A diagnostic primer pair to distinguish between wMel and wAlbB *Wolbachia* infection

- A simplified real-time PCR approach for use in field collected *Aedes aegypti* in dengue reduction trials

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Abstract

Detection of the *Wolbachia* endosymbiont in *Aedes aegypti* mosquitoes through real-time PCR assays is widely used during and after *Wolbachia* releases in dengue reduction trials involving the wMel and wAlbB strains. However, primers applied in current successful *Wolbachia* releases cannot be used in a single assay to distinguish between these strains. Here, we developed a new diagnostic primer pair wMA which can detect wMel or wAlbB infection in the same assay. We also tested current *Wolbachia* primers and show that there is variation in their performance when they are used to assess the relative density of *Wolbachia*. The new wMA primers provide an accurate and efficient estimate of the presence and density of both *Wolbachia* infections.

Keywords

Primers; *Wolbachia*; *Aedes aegypti*

Introduction

The bacterium, *Wolbachia*, is providing an increasingly popular method to inhibit dengue virus transmission in the mosquito, *Aedes aegypti*. *Wolbachia*-infected populations involving the wMel strain have now been successfully established in *Ae. aegypti* in regions including northern Australia, Brazil and Indonesia [1-3], while wAlbB-infected *Ae. aegypti* have been established in Malaysia [4]. Detection of the *Wolbachia* endosymbiont in *Ae. aegypti* mosquitoes is a standard requirement for good laboratory practice during *Wolbachia* mosquito releases in dengue reduction programs and for tracking *Wolbachia* invasions in the field [4, 5]. Real-time PCR and High Resolution Melt (HRM) assays (SYBR[®] equivalent/non-probe) have been developed that enable detection and *Wolbachia* density estimation for the strain of interest [6-8]. However, difficulties can arise in using these assays when there is a need to detect *Wolbachia* and distinguish between multiple

Wolbachia strains. In experiments where superinfected lines are used [9], or where mosquitoes carrying different single infections need to be distinguished for experiments or in field collected samples [10], different assays are currently required. Given that both *wMel* and *wAlbB* strains are now actively being used in field releases and that each strain may have advantages in particular situations, the requirement for multiple strain identification is likely to increase in the foreseeable future.

In previous work, we have used a *Wolbachia*-specific primer pair, *w1* [7], which targets a conserved locus VNTR-141 containing tandem repeats [11]. This pair of primers works efficiently in amplifying *wMel* and *wMelPop* infections in a real-time PCR and HRM assay, but achieves only poor amplification of *wAlbB* [10]. As well as being used for *Wolbachia* detection, primers are needed for quantification of *Wolbachia* density in mosquitoes. There are various *Wolbachia* specific primers for *wMel*, *wAlbB* or *wMelPop* [9, 12-14], but currently there is no standardised assay for *Wolbachia* screening that is comparable between strains and that can be used to compare results between laboratories. Although cross-laboratory comparability may not be a realistic aim when using a SYBR® equivalent/non-probe-based assay, the use of extra internal controls can make these assays robust for relative density estimates, improving consistency within laboratory experiments [7, 10].

In this study, we developed a diagnostic primer pair that can detect and distinguish between the *wMel* and *wAlbB* infections and also provides an estimate of *Wolbachia* density. In addition, we assessed primer efficiency of some other published primers for *Wolbachia* in mosquitoes and looked for cross-amplification between strains.

Materials and methods

To develop the new primers, we screened for differences between the *wAlbB* and *wMel* strains and then focussed on the sequences of gene *WD_RS06155* in *wMel* and its analogue in *wAlbB*. We then developed a new pair of primers designated *wMA* (Table 1) to distinguish *Wolbachia wMel* and *wAlbB* in a single run of a qPCR assay, based on two base-pair mismatches at the 3'-end of each primer, which resulted in the *T_m* peak for *wAlbB* being separated from that of *wMel*.

Sample preparation and LightCycler® efficiency test

The *wMel* and *wAlbB*-infected *Ae. aegypti* were tested against lines transinfected previously [15, 16]. The *wMel* line was collected from Cairns, Australia in 2019 from regions that had been invaded several years earlier [2, 12], while the *wAlbB* line was derived from a *wAlbB* infected line crossed to an Australian background and maintained in the laboratory.[13]. An uninfected line which was developed from *wMel*-infected line after tetracycline treatment was also used in this study.

Mosquitoes were reared with TetraMin® fish food tablets in reverse osmosis (RO) water until the adult stage [17]. We used a mixture of young (4 ± 1 days since eclosion) and old (38 ± 1 days since eclosion) mosquitoes from *wMel*-infected [16], *wAlbB*-infected [15] and uninfected strains that were killed in absolute ethanol before Chelex® DNA extraction. In the

standard procedure, DNA was extracted in 250 µL 5% Chelex® 100 Resin (Bio-Rad Laboratories, Hercules, CA) and 3 µL of Proteinase K (20 mg/ mL, Bioline Australia Pty Ltd, Alexandria NSW, Australia). The DNA concentration in the Chelex® 100 Resin solution was measured using a Quantitation Qubit™ 1X dsDNA HS Assay Kit, and then diluted ten times before making a three-fold dilution series to test the efficiency of currently-used *Wolbachia* primers in a real-time PCR assay. We also diluted the solution six times before making a three-fold dilution series to investigate the influence of DNA and Chelex® concentration.

For the real-time PCR and HRM, we used a LightCycler® 480 High Resolution Melting Master (HRMM) kit (Roche; Cat. No. 04909631001, Roche Diagnostics Australia Pty. Ltd., Castle Hill New South Wales, Australia) and IMMOLASE™ DNA polymerase (5 U/µl) (Bioline; Cat. No. BIO-21047) as described by Lee et al. (2012). The PCR conditions for DNA amplification began with a 10 minute pre-incubation at 95°C (Ramp Rate = 4.8 °C/s), followed by 40 cycles of 95 °C for 5 seconds (Ramp Rate = 4.8°C/s), 53°C for 15 seconds (Ramp Rate = 2.5°C/s), and 72°C for 30 seconds (Ramp Rate = 4.8°C/s).

Three technical replicates were run for each sample of each dilution and a graph was plotted showing the log₃ [dilution factor] (x-axis) against mean Cp (y-axis) and a linear trend line ($y = mx + c$) was fitted. Slope (m) and R² values were recorded so that PCR amplification efficiency (E) could be evaluated with the equation:

$$E = \left(3^{\frac{1}{\text{slope}}} - 1 \right) \times 100\%$$

Table 1. Primers for detection of *Wolbachia* strains and estimation of density

Primer name	<i>Wolbachia</i> status target	Molecular target	Forward	Reverse	Source
<i>mos</i>	uninfected	RpS6 mosquito	AGTTGAACGTATC GTTTCCCGCTAC	GAAGTGACGCAGCT TGTGGTCGTCC	[7]
<i>aeg</i>	uninfected	RpS6 <i>Aedes aegypti</i>	ATCAAGAAGCGCC GTGTCG	CAGGTGCAGGATCT TCATGTATTCTG	[7]
<i>w1</i>	wMel	wMel genome VNTR 141	AAAATCTTTGTGAA GAGGTGATCTGC	GCACTGGGATGACA GGAAAAGG	[7]
<i>wsp</i>	wMel, wAlbB-infected	<i>Wolbachia</i> surface protein	GCATTTGGTTAYAA AATGGACGA	GGAGTGATAGGCAT ATCTTCAAT	[9]
<i>wMel</i>	wMel-infected	wMel genome WD_0513	CAAATTGCTCTTGT CCTGTGG	GGGTGTTAAGCAGA GTTACGG	[9]
<i>wAlbB</i>	wAlbB-infected	wAlbB genome DEJ70_0111 5	CCTTACCTCCTGCA CAACAA	GGATTGTCCAGTGG CCTTA	[9]
<i>wMA</i>	wMel, wAlbB-infected	wMel genome	GAAGTTGAAGCAC AGTGACCTT	GCTTGATATTCCTGT AGATTCATC	Newly designed

Primer crossing points comparison and density estimation

Following the efficiency study, we tested for Cp value differences between primers for different *Wolbachia* strains to assess suitability for relative *Wolbachia* density estimation from a total of 16 *Wolbachia*-infected mosquito samples, which were extracted using Chelex[®] resin and then diluted ten times before real-time PCR.

Results and Discussion

Diagnostic primer design

In this study, we developed a diagnostic primer pair that can detect and distinguish between the wMel and wAlbB infections in *Ae. aegypti*, which is important in simplifying current approaches for *Wolbachia* identification. The newly designed primer pair wMA has distinctive Tm values between *Wolbachia* wMel (82.6 ± 0.03 °C) and wAlbB (80.4 ± 0.02 °C) screening (Figure 1c). The high resolution melt produces two joined peaks when the template contains both *Wolbachia* wMel and wAlbB DNA (Figure 1d).

Figure 1. Development of primers to detect *Wolbachia* wMel and wAlbB infection in *Ae. aegypti*. (a) The new primer pair wMA aligns to a region in gene WD_RS06155 of wMel, and aligns to its analogue in wAlbB genome which had two base-pair mismatches at the 3'- end; (b) the wMA primers showed distinct Tm values for *Wolbachia* wMel (82.6 ± 0.03 °C) and wAlbB (80.4 ± 0.02 °C), the x axis represents the crossing point and the y axis represents the amplicon melting temperature; (c) the wMA primers showed distinct Tm values for *Wolbachia* wMel and wAlbB; (d) the wMA primers showed two Tm peaks when mixing DNA template of wMel and wAlbB-infected *Ae. aegypti*.

Primer efficiency test and Cp values comparisons

We tested the efficiency of each of the primers used for screening *Wolbachia* in *Ae. aegypti* (Table 2) by using a threefold dilution series. The efficiencies of all primers were within the acceptable range (85% -110%, Table 2, Figure 2) [18] and the efficiency curves all showed an R^2 valued greater than 0.99.

