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Detection of *Wolbachia* in wild mosquito populations from selected areas in Peninsular Malaysia by loop-mediated isothermal amplification (LAMP) technique

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Abstract. *Wolbachia*, a naturally endosymbiotic bacteria, has shown its potential as one of biological control agents for vector borne diseases. Due to large number of mosquitoes involved in *Wolbachia* screening, Loop-mediated isothermal amplification (LAMP) is recommended as a convenient and time-saving technique. This study aimed to evaluate a newly developed LAMP assay for detection of *Wolbachia* by targeting 16S rDNA gene in samples of wild mosquito populations. The LAMP products were confirmed by colorimetric detection using hydroxy naphthol blue (HNB), digestion with *Rsa*I restriction enzyme and gel electrophoresis. The restriction enzyme digestion of PCR products was performed to differentiate between *Wolbachia* supergroups A and B. Out of 765 mosquito samples tested, 349 (45.6%) and 237 (31%) of the samples were positive for LAMP and PCR techniques respectively. The prevalence of *Wolbachia* detected in mosquitoes was significantly higher using LAMP as compared to PCR. There is significant association between numbers of mosquitoes positive with *Wolbachia* detected using LAMP and PCR (χ^2=61.31; df=1; $p < 0.05$) with a kappa (κ) value of 0.27 and *Phi* value, 0.283. This study highlighted the potential of LAMP as a sensitive, specific and rapid tool for screening of *Wolbachia* in mosquitoes, thus it presents as an alternative to PCR-based assays.

INTRODUCTION

Dengue viruses (DENV) are mosquito-borne viruses of the genus *Flavivirus* and family *Flaviviridae*, which cause dengue and dengue hemorrhagic fever. Almost half the worlds populations are at high risk for dengue and 1.8 billion of them reside in Asia-Pacific countries (WHO, 2016). In Malaysia, an increasing trend in the number of dengue cases was observed since 2000 until 2014 (Mudin, 2015) and same trend continues until recently (Ministry of Health Malaysia [MOH], 2017). Several factors contributed to the increase of dengue transmission including DENV serotype/genotype shifts, climate change and rapid urbanization (Mudin, 2015). In order to increase the effectiveness of dengue prevention and control, the Ministry of Health Malaysia has implemented the Integrated Vector Management (IVM) strategies involving a combination of source reduction, vector control (chemical and biological control) and social mobilizations (MOH, 2009). However, these strategies may not have a significant impact on the reduction of dengue vector populations because of several factors, including limited human resources for vector control activities, lack of community participation and non-sustainable methods due to high costs (Azil *et al.*, 2011; Mariam *et al.*, 2014; Packierisamy *et al.*, 2015). In addition, dengue vectors have shown

resistance towards the insecticides used, for example temephos (Hamdan *et al.*, 2005; Chen *et al.*, 2013). Therefore, newer, safer and more effective methods need to be integrated into the current vector control program in order to reduce dengue vector populations.

The use of endosymbiotic bacteria *Wolbachia* as a biological agent has been postulated to reduce transmission of dengue viruses. *Wolbachia* is a maternally inherited Gram-negative bacteria that are found naturally living in body tissues and reproductive system of infected arthropods, and some filarial nematodes (Werren, 1997). It has the ability to induce reproductive abnormalities, known as cytoplasmic incompatibility (CI), which enables replacement of uninfected mosquito populations that will help *Wolbachia* to successfully establish in vector populations. On the other hand, CI may facilitate population suppression through reduction of viable mosquito eggs (Bourtzis *et al.*, 2014). For example, bidirectional-CI involving crosses between both males and females infected with different strains of *Wolbachia* may prevent progeny from being produced, thus, the introduced strain cannot establish in the population (Sinkins, 2004; Bourtzis *et al.*, 2014).

In early January 2011, the first field trial involving the release of *Aedes aegypti* artificially infected (i.e. transinfected) with *w*Mel *Wolbachia* strain (from *Drosophila melanogaster*, a fruit fly) was conducted in several areas near Cairns, Australia. Besides the capability to induce CI, the *w*Mel strain was selected due to its ability to interfere with dengue virus propagation throughout the mosquito body, which will reduce the transmission of the virus. The release of transinfected mosquitoes showed a successful establishment of the mosquitoes replacing more than 90% of the uninfected *Aedes* population (Hoffmann *et al.*, 2011). Recently, such releases were also performed in Indonesia, Vietnam, Colombia, Brazil and Malaysia. In these studies, field surveys were continuously conducted to monitor the establishment of infected mosquitoes in wild mosquito populations.

Wolbachia is naturally present in some disease vectors, for instance, *Ae. albopictus*, *Culex quinquefasciatus* and *Mansonia* spp., whilst absent in others such as *Ae. aegypti* and *Anopheles* spp. (Kittayapong *et al.*, 2000). However, a *Wolbachia* strain named *w*Anga was isolated from *Anopheles gambiae* collected from West Africa (Baldini *et al.*, 2014). There is a possibility for *Wolbachia* to be transferred through horizontal transmission between mosquito populations, albeit the mechanism is not fully understood (O'Neill *et al.*, 1992). Therefore, it is important to investigate the prevalence of natural *Wolbachia* infections from various mosquito species to help us understand the challenges in using *Wolbachia* as a biological control for vector-borne diseases.

