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Molecular detection and screening of *Wolbachia* infections in tropical bed bugs *Cimex hemipterus* (Hemiptera: Cimicidae) from peninsular Malaysia populations

Nur Hassanah Mohd Hassan[1,2], Dinie Eyldira Ismail[1,3], Siti Nor Ain Seri Masran[1,4] and Abdul Hafiz Ab Majid[1*]

[1]Household and Structural Urban Entomology Laboratory, Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, 11800, Gelugor, Malaysia.
[2]Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia,11800, Gelugor, Malaysia.
[3]School of Biological Sciences, Universiti Sains Malaysia, 11800 Gelugor, Malaysia.
[4]Department of Environmental Health, Faculty of Health Sciences, Universiti Teknologi MARA, 94300, Kota Samarahan, Sarawak, Malaysia.
Email: abdhafiz@usm.my

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ABSTRACT

Aims: *Wolbachia* is an endosymbiont and a gram-negative genus bacterium which has received the spotlight in the field of research studies due to its multiple capabilities to affect it hosts, including the bed bugs (Hemiptera: Cimcidae). While most investigations concentrated on the common bed bugs (*Cimex lectularius*), no published studies have yet to be done on molecular screenings of *Wolbachi a*ssociated with tropical bed bugs (*C. hemipterus*). The present study was undertaken to screen Wolbachia infection from tropical bed bugs from Peninsular Malaysia.
Methodology and results: We attempted to screen and characterize *Wolbachia* infections in tropical bed bugs from 22 different localities throughout Peninsular Malaysia using a molecular approach; multiple Polymerase Chain Reaction (PCR) assays with four sets of primer sequences.
Conclusion, significance and impact of study: Our findings yielded negative results of *Wolbachia*infectionsand, therefore,further confirmed that all bed bug samples from all localities in Peninsular Malaysia are free from *Wolbachia* infections. Our findings also suggested that the prevalence of *Wolbachia*in tropical bed bug populationsin Peninsular Malaysia is very unlikely.

Keywords: Tropical bed bugs, *C. hemipterus*, PCR, *Wolbachia*, molecular detection

INTRODUCTION

The emergence of bed bug infestation in buildings and residential areas has caused a public nuisance worldwide. Two most common species of these blood-sucking parasites are *Cimex lectularius* (common bed bug) notably lives in the temperate zone and *C. hemipterus* (tropical bed bugs) that can be found in the tropical zone (Seri Masran and Ab Majid, 2017). These notorious ecto parasites are members in order Hemiptera that feed exclusively on the bloods of humans, birds and bats (Delauney *et al*., 2011). Although the bite of these miniscule creatures may cause skin irritations (Seri Masran and Ab Majid, 2017), their attacks, however, have not been associated with a disease transmission (Montes *et al*., 2002). Blow *et al*. (2001) reported that bed bugs are likely to involve in mechanical transmission of Hepatitis B virus despite Jupp *et al*. (1983) hypothetically stated that the spread of Hepatitis B may occur due to Hepatitis B-contaminating bed bugs or infected faeces. The spread of bed bugs usually comes from international travelers, public places and even residential areas (Ab Majid and Kee, 2015). Despite all the above-mentioned facts, there is no case report stated that bed bugs acted as a vector of pathogens to humans (Meri weather *et al*., 2013).

In animal-microbial interactions, bed bugs are frequently associated with *Wolbachia*, a cytoplasmic intracellular symbiotic and maternally-inherited rickettsia-like bacterium that is widely known to infect tremendous species of arthropods and nematodes (Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000; Hilgenboeker *et al*., 2008; Choi *et al.,* 2015; Akhoundi *et al.,* 2016). The parasitic *Wolbachia* selfishly initiates adverse effects to its hosts by influencing the reproductive alternation through cytoplasmic incompatibility, male killing, parthenogenesis, speciation and feminization (O'Neil *et al.*, 1992; 1997; Weeks and Breeuwer, 2001; Hilgenboeker *et al.*, 2008; Werren*et al*., 2008; Salunkhe*et al*., 2014). Owing to these abilities, *Wolbachia* has been promoted as a promising candidate to control and end the spread of viral and

