RESEARCH ARTICLE

Direct nucleic acid analysis of mosquitoes for high fidelity species identification and detection of *Wolbachia* using a cellphone

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Abstract

Manipulation of natural mosquito populations using the endosymbiotic bacteria *Wolbachia* is being investigated as a novel strategy to reduce the burden of mosquito-borne viruses. To evaluate the efficacy of these interventions, it will be critical to determine *Wolbachia* infection frequencies in *Aedes aegypti* mosquito populations. However, current diagnostic tools are not well-suited to fit this need. Morphological methods cannot identify *Wolbachia*, immuno-assays often suffer from low sensitivity and poor throughput, while PCR and spectroscopy require complex instruments and technical expertise, which restrict their use to centralized laboratories. To address this unmet need, we have used loop-mediated isothermal amplification (LAMP) and oligonucleotide strand displacement (OSD) probes to create a one-pot sample-to-answer nucleic acid diagnostic platform for vector and symbiont surveillance. LAMP-OSD assays can directly amplify target nucleic acids from macerated mosquitoes without requiring nucleic acid purification and yield specific single endpoint yes/no fluorescence signals that are observable to eye or by cellphone camera. We demonstrate cellphone-imaged LAMP-OSD tests for two targets, the *Aedes aegypti* cytochrome oxidase I (*coi*) gene and the *Wolbachia* surface protein (*wsp*) gene, and show a limit of detection of 4 and 40 target DNA copies, respectively. In a blinded test of 90 field-caught mosquitoes, the *coi* LAMP-OSD assay demonstrated 98% specificity and 97% sensitivity in identifying *Ae. aegypti* mosquitoes even after 3 weeks of storage without desiccant at 37˚C. Similarly, the *wsp* LAMP-OSD assay readily identified the *w*AlbB *Wolbachia* strain in field-collected *Aedes albopictus* mosquitoes without generating any false positive signals. Modest technology requirements, minimal execution steps, simple binary readout, and robust accuracy make the LAMP-OSD-to-cellphone assay platform well suited for field vector surveillance in austere or resource-limited conditions.

Author summary

Mosquitoes spread many human pathogens and novel approaches are required to reduce the burden of mosquito-borne disease. One promising approach is transferring *Wolbachia* into *Aedes aegypti* mosquitoes where it blocks transmission of arboviruses like dengue, Zika and Yellow fever viruses and spreads through mosquito populations. For effective evaluation of this approach, regular surveillance of *Wolbachia* infections in *Ae. aegypti* is required. However, current diagnostic tools, such as real time polymerase chain reaction, are not well suited to support these critical surveillance needs in resource poor settings due to their dependence on expensive instruments and technical expertise. To fill this need we developed a simple, robust and inexpensive assay to identify *Ae. aegypti* mosquitoes and *Wolbachia* using our unique one-pot assay platform, LAMP-OSD, which uses loop-mediated isothermal amplification to amplify nucleic acid targets at a single temperature. Unlike other LAMP-based tests, our assays assure accuracy by coupling amplification with novel nucleic acid strand displacement (OSD) probes that hybridize to specific sequences in LAMP amplification products and thereby generate simple yes/no readout of fluorescence readable by human eye and by off-the-shelf cellphones. To facilitate field use, we developed our assays so they are compatible with crushed mosquito homogenate as the template, meaning no nucleic acid extraction is required. In blinded tests using field collected mosquitoes, LAMP-OSD-cellphone tests performed robustly to identify 29 of 30 *Ae. aegypti* even after 3 weeks of storage at 37°C while producing only one false positive out of 60 non-specific mosquitoes. Similarly, our assay could identify *Wolbachia* in field-caught *Aedes albopictus* without producing any false positives. Our easy to use and easy to interpret assays should facilitate widespread field mosquito surveillance with minimal instrumentation and high accuracy.

Introduction

Mosquitoes are vectors that can transmit an array of pathogens that often cause devastating human diseases [1]. Traditionally considered a problem for tropical regions, mosquitoes are increasingly becoming a global public health challenge [2, 3] due to a changing global environment, urbanization, increases in the global movement of populations, and the emergence of insecticide resistance [4]. Estimates suggest nearly half the world's population is at risk for mosquito-borne diseases [5, 6], and as such, there is an urgent need for novel approaches to reduce the burden of disease.