A number of molecular techniques have been used for the detection of *Wolbachia* including polymerase chain reaction (PCR) (Werren & Windsor, 2000), real-time PCR (de Oliveira *et al.*, 2015) and followed with various sequencing analysis method such as multi-locus sequence typing (MLST) system (Osei-Poku *et al.*, 2012) and high-resolution melting (HRM) analysis (Henri & Mouton, 2012). However, these methods require well-equipped laboratories which might not be affordable in some regions of the world. Alternative methods of detection which are less laborious are required. Thus, LAMP is proposed as a highly specific and sensitive method with faster time to obtain results and high DNA copies. The amplification product can be seen with naked eyes through turbidity formation or with the help of colour indicators such as HNB, SYBR Green I and calcein (fluorescent detection reagent) (Goto *et al.*, 2009; Fischbach *et al.*, 2015). In addition, LAMP uses a unique DNA polymerase with the ability of strand-displacement and only requires two sets of primer targeting six different regions of target gene (Notomi *et al.*, 2000). LAMP can be easily performed through incubation using a heating block or water bath at a constant temperature for at least one hour. LAMP has therefore been widely used in the detection of various pathogens, including detection of *Wolbachia* in mosquitoes (Gonçalves *et al.*, 2014).

Detection of *Wolbachia* was conducted previously by amplification of various genes, such as *wsp*, *fts*Z, and 16S rDNA, using PCR technique (Kittayapong *et al.*, 2000; Werren & Windsor, 2000). Based on Marcon *et al.* (2011), a combination of two DNA target regions namely 16S rDNA and *wsp* will increase the efficiency of detection and differentiation into supergroups, whereas, *ftsZ* primers are proved to be less sensitive for detection of *Wolbachia* and can lead to false-positive results (Marcon *et al.*, 2011). In the current study, 16S rDNA was chosen as a target DNA, instead of *wsp*, due to its highly conserved regions. The designed LAMP primers are expected to be highly specific because the primers were located within approximately 300 bp sequences of the conserved region. In contrast, *Wolbachia wsp* gene consists of hypervariable regions (Baldo *et al.*, 2010). Therefore, it is unsuitable to be used as a target region in designing of LAMP primers due to production of non-specific amplifications. The objectives of this study were (i) to evaluate LAMP as a method for detection of *Wolbachia* in samples of wild mosquito populations in comparison with PCR, and (ii) to determine supergroups of *Wolbachia* in the samples based on restriction enzyme digestion of LAMP and PCR products.

MATERIALS AND METHODS

Mosquito collections

Mosquito collections were performed using various techniques including Mosquito Larvae Trapping Device (MLTD), larval survey, BG-Sentinel trap and human landing catch (HLC). Mosquitoes were collected from selected areas in several states of Peninsular Malaysia (Selangor, Kuala Lumpur, Pahang, Perak, Kelantan and Terengganu) from March 2014 until May 2015. Some of the mosquito larvae samples were provided by Kuala Lumpur City Hall (DBKL) staff who collected the larvae from MLTD monitoring sites located in dengue-endemic areas in Kuala Lumpur. All selected areas of this study were not involved in *Wolbachia*-infected mosquitoes release project which is currently conducted in Malaysia.

All mosquito collections were brought back to the entomology laboratory at the Department of Parasitology and Medical Entomology, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). Larvae were reared to adulthood in the insectarium. After emergence, the mosquitoes were sorted based on species. Species of adult mosquitoes were determined using identification keys for mosquitoes of Peninsular Malaysia (Jeffery *et al.*, 2012) and other keys as listed here; Mattingly (1958), Stojanovich & Scott (1965), Reinert (1981), Rattanarithikul *et al.* (2005) and Rattanarithikul *et al.* (2006). Subsequently, these mosquitoes were preserved in 95% ethanol and stored at -20°C prior to DNA extraction.

Preparation of DNA templates

DNA extractions were conducted as described in manufacturer's protocols with several modifications. The extraction was performed by homogenizing the whole body of adult mosquito using 100 µL of DNAzol® reagents (Life Technologies, USA) by manually crushing it using pipette tip. The homogenate was centrifuged at 4°C for 15 min at 10 000 r.p.m. DNA was precipitated from the supernatant by the addition of 50 µL of absolute ethanol AR (Ajax Finechem Pty. Ltd., Australia), mixed well and centrifuged for 12 000 r.p.m for 5 min. The DNA precipitate was washed twice with 75% ethanol. Wash solution was discarded and the opened tubes were left upside down at room temperature until the ethanol was evaporated. Finally, the DNA was eluted with 50 µL of sterilized distilled water and stored at -20°C. *Drosophila simulans* naturally infected with *Wolbachia* was used as an internal control to determine the success of DNA extraction (obtained from *Drosophila* Stock Centre, University of California San Diego with a stock number of 14021-0251.291). These adult *D. simulans* were received in 1 mL microcentrifuge tube containing 95% ethanol.

PCR reactions

PCR was conducted according to the standard manufacturer's protocol with some modifications. Each reaction contains 25 µL consisting of 1× PCR buffer (Invitrogen), 2 mM $MgCl_2$ (50 mM), 0.32 mM dNTPs (10 mM each), 20 pmol/µL forward and reverse primers, 3 µL of DNA template and 1.5 units of *Taq* DNA polymerase (5U/µL) (Invitrogen, USA). The PCR were performed using Eppendorf Pro-S thermal cycler (Hamburg, Germany) with initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 90 s and with final elongation at 72°C for 10 min. Primers used for forward [(5'-CATACCTATTCGAA GGGATAG-3') and reverse (5'-AGCTTCGAG TGAAACCAATTC-3') were targeting approximately 438 bp of 16S rDNA of *Wolbachia* gene (Werren & Windsor, 2000). The primers were synthesized by Biobasic, Canada. The size of DNA amplification was confirmed by 2% gel electrophoresis (stained with SYBR® Safe stains) at 90 V for 90 min. The expected bands were visualized by Gel Doc EZ Imaging System (Bio-Rad, Hercules, CA, USA) and captured with Image Lab™ Software. PCR products were send for sequencing and followed by digestion with a restriction enzyme, *Rsa*I to differentiate between supergroup A and supergroup B. Successful PCR digestion will produce three different fragments (311, 83 and 46 bp) for supergroup A, and five fragments for supergroup B (165, 146, 67, 46 and 16 bp) (Pourali *et al.*, 2009) (Figure 1).