***Corresponding author**

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parasitic pathogens of various mosquito-transmitted illnesses such as dengue fever and malaria (Shaw *et al*., 2016). Of further note, observations on a wide range of insect hosts have demonstrated that they can be infected by a single or multiple strain of *Wolbachia* (Kikushi and Fukatsu, 2002; Raychoudhury*et al*., 2009). For instance, a previous study by Choi *et al*. (2015) found that three species of butterflies were infected by different strains of *Wolbachia* in sequence typing.

The screening of *Wolbachia* has been done mostly in bed bugs of temperate zone, *C. lectularius*, although the presence of *Wolbachia* in bed bugs of tropical zone, *C. hemipterus* is possible. Hence, the purpose of this study was to screen *Wolbachia* infections in tropical bed bugs, *C. hemipterus* throughout Peninsular Malaysia by applying molecular techniques. A few modifications on PCR programs and primers were made to detect the presence of *Wolbachia* in bed bug samples. Results of this study were reported at the end of the paper.

MATERIALS AND METHODS

Bed Bug Samples

Samples of the tropical bedbug (*C. hemipterus*) collected during 2013 to 2015 from 22 localities throughout Peninsular Malaysia were obtained from Household and Structural Urban Entomology Laboratory, School of Biological Sciences, UniversitiSains Malaysia (Figure 1). Five individuals per localities were selected. The samples were labelled (Table 1) and stored in RNA later reagent in -20ºC to preserve the DNA sample from degraded (Seri Masran and Ab Majid, 2017).