One biocontrol countermeasure gaining traction for mosquito control is the release of *Wolbachia*-infected mosquitoes [7–9]. *Wolbachia* is a maternally-transmitted endosymbiont that can rapidly become established in the natural mosquito populations and can inhibit a variety of pathogens, including arboviruses, malaria parasites, and filarial nematodes [10–15]. *Wolbachia* control strategies are currently being deployed into the field to alter the capacity of *Aedes aegypti* to transmit arboviruses or to suppress mosquito populations [16–18]. Surveillance of transinfected mosquitoes as well as natural vector populations is crucial to evaluate the efficacy of these interventions [19]. However, most current screening methods rely on PCR, which is expensive and relies on laboratory facilities. In addition to screening for *Wolbachia* infection, it would also be desirable to identify the host mosquito species using these assays since different mosquito species differ in their ability to transmit pathogens [20]. Knowledge of vector species, and prevalence and stability of *Wolbachia* is essential for effective vector control and pre-emption of disease outbreaks with public health measures [21].

Unfortunately, mosquitoes are most commonly identified using morphological taxonomic keys. This process can be tedious, and requires highly trained personnel and undamaged mosquitoes. Alternative morphological methods such as the identification of morphometric wing characters [22] are low throughput and require microscopes and complex imaging instruments. Moreover, traditional morphological-based approaches cannot detect associated symbionts or pathogens. These limitations restrict widespread accessibility and necessitate sample preservation and transport. On the other end of the spectrum, immunoassay-based tools for identifying pathogen-infected mosquitoes, such as VecTest dipsticks (Medical Analysis Systems Inc.), are portable and inexpensive. However, these tests have poor sensitivity [23–25] and are not necessarily available for distinguishing mosquito species or identifying *Wolbachia* endosymbionts.

Nucleic acid tests can provide the necessary sensitivity and versatility for identifying both *Wolbachia* and mosquito species. However, since molecular testing is currently heavily reliant on PCR [26–28], opportunities for field-based determinations are limited, leading to significant delays and gaps in actionable surveillance. To support widespread vector surveillance inexpensive, portable, nucleic acid diagnostic platforms are needed that rapidly produce accurate results without requiring complex procedures, instruments, and laboratory infrastructure. In this regard, isothermal nucleic acid amplification assays such as loop-mediated isothermal amplification (LAMP) have begun to be employed because they do not require complex thermocycling instruments [29–31]. However, although LAMP can rival PCR for sensitivity it often produces spurious amplicons, which in turn lead to false positive readouts with non-specific reporters such as Mg^{2+} precipitation or fluorescent dye intercalation [32–34].

To mitigate the spurious signals that arise with LAMP, we have previously applied principles that were developed for nucleic acid strand exchange circuits [35–38] to the design of short hemiduplex oligonucleotide strand displacement (OSD) probes for LAMP [39]. The single stranded 'toehold' regions of OSD probes bind to LAMP amplicon loop sequences, and then signal via strand exchange [40] that leads to separation of a fluorophore and quencher [39]. OSDs are the functional equivalents of TaqMan probes and can specifically report single or multiplex LAMP amplicons without interference from non-specific nucleic acids or inhibitors [39, 41]. OSDs significantly enhance the diagnostic applicability of LAMP, allowing it to match the allelic specificity of real-time PCR. Recently, we engineered these molecular innovations to function fluently in one-pot LAMP-OSD reactions that can directly amplify a few tens to hundreds of copies of DNA and RNA analytes from minimally processed specimens and produce sequence-specific fluorescence signals that are easily observable by the human eye or (more importantly) by unmodified cellphone cameras [42]. The fluorescence endpoints that are produced can be used for yes/no determinations of the presence of an analyte, and also estimation of analyte copies on an order of magnitude scale [42, 43].

Here, we have adapted our smartphone-read one-pot LAMP-OSD system to directly amplify target nucleic acids from crudely macerated mosquitoes and to sequence-specifically report both mosquito and symbiont amplicons as visually readable fluorescence. In particular, we have developed two LAMP-OSD assays–one targeting the *Ae. aegypti* cytochrome oxidase I gene (*coi*), and the other the *Wolbachia* wAlbB surface protein (*wsp*) gene. Using a blinded set of field-caught mosquitoes, we demonstrate the exquisite sensitivity and specificity of our LAMP-OSD platform for identifying mosquito species and detecting *Wolbachia* infections.