LAMP conditions

The LAMP reaction was performed using two pairs of primer targeting six distinct regions of 16S rDNA of *Wolbachia*, approximately 316 bp of target sequence, as previously designed (Gonçalves *et al.*, 2014). In this study, loop primers were not included. We used OmniAmp DNA polymerase instead which proves to provide shorter time to result. The nucleotide sequences of each LAMP primers are located on the sequence of target gene as shown in Figure 2(A). Briefly, the LAMP reaction mixtures were prepared using OmniAmp™ RNA & DNA LAMP kit (Lucigen Corporation, USA) consisting of 1 M betaine (5 M), 2 mM of $MgSO_4$ (100 mM), 2.5 µL of 10× DNA polymerase buffer C, 0.32 mM dNTP (10 mM), 1 µL each of inner primers FIP and BIP (20 pmol/µL), 1 µL each of outer primers F3 and B3 (5 pmol/µL), 0.3 µL of OmniAmp™ DNA polymerase (50×), 2 µL of 180 µM hydroxy naphthol blue (HNB) dye, 3 µL of DNA template and addition of distilled water to make the final volume of 25 µL. The HNB (molecular weight 620.47 g/mol) was purchased separately from Sigma-Aldrich, Germany.

The reaction mixtures were placed in Loopamp real-time turbidimeter LA-500 (Eiken Chemical Co., Ltd., Tokyo, Japan) at 68°C for 1 hour and followed by enzyme

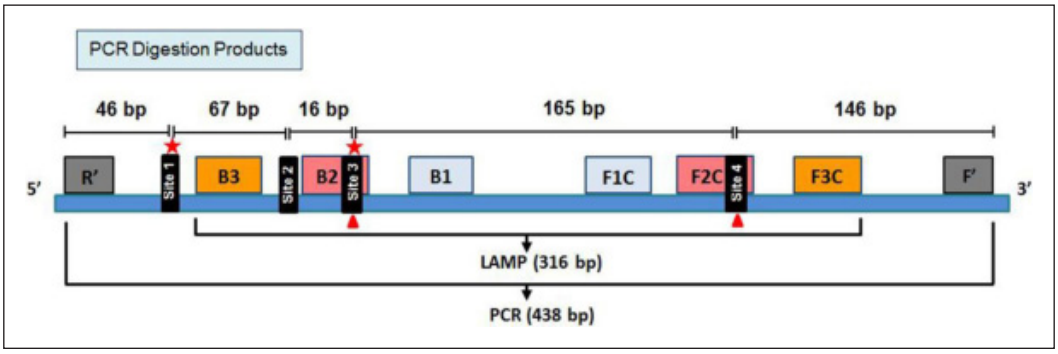

Figure 1. Schematic diagram shows the position of LAMP and PCR primers on the sequence of *Wolbachia* 16S rDNA and the cleavage sites of *Rsa*I for both target regions. For LAMP, two crucial cleavage sites are involved; which are Sites 3 and 4 whereas for PCR, four sites are required in order to differentiate *Wolbachia* supergroup. Size of DNA fragments by PCR digestion are shown and the star symbol (★) represents the *Rsa*I site for *Wolbachia* supergroup A.

Figure 2. Schematic diagram represents the LAMP primers and the expected LAMP products. (A) Location of LAMP primers on the target sequences of *Wolbachia* 16S rDNA gene (confirmed as supergroup AB). Bold type "GTAC" indicates the restriction site of *Rsa*I presence in *Wolbachia* supergroup A, whereas boxes represent the additional restriction site in supergroup B. False priming sites are marked with single nucleotide boxes both in DNA sequence and LAMP primers, and circles represent primer-mismatches in GTAC site, (B) Expected size of amplified products expressed in term of B+, B-, F+ and F- (Terms as described in Notomi *et al.*, 2000). The size of expected digestion products are shown in the boxes.

reaction termination at 80°C for 3 min. For confirmation of amplified *Wolbachia* 16S rDNA, LAMP products were analyzed based on colour changes of HNB dye (violet to sky blue) and 2% agarose gel electrophoresis followed by digestion with a restriction enzyme, *Rsa*I. For the digestion, 8 µL of LAMP products were digested with 1 µL of *Rsa*I (New England Biolabs, USA), 2.5 µL of CutSmart® buffer (10×) and incubated for 16 hour on 37°C. The digested LAMP products were analyzed using 3% agarose gel electrophoresis. PCR was also conducted using LAMP outer primers (F3 and B3 primer) for several samples to validate that the correct target DNA sequence had been amplified. The PCR products were sent for sequencing and confirmed as *Wolbachia* 16S rDNA with the formation of 316 bp DNA band size.

LAMP primers specificity

The specificity of LAMP primers were tested on other gram negative bacteria, *Escherichia coli*, *Serratia* sp., *Pseudomonas* sp. and *Klebsiella* sp. which were previously identified in *Aedes* mosquitoes (Gusmão *et al.*, 2007; Gusmão *et al.*, 2010). All colonies of bacteria were obtained from the Department of Medical Microbiology and Immunology, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). The bacteria DNA were also extracted using DNAzol® reagent.

LAMP and PCR detection limit

PCR reaction (50 µL) was conducted from samples of *Ae. albopictus*. The expected size of *Wolbachia* gene 16S rDNA (438 bp) was analysed with 2% agarose gel electrophoresis. PCR products on agarose gel were purified using DNA Purification kit (Norgen Biotek Corporation, Canada) and the concentration of DNA was measured using a spectrophotometer (7300 model, Jenway, UK). The *Wolbachia* DNA was diluted with distilled water to a final concentration of 1 ng/µL. From 1 ng/µL, the DNA was serially diluted (10-fold) ranging from 10^{-1} to 10^{-6} (0.1 ng/µL to 1.0 ρg/µL). Each dilutions of 16S rDNA gene were tested with PCR and LAMP.