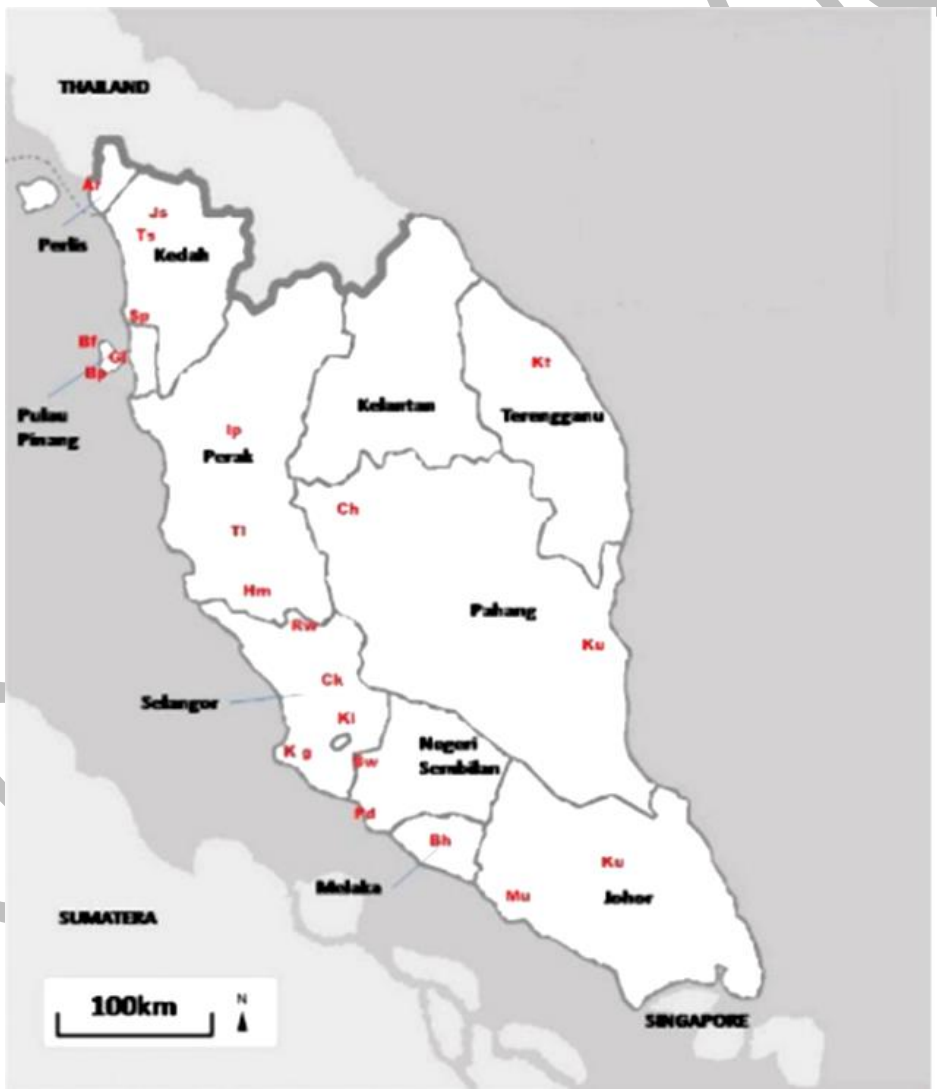

Figure1: Sampling locations throughout Peninsular Malaysia (Seri Masran and Ab Majid, 2017).

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Table 1: Collection sites, isolate codes, groups, and accession numbers of each bed bug in this study (Seri Masran and Ab Majid, 2017).

No.	Isolate code	Collection site	State	Collection date	Isolation source	Lat-Lon
1	KL	KLIA	Selangor	25 Feb. 2014	Lounge seat	2.75 N 101.70 E
2	KT	Kuala Terengganu	Terengganu	20 Aug. 2014	Apartment room (bedding)	5.32 N 103.15 E
3	IP	Ipoh	Perak	17 Oct. 2014	Worker's dormitory (bedding)	4.59 N 101.09 E
4	SP	Sungai Petani	Kedah	26 Sept. 2014	Worker's dormitory (bedding)	5.61 N 100.53 E
5	JS	Jalan Sekerat, Alor Setar	Kedah	25 Sept. 2014	Worker's dormitory (bedding)	6.12 N 100.36 E
6	TS	Taman Saga, Alor Setar	Kedah	25 Sept. 2014	Residential room (bedding)	6.09 N 100.38 E
7	KG	Klang	Selangor	25 Nov. 2014	Residential room (bedding)	3.03 N 101.44 E
8	TI	Teluk Intan	Perak	18 Oct. 2014	Worker's dormitory (bedding)	4.04 N 101.04 E
9	HM	Hutan Melintang	Perak	18 Oct. 2014	Worker's dormitory (bedding)	3.89 N 100.93 E
10	CK	Cheras	Selangor	27 Nov. 2014	Low cost apartment room (bedding)	3.11 N 101.72 E
11	SW	Senawang	Negeri Sembilan	29 Nov. 2014	Low cost apartment room (bedding)	2.71 N 102.01 E
12	BF	BatuFeringghi	Penang	25 Nov. 2014	Apartment room (bedding)	5.28 N 100.15 E
13	PD	Port Dickson	Negeri Sembilan	29 Nov. 2014	Worker's dormitory (bedding)	2.56 N 101.81 E
14	AR	Arau	Perlis	24 Sept. 2014	Worker's dormitory (bedding)	6.41 N 100.28 E
15	MU	Muar	Johor	25 Dec. 2014	Worker's dormitory (bedding)	2.04 N 102.56 E
16	KU	Kluang	Johor	24 Dec. 2014	Worker's dormitory (bedding)	2.03 N 103.30 E
17	RW	Rawang	Selangor	25 Nov. 2014	Worker's dormitory (bedding)	3.32 N 101.57 E
18	GL	Gelugor	Penang	03 Nov. 2014	Residential room (bedding)	5.37 N 100.30 E
19	CH	Cameron Highland	Pahang	23 Aug. 2014	Worker's dormitory (bedding)	4.49 N 101.38 E
20	KU	Kuantan	Pahang	22 Aug. 2014	Worker's dormitory (bedding)	3.82 N 103.30 E
21	BH	Bandar Hilir	Melaka	26 Dec. 2014	Worker's dormitory (bedding)	2.19 N 102.25 E
22	BP	BalikPulau	Penang	13 May. 2014	Residential room (bedding)	3.00 N 101.53 E