Methods

Chemicals and reagents

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise indicated. All enzymes and related buffers were purchased from

New England Biolabs (NEB, Ipswich, MA) unless otherwise indicated. All oligonucleotides and gene blocks (summarized in **S1 Table**) were obtained from Integrated DNA Technologies (IDT, Coralville, IA, U.S.A.).

Preparation of amplification targets

Ae. aegypti coi LAMP-OSD target sequence was amplified by PCR from mosquito genomic DNA. LAMP-OSD target region of the *Wolbachia w*AlbB *wsp* gene was purchased as a gBlock fragment. Both amplification targets were cloned into the pCR2.1-TOPO vector (Fisher Scientific, Hampton, NH) by Gibson assembly according to manufacturer's (NEB) instructions [44]. Cloned plasmids were selected and maintained in an *E. coli* Top10 strain. Plasmid minipreps were prepared from these strains using the Qiagen miniprep kit (Qiagen, Valencia, CA, USA). All target inserts were verified by sequencing at the Institute of Cellular and Molecular Biology Core DNA Sequencing Facility.

LAMP primer and OSD probe design

*Wolbachia w*AlbB and *w*Pip strain *wsp* genes and *Ae. aegypti coi* gene sequences were obtained from NCBI GenBank. Consensus signature sequences were derived following MUSCLE (MUltiple Sequence Comparison by Log-Expectation) alignment analysis of each gene set. Target specificity of these signature sequences was evaluated by comparing them to respective *wsp* or *coi* gene sets from phylogenetically-related strains and species such as *Wolbachia w*Mel and *Ae. albopictus*, respectively. Both MUSCLE alignment as well as NCBI BLAST [45, 46] analysis were used for this *in silico* specificity analysis.

The Primer Explorer v5 primer design software (Eiken Chemical Co., Japan) was used for generating several potential LAMP primer sets composed of the outer primers F3 and B3 and the inner primers FIP and BIP. Primer design was constrained to include at least a 40 bp gap between the F1 and F2 or between the B1 and B2 priming sites. Primer specificity for targeted sequence and a corresponding lack of significant cross-reactivity to other nucleic acids of human, vector or pathogenic origin were further assessed using NCBI BLAST.

These primer sets were functionally tested in LAMP assays using zero to several hundred copies of purified plasmids as templates. Amplification kinetics were measured in real time using the fluorogenic intercalating dye Evagreen and the LightCycler 96 real-time PCR machine (Roche). The fastest primer sets that detected the fewest template copies with negligible spurious reactivity in the absence of templates were selected for further assay development.

Fluorogenic OSD probes were then designed to undergo toehold-mediated strand exchange with these *Ae. aegypti coi* and the *Wolbachia wsp* LAMP amplicons. Of the two target derived loop regions (between the F1c and F2, and the B1c and B2 primer binding sites) the regions between F1c and F2 were chosen as *wsp* and *coi* OSD binding regions (**S1 Fig**). Fluorogenic OSD probes were designed using the NUPACK software and our previously described engineering principles [39]. Briefly the hemiduplex OSDs were designed to display 11–12 nucleotide long single-stranded toeholds on the longer, fluorophore-labeled strands. All free 3'-OH ends were blocked with inverted dT to prevent extension by DNA polymerase.

Single loop primers were designed to bind the second loop region (between B1c and B2 primer binding sites) of the *wsp* and *coi* LAMP amplicons and accelerate LAMP amplification.

LAMP assay with synthetic DNA template

LAMP assays were assembled in a total volume of 25 µl of 1X Isothermal buffer (NEB; 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, pH 8.8 at 25˚C). The buffer was supplemented with 0.4 mM dNTPs, 0.8 M betaine, 2 mM additional $MgCl_2$,

1.6 μM each of FIP and BIP, 0.8 μM of loop primer, 0.4 μM each of F3 and B3 primers, and 16 units of *Bst* 2.0 DNA polymerase. Plasmid DNA templates were serially diluted in TE buffer (10 mM Tris-HCl, pH 7.5:0.1 mM EDTA, pH 8.0) immediately prior to use. Zero to several hundred copies of synthetic plasmid or gBlock templates were added to the LAMP reaction mixes followed by 90 min of incubation at 65˚C.

