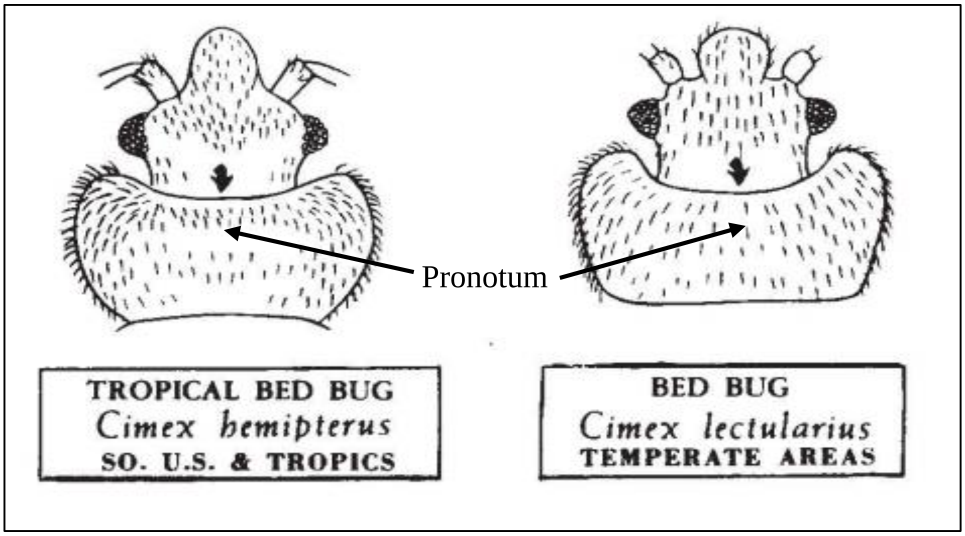

Figure 2.1 Difference between tropical bed bugs and common bed bugs. Retrieved from "Bugs: Pictorial Key to Some Species That May Bite Man", Pratt and Stojanovich (1967).

Geographical range may help but identification of the insect must be done before any control actions can be taken. Foreign countries like Australia, United States, Denmark, Nigeria, Iran and Israel has been seriously infested with *C. lectularius*, causing disturbance to residents which allowed lawsuit action to be filed to the hotels that having bed bugs (Doggett et al. 2012). Unlike tropical bed bugs in Southeast Asia, reports regarding their infestation are lesser compared to the foreign countries (Potter et al. 2008, Mumcuoglu et al. 2010, Seidel and Reinhardt, 2013).

Bed bugs are often seen clumping and aggregating together in one place, regardless of their nymphal stages. During this aggregation, multiple pheromones were found by these insects as a form of communication, defense mechanism aside from finding suitable harborages in their colony which is similar in both species of bed bugs (Siljander et al. 2008). E-(2)-hexenal and E-(2)-octenal were alarm pheromones detected the most in late instar nymphs than in adult stage (Liedtke et al. 2011, Harraca et al. 2010), protecting them from being sexually assaulted by males. Meanwhile, secretion of odor consisting of airborne aggregation pheromones had a positive relationship with the alarm pheromones which function as an attractant in all stages of bed bug except in mated females. Injuries in gravid females due to the traumatic insemination cause them to negatively respond towards the pheromones in the aggregation. Therefore, mated females are classified under a category of non-aggregated individuals since they would avoid repeated mating, making them to disperse even farther from the colony (Liedtke et al. 2011).

Researches on tolerance level of *C. lectularius* have been further studied including regulation of heat shock proteins (Hsps), dehydration, and extreme high and low temperatures (Benoit et al. 2009). Survival of the insect drop gradually when they are exposed below freezing point approximately at -20°C whereas temperature above 44°C may kill more than half of the population depending on their tolerance level. They are also able to adapt very well in the environment with low water balance especially generations of the first newborn in about 50-70% relative humidity (RH) of a human dwelling. Their longevity against this cold and high temperature is mainly controlled by the expression of Hsps in maintaining water balance in their body. Issues of dehydration are interconnected with the release of pheromones in aggregation of juveniles but somehow can be reduced by responding towards thigmotatic stimuli which causes stresses on other individuals so that larger aggregations can be made (Benoit 2011).

