A diagnostic primer pair to distinguish between \textit{wMel} and \textit{wAlbB} \textit{Wolbachia} infections

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Abstract

Detection of the \textit{Wolbachia} endosymbiont in \textit{Aedes aegypti} mosquitoes through real-time polymerase chain reaction assays is widely used during and after \textit{Wolbachia} releases in dengue reduction trials involving the \textit{wMel} and \textit{wAlbB} strains. Although several different primer pairs have been applied in current successful \textit{Wolbachia} releases, they cannot be used in a single assay to distinguish between these strains. Here, we developed a new diagnostic primer pair, \textit{wMwA}, which can detect the \textit{wMel} or \textit{wAlbB} infection in the same assay. We also tested current \textit{Wolbachia} primers and show that there is variation in their performance when they are used to assess the relative density of \textit{Wolbachia}. The new \textit{wMwA} primers provide an accurate and efficient estimate of the presence and density of both \textit{Wolbachia} infections, with practical implications for \textit{Wolbachia} estimates in field collected \textit{Ae. aegypti} where \textit{Wolbachia} releases have taken place.

Introduction

The bacterium, \textit{Wolbachia}, is providing an increasingly popular method to inhibit dengue virus transmission in the mosquito, \textit{Aedes aegypti}. \textit{Wolbachia}-infected populations involving the \textit{wMel} strain have now been successfully established in \textit{Ae. aegypti} in regions including northern Australia, Brazil and Indonesia [1–3], while \textit{wAlbB}-infected \textit{Ae. aegypti} have been established in Malaysia [4]. Detection of the \textit{Wolbachia} endosymbiont in \textit{Ae. aegypti} mosquitoes is a standard requirement for good laboratory practice during \textit{Wolbachia} mosquito releases in dengue reduction programs and for tracking \textit{Wolbachia} invasions in the field [4, 5]. Real-time polymerase chain reaction (real-time PCR) and High Resolution Melt (HRM) assays (SYBR® equivalent/non-probe) have been developed that enable detection and \textit{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 \textit{Wolbachia} and distinguish between multiple \textit{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], several real-time PCR assays using different primer pairs are currently required. Given that both \textit{wMel} and
AlbB 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 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 standardized 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. We also tested quantification cycle (Cq) [15] value differences between primers for different Wolbachia strains to assess primer suitability for relative Wolbachia density estimation.

Materials and methods

Diagnostic primer design

To develop the new primers, we screened for sequence differences between the wMel and wAlbB strains and then focused on the sequences of a DNA-directed RNA polymerase subunit beta/betagene with locus tag WD_RS06155 in wMel and its analogue in wAlbB. We then developed a new pair of primers designated wMwA (Table 1) to distinguish Wolbachia wMel and wAlbB in a single run of a real-time PCR assay, based on two base-pair mismatches at the 3’-end of each primer, which resulted in the Tm peak for wAlbB being separated from that of wMel. We checked the specificity of this primer pair by an initial test of six males and six females for each strain with different Wolbachia infection type (wMel- or wAlbB-infected or uninfected). Subsequent testing was done with female mosquitoes only.

Sample preparation

The wMel and wAlbB-infected Aedes aegypti were tested for strains transinfected previously [16, 17]. The wMel strain was collected from Cairns, Australia in 2019 from regions that had been invaded several years earlier [2, 12], while the wAlbB strain was derived from a wAlbB infected strain crossed to an Australian background and maintained in the laboratory [13]. An uninfected strain was developed from Ae. aegypti eggs collected in Cairns, Queensland, Australia prior to Wolbachia releases [10, 18].

Female mosquitoes of wMel-infected [17], wAlbB-infected [16] and uninfected were reared with TetraMin® fish food tablets in reverse osmosis (RO) water until the adult stage [19], and then were killed in absolute ethanol before Chelex® DNA extraction. In the standard procedure, DNA of an individual female 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 Chelex® 100 Resin solution containing DNA was centri-fuged at 12500 rpm for 5 min and DNA solution was pipetted from the supernatant.
LightCycler® efficiency test