Real-time detection of LAMP amplicons using intercalating dyes

1X EvaGreen (Biotium, Hayward, CA, USA) was included in LAMP assays that were then analyzed using the LightCycler 96 real-time PCR machine (Roche, Basel, Switzerland). Reactions were subjected to 45 cycles of two-step incubations–step 1:150 sec at 65˚C, step 2: 30 sec at 65˚C. EvaGreen signal was measured in the FAM channel during step 2 of each cycle. Subsequently, amplicons were subjected to a melt analysis by incubation at 65˚C for 1 min followed by incremental rise in temperature to 97˚C. Amplicon melting was monitored by measuring fluorescence at the rate of 10 readings per˚C change in temperature. The resulting data was analyzed using the LightCycler 96 analysis software to measure Cq values for amplification and amplicon melting temperatures.

Real-time detection of LAMP amplicons using OSD probes

LAMP reactions monitored in real time using OSD probes were assembled and analyzed as above with the following changes. First, OSD probes were prepared by annealing 1 μM of the fluorophore-labeled OSD strand with 5 μM of the quencher-labeled strand in 1X Isothermal buffer. Annealing was performed by denaturing the oligonucleotide mix at 95˚C for 1 min followed by slow cooling at the rate of 0.1˚C/s to 25˚C. Excess annealed probe was stored at -20˚C. Annealed OSD probes were added to the LAMP reactions at a final concentration of 100 nM of the fluorophore-bearing strand.

Endpoint visual readout and smartphone imaging of LAMP-OSD assays

LAMP-OSD assays intended for visual readout and smartphone imaging were assembled in 0.2 ml optically clear thin-walled tubes with low auto-fluorescence (Axygen, Union City, CA, USA). Following 90 min of amplification at 65˚C, LAMP-OSD reactions were incubated at 95˚C for 1 min followed by immediate transfer to room temperature and fluorescence imaging. Images were acquired using an unmodified iPhone 6 and an UltraSlim-LED transilluminator (Syngene, Frederick, MD, USA). In some experiments, our previously described in-house 3D-printed imaging device [42] was used for LAMP-OSD fluorescence visualization and smartphone imaging. Briefly, this device uses Super Bright Blue 5 mm light emitting diodes (LED) (Adafruit, New York, NY, USA) to excite OSD fluorescence. Two cut-to-fit layers of inexpensive >500 nm bandpass orange lighting gel sheets (Lee Filters, Burbank, CA, USA) on the observation window filter the OSD fluorescence for observation and imaging.

Rearing and collection of mosquitoes

Ae. aegypti, *Ae. albopictus*, *Culex tarsalis*, *Cx. quinquefasciatus* (Houston), and *Cx. quinquefasciatus* (Salvador) mosquitoes were reared under conventional conditions in the insectary at the University of Texas medical Branch, Galveston, TX, USA. Four to seven day old mosquitoes were collected and immediately frozen for shipment, storage and subsequent testing. To obtain blood fed insects, *Aedes* mosquitoes were starved for a period of 24 hours then offered a sheep blood meal (Colorado Serum Company, Denver, CO, USA) using a hemotek membrane system (Hemotek). Unfed mosquitoes were separated and mosquitoes that were engorged were

collected 24 hours post feeding and processed in the same manner as unfed mosquitoes. For field collections, female mosquitoes were trapped using Fay prince trap (John W. Hock) baited with CO_2 in Galveston, Texas. 90 mosquitoes were morphologically identified and sorted into three blinded groups that were stored at -20°C, 4°C and 37°C, respectively for up to 3 weeks prior to LAMP-OSD analysis.

LAMP-OSD analysis of mosquitoes

For LAMP-OSD analysis individual mosquitoes were prepared either in 1 cc syringes or in 1.5 ml microcentrifuge tubes as follows. In-syringe preparation: The plunger was removed from a 1 cc syringe and a 0.5 μM pore size 1/8th inch diameter frit (catalog # 59037, Sigma-Aldrich, St. Louis, MO, USA) was placed inside the syringe. A single mosquito was placed on top of the frit and macerated thoroughly using the syringe plunger. 100 μl of water was aspirated into the syringe to fully re-suspend the macerated mosquito prior to evicting this mosquito-containing water from the syringe into a microcentrifuge collection tube. A 2 μl aliquot of this sample was directly tested by LAMP-OSD assays. In-tube preparation: A single mosquito was placed in a 1.5 ml microcentrifuge tube and manually macerated using a disposable micropestle (Fisher-brand RNase-Free Disposable Pellet Pestles, Cat # 12-141-364, Fisher Scientific, Hampton, NH, USA). Each macerated mosquito was resuspended in 100 μl water. A 2 μl 1:10 diluted aliquot of this mosquito sample was directly assessed by LAMP-OSD analysis.