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DNA Extraction

Bacterial DNA was extracted from the entire body of each *C. hemipterus* specimen following a method of Sakamoto and Rasgon (2006) with a minor adjustment. The specimens were left to be digested overnight (≈20 h) in the mixture of 200 µL of 1× phosphate buffered saline, 20 µL of Proteinase K and 200 µL of QGT buffer at 60 ºC. Then, the lysate was vigorously shaken with 200 µL of QCB buffer and 200 µL of absolute ethanol to ensure the cells were completely lysed. The DNA binding, washing and elution steps were executed according to the manufacturer's protocol (Real Biotech Corporation, RBC, Taipei) with a minor adjustment where the elution steps were repeated twice to obtain a final volume of 70 µL. The DNA concentration was quantified on an Optizen NanoQ Spectrophotometer (Optizen, Korea). The extracted DNA was stored at ─20 ºC until use. DNA samples were verified by electrophoresis in 1% agarose gel prior to PCR amplification.

PCR Amplification

The extracted DNA samples were amplified by using PCR standard protocols and four sets of *Wolbachia* primers (Table 2). A total of 25 µL aliquot containing 12.5 µL of Master Mix (Econotaq/Plus green, Lucigen Corporation), 0.25 µL of each forward and reverse primer, 11.0 µL of nuclease free water and 1.0 µL of DNA template were used by following the manufacturer's protocol. The DNA fragments were amplified by PCR thermocycler (G-Storm, Gene Technologies Ltd, England) using various adjusted programs (Table 3). The amplified products were then evaluated in 1% agarose gel electrophoresis stained with 2.0 µL red safe before visualizing the gel under the UV light.

Table 2: Primers used to screen *Wolbachia* infections.

Primer (F/R)	Short names	Primer sequence (5'-3')	Product size (bp)	Reference
INTF2 INTR2	INTF2 INTR2	AGTCATCATGGCCTTTATGGA TCATGTACTCGAGTTGCA	136	Sakamoto and Rasgon, 2006
81F 691R	81F 691R	TGGTCCAATAAGTGATGAAGAAAC AAAAATTAAACGCTACTCCA	600	Akhoundi*et al*., 2016
553F-W 1334R-W	553F-W 1334R-W	CTTCATRYACTCGAGTTGCWGAGT GAKTTAAAYCGYGCAGGBGTT	781	Simoes*et al.,* 2011
W-Specf W-Specr	W-Specf W-Specr	CATACCTATTCGAAGGGA AGCTTCGAGTGAAACCAATTC	438	Shaw *et al*., 2016

Table 3: PCR programs involving steps of pre-denature, denature, annealing, extension and final extension with respective cycles.

Primer	Pre-denature		Denature		Annealing		Extension		Number of cycles	Final extension		Reference
	T (ºC)	D (sec)	T (ºC)	D (s)	T (ºC)	D (s)	T (ºC)	D (s)		T (ºC)	D (sec)	
INTF2/INTR2	95	320	95	60	55	60	72	60	40	72	320	Sakamoto and Rasgon, 2006
81F/691R	94	320	94	60	55	60	72	60	35	72	320	Zhou and Rousset, 1998
INTF2/INTR2 and 81F/691R	94	240	94	30	60	30	72	60	30	72	600	Seri Masran and Ab Majid, 2017
INTF2/INTR2 and 81F/691R	95	120	95	30	60	60	72	45	35	72	320	Siddiqui and Raja, 2015
553F-W/1334R-W	94	240	94	40	50	40	72	60	30	72	600	Zha*et al*., 2014
553F-W/1334R-W	94	120	94	60	62	60	72	60	35	72	600	Simoes *et al*., 2011
W-Specf/W-Sper	95	920	95	15	66	25	72	30	35	72	320	Shaw *et al*., 2016

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Table 4: Screening results for *Wolbachia* infections from tropical bed bug specimens based on PCR programs and primers proposed by numerous researchers.