After extraction, DNA concentration was measured using a Qubit™ 1X dsDNA HS Assay Kit and Qubit™ 2.0 fluorometer (ThermoFisher Scientific, Waltham, MA USA), 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 (Table 1). We also diluted the solution six times before making a three-fold dilution series to investigate the influence of Chelex®-extracted DNA 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) (S1 Table). We used 384-well plates with white wells (SSI Bio, Lodi CA USA, Cat. No. 3430–40), and the PCR conditions for DNA amplification beginning 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 produced showing the log3 [dilution factor] (x-axis) against mean Cq (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:

\[ E = \left(3^{\frac{1}{m}} - 1\right) \times 100\% \]

Compare with Chelex® extraction, we also purified DNA from the above Chelex® 100 Resin solution using the PureLink® Quick PCR purification Kit (Invitrogen Cat. No. K3100-01), in which the binding buffer B2 was used. In addition, a different DNA extraction method was used: female mosquitoes were homogenized individually in 100 μL STE buffer (10 mM Tris-HCl pH8, 100 mM NaCl, 1mM EDTA), and then incubated at 95°C for 10 minutes. After these extractions, 10 μL supernatant was pipetted into 90 μL ddH₂O and made a three-fold dilution series.

Primer quantification cycle comparison and density estimation

Following the efficiency study, we used a mixture of young (4±1 days since eclosion) and old (38 ±1 days since eclosion) female mosquitoes and tested for Cq value differences between primers for different Wolbachia strains to assess suitability for relative Wolbachia density
estimation. A total of 16 Wolbachia-infected mosquito samples 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, \( wMwA \), that can detect and distinguish between the \( w \)Mel and \( w \)AlbB infections in \( Aedes aegypti \) (Fig 1), which is important in simplifying current approaches for Wolbachia identification. In the initial test for the specificity of this primer pair, all uninfected samples were negative, and all Wolbachia-
infected samples were positive with distinctive Tm values from *Wolbachia w*Mel (82.6 ± 0.03˚C) and *w*AlbB (80.4 ± 0.02˚C) screening (Fig 1C). The high-resolution melt produces two joined peaks when the template contains both *Wolbachia w*Mel and *w*AlbB DNA (Fig 1D).

**Primer efficiency test**

We tested the efficiency of each of the primers for screening *Wolbachia* in *Ae. aegypti* by using a threefold dilution series. When template DNA was extracted in Chelex® 100 Resin solution, the efficiencies of all primers ranged from 86.4% to 104.9%, (Table 2 and Fig 2) and the efficiency curves all showed an R² valued greater than 0.99.

However, we found the amplification curve increase showed inhibition at the first dilution (Fig 3) for each of the primers, particularly when DNA was first diluted six times instead of ten times, resulting in outliers (S1 and S2 Figs and S2 Table). These results highlight a potential risk of lowering the relative density estimate in *Wolbachia* screening when using a highly concentrated Chelex®-extracted DNA solution. We also found differences between primer efficiency when a different DNA extraction method was used, with changes ranging from -22.2% to 29% (S3 Table). Different DNA extraction methods may affect DNA yield and quality, and/or change PCR inhibitors and their effects, which can increase variation between host and parasite DNA [20–22]. It is, therefore, worth noting that new standard curves should be run when changing to a different DNA extraction method, given that the efficiency of primers can deviate substantially from recommendations (90% - 110%) [23, 24] to prevent an inaccurate estimate of relative density being made.

**Cq value comparisons in Chelex® 100 Resin**

We noticed that primers had different Cq values even when screening the same individual organism/endo symbiont (*Ae. aegypti*, *w*Mel or *w*AlbB) and using the same DNA concentration, despite the efficiency of these primers all falling within 85% - 110%. We therefore tested the Cq ranges of the primers and correlated them with *wsp*. We found variation between these primers (Fig 2), which would be expected to result in differences in relative density estimates. The relationship between Cq values of different primers all fit into a linear relationship, with R² greater than 0.97, whereas the coefficient varies from 0.83 to 1.05 (Fig 4). For the newly-designed primer pair *w*MwA, the coefficients for *w*Mel and *w*AlbB are similar (0.97 for *w*Mel and 1.04 for *w*AlbB).

| Colony   | Primers | Slope of graph | R²   | Efficiency  | DNA concentration* (ng/μL) | Efficiency curve |
|----------|---------|----------------|------|-------------|-----------------------------|-----------------|
| Uninfected | *mos*   | -1.566         | 0.999| 101.659%    | 5.12                        | Fig 2A          |
| Uninfected | *aeg*   | -1.531         | 0.999| 104.946%    | 5.12                        | Fig 2B          |
| *w*Mel   | *w*1    | -1.644         | 0.999| 95.109%     | 5.24                        | Fig 2C          |
| *w*Mel   | *w*M    | -1.677         | 0.998| 92.559%     | 5.24                        | Fig 2D          |
| *w*Mel   | *w*sp*  | -1.767         | 0.999| 86.195%     | 5.24                        | Fig 2E          |
| *w*Mel   | *w*MwA  | -1.769         | 0.999| 86.107%     | 5.24                        | Fig 2F          |
| *w*AlbB  | *w*A    | -1.741         | 0.993| 87.953%     | 6.87                        | Fig 2G          |
| *w*AlbB  | *w*sp*  | -1.755         | 0.999| 87.032%     | 6.87                        | Fig 2H          |
| *w*AlbB  | *w*MwA  | -1.730         | 0.997| 88.732%     | 6.87                        | Fig 2I          |

*Template DNA was extracted in 250 μL 5% Chelex® 100 Resin and then diluted ten times before making a three-fold dilution series. Concentration was measured before dilution.*

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These primer differences could not be explained fully by pipetting error and PCR inhibition [25, 26]. Inhibition effects on DNA amplification can vary when using different primers, and/or when the DNA concentration varies. Intercepts of these C_q values ranged from -1.52 to 1.37 though all primers used in this study only have one copy based on their genomic sequences. However, it is possible that there may be different copies of Wolbachia genes inside mosquito cells [27, 28], such as is documented for the octomom region [29, 30] which can be variable under different environmental conditions [31, 32]. As a result, care is needed when choosing primers for assessing the relative concentration of Wolbachia.

In our study, the wsp primers represent a useful pair of universal primers for amplifying the Wolbachia surface protein gene which has been applied as a Wolbachia diagnostic for decades [14]. Given potential variation between Wolbachia primers, comparisons with universal Wolbachia primers should be undertaken before using the newly-designed primers in Wolbachia density calculations. Our newly-designed primer pair, wMwA, correlated with density estimates based on wsp, with coefficients for both wMel and wAlbB close to 1. Thus, this new primer pair has the potential to be accurate and efficient for large-scale Wolbachia detection and relatively density estimate.
Conclusions

Chelex® DNA extraction and real-time PCR provide an easy and economical approach for detecting both currently-released *Wolbachia (wMel and wAlbB)* infections in *Aedes aegypti*, while other options like multiplex probe assays and the use of DNA extraction kits are likely to cost more. Here, we designed a new primer pair, *wMwA*, which not only identifies *wMel* and *wAlbB* infections in *Aedes aegypti*. The primers are defined in Table 2.

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**Conclusions**

Chelex® DNA extraction and real-time PCR provide an easy and economical approach for detecting both currently-released *Wolbachia (wMel and wAlbB)* infections in *Aedes aegypti*, while other options like multiplex probe assays and the use of DNA extraction kits are likely to cost more. Here, we designed a new primer pair, *wMwA*, which not only identifies *wMel* and
wAlbB at the same time, but is also correlated with density estimates based on a universal Wolbachia primer wsp. We demonstrated this new primer pair has the potential to be accurate and efficient for large-scale Wolbachia detection and relatively density estimates, especially for use in field collected Aedes aegypti.

**Supporting information**

S1 Table. Real-time PCR reagents and volume in 384-well plates with white wells. (DOCX)

S2 Table. Primer efficiency when sample DNA was first diluted six times. (DOCX)

S3 Table. Primer efficiency when sample DNA was extracted using different methods. (DOCX)

S1 Fig. Primer efficiency when sample DNA was first diluted six times. DNA is extracted in 250 μL 5% Chelex® 100 Resin and then diluted six times before making a three-fold dilution series. Outliers are marked with red colour and are excluded from the efficiency curve. The primer names are defined in S3 Table. (PNG)

S2 Fig. Variation in the shape of the PCR amplification curves when sample DNA was first diluted six times. The curves from left to right represent amplification curves of 1/6, 1/18, 1/54, 1/162 and 1/486 DNA dilution from 250 μL 5% Chelex® 100 Resin. The primers are defined in S2 Table. (PNG)
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