For LAMP-OSD analysis of pools of mosquitoes 25, 50, or 100 mosquitoes were placed in 1.7 ml microcentrifuge tubes and processed by the 'in-tube' method. Briefly, the mosquito pools were macerated manually using a disposable micropestle. The macerated pools of 25, 50, or 100 mosquitoes were re-suspended in 250 μl, 500 μl, or 1000 μl of water, respectively. 2 μl aliquots of 1:10 and 1:100 dilutions in water of these mosquito pool macerates were then assayed by LAMP-OSD assays. Mosquito pools tested included Pool A (99 un-infected *Ae. aegypti*); Pool B (99 un-infected *Ae. aegypti* and one *Wolbachia*-infected *Ae. albopictus*); Pool C (49 un-infected *Ae. aegypti* and one *Wolbachia*-infected *Ae. albopictus*); and Pool D (24 un-infected *Ae. aegypti* and one *Wolbachia*-infected *Ae. albopictus*).

Statistical analysis

The paired results of morphological identification and LAMP-OSD analysis were compared using 2x2 contingency tables. Sensitivity or true positive rate was calculated by using the formula TP/(TP+FN) where TP are true positive samples, and FN are false negative samples. Specificity or true negative rate was calculated using the formula TN/(TN+FP) where TN are true negative samples and FP are false positive samples.

Results

Development of visually read LAMP-OSD assays to identify mosquito species and *Wolbachia* infection

LAMP uses two inner (FIP and BIP) and two outer (F3 and B3) primers specific to six consecutive target sequences (B3, B2, B1, F1c, F2c and F3c) (S1 Fig) [47]. *Bst* DNA polymerase extends these primers by strand displacement DNA synthesis to form 10^9 to 10^{10} copies of concatemerized amplicons with loops between the F1 and F2, B1 and B2, F1c and F2c, and B1c and B2c regions. We use an additional fifth primer that binds to one of these loop regions and accelerates amplification [41]. OSD probes, with blocked 3'-ends that prevents spurious signaling from polymerase-mediated extension, hybridize to the second loop region (S1 Fig).

To enable molecular identification of *Ae. aegypti* mosquitoes, we designed a smartphone-imaged LAMP-OSD assay to amplify and detect a signature sequence in the mitochondrial cytochrome c oxidase I (*coi*) gene. Each cell has multiple mitochondria and hence several hundred copies of the *coi* gene, which should enable detection from a very small amount of sample. Moreover, mitochondrial *coi* gene sequences are commonly used as barcodes for molecular identification of animal species including distinction of mosquito species; our chosen *coi* signature sequence was assigned to *Ae. aegypti* when queried against the Barcode of Life Data Systems (BOLD; http://www.boldsystems.org/index.php) *coi* signature sequence database [48–51].

We developed a second visually read LAMP-OSD assay targeting the *Wolbachia* surface protein (*wsp*) gene to identify *Wolbachia*-infected insects. The *wsp* gene is widely used as a marker for strain typing and screening for infected insect vectors [28, 52]. We engineered our *wsp* LAMP outer and inner primers to be complementary to, and hence amplify, two *Wolbachia* strains, the *w*AlbB and the closely related *w*Pip (**S2 Fig**). We deliberately designed our assay to detect both strains in order to ensure that we could assess field-collected mosquitoes irrespective of temporal and spatial variation in relative abundance of *w*AlbB-infected *Ae. albopictus* and *w*Pip-infected *Cx. quinquefasciatus* mosquitoes in our collection area [53, 54]. Significant nucleic acid sequence variation should prevent amplification of the *wsp* gene from all other *Wolbachia* groups (**S2 Fig**). To enable transduction of both *w*AlbB and *w*Pip *wsp* LAMP amplicons to visible fluorescence we designed an OSD probe that is specific to an identical loop sequence present in both amplicons.