Statistical analysis

A chi-square (χ^2), kappa agreement (κ) and *Phi Cramer's V* tests were used to calculate the association and degree of agreement between PCR and LAMP. The tests were run using SPSS software programme (IBM, SPSS Statistics 23). Kappa result is interpreted as: κ value above 0.90 considered as almost perfect, κ = 0.8–0.9 strong agreement, κ = 0.6–0.79 as moderate, κ = 0.4–0.59 as weak, κ = 0.21–0.39 as minimal agreement and value ≤ 0 as none agreement (McHugh, 2012). The interpretation of *Phi* value is based on Cohen (1988), *Phi* = 0.1 as small effect, 0.3 as medium and 0.5 considered as large effect (Allen & Bennett, 2008). *P* values of < 0.05 were considered statistically significant.

RESULTS

A total of 1,606 mosquitoes (males and females) studied consisted of *Ae. albopictus* (74.7%) followed by *Ae. aegypti* (11.6%), *Armigeres subalbatus* (4.6%), *Culex sitiens* (1.7%), *Cx. quinquefasciatus* (1.0%), *Cx. hutchinsoni* (1.0%) and *Cx. mimeticus* (0.6%). Of the total mosquitoes collected, 765 mosquito samples were processed and screened for the presence of *Wolbachia* using PCR and LAMP by targeting 16S rDNA. A total of 349 (45.6%) of the samples were positive by LAMP whereas 237 (31%) of the samples were positive by PCR (Table 1). A significant association was found between numbers of mosquitoes positive with *Wolbachia* detected using LAMP and PCR (χ^2=61.31; df=1; $p < 0.05$) with a minimal kappa agreement (κ), 0.27 and small effect size at *Phi* value, 0.283.

Both techniques were found to be congruent in 495 samples [64.7%; 158 (both LAMP and PCR positive) plus 337 (both LAMP and PCR negative)], whilst incongruent in 270 samples [35.3%; 79 (LAMP negative-PCR positive) plus 191 (LAMP positive-PCR negative)]. For these LAMP positive-PCR negative samples, LAMP products were confirmed with (i) the formation of various sizes of DNA (ladder-like pattern) on the agarose gel, (ii) colour change of HNB dye (from violet to sky blue) (Figure 3) and (iii) digestion of several positive samples with restriction enzyme *Rsa*I (Figure 4). Meanwhile, for the LAMP negative-PCR positive

Table 1. Comparison of LAMP results with PCR for *Wolbachia* detection

Technique and Results	PCR		Total
	Positive	Negative	
LAMP			
Positive	158 (20.7%)	191 (25.0%)	349 (45.6%)
Negative	79 (10.3%)	337 (44.0%)	416 (54.4%)
Total	237 (31.0%)	528 (69.0%)	**765**

* Association between the techniques shows a chi-square value, χ^2 = 61.31 with significant value, $p < 0.05$, kappa value (κ) = 0.27, *Phi* value = 0.283. The percentages are calculated from the number of positive/negative samples over the total 765 samples.

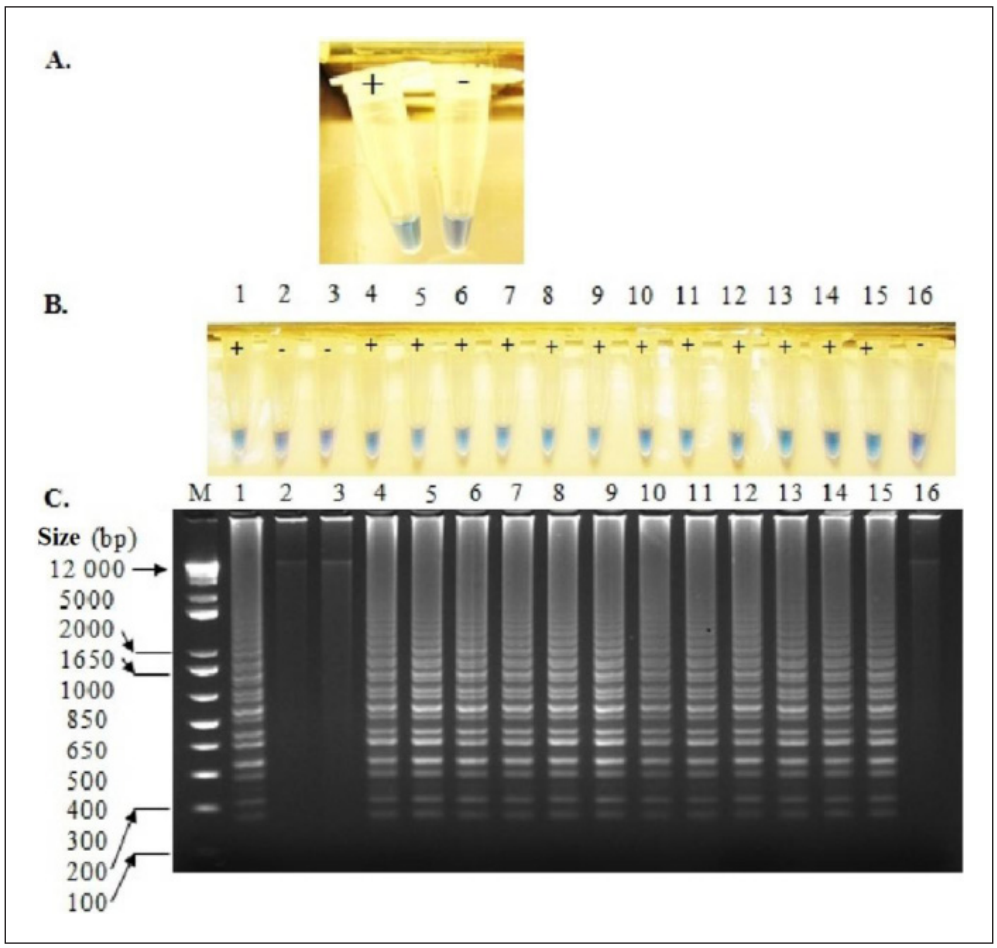

Figure 3. Confirmation of LAMP reaction (A) using hydroxy naphthol blue (HNB) dye by examining the changes of colour under warm white light or yellow LED light; on the left – positive control (blue) and on the right – negative control without template (purple/violet), and (B) shows several samples of mosquito tested; tube 1, positive control; tubes 2–15, mosquito samples; tube 16, negative control and was double confirmed through (C) agarose gel electrophoresis; Lane M, 1kb Plus DNA ladder (Invitrogen, USA); Lane 1, positive control (genomic DNA of *Wolbachia*-infected *D. simulans*); Lanes 2–4, *Ae. albopictus* samples; Lanes 4–15, *Armigeres subalbatus* samples (All these samples were reported negative by PCR).