2.3 Resurgence and Survey of Bed Bugs Infestation

Alarming rates of bed bugs cases have been detected in many developed countries. Although laborious studies resulted in disease transmission among bed bug colonies, this, however, were not established in the field (Doggett and Russell 2008, Williams and Willis

2012). Skin reactions due to their bites may be varied depending on the sensitivity of each individual. From the small papules, wheals, and bullous bite reactions to severe anemia, making bed bug is considered as medical importance pest as well (Leverkus et al. 2006, de Shazo et al. 2012, Paulke-Korinek et al. 2012, Quach and Zaenglein 2014, Abd Hafis et al. 2016). Mental health symptoms like psychological distress, insomnia and anxiety including post-traumatic disorders were rising in most developed countries based on the recent reported cases (Heukelbach and Hengge 2009, Goddard and de Shazo 2012, Ashcroft et al. 2015).

Bed bugs have been linked to human beings since prehistoric ages after they inhabited on bats previously (Usinger 1966, Potter 2011). Poor sanitation and migration of residents during the World War II worsened bed bugs cases around the world (Boase 2001, Hwang et al. 2005). Since then, the pest was able to be controlled then and the population dropped until their reemergence due to improper usage of insecticides and international travelling activities in the 1990s (Boase 2001). Reported infestations by *C. lectularius* were seen mostly in foreign countries including United States, Australia, and several European countries like France and Italy (Bencheton et al. 2011, Masetti and Fabrizio 2007, Hwang et al. 2005, Doggett et al. 2004). Cases of infestation were received from the hospitals, office buildings, homeless shelter, and short-stay lodges (Baumblatt et al. 2014, Williams 2013, Hwang et al. 2005, Ryan et al. 2004).

Continuous studies and surveys on bed bugs were also conducted in other countries reported their sudden resurgence at its highest peak. This includes survey in homes and hostels in Nigeria by Emmanuel et al. (2014), which they concluded that poor hygienic practices and lack of knowledge in controlling bed bugs were some of the factors to cause resurgence. The pest resurgence was also reported by a survey conducted in residential area of Bahnamir, Iran (Haghi et al. 2014). In Pennsylvania, Wu et al. (2014) investigated row homes in Philadelphia to know the pattern of infestations of common bed bugs. Surveys on infested premises in public accommodations like hotels and worker quarters mostly found tropical bed bug, *C. hemipterus*, in the tropics and subtropics suggested that migrated workers brought the pest to reinvade other places as well (Wang et al. 2013, 2015, Wang and Wen, 2011, How and Lee 2010a, Suwannayod et al. 2010).

Medium or instrument normally used on bed bug surveys are visual inspection, intercepting devices as baits to detect presence of the pest, questionnaires and interviews with residents in the study area. For example, Wang et al. (2010) monitored bed bugs dispersal in apartment building in Indianapolis via interceptors and inspected manually for any signs of infestations in the perimeter of the building and among other apartments. Interviews in Germany showed that 60% of the respondents were able to identify the insect while the remaining knew the pest from other sources like families, friends, and educational lessons in schools or colleges (Seidel and Reinhardt 2013). According to Ralph et al. (2013), telephones survey in New York City concluded that bed bugs scattering is also associated with the poverty in the neighborhoods and areas with multiple units of houses as there were reports of infestations about seven percent of the adult bug in 2009.

2.4 History of Bed Bugs Control

Various hypotheses have been ascribed to bed bugs resurgence including worldwide travelling activities by locals and foreigners, used of second-hand furniture, poor knowledge among the professionals in handling them and development of insecticide resistance in the pest themselves. Pesticides such as dichlorodiphenyltrichloroethane (DDT) were once believed to cause a reduction in the pest number until their usage were banned in most countries as they were harmful to the environment and also soil degradation in crop yield (Davies et al. 2012). Following up to this matter, a study by Busvine (1958) discovered resistance in bed bugs by testing them using impregnated papers with insecticides especially DDT and organochlorine classes. Synthetic pyrethroid resistance then were brought up in later years which demonstrate field-collected strains in United Kingdom were highly resistant towards deltamethrin and lambda-cyhalothrin (Romero et al. 2007).

Inconsistent usage of vary chemical insecticides were revealed in latest reports due to low mortality rate in the pest population. For instance, laboratory strain of *C. lectularius* displayed high resistant in chlorfenapyr-based insecticide in comparison to pyrethroid products according to Moore and Miller (2006). Other experimental design using mixtures of different active ingredients with varying mode of action showed similar outcomes as bed bug are able to modify their defense mechanism while adapting to the exposure of pesticides. Zhu et al. (2013) found that kdr mutation and multiple resistance existed in field strain bed bugs after treating them with Temprid® product consisting of beta-cyfluthrin and imidacloprid, resulting in low percentage of mortality. Although laboratory strain showed effectiveness of the product in controlling bed bug populations especially in adults (Abdul Hafiz and Zulaikha 2015a), but their systemic activities may be varied in the field condition causing defensive mechanism to develop. Hence, professionals should examine the mode of action of active ingredients in insecticide thoroughly to prevent more resistant strain in bed bugs.