PCR Program (C)	Primers used	Result
Sakamoto and Rasgon (2006)	Sakamoto and Rasgon, 2006	Negative for *Wolbachia* detection
Zhou and Rousset, 1998	Akhoundi *et al*., 2016	Negative for *Wolbachia* detection
Seri Masran and Ab Majid, 2017	Sakamoto and Rasgon, 2006	Negative for *Wolbachia* detection
Seri Masran and Ab Majid, 2017	Akhoundi *et al*., 2016	Negative for *Wolbachia* detection
Siddiqui and Raja, 2015	Sakamoto and Rasgon, 2006	Negative for *Wolbachia* detection
Siddiqui and Raja, 2015	Akhoundi *et al*., 2016	Negative for *Wolbachia* detection
Zha *et al*, 2014	Simoes *et al*., 2011	Negative for *Wolbachia* detection
Simoes *et al*, 2011	Simoes *et al*., 2011	Negative for *Wolbachia* detection
Shaw *et al*, 2016	Shaw *et al*., 2016	Negative for *Wolbachia* detection

RESULTS

We screened 22 specimens of the tropical bed bugs for *Wolbachia* infections collected from different localities in Peninsular Malaysia. Our results found that all PCR amplification of the bacterial genes were negative although four different sets of specific primers were used (Table 4). The amplification was initially attempted using a set of internal primers (INTF2 and INTR2) that amplifies approximately 136 bp fragment from *Wolbachia*16S rDNA. Amplification was subsequently done for the surface protein (wsp) gene of *Wolbachia* sp. using primers 81F and 691R.However, the amplification with these primers also yielded a negative result. Next, we attempted to amplify the 16S rRNA gene (781 bp) using primers 553F-W and 1334R-W but was also not successful. A similar result was obtained in the amplification of an approximately 438 bp fragment from 16S rRNA gene when we attempted with primers WSpecF and WSpecR, where the amplification failed to produce any amplicons. Based on the results, we hereby assumed that our specimens are free from *Wolbachia* infections.

DISCUSSION

With the advent of PCR, molecular techniques have been used to partially circumvent traditional bacteriological methods (Dobson *et al*., 2002), several attempts were made to detect any signs of *Wolbachia* infections in tropical bed bugs samples using a few published primers during PCR amplification assays. These primer sequences were adopted in previous literature to prime the genetic sequences of *Wolbachia* in *C. lectularius* (Sakamoto and Basgon, 2006; Simoes *et al*., 2010; Siddiqui and Raja, 2015; Akhoundi *et al*., 2016). Numerous studies have been done to elucidate *Wolbachia* infections in multiple arthropods (Jigginset *al*., 2001; Konecka and Olszanowski, 2015). These studies stand as huge contributing factors to understanding the physiological and biochemistry effects of thisendosymbiont bacterium to their hosts. It manipulates the hosts' reproduction system by male-killing (Jiggins *et al*., 2001), induces cytoplasmic incompatibility (Poinsont *et al*., 2003) and possesses the capability to feminize the genetics of the male hosts (Hiroki *et al*., 2002). Knowledge of *Wolbachia* is, therefore, fundamental as it plays a potential role in controlling the population size of the hosts. For this reason, multiple research studies have been extensively done to screen the prevalence of *Wolbachia* in different arthropod hosts, including bed bugs.