samples, *Wolbachia* detection was repeated using both PCR and LAMP and the results were the same.

In order to confirm the specificity of LAMP reactions in targeting the correct sequence, LAMP products were digested with *Rsa*I which cut the restriction sites of GT^AC located on B2 and complementary sequence of F2 primers (Figure 2(A)). The schematic diagram on Figure 2(B) shows the expected size of LAMP final products and the digestion products. Theoretically, based on *Rsa*I restriction sites that are present in the target gene 16S rDNA, we believed that *Wolbachia* can be differentiated into supergroup A and supergroup B through the digestion of LAMP products. *Wolbachia* supergroup A contains one restriction site on primer B2 region of predicted LAMP products which would give rise to three different fragments, 277, 232 and 187 bp. Supergroup B in the presence of two restriction sites on primers B2 and also F2C, would produce four different fragments,

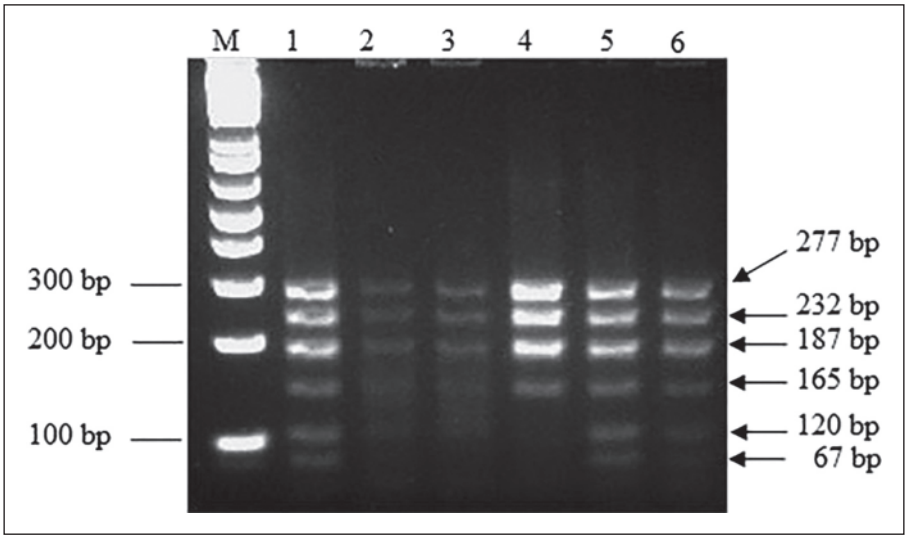

Figure 4. Agarose gel electrophoresis (3%) shows all expected sizes of digestion products of LAMP (*Wolbachia* 16S rDNA confirmed but unable to differentiate supergroups). Lane M, 1kb Plus DNA ladder; Lane 1, *D. simulans* as positive control (supergroup A); Lanes 2–3, *Ae. albopictus* (supergroup AB); Lane 4, *Culex quinquefasciatus* (supergroup B); Lanes 5–6, *Armigeres subalbatus* (supergroup A).

165, 120, 112 and 67 bp. The expected sizes of fragmented DNA of LAMP products were shown on the gel electrophoresis (Figure 4) which proves that the LAMP reactions have amplified the correct target sequence, 16S rDNA of *Wolbachia*. However, as shown in Figure 4, all of the mosquito samples and *D. simulans* which have been confirmed to be infected with *Wolbachia* supergroup A (act as internal control) showed the presence of almost all predicted sizes of fragmented DNA, 277, 232, 187, 165, 120 and 67 bp. These indicate that the digestion of LAMP products with *Rsa*I cannot be used to differentiate *Wolbachia* into supergroups A and B.

In terms of sensitivity of LAMP assay, it is important to determine the lowest amount of DNA concentration (i.e. detection limit) that can be detected for both PCR and LAMP techniques, and to determine the sensitivity of LAMP primers. As shown in Figure 5, the lowest detection limit for PCR is 1×10^{-4} ng/µL of DNA concentration. On the other hand, LAMP can detect the DNA up to 1×10^{-5} ng/µL, although the ladder-like band pattern on agarose gel 2% was not clearly visible. Specificity of LAMP reaction was tested on other genera of gram negative bacteria; *Serratia* sp., *Escherichia coli*, *Pseudomonas* sp., *Klebsiella* sp. and genomic DNA extracted from *Ae. aegypti* (not naturally found to be infected with *Wolbachia*). All resulted in no amplification (figure not shown) which substantiates the specificity of our LAMP assay.

In this study, 189 out of 237 PCR-positive mosquitoes were subjected to *Rsa*I digestion for differentiation of *Wolbachia* supergroups. Only 189 PCR products were chosen for PCR digestion, due to cost and time constraints. Results of 16S rDNA digestion were examined by the presence of expected size of DNA fragments (Figure 6). Out of 189 samples, 147 of the samples were *Ae. albopictus* (Table 2). Overall, approximately 52.4% (99/189) of the mosquitoes positive for *Wolbachia* were superinfected by both supergroups, whereas 27% (51/189) and 20.6% (39/189) were singly infected with supergroup A and B respectively. Single infection with either supergroup A or B was found in most of the *Ar. subalbatus* and *Culex* spp. collected respectively.

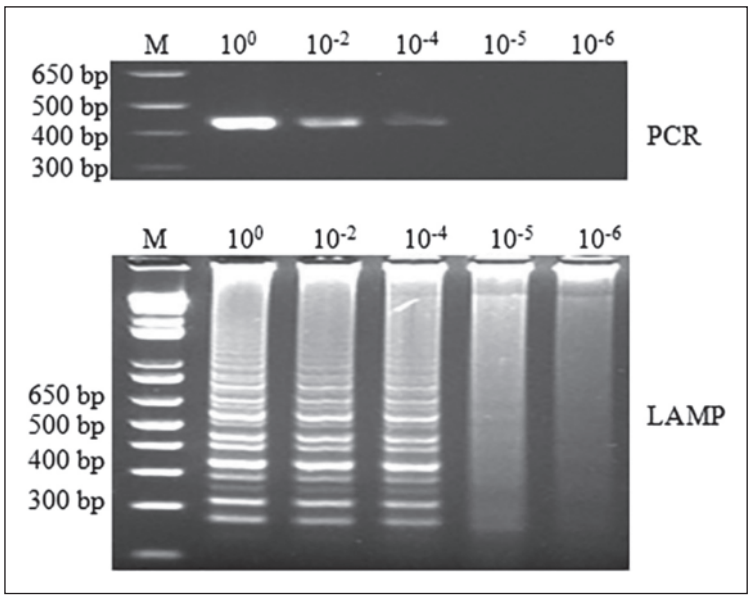

Figure 5. Agarose gel electrophoresis profile (2%) shows the amplified products of 16S rDNA of *Wolbachia* gene to determine the lower detection limit of the PCR and LAMP techniques, Lane M, 1kB Plus DNA ladder; Lanes 1–5, 1 ng/µL (10^0), 10^{-2}, 10^{-4}, 10^{-5} and 10^{-6}.

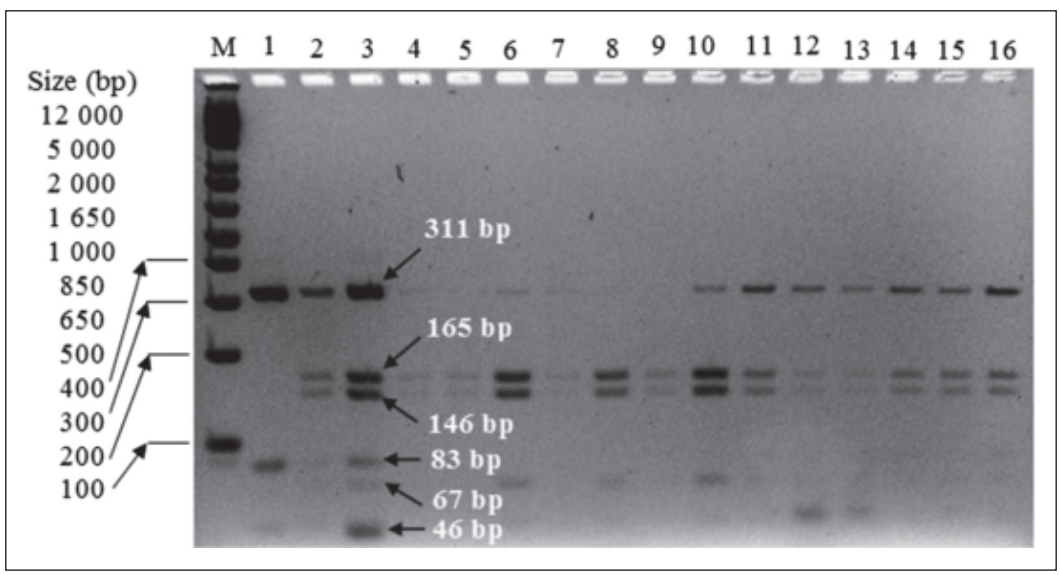

Figure 6. Agarose gel electrophoresis (3%) shows the results of PCR digestion conducted on representatives of mosquito samples (*Aedes albopictus*) positive for *Wolbachia*. Lane M; 1kB Plus DNA ladder; Lane 1, *D. simulans* (supergroup A); Lanes 2–3, *Ae. albopictus* infected with supergroup AB; Lanes 4–16, infected with supergroup AB and also single infection with supergroup B (Lanes 8 and 9).

Table 2. Screening of 16S rDNA gene of *Wolbachia* from different species of mosquitoes and *Wolbachia* supergroups by PCR digestion

Species of mosquito	Samples positive *Wolbachia* by PCR	*Wolbachia* supergroups		
		A	B	AB
Aedes albopictus	147	21	28	98
Armigeres subalbatus	30	29	–	1
Armigeres spp.	1	1	–	–
Culex quinquefasciatus	9	–	9	–
Culex mimeticus	2	–	2	–
Total	189	51	39	99

DISCUSSION

Thus far, the field releases of *Wolbachia*-transinfected mosquitoes have shown promising result, as the *w*Mel *Wolbachia* strain was able to establish in local mosquito populations (Hoffmann *et al.*, 2011). In this CI-based biological control, the successful establishment of *Wolbachia*-transinfected mosquitoes depends on the ability of the introduced strain to induce or rescue CI. Multiple infections of *Wolbachia* in a population will cause various incompatibility crosses between individual mosquitoes with different supergroups or strains, which leads to non-viable progeny being produced (Sinkins, 2004). Therefore, before commencing the releases of *Wolbachia* transinfected mosquitoes, it is important to conduct a baseline study on the natural infections of *Wolbachia* that are present in local mosquito populations in dengue-endemic or epidemic areas.

