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High-Throughput PCR Assays To Monitor *Wolbachia* Infection in the Dengue Mosquito (*Aedes aegypti*) and *Drosophila simulans*

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We have developed and validated two new fluorescence-based PCR assays to detect the *Wolbachia* *w*Mel strain in *Aedes aegypti* and the *w*Ri and *w*Au strains in *Drosophila simulans*. The new assays are accurate, informative, and cost-efficient for large-scale *Wolbachia* screening.

The intracellular bacterium *Wolbachia pipientis* parasitizes and spreads in many arthropod hosts (13, 14). One classic example is the rapid sweep of the *Wolbachia* Riverside strain (*w*Ri) across populations of *Drosophila simulans* in California (11). This evolutionarily optimized mechanism has inspired the use of *Wolbachia* as a driver to alter insect population structure (10). The recent establishment of *w*Mel-infected, dengue virus-suppressing *Aedes aegypti* populations in Australia paves the way for similar programs in other countries (5, 12). In future release operations, rapid monitoring of *w*Mel in *Ae. aegypti* will remain an ongoing requirement.

Current molecular methods to detect *Wolbachia* in *Ae. aegypti* are based on PCR followed by electrophoresis (2, 3, 5, 7–9, 15). These assays might be adequate for routine applications, but they are not ideal for large-scale field experiments. Under field conditions, mosquito specimens often comprise a mixture of *Wolbachia*-infected and uninfected individuals. One potential problem is the amplification of trace amounts of exogenous *Wolbachia* DNA from *Wolbachia*-negative samples. It is therefore desirable to develop a robust screening assay that can simultaneously detect *w*Mel infection and quantify *w*Mel density.

Three sets of primers were developed for the *Ae. aegypti* assay: (i) *Aedes* universal primer pair *mRpS6*_F (5′-AGTTGAACGTATCGTTTCCCGCTAC) and *mRpS6*_R (5′-GAAGTGACGCAGCTTGTGGTCGTCC), which target the conserved region of the *RpS6* gene, to detect the presence of *Aedes* DNA (Fig. 1a); (ii) *Ae. aegypti* primers *aRpS6*_F (5′-ATCAAGAAGCGCCGTGTCG) and *aRpS6*_R (5′-CAGGTGCAGGATCTTCATGTATTCG), which target the *Ae. aegypti*-specific polymorphisms within the variable region of *RpS6*, to distinguish *Ae. aegypti* from non-*Ae. aegypti* specimens (Fig. 1a); (iii) *Wolbachia*-specific primers *w1*_F, (5′-AAAATCTTTGTGAAGAGGTGATCTGC) and *w1*_R (5′-GCACTGGGATGACAGGAAAAGG), to detect the presence of *Wolbachia* DNA (Fig. 1b).

PCR was carried out using the Roche LightCycler 480 system in a 384-well format (see the supplemental material). *Aedes aegypti* mosquitoes infected with *Wolbachia* (*w*Mel) produced robust amplification for all three markers (Fig. 2a). *Ae. aegypti* mosquitoes that were *Wolbachia* negative supported amplification of *mRpS6* and *aRpS6*, but not *w1* (Fig. 2b). *Aedes notoscriptus* mosquitoes showed strong amplification for the generic mosquito marker (*mRpS6*) but failed to support amplification of the *Ae. aegypti*-specific marker (*aRpS6*) and the *Wolbachia* marker (*w1*) (Fig. 2c). These results indicate that the assay is able to simultaneously distinguish (i) between *Ae. aegypti* and *Ae. notoscriptus* and (ii) between *Wolbachia*-infected and uninfected *Ae. aegypti* mosquitoes. We have termed this new genotyping method the RT/HRM (real-time PCR/high-resolution melt) assay. The RT/HRM assay results were consistent with the two traditional PCR/electrophoresis-based assays, namely, the Braig assay (2) and the Caragata assay (3).

The ability to quantify *w*Mel in *Ae. aegypti* is important for preventing detection of false positives in field samples. Primer efficiencies were not significantly different from 100% based on standard curve analysis of four *w*Mel$^+$ genomic DNA dilutions (0.1×, 0.05×, 0.025×, and 0.0125×). We used the crossing point (Cp) difference between the *aRpS6* and *w1* markers to estimate *Wolbachia* load. The average density, estimated as $2^{[(\text{Cp of } aRpS6) - (\text{Cp of } w1)]}$, was ~6 copies of *w*Mel per copy of *RpS6* of the host genome. We also subjected the same DNA dilutions to the traditional electrophoresis-based PCR method. All dilutions produced a single expected PCR product of similar intensity (see Fig. S3 in the supplemental material). This indicates that the RT/HRM method is able to detect and quantify *w*Mel at low concentrations, whereas the traditional assay is less quantitative.