With a single endpoint visual 'yes/no' readout of OSD fluorescence (either directly observed or imaged using cellphone camera), the *Ae. aegypti* *coi* LAMP-OSD assay could reliably identify the presence of as few as 4 copies of synthetic target DNA (**Fig 1**). Similarly, the cellphone-imaged *wsp* LAMP-OSD assay produced bright visible fluorescence when presented with only 40 copies of its target *wsp* sequences while remaining dark in the presence of synthetic *w*AlbA, *w*Aus, *w*Mors, and *w*Ana *wsp* templates (**Fig 1** and **S2 Fig**). In the absence of target DNA, neither assay generated spurious signal.

Direct LAMP-OSD analysis of crudely crushed individual mosquitoes

Our next goal was to demonstrate the ability of these LAMP-OSD assays to detect naturally occurring target sequences in mosquitoes. At the same time, we wanted to ensure that minimally processed samples would be compatible with our detection platform in order to facilitate rapid in-field vector testing with fewest instruments and user-required steps.

Therefore, as an initial approach, we developed the 'in-syringe' method for rapid sample preparation wherein individual mosquitoes were crushed inside 1 cc syringes using the syringe plunger as a pestle. A small chromatography column frit placed inside the syringe served as a pedestal that aided maceration and removed larger particulates when the macerated sample was re-suspended in water and recovered. Small portions (up to 8% of a LAMP-OSD reaction) of these macerated samples were added directly to LAMP-OSD reactions, which were then incubated for 90 min at 65°C to initiate and sustain amplification.

Endpoint visual examination of these assays for the presence or absence of OSD fluorescence revealed that our visually read LAMP-OSD system is compatible with direct analysis of crudely processed mosquitoes (**Fig 2**). The *coi* LAMP-OSD assay generated bright fluorescence readily distinguishable from sample auto-fluorescence when seeded with crudely prepared *Ae. aegypti* mosquitoes. In contrast, closely related *Ae. albopictus* failed to instigate false positive signal. Similarly, the *wsp* LAMP-OSD assay generated bright fluorescence in response to *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes, which are naturally infected with *w*AlbB and
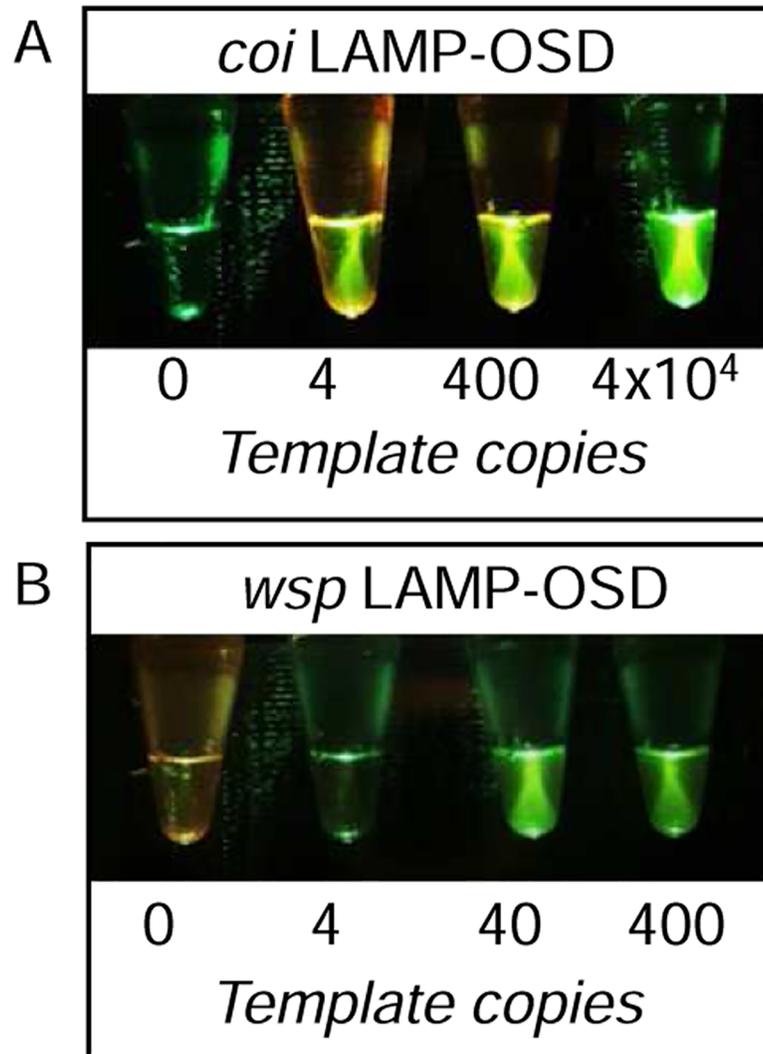
Fig 1. Detection of *Ae. aegypti coi* gene and *Wolbachia w*AlbB *wsp* gene using visually read LAMP-OSD assays. Indicated copies of recombinant plasmids bearing *coi* or *wsp* target sequences were amplified by the *coi*-specific (**A**) or *wsp*-specific (**B**) LAMP-OSD assays, respectively. OSD fluorescence was imaged at endpoint using a cellphone.