In this study, we report on a newly developed LAMP assay as an alternative to the common method used for *Wolbachia* detection, PCR. In addition the application of LAMP method also provides baseline information regarding distribution of *Wolbachia* in wild mosquito populations by amplification of 16S rDNA gene. LAMP is highly efficient in the sense that it produces a high copy number of amplified target DNA within 60 minutes. Our study found a higher detection rate of *Wolbachia* by LAMP (45.6%; 349/765) as compared to PCR (31%; 237/765). There is a significant association between LAMP and PCR techniques, albeit the minimal agreement between these two techniques. In this study, LAMP is shown to be more sensitive and specific in detecting *Wolbachia* due to a higher number of positive samples detected compared to PCR. A total of 191 mosquito samples that were LAMP(+)/PCR(–) were confirmed for *Wolbachia* by the presence of expected sizes of fragmented DNA. Some of the LAMP products were digested with *Rsa*I, to exclude the possibility of false positive and as a validation that LAMP products have targeted the correct gene. The discrepancies between PCR and LAMP positive samples are possibly due to the concentration of *Wolbachia* DNA falling below the threshold for PCR detection but not for detection by LAMP. Based on sensitivity test, LAMP can detect DNA concentration up to 1×10^{-5} DNA dilution, as compared to PCR (1×10^{-4} ng/µL). A study conducted by Gonçalves *et al.* (2014) also demonstrated the sensitivity of the designed LAMP primers which can detect up to 10^{0} dilution (i.e. 1 copy of DNA plasmid). Besides that, an advantage of LAMP assay is that the product of amplification can be seen clearly through the formation of ladder-like pattern shown on agarose gel. While PCR shows only a single band of the expected size (Figure 5). When there is a very low infection (i.e. low amount of DNA in PCR product), it will show a very faint DNA band which is difficult to be observed. The specificity of LAMP primers were also tested on other Gram-negative bacteria that may be present in *Aedes* midgut (Gusmão *et al.*, 2007;

Gusmão *et al.*, 2010). As a result, DNA amplification was not detected in all of the samples and thus indicates that the LAMP primers used is highly specific for the detection of *Wolbachia* 16S rDNA gene.

A total of 79 samples of LAMP(–)/PCR(+) samples were subjected to another run for both techniques and the results remain the same. The patterns of LAMP–/PCR+ were randomly spread among the samples collected from various localities and not constrained to only one species of mosquitoes. In order to confirm the presence of 16S rDNA gene, some of the PCR products were sent for sequencing. Out of 237 samples that were positive for PCR, 189 were re-run for PCR before proceeding with *Rsa*I digestion. Results are similar with the initial run (438 bp DNA band on agarose gel). The LAMP–/PCR+ samples might be due to our genomic DNA extraction products which could be contaminated with mosquito tissue debris as indicated by a low value of A260/A230 ratio from spectrophotometer reading. An absorbance ratio of less than 2.00 indicates that the DNA extracts were contaminated with other substances such as chemicals, carbohydrates, phenols and peptides (Ferreira *et al.*, 2016). A study conducted by Aonuma *et al.* (2009) shows that mosquito debris could potentially affect the PCR reaction. In our case, presence of these inhibitors may reduce the effectiveness of OmniAmp™ DNA polymerase. Our results are in agreement with previous studies that also reported unsuccessful amplification of target region using LAMP as compared to PCR and they hypothesized reasons as follow: (1) DNA extract from ticks contains PCR-inhibitors that had inhibited LAMP reactions (Nakao *et al.*, 2010) and, (2) Presence of salt in reactions affected the sensitivity of LAMP polymerase; especially when OmniAmp was used (Jevtuševskaja *et al.*, 2017).

Therefore, extra steps are needed to improve DNA purification when using DNAzol; such as the addition of incubation step to the homogenate to enhance mosquito tissue lysis, and the use of Polyacryl carrier reagent during DNA isolation to increase yield of low molecular weight DNA (Chomczynski *et al.*, 1997; Rider *et al.*, 2012). We believe that DNAzol is the best DNA extraction kit for our study due to its time- and cost-saving features (Lickfeldt *et al.*, 2002). Together with OmniAmp polymerase, it will reduce time for detecting *Wolbachia* in a large number of mosquito samples. Precautions were taken in our study to prevent contamination. These include the uses of laminar flow for the preparation of LAMP reaction and different working rooms for other downstream tests involving LAMP products, such as preparation for digestion (master mix) and running the agarose gel electrophoresis. In resource-poor settings, use of separate rooms during LAMP reaction preparation is highly recommended in order to prevent contamination.

Collectively, our findings show that the *Wolbachia*-LAMP detection method can be applied in resource-poor setting area without requiring a thermocycler machine. DNA amplification by LAMP is shown through the formation of white precipitate (turbidity), known as magnesium pyrophosphate, in reaction solution. Positive reaction of LAMP can be visualized by naked eyes with the help of HNB without the need for gel electrophoresis. HNB is a metal ion indicator categorized as one of the colorimetric assays that can detect the production of LAMP byproduct (Goto *et al.*, 2009). Other methods of detection include fluorescence dyes such as SYBR Green I. However, there is a possibility to get false-positive results due to unspecific binding of dye on random dsDNA (primer-dimers) when such fluorescence dyes are used (Njiru, 2012). HNB, added during pre-reaction steps of LAMP, is able to prevent cross contamination between LAMP reaction solutions by eliminating the post-reaction opening of tubes which is required for gel electrophoresis. In addition, OmniAmp polymerase used in this study has the advantage to be kept under room temperature for a long period of time (Chander *et al.*, 2014). Many studies were done to improve the application of LAMP in resource-poor settings including on its ability to amplify nucleic acids directly from partially processed samples (Kaneko *et al.*, 2007; Notomi *et al.*, 2015).