Unlike in *Ae. aegypti*, in which *Wolbachia* has been artificially introduced, some populations of *D. simulans* in Australia are naturally infected with *Wolbachia*. One such strain is *w*Au, which does not induce host cytoplasmic incompatibility (4). The distribution of *w*Au in Australia has been documented, and the infection is generally found at low frequencies in populations of *D. simulans* on the east coast of Australia (4). Recently, sequencing of the *Wolbachia wsp* gene from *D. simulans* isofemale lines collected at Coffs Harbour in 2008 suggested that the *w*Ri strain (11) might be present in this population (A. R. Weeks, unpublished data).

To confirm the presence of *Wolbachia* *w*Ri and *w*Au strains in *Drosophila simulans*, we developed a new assay (Fig. 1c and d). We designed a pair of *RpS6* primers (*Dsim_RpS6*_F, 5′-CCAGATCGCTTCCAAGGAGGCTGCT-3′; *Dsim_RpS6*_R, 5′-GCCTCCTCGCGCTTGGCCTTAGAT-3′) to check for successful DNA isolation (Fig. 1c). To detect and differentiate *Wolbachia* *w*Ri and *w*Au infection in *D. simulans*, we designed a set of *Wolbachia*-specific

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FIG 1 Development of gene markers to detect and quantify *Wolbachia* infection in *Ae. aegypti* and *D. simulans.* (a) Coding sequences of the *RpS6* gene from *Ae. aegypti* and *Anopheles* (*An.*) *gambiae* were aligned; a conserved region was selected to place the universal *Aedes* primers *mRpS6*_F and *mRpS6*_R. Nucleotide alignments of the coding sequences from *Ae. aegypti* and *Ae. albopictus RpS6* were used to identify a variable region in which to position a pair of *Ae. aegypti*-specific primers (*aRpS6*_F and *aRpS6*_R), with 1 to 2 diagnostic nucleotides at the 3′ end of each primer. (b) The complete genome sequence of *Wolbachia w*Mel was used to design a *Wolbachia*-specific marker at the *VNTR-141* locus (see reference 9). The GenBank identifiers (GI) of the source sequences are given. Solid circles indicate polymorphic sites. (c) The variable region of the coding sequences in the *RpS6* gene between *Drosophila melanogaster* and *D. simulans* is shown. Primers were designed to amplify *D. simulans* but not *D. melanogaster.* Solid circles indicate polymorphic sites. (d) Universal primers were placed at the conserved regions between the *w*Ri and the *w*Au strains. These primers flank an ~290-bp highly variable region that contains 22 polymorphic sites. The GI numbers of the source sequences are given. Note: reverse primers (*mRpS6_R*, *aRpS6_R w1_R*, *Dsim_RpS6_R*, and *w1_R*) are illustrated in the sense direction; their 5′-to-3′ sequences are described in the text.