In general, *C. lectularius* and *C. hemipterus* are two anthropophilic species with different abilities in tolerating several environmental factors especially the surrounding temperature (Omori, 1941). As such, the tropical bed bugs *per se* have a higher tolerance towards a high temperature due to its common exposure in tropical countries (Omori, 1941). In the same point of view, How and Lee (2010) also expanded the existing knowledge on water balance profile of common bed bugs examined by Benoit *et al* (2007) to tropical bed bugs, assuming that the environmental factors would contribute differently to the bed bugs. Thus, it can be suggested that the geographic distribution of *Wolbachia* differed in both species as well. A similar stand was also reported by Ahmed *et al*. (2015) in *Wolbachia* abundance residing in the Lepidoterans. Their study covered up a larger latitude range where their samples were collected from 36 countries including all continents in the exclusion of Antarctica. They concluded that climate and geography are strong predictors of *Wolbachia* infection frequency. Corresponding to this, Toju and Fukatsu (2010) inferred the same statement where their study substantiated a significant correlation between endosymbionts infection frequencies and climatic or ecological factors. However, this statement is contradicted with Akhoundi *et al*. (2016) where they suggested that there are no significant differences in infection frequencies and geographic ranges. Akhoundi *et al* (2015) suggested this conclusion from a pool of bed bugs they collected from eight localities within the perimeter of France. Hence, it might be inappropriate and is arguable that the utilization of their methodology is not reproducible in the study of tropical bed bugs due to insufficient sample collections.

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To validate our study, the PCR thermal settings were used according to the primer sequences adopted, while the annealing temperature was set as per recommended by the manufacturer (Sakamoto and Basgon, 2006; Simoes *et al*., 2010; Siddiqui and Raja, 2015; Akhoundi *et al*., 2016;). All experimentations were carried out on samples preserved in RNA later reagent. However, as a measure of qualitative comparative towards other samples, fresh samples and samples stored in alcohol were also tested and they resulted in negative *Wolbachia* DNA detection as well, which, therefore, suggesting that the preservation method did not affect or degrade the DNA of the samples.This is strongly supported by Basnet *et al*. (2017) where the data in their studies provided an evidential data demonstration in measuring DNA quality from various extraction techniques. Through an optical density measurement using a spectrophotometer, it is suggested that DNA can be extracted regardless of the preservation technique (Basnet *et al*, 2017). Our samples were kept frozen at -20 °C as per according to Mohammed *et al*. (2017). A similar preservation technique was employed by Simoes *et al*. (2010) and Akhoundi *et al.* (2016).

No *Wolbachia* infections were detected in our samples after amplification using four sets of specific primers in multiple PCR settings. We hypothesized that the gene pool of bed bugs that we screened are all *Wolbachia*-free and it seems that *Wolbachi a*re sidedin their bodies are unlikely. We also believed that *Wolbachia* is more prevalent in *C. Lectularius* compared to *C. hemipterus* as has been reported earlier (Akhoundi *et al*.,2016; Sakamoto and Basgon, 2006; Siddiqui and Raja, 2015; Simoes *et al*., 2010). However, we assumed that the higher individuals pooled per locality, the higher it is to detect the infected ones. Thus, a broader sample of bed bugs from various localities should be collected to robustly evaluate the prevalence of this bacterium. To our knowledge, the current study is the first to report on *Wolbachia* infections in *C. hemipterus* populations in tropical regions. In an informative manner, this pioneered report is useful in highlighting the status of *Wolbachia* infections in tropical *C. hemipterus* and further recognizing its occurrence in this region.

CONCLUSION

The present study allowed us to screen and analyse *Wolbachia* infections in a pool of five randomized individuals of bed bugs per 22 localities throughout Peninsular Malaysia. We concluded that our samples are free from *Wolbachia* infections as no DNA of this reproductive parasite was detected in our samples. Therefore, it can be further concluded that the prevalence of *Wolbachia* in tropical bed bugs, *C. hemipterus* is unlikely. However, we recommend a further analysis such as high throughput DNA sequencing such as Next Generation Sequencing (NGS) to better determine a much more accurate status of this bacterium in tropical bed bugs.

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