However, we found the amplification curve increase showed inhibition at the first dilution (Figure 3) for each of the primers. We also found the same phenomenon when DNA was first diluted six times instead of ten times which resulted in outliers occurring in efficiency curves (Sup. Figure 1, 2). These results highlight a potential risk of lowering the relative density estimate in *Wolbachia* screening when using highly concentrated Chelex[®]-extracted DNA solution.

Table 2. Primer efficiency for primers used in detection of *Wolbachia* strains and estimation of density. DNA is extracted in 250 μ L 5% Chelex[®] 100 Resin and then diluted ten times before making a three-fold dilution series.

Colony	Primers	Slope of graph	R^2	Efficiency	DNA concentration in Chelex [®] 100 Resin (ng/ μ L)
Uninfected	<i>mos</i>	-1.566	0.999	101.659%	5.12
Uninfected	<i>aeg</i>	-1.531	0.999	104.946%	5.12
wMel	<i>w1</i>	-1.644	0.999	95.109%	5.24
wMel	<i>wM</i>	-1.677	0.998	92.559%	5.24
wMel	<i>wsp</i>	-1.767	0.999	86.195%	5.24
wMel	<i>MA</i>	-1.769	0.999	86.107%	5.24
wAlbB	<i>MA</i>	-1.730	0.997	88.732%	6.87
wAlbB	<i>wsp</i>	-1.755	0.999	87.032%	6.87
wAlbB	<i>wA</i>	-1.741	0.993	87.953%	6.87

Figure 2. Primer efficiency for detection of *Wolbachia* strains and estimation of density. DNA is extracted in 250 μ L 5% Chelex® 100 Resin and then diluted ten times before making a three-fold dilution series. The primer names are defined in Table 2.

Figure 3. Variation in the shape of the PCR amplification curves when Cp values are fitted with the standard curve. The curves from left to right represent amplification curves of 1/10, 1/30, 1/90, 1/270 and 1/810 DNA dilution from 250 μ L 5% Chelex® 100 Resin. The primers are defined in Table 2.

We noticed that primers had different Cp values even when screening the same individual organism (*Ae. aegypti*, wMel or wAlbB) using the same DNA concentration, even though these primers all have a high efficiency. We therefore tested the Cp ranges of the primers and confirmed variation when using them (Figure 3), which would be expected to result in differences in relative density estimates. The relationship between Cp values of different primers all fit into a linear relationship, with R^2 greater than 0.97, whereas the coefficient varies from 0.83 to 1.05 (Fig. 4).

This finding could not be explained fully by pipetting error and PCR inhibition [19, 20] as PCR efficiency was always high. Inhibition effects on the DNA amplification can vary not only when using different primers, but also when the DNA concentration varies. The intercepts of these Cp values differ from -1.52 to 1.37, indicating that there may be different copies of genes inside mosquito cells [21, 22], which can be variable under different environmental conditions [23, 24]. As a result, care is needed when choosing primers for assessing the relative concentration of *Wolbachia*

Figure 4. Variation in Cp values when using different *Wolbachia* primers for the same samples. (a) wA and wsp primers in *Wolbachia* wAlbB screening; (b) wMA and wsp primers in *Wolbachia* wAlbB screening; (c) mos and aeg primers; (d) wM and wsp primers in *Wolbachia* wMel screening; (e) wMA and wsp primers in *Wolbachia* wMel screening; (f) w1 and wsp primers in *Wolbachia* wMel screening.

Conclusions

The primers provide a useful approach for detecting both of the currently released *Wolbachia* infections. Other options like multiplex probe assays and using DNA extraction kits to purify DNA are likely to cost more, and the Chelex[®] DNA extraction remains an easy and economic method for high-throughput *Wolbachia* screening [4]. Given potential variation within infections, we would still recommend undertaking comparisons between *Wolbachia* primers described in our study and other *Wolbachia*-specific primers in different field contexts. Comparisons with universal *Wolbachia* primers should also be undertaken before using the newly-designed primers in *Wolbachia* density calculations. The *wsp* primers still represent a useful pair of universal primers for amplifying the *Wolbachia* surface protein gene which has been used as a *Wolbachia* diagnostic for decades [14]. For the *Wolbachia* wMel or wAlbB infection in *Ae. aegypti*, our newly-designed primer pair, *wMA*, not only identifies wMel and wAlbB at the same time, but is also correlated with density estimates based on *wsp*, with correlation estimates for both wMel and wAlbB close to 1. Thus, the new primers have the potential to be an accurate and efficient approach for large-scale *Wolbachia* screening.

Acknowledgements

We thank Perran Stott-Ross and Jason Axford for providing the mosquito samples. We also thank support of Jasper Loftus-Hills award offered by the Faculty of Science, the University of Melbourne. This research is also funded by the National Health and Medical Research Council (1132412, 1118640, www.nhmrc.gov.au) and the Wellcome Trust (108508, wellcome.ac.uk).

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