For LAMP digestion, supergroup A (internal control: *D. simulans*) shows the same fragment sizes as supergroup B. One possible reason causing this phenomenon is primer-template mismatches which cause polymorphism of the LAMP products. We have noticed that the sequence of internal primers (FIP and BIP) designed by Gonçalves *et al.* (2014), is not exactly a complement to our DNA template. As shown in Figure 2(A), there are six locations of primer-template mismatches found. The study on the effect of internal LAMP primer-template mismatches on LAMP reactions has been reported by Wang (2016). As illustrated in Figure 1, supposedly there are three *Rsa*I cleavage sites that are present in LAMP target region. However, only two restriction sites are present in LAMP final products. Unlike PCR, LAMP produces DNA copies in a much more complicated way which involved the formation of stem loop sequence and repeated sequence of 16S rDNA between B2 and F2C region for one strand of DNA copy (the linearized sequence of LAMP product was arranged as described by Notomi *et al.* (2000), Figure 2(B)). The two restriction sites on Sites 3 and 4 (Figure 1) are the crucial cleavage sites for differentiation of supergroups A and B. The restriction enzyme will cut at Site 3 only (three fragments DNA: 277, 232 & 187 bp) for supergroup A while both Sites 3 and 4 for supergroup B (four fragments DNA: 165, 120, 112 & 67 bp). However, practically, all of the digested samples showed the expected fragments size for both supergroups (Figure 4). Therefore, we acknowledge the limitation of using restriction enzyme for LAMP in order to determine *Wolbachia* supergroups. Although further investigation is needed for this, we have proved that the restriction enzyme, *Rsa*I can be used to validate the successful amplification of *Wolbachia* 16S rDNA using LAMP. Using PCR method, we have successfully differentiated the supergroups through *Rsa*I digestion and the different sizes of fragmented DNA between supergroups were seen (Figure 6). Several studies had conducted *Rsa*I digestion of 16S rDNA using PCR products and similar result on the sizes of fragmented DNA was obtained (Mikac, 2007; Pourali *et al.*, 2009).

Interestingly, our study has demonstrated that more than half of the infected *Ae. albopictus* (66.7%; 98/147) were superinfected with supergroup AB, and singly infected with supergroup A (14.3%; 21/147) and B (19.0%; 28/147). Recently, Joanne *et al.* (2015) have reported the prevalence of *Wolbachia* in *Ae. albopictus* collected from selected areas of Malaysia, obtained using multiplex PCR targeting *wsp* gene of *Wolbachia*. The study showed that out of 286 *Ae. albopictus* positive for *Wolbachia*, 262 (91.6%) were superinfected with both supergroup A (*w*AlbA) and B (*w*AlbB). A study conducted by Noor Afizah *et al.* (2015) showed that, all 104 samples of *Ae. albopictus* caught were found to be infected with *Wolbachia*. They reported that 70.2% of samples were superinfected with supergroup AB (*w*AlbA and *w*AlbB) and singly infected with supergroup B (29.8%). There is no single infection of *Wolbachia* supergroup A reported in their study (Noor Afizah *et al.*, 2015). These studies indicated that *Wolbachia* superinfection predominantly exists in Malaysian *Ae. albopictus* populations. The supergroup variations may occur due to expression of CI in the mosquito populations. The compatible crosses involving superinfected females with single infected or uninfected males, lead to the spread of superinfected population and replacement of *Wolbachia* single infection population (Dobson *et al.*, 2004; Sinkins, 2004). Based on Tortosa *et al.* (2010), the individual mosquito's age, sex, type of *Wolbachia* strains infected and geographical distribution affect the density of *Wolbachia* in individual mosquitoes. Nonetheless, these criteria were not observed in this study as the ages of mosquitoes collected from the field were undetermined.

Surveys on the diversity of *Wolbachia* infection in wild mosquito populations were also conducted in Brazil (de Oliveira *et al.*, 2015), California, USA (Rasgon & Scott, 2004) and Southeast Asia countries such as in

Thailand (Kittayapong *et al.*, 2000). The Thailand study showed 28.1% of the wild mosquitoes were naturally infected, and six out of eight mosquito genera found positive with *Wolbachia* were disease vectors (Kittayapong *et al.*, 2000). Among these are *Aedes*, *Culex* and *Mansonia*. Our study has proved that the newly developed LAMP assay targeting *Wolbachia* 16S rDNA is more sensitive when compared with PCR technique. Furthermore, through the use of LAMP, the number of mosquitoes positive with *Wolbachia* from each sampling location is higher. Several species of mosquitoes that were reported negative for *Wolbachia* by PCR were found to be positive *Wolbachia* by LAMP. The species are namely *Coquillettidia crassipes*, *Cx. gelidus*, *Cx. hutchinsoni*, *Cx. sitiens* and *Cx. sinensis*. All of these species except *Cx. sinensis* were reported positive for *Wolbachia* in the Thailand study (Kittayapong *et al.*, 2000). Other studies also found natural infection of *Wolbachia* in mosquitoes of the following genera: *Aedes*, *Armigeres*, *Culex*, *Coquillettidia*, *Hodgesia*, *Mansonia*, *Tripteroides* and *Uranotaenia* (Kittayapong *et al.*, 2000; Ricci *et al.*, 2002; Tsai *et al.*, 2004). However, we found that several mosquito species were negative for *Wolbachia* infection both by PCR and LAMP; namely *Mansonia annulata*, *Ma. indiana*, *Ma. uniformis* and *Ae.* (*Paraedes*) *collessi*.

This study substantiates the suitability of LAMP technique as a screening method for *Wolbachia*, especially when a large number of mosquito samples and resource-poor settings are involved. We suggest LAMP to be used in the screening of natural infection of *Wolbachia* involving a variety of mosquito genera/species and to monitor the prevalence of *Wolbachia* after releases of transinfected mosquitoes. Current findings support that LAMP, after several modifications and optimizations, can be more effective in detecting *Wolbachia* compared to PCR. Finding from our study supports the use of restriction enzyme digestion on 16S rDNA PCR product as a method to explore the distribution of *Wolbachia* supergroups in naturally infected mosquitoes.

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