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*w*Pip *Wolbachia,* respectively, but remained negative in the presence of unrelated *Wolbachia w*Mel and uninfected mosquitoes (**Fig 2** and **S3 Fig**).

These results indicate that the *Wolbachia wsp* and *Ae. aegypti coi* LAMP-OSD assays are able to specifically amplify and signal the presence of their target DNA directly from crudely crushed mosquito samples without requiring any extraction and purification of nucleic acids. Furthermore, the large burden of non-specific nucleic acids as well as other molecular and macroscopic components present in a crude mosquito sample did not compromise signal accuracy. We also confirmed the absence of significant inhibition of amplification and signaling by recapitulating the detection limit of synthetic DNA targets in a background of crude non-specific mosquito sample. The *coi* and *wsp* LAMP-OSD assays could detect 4 and 40 target copies, respectively, even in the presence of 8% reaction volume of crude mosquito sample (**S4 Fig**).
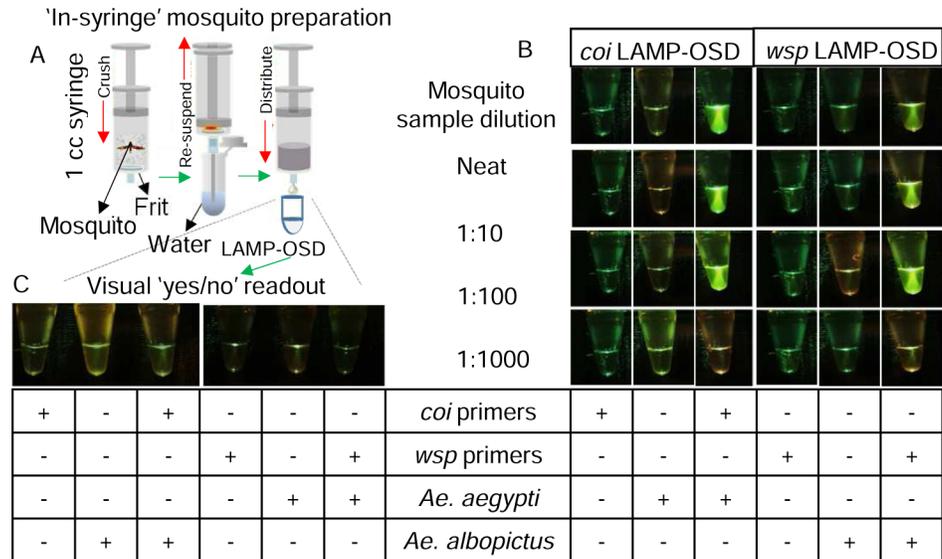
Fig 2. Visually read LAMP-OSD analysis of crudely processed individual mosquitoes. (A) Schematic depicting rapid preparation of crude mosquito samples for direct LAMP-OSD analysis by performing in-syringe individual mosquito maceration. **(B)** Analysis of individual *Ae. aegypti* and *w*AlbB *Wolbachia*-infected *Ae. albopictus* mosquitoes by *coi* and *wsp* LAMP-OSD assays, respectively. 2 μL of mosquito samples were either added directly to LAMP-OSD assays or subjected to 10-fold serial dilution in water prior to introduction in LAMP-OSD reactions. LAMP-OSD reactions without any macerated mosquito samples served as negative controls. Assays lacking LAMP primers but containing macerated mosquito samples served as controls for monitoring background fluorescence. **(C)** Specificity analysis of *coi* and *wsp* LAMP-OSD assays. The *coi* LAMP-OSD assay was challenged with 2 μL of *Ae. albopictus* in-syringe prepared crude mosquito sample. Similarly, the *wsp* LAMP-OSD assