Detecting wMel Wolbachia in field-collected Aedes aegypti mosquitoes using loop-mediated isothermal amplification (LAMP)

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Abstract

Background: The World Mosquito Program uses Wolbachia pipientis for the biocontrol of arboviruses transmitted by Aedes aegypti mosquitoes. Diagnostic testing for Wolbachia in laboratory colonies and in field-caught mosquito populations has typically employed PCR. New, simpler methods to diagnose Wolbachia infection in mosquitoes are required for large-scale operational use.

Methods: Field-collected Ae. aegypti mosquitoes from North Queensland were tested using primers designed to detect the Wolbachia wsp gene, specific to the strain wMel. The results were analysed by colour change in the reaction mix. Furthermore, to confirm the efficiency of the LAMP assay, the results were compared to the gold-standard qPCR test.

Results: A novel loop-mediated isothermal amplification (LAMP) colorimetric test for the wMel strain of Wolbachia was designed, developed and validated for use in a high-throughput setting. Against the standard qPCR test, the analytical sensitivity, specificity and diagnostic metrics were: sensitivity (99.6%), specificity (92.2%), positive predictive value (97.08%) and negative predictive value (99.30%).

Conclusions: We describe an alternative, novel and high-throughput method for diagnosing wMel Wolbachia infections in mosquitoes. This assay should support Wolbachia surveillance in both laboratory and field populations of Ae. aegypti.

Keywords: wMel, Wolbachia, LAMP, Diagnostics

Background

Arboviral diseases transmitted by the mosquito Aedes aegypti such as dengue, chikungunya and Zika constitute a significant burden to human health and economic development worldwide [1, 2]. This is reflected in the nomination by the World Health Organisation of dengue as one of the top ten global health threats in 2019. There is an urgent need for novel and efficient strategies to control these diseases [3].

The World Mosquito Program (WMP, https://www.worldmosquitoprogram.org, formerly known as the Eliminate Dengue Program) has developed a novel arboviral disease biocontrol strategy utilising the endosymbiotic bacterium Wolbachia pipientis. This maternally transmitted bacterium [4] is found in 40–60% of insect species worldwide [5–7]. WMP reported the successful introduction of the wMel strain of Wolbachia into Ae. aegypti in northern Australia since 2011 [8]. Subsequently, numerous studies demonstrated that the presence of wMel reduces dengue virus [9], Zika [10], chikungunya virus [11] yellow fever [11] and Mayaro virus [12] infection and replication and, in turn the virus transmission potential of the mosquito [13, 14]. wMel has been established in field populations of Ae. aegypti in five countries and it...
has been expanded to more than ten worldwide [15]. The mitigation of local dengue outbreaks in northern Australia [16] following the establishment of wMel is consistent with the laboratory and modelling expectations of this intervention [17].

A duplex TaqMan™ qPCR assay has been considered the gold-standard reference method for diagnosing Wolbachia infection in mosquitoes [5, 18]. The advantages of qPCR are clear: it is able to detect multiple genes of interest, produce quantitative or qualitative data [19] and is scalable. However, these advantages are significantly offset by high initial start-up costs and on-going maintenance of equipment, and the complexity of interpreting threshold values and amplification curves requires extensive training [20]. This is exacerbated in low-resource settings where the sourcing of laboratory equipment can be challenging, and technical expertise is not readily available. Given the rapid expansion of the World Mosquito Program [21, 22], there is a need to develop diagnostic tools suitable for resource limited settings.

Loop-mediated isothermal amplification (LAMP) [23, 24] is a technology potentially suitable for Wolbachia diagnostics in resource-limited laboratories [25, 26]. LAMP has been adapted as a nucleic acid test with multiple direct and indirect methods to detect several pathogens [27–30]. Hence the purpose of this study was to develop and validate the diagnostic accuracy of a colorimetric LAMP assay for the wMel strain of Wolbachia.

**Methods**

**Mosquito samples**

Field-collected *Ae. aegypti* mosquitoes (n = 3585) were collected from BG-Sentinel traps during and after Wolbachia establishment in Cairns, Townsville and Innisfail, Australia, over an eight-month period from June 2017. The DNA from each mosquito was individually crudely extracted in the laboratory as previously described [31] before being tested by both TaqMan™ qPCR [32] and LAMP assays.

**wMel LAMP reactions**

LAMP primers (Integrated DNA Technologies, Singapore, Singapore) were designed to detect the wsp gene from wMel and wMelPop-CLA strains using the software LAMP Designer 1.02 (PREMIER Biosoft International, Horsham, UK). Individual reaction consisted of 2X WarmStart® Colorimetric LAMP Master Mix (New England BioLabs, Ipswich, USA; Cat# M1800S), primers according to the manufacturer recommendation (Table 1), and 1 µl of target DNA in a total reaction volume of 17 µl. Reactions for individual samples were performed in 96-well PCR plates (LabAdvantage, Tingalpa, Australia; 96-well PCR plates, full skirt, clear). Plates were incubated in a thermocycler (BioRad C1000) at 65 °C for 30 min then held at 12 °C until scoring. Within one hour after incubation, colour changes of individual samples were recorded where pink indicates negative, yellow as positive and orange as equivocal (see Additional file 1: Figure S1). Results were interpreted by the naked eye directly from the reaction plates and also captured with a smartphone for data storage.

**wMel LAMP assay performance**

**Target specificity**

To assess the specificity of the LAMP assay for wMel, multiple Wolbachia trans-infected *Ae. aegypti* lines (wMel, wMelPop-CLA, wRi, wPip and wAlbB) were tested, in addition to two tetracycline-treated lines (wMel.tet and wAlbB.tet). For each mosquito line, three mosquitoes were tested and three technical replicates were performed, totalling nine reactions per line. Mosquitoes were reared and maintained as described [32], DNA was crudely extracted as previously described [31] and tested in the wMel LAMP assay.

**Diagnostic performance comparison**

Field-collected samples were tested using both the wMel LAMP and the reference TaqMan™ qPCR assays [32]. The qPCR assay was modified by the use of an LC-640-tagged wsp gene probe (fluorescence bandwidth

| Primer name | Primer sequence (5′–3′) | Length (bp) | Final primer concentrations (µM) |
|-------------|--------------------------|-------------|-------------------------------|
| FIP_wMel/wPop | TGTATGC GGCTGCATCAGCTCCGGTGTTCAATGTTGCTAA | 39 | 1.6 |
| BIP_wMel/wPop | GCAGAACTGGAAGGTTGGTTGATGCATGCGACTTGAATCG | 40 | 1.6 |
| F3_wMel/wPop | TGATGAACTTCGCAAAGTGCA | 20 | 0.2 |
| B3_wMel/wPop | CTAATGGAACAAAGCAGATCG | 21 | 0.2 |
| LpF_wMel/wPop | AGGCTGCCGGTGAATT | 18 | 0.4 |
| LpB_wMel/wPop | CAGCTCTGTATCCCAGGTGAGT | 22 | 0.4 |
Results

wMel LAMP assay performance

Target specificity

The wMel LAMP assay consistently detected only the wsp target sequence from the wMel strain in Ae. aegypti, and did not amplify the other six Wolbachia strains tested (Additional file 2: Figure S2).

Diagnostic performance on field-caught mosquitoes

A total of 3585 individual field-collected adult mosquitoes, sampled between June 2017 to February 2018 from Cairns, Townsville and Innisfail, were tested by both TaqMan™ qPCR and LAMP and the comparison between the results from each assay is shown in Table 2. Amongst the field-caught mosquitoes, 24 (0.7%) were found to be non-Ae. aegypti due to non-amplification of Ae. aegypti housekeeping Rps17 gene in duplex TaqMan™ qPCR assay, and hence excluded from the analysis.

Relative to the qPCR reference method, LAMP false positives were more likely (n=70) to occur than false negatives (n=8). Equivocal LAMP results were more likely to result from qPCR-negative samples than qPCR-positive samples. LAMP increased the estimation of wMel positivity by 2%, with an additional 1% of total samples producing equivocal results. The sensitivity of LAMP assay was close to 100% (Table 3) and this parameter was confirmed when performed in serially diluted field samples up to 1:1000 (data not shown). Also, wMel LAMP diagnostic had high positive and negative predictive values in relation to the wMel TaqMan™ qPCR (Table 3).

Discussion

TaqMan™ qPCR has been a mainstay for diagnosing Wolbachia infection in mosquitoes despite utilising expensive reagents and sophisticated equipment that require specialised training and maintenance [20]. Previous work has shown that there is potential to use LAMP for detecting Wolbachia in Ae. aegypti, either for any strain, targeting the 16S rRNA gene [25], or specifically targeting the wsp gene of the strains wAlbB and wPip [26]. Here, we have taken this framework and built on it by utilising a pH indicator that possesses the same characteristics but gives a greater resolution to differentiate between positive and negative results. The colorimetric LAMP assay in this study is an attractive candidate to replace qPCR because it does not require sophisticated equipment, is qualitative in nature, can easily be analysed by visual inspection and can be more cost-effective [33–35]. In addition, LAMP has been shown to be a reliable and robust assay across a range of DNA matrices [36] making it ideal for field-caught mosquito homogenates that can be highly variable. A small number of results were scored as false negatives. These could be explained by pipetting errors, or the presence of inhibitors of DNA amplification. Inhibitors such as EDTA, or human blood in blood-fed female mosquitoes, could block enzyme activity [37]. The frequency of false negatives was very low (0.22%), and does not affect the robustness of our assay.

When considering the implementation of colorimetric LAMP as the primary diagnostic method for monitoring the establishment of wMel, certain trade-offs should be recognised. First, compared to qPCR there may be an increased likelihood of contamination due to the high amplification efficiency of LAMP [23, 38]. Secondly, as the colorimetric LAMP assay is a single target nucleic acid test, it relies on entomologists to accurately identify Ae. aegypti mosquitoes from other species and insects that might be collected from the field. Thirdly, despite

Table 2: wMel LAMP positivity and negativity compared to qPCR

|       | qPCR-positive | qPCR-negative | Total |
|-------|---------------|---------------|-------|
| LAMP-positive | 2327          | 70            | 2397  |
| LAMP-negative  | 8             | 1128          | 1136  |
| LAMP-equivocal | 2             | 26            | 28    |
| Total          | 2337          | 1224          | 3561  |

Table 3: LAMP diagnostic parameters of LAMP-qPCR parallel testing

| Diagnostic parameter | Value  | 95% CI   |
|----------------------|--------|----------|
| Specificity          | 94.16  | 92.67–95.42|
| Sensitivity          | 99.66  | 99.33–99.85|
| Accuracy             | 97.79  | 97.25–98.25|
| Positive predictive value | 97.08  | 96.36–97.66|
| Negative predictive value | 99.30  | 98.60–99.65|

Abbreviation: CI, confidence interval
its robustness, the LAMP assay can produce equivocal results occasionally, presenting as wells with varying hues of the colour orange. However, equivocal results were rare (typically 1% of the samples) and did not significantly impact on the predictive ability of the assay. In general, this rate of equivocal findings should not adversely affect the chronological and geographical picture of Wolbachia establishment. Finally, the wMel LAMP assay is scored visually which may be subject to interpretation bias. To avoid possible visual biases, a smartphone application has been developed to conveniently and reliably score positivity and negativity, and this can promote consistency across multiple and international settings.

Conclusions

In conclusion, the wMel LAMP assay described here was sensitive, specific and suitable for high throughput application. With these results, we believe the assay is an appropriate tool to monitor the progress of wMel Wolbachia establishment in field Aedes aegypti populations worldwide in order to protect local communities from mosquito-borne diseases.

Additional files

- **Additional file 1: Figure S1.** Example of colorimetric LAMP result interpretation. Results are scored based on colour change. Samples (1) and (2) in yellow are positive for wMel Wolbachia; (3) and (4) in pink are negative; and (5) and (6) in orange considered equivocal.

- **Additional file 2: Figure S1.** Specificity of the wMel LAMP assay. LAMP reactions were performed using a number of Ae. aegypti lines infected with different Wolbachia strains per column, as follows: (1) wMel-infected, field-collected; (2) wMel, purified qDNA; (3) wAlbB; (4) Ae. aegypti tetracycline treated (without wAlB); (5) wMelPop-CLA; (6) wPip; (7) wRi; (8) wMelCS; (9) Ae. aegypti tetracycline treated (without wMel); (10) wild type uninfected Aedes aegypti from Townsville, Australia; (11) water; and (12) extraction buffer negative control. Eight technical replicates were run for controls.

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Authors’ contributions

DSG, DJH, NB and PK participated to the study design, performed experiments, contributed to obtaining the results and drafted the manuscript. YD contributed to the study design and drafted the manuscript. IOO, CPM and SLO participated on the study design. CPM and SLO coordinated the work and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All relevant data generated or analysed during this study are included in this published article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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