Due to high resistance level in pyrethroid and organophosphate insecticides, carbamate pesticide such as propoxur (2-Isopropoxyphenyl N-methylcarbamate) has been proposed in controlling bed bug populations in 1980s (Berg 2010). Propoxur has been applied in agricultural field mostly to control pests like ants, flies, snails and mosquitoes that may destroy flowering plants and trees. This chemical inhibits production of enzyme cholinesterase in the nervous system. It gives a rapid-knockdown effect to the insects as the pesticide paralyzes the nervous system, forcing them to die immediately (Metcalf 1984). Studies have shown that time interval for the systemic action and higher lethal dose prove the efficacy of the use of propoxur in eliminating bed bugs (Miller 2013). Nevertheless, the usage of propoxur is withdrawn by the authorities due to its high toxicity both to insects and humans (Busvine 1958, Berg 2010). In addition, the residual effect in the environment after the first application lasted for several weeks.

Natural products such as diatomaceous earth and other non-chemical ingredients are greatly commercialized in order to curb bed bug resistance issues. However, their effectiveness may take up to six months or even longer depending on how they are applied on the surfaces. These insecticides are available in the form of spray or aerosol, liquid, dust or formulated with insect growth regulator (IGR) (Drisdelle 2013). Powdered diatomaceous earth can only be applied to unreachable spots with no air movement. In comparison to integrated chemical pesticides, the natural producing insecticides, mortality rate of bed bugs is only 50%, thus are ineffective in controlling bed bugs especially for a long period of time (Goddard 2014).

In contrast of chemical pesticides, non-chemical treatments are widely practiced to avoid presence of bed bugs in residential areas. This involves vacuuming, steaming, freezing, trapping and encasements of furniture (Meek 2003, Kells 2006). Integrated pest management (IPM) practices using diatomaceous earth interceptors to trap bed bugs produced a better reduction when compared to the use of chlorfenapyr residual spray (Wang et al. 2009, Akhtar and Isman 2013). Thermal control of bed bugs however, did not killed large population of bed bugs although mattress were encased in black plastic bag and laid under the sun (Doggett et al. 2006). Results indicated that extreme temperature forced them to flee to cooler areas around the mattress and survive. Still, encasing mattresses can be carried out as a precautionary step to prevent small insect infestation. Nonetheless, heavy infestation require in depth study in controlling bed bugs using appropriate treatment methods giving benefit not only to human but environment as well.

2.5 Insecticide Resistance in Insects

A common nature in insects is to excrete or enhance their defense mechanisms once they have been threatened. For instance, some may develop their metabolic activity, modified their gene structure and gene mutation that resulted in resistance in them (Hemingway and Ranson 2000). Survival of insects after the exposure of certain concentration of insecticide that normally kills a population is called resistance (Miller 2013). Studies have shown that bed bugs slowly enhance their resistance level against chemical insecticides, mostly against pyrethroid class (Busvine 1958, Drisdelle 2013). Due to bed bugs massive reduction, insecticide products for bed bugs have declined and contain similar active ingredients (Moore and Miller 2006). This causes management and control of the insect to be delayed and infestation cases on the rise.

Target-site mechanism is defined as changes in sequences of amino acids which affected the binding activity of insecticide to their target-site (Brogdon and McAllister 1998). These alterations will inhibit the insecticide from binding to its target site which causes such products to leave no impact against the pest. Basically, the four major groups of insecticide, namely organophosphate, organochloride, carbamate, and pyrethroid are believed in causing systemic failures in insect vectors. Each class of insecticide has their own target-site to disrupt the nerve system in an individual in order to reduce the pest population within a shorter period (Mamidala et al. 2011). For instance, organophosphate and carbamate actively attacked its main target site, acetylcholinesterase in the synapse. The insecticide will block the binding site by inhibiting acetylcholinesterase, that prevent the enzyme to be pulled from the receptor, thus allowing unlimited sending of impulses to the system (Hemingway and Ranson 2000). As a result, the insect will suffer from paralysis and die eventually.

To this date, insect vectors such as mosquitoes, sandlflies, biting midges and body lice have developed their insensitivity to insecticides, making them difficult to be eradicated. Upgrading of mechanism and adjusting to new surroundings with maximum dose of insecticides have failed in reducing the pest populations. Excessive exposure to DDT in curbing pest population back then leaves adverse effects on humans and nature. Mosquitoes were the first insect to develop resistance to DDT which showed negative effect on other insecticides during high peak malaria cases (Moreno et al. 2008). As a result, DDT had been banned and as a substitute, artificial insecticides are continuously produced in most part of the world (Onstad 2008). However, poor knowledge among pest control companies in both mode of action of the insecticides and their target-site mechanisms increased the resistance of the pest. According to Hemingway and Ranson (2000), enzyme-based resistance, gamma-aminobutyric acid (GABA) receptors and sodium channels were responsible in preventing resistance develop in the insect.

While there are many resistance studies have been carried on vectors of diseases, few research are developed on urban insect including tropical bed bug where knockdown resistance (kdr) mutations have recently been found in them, (Dang et al. 2015). The kdr mutation in insect is commonly associated with pyrethroid class insecticide that attack voltage-gated sodium channel (VGSC) in nervous system. Alteration of sequences of binding site in sodium channel counteracted the mode of action of pyrethroid and DDT, resulting in insensitivity to both classes of insecticides (Soderlund and Knipple 2003). The mutations have been proven in studies of several hematophagous insect like mosquitoes, *Anopheles gambiae, Culex pipiens,* cat flea, *Ctenocephalides felis* and *C. hemipterus* (Bass et al. 2004, Dang et al. 2015, DeVries et al. 2015).

In terms of species comparison, *C. hemipterus* is more susceptible than *C. lectularius,* but still some of them have higher tolerance level towards insecticides which can be passed on to their progeny (Busvine 1958). Pest control companies in Southeast Asia relied mostly on chemical and spray method in their treatments (Wang et al. 2011, Abdul Hafiz and Zulaikha 2015b). However, due to resistant factors, the companies find it difficult to reduce the pest population even though there are many formulations of insecticides can be found recently in the market. Pyrethroid class which has been used widely to kill bed bugs and other insect vectors, eventually leave no effect to them (Wood et al. 2010, Miller 2013). In later years, bed bugs may also develop their resistance to other classes of insecticides with similar active ingredients if there is no alternative measure taken to avoid the circumstances (Moore and Miller 2006).

There are findings related to chemical and non-chemical method that resulted in effective control of bed bugs. These include application of diatomaceous earth and spraying of insecticides such as chlorfenapyr, propoxur and other classes as well (Wang et al. 2009, Manuel 2010). Bed bugs, unlike termites do not have grooming activity to affect other members in a population but horizontal transfer can occur through contact and ingestion of insecticide (Akhtar and Isman 2013). Simultaneous practices of the two control methods can eliminate resistant populations effectively compared to implementation of single method, either spraying or dust application only (Wang et al. 2009, Akhtar and Isman 2013).

2.6 Treatment Methods Using Biological Control

Mosquitoes, flies, biting midges, cockroaches and termites are common with various types of control methods including biological control. Biological control is one of the approaches in Integrated Pest Management (IPM) program that stresses on the use of natural enemies in the environment to suppress pest population. For example, biological agents like predators, pathogens, microorganisms and parasitoid work effectively against their prey although the process is time consuming. The method also can overcome resistant populations regardless of any stages of the insect.

Entomopathogenic fungi are very popular in most studies in relation to biological control (Nicolas et al. 2009, Ansari et al. 2011). The topmost fungi tested are *Metarhizium anisopliae* and *Beauvaria bassiana,* usually in spraying forms as it is more practical to use in the field. Traps containing fungus also managed to kill the insect pest while another method used fungus as contact or stomach poison (Nicolas et al. 2009). The infected insect might result in the collapse of the whole colony depending on the virulence of pathogen as in *M. anisopliae* (Ansari et al. 2011).

Biological control is most successful in most insect vectors to prevent transmission of diseases but not in bed bugs populations. Since bed bugs do not carry disease up to this date (Haynes et al. 2010), approaches using biological agents are yet to be discovered. Treatments normally involve application of insecticides and mechanical control such as soaking infested items in hot water, laying mattresses in the sun or even self-treatment methods (Hwang et al. 2005, Seidel and Reinhardt 2013). The treatments however, are less effective due to some individuals that escaped to begin a new infestation (Wang et al. 2009). It can be concluded that studies using biological agents to eradicate bed bug populations should be explored widely to balance chemical and non-chemical methods.

2.7 Introduction of Fungus as Biocontrol Agent

Application of entomopathogenic fungi lately has become an alternative source to chemical insecticides in the laboratory as well as in the outdoor field. To be concise, entomopathogenic fungi are fungi that are capable of producing toxins which kills an insect host once they are infected (Singkaravanit et al. 2010). There are several divisions or phyla of entomopathogenic fungi. Most researchers found that infection from Phylum

Ascomycota had convincingly shown positive effect against pest host. Commercialized insect pathogens such as *Beauvaria bassiana* and *Metarhizium anisopliae* are well-known biological control agents for wide variety of insect pest including termites, mosquitoes, biting midges, houseflies, and bed bugs (Hafiza et al. 2014). Other divisions are also regarding their biological and toxicity effect on insect or plants but unstable efficacy results reflect more in-depth research should be carried out so that in future, the mycoinsecticide would be able to possibly replace the existing resistant pesticides.

Typical mold species attacking food storage of humans and animal livestock, *Aspergillus* sp. also from Phylum Ascomycota (Hafiza et al. 2014). *Aspergillus* colonies are fast-growing conidiophores, mainly composed of vesicle, phialides and conidia making the conidial head. They tend to contaminate 50% of the crop plantation worldwide whilst in tropical regions around 80% probably due to wet and dry climatic pattern(Abdin et al. 2010). Besides from being insect pathogens, majority of the species found in yield crops and plantation also causes infection especially in starchy food when proper sanitation are less practiced. These molds are actually present in an aerobic environment, areas with high oxygen content and members of this species are able to proliferate rapidly in high concentration of salt and sugar compared to others. Some species are used in breaking down fat soluble and sugar molecules in food making process as fermentation products like *A. oryzae* and *A. niger*. On the other hand, they also produce toxins which may become carcinogenic to humans, animals and insects, leading to deadly effects (Sheppard 2011).

Another category of mold species, *Trichoderma* sp. is considered to be in divisions of Ascomycota, natural fungi in almost all soils and in a symbiotic relationship with the plant roots. Since 1930s, they are known to be control agents to plant diseases due to their ability in parasitizing other fungal species that infected plant host, also termed as mycoparasitism (Weindling 1932). In fact, they are also able to survive in unfavorable conditions. Colony members are culturable in most soil and organic matters ranging with temperature about 25-30°C for their rapid growth activity (Howell 2003). *Trichoderma* sp. differ in several measurements of the colony including colony appearance, colours, growth rate, conidial and phialides morphology based on study by Shah et al. (2012). For instance, *T. harzianum* on potato dextrose agar (PDA) media, the colony appeared to be dark green-coloured conidia in dense form of concentric rings towards the margins of the plate. Green conidia can be greatly produced with one-celled in the center of inoculum with less dense of mycelial.

Meanwhile, *T. harzianum* on malt extract agar (MEA) showed white mycelium growth with green conidia scattered around two concentric rings but become denser as they approach to the rings. Apart from being saprophytic fungi to the trees, this mold species produce secondary metabolites that can be used as one of the biological control agents of plant roots. This was confirmed in the study conducted by Shakeri and Foster (2007) where they found that entomopathogenic fungi of *T. harzianum* produced chitinase and antibiotic activity which help in controlling larvae of mealworm beetle. Nevertheless, reports also stated that some *Trichoderma* sp. was able to induce resistance on several plants resulting in detrimental effects like water and salt deficiency (Howell 2003). Therefore, findings related to their roles as control agents on other plant pathogens and insect should be viewed in a wide range of aspects so that their benefits can be derived and applied in the future.

2.7.1 Mechanism of Entomopathogenic Fungi

It is essential to know and understand the activity of conidia (Plate 2.2) in the insect host before being introduced to the world as a biological control agent. This pathway will ensure that the fungal strains are potent enough in reducing insect vectors for a long term and allow improvements to be made. In general, entomopathogenic fungi attack the insect host via direct contact or penetration of the fungal strains to the cuticle regardless of specific body parts. Once the conidia safely attached to the host cuticle, it will proliferate on the surface while releasing enzymatic activities for further growth rate of the colony. A modified structure, called hyphae, used to penetrate through the integument layers of the insect resulting in systemic failure (Ortiz-Urquiza and Keyhani 2013). The success of the fungal growth is dependent on temperature and moisture content. The mechanisms are also accompanied by the production of carcinogenic toxins, metabolites, chitinase, protease and antibiotic activity which are the useful 'weapons' in penetrating the immune system in insect.

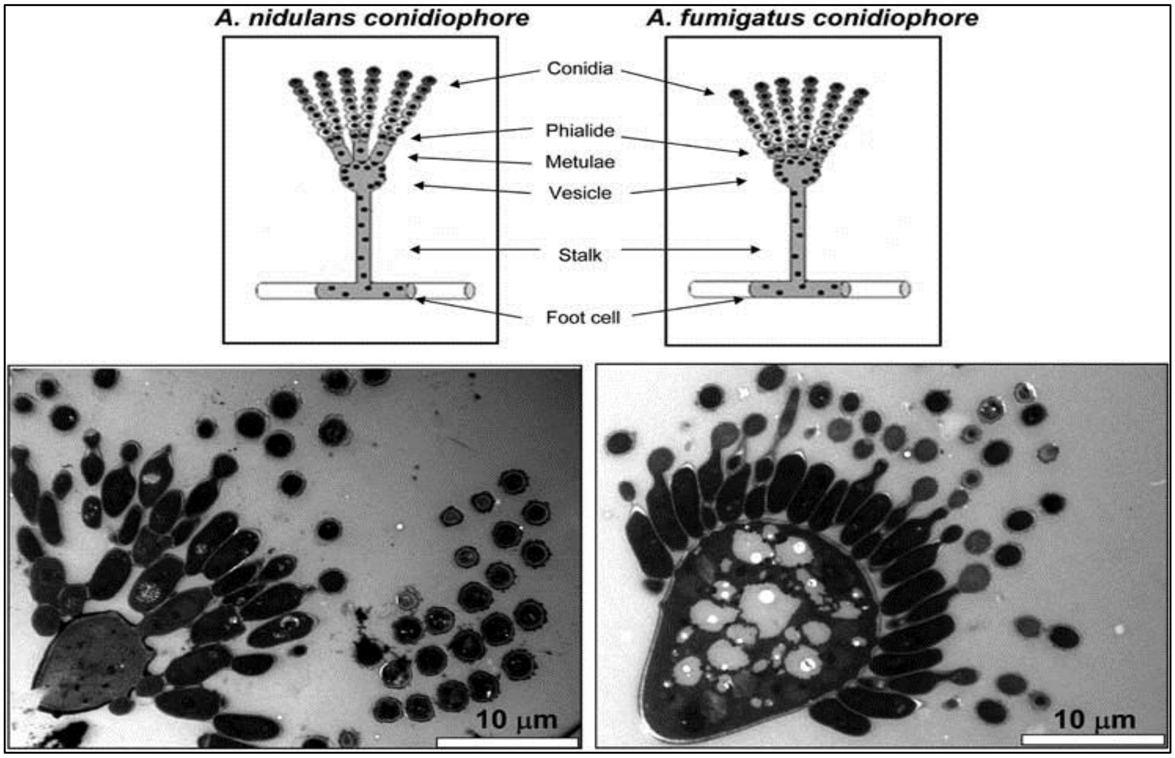

Plate 2.2. Morphology of conidiophores of *Aspergillus nidulans* and *Aspergillus fumigatus* by Yu, Departments of Bacteriology and Genetics, University of Wisconsin-Madison, Madison, WI 53706, USA. 2010. Retrieved from Regulation of Development in *Aspergillus nidulans* and *Aspergillus fumigatus,* Mycobiology 38(4): 229-237.

Mycotoxin in *Aspergillus* sp. for example, is a secondary metabolite produced by these filamentous fungi often attack food storage of animal livestock (Abdin et al. 2010). Each species produces various types of mycotoxin such as aflatoxin, ochratoxins, zeralenone, trichothecenes, gliotoxin and fumonisins, that may also affect public health. Aflatoxin is known to be the most carcinogenic mycotoxin produced by *A. nomius* (Plate 2.3) and *A. parasiticus* species commonly found in agricultural orchard (Feibelman et al. 1998). Aflatoxin B1 is the most carcinogenic among the other three major groups of aflatoxins (Klich 2007). Moreover, these fungi are considered to be opportunistic pathogens and they affect human health by causing aspergillosis and aspergillus-related