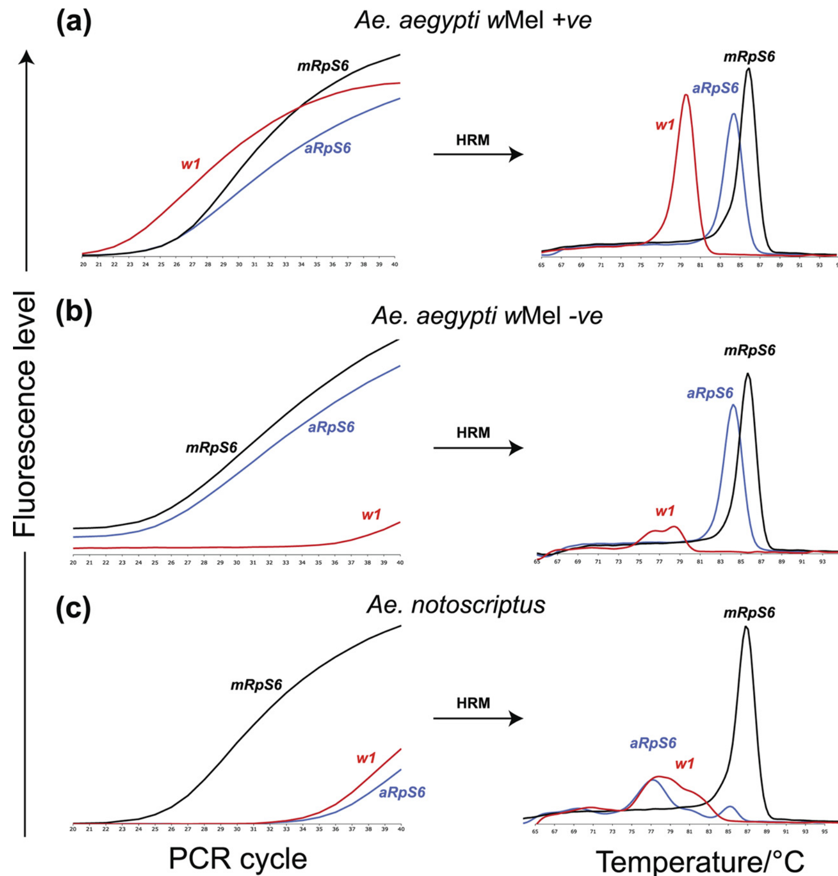

FIG 2 Performance and expected outcomes of the *Aedes* RT/HRM assay. (Left graphs) PCR amplification profile; (right graphs) amplicon-specific melting peaks (i.e., T_m). (a) *Ae. aegypti* that carries *w*Mel supports robust amplification of all three markers, with average Cp values (means ± 95% confidence intervals) of 27.86 ± 0.49 for *mRpS6*, 27.54 ± 0.49 for *aRpS6*, and 24.97 ± 0.83 for *w1*. The amplicons of these three markers had distinct T_m values: 85.65 ± 0.03°C for *mRpS6*, 84.33 ± 0.02°C for *aRpS6*, and 79.47 ± 0.02°C for *w1*. (b) *Ae. aegypti* that does not carry *w*Mel supports robust amplification of the *mRpS6* and *aRpS6* markers from mosquito host DNA but not of the *w1* primer from the *Wolbachia* DNA. (c) An *Ae. notoscriptus* mosquito could support amplification of only the universal *Aedes* marker (*mRpS6*), and not the *Ae. aegypti*-specific (*aRpS6*) or the *w*Mel-specific (*w1*) marker.

primers (*wsp*_validation_F, 5′-TTGGTTACAAAATGGACGACATCAG-3′; *wsp*_validation_R, 5′-CGAAATAACGAGCTCCAGCATAAAG-3′). The priming sites of the *Wolbachia* primers are located at conserved sequences flanking a variable region (22 polymorphisms) of the *wsp* gene between the *w*Au and the *w*Ri sequences (Fig. 1d). Among the 28 *D. simulans* flies from Coffs Harbor successfully genotyped, 17 were *Wolbachia* positive. A closer inspection of the melting temperatures (T_m) of the *wsp* products revealed two distinct T_m clusters that differed by ~0.5°C (Fig. 3). Sequencing of amplicons confirmed that the high-T_m cluster (~82.7°C) was the *w*Ri allele and the lower-T_m cluster (~82.2°C) was *w*Au. Since amplicon T_m is condition dependent, we believe that the 0.5°C T_m difference between *w*Ri and *w*Au is a more useful diagnostic than their respective T_m's. Based on this T_m-based genotyping method, there were 4 occurrences of *w*Ri (14.3%) 13 of *w*Au (46.4%), and 11 flies uninfected (39.3%) in Coffs Harbor, Australia.

The *w*Ri strain of *Wolbachia* has not previously been detected in Australian *D. simulans* populations (1, 4) and likely represents a new infection. The origin of the infection is unclear, but given the strong cytoplasmic incompatibility associated with this strain (6) and its incompatibility with the endemic *w*Au strain (4), it is likely

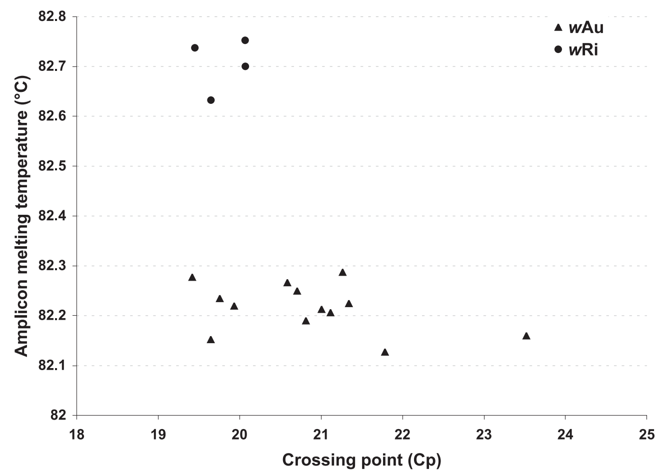

FIG 3 Classification of *Wolbachia w*Ri and *w*Au infection status based on amplicon melting temperature differences. The graph shows a plot of melting temperature against the crossing point (Cp) of the *wsp* validation PCR amplicon in 17 *Wolbachia*-positive individuals. The proposed *Wolbachia* genotypic clusters are indicated by triangles (*w*Au) and circles (*w*Ri).

that the distribution of the Australian *Wolbachia* infections in *D. simulans* populations will change over time.

While the traditional assays are sufficient for small-scale *Wolbachia* screening, the new RT/HRM assays provide high-throughput options to detect and quantify *Wolbachia* infection in *Ae. aegypti* and *D. simulans* at all life stages. We have successfully reduced the unit cost of genotyping such that large-scale field monitoring can be more feasible. Although the reagent costs have been minimized, the RT/HRM assay does require an initial capital investment in (or access to) an RT-PCR instrument capable of performing HRM analysis. In addition, the current specificity of the RT/HRM assays means that further adjustments will be needed if additional *Wolbachia* strains (e.g., *w*Melpop) are introduced into *Ae. aegypti* or *D. simulans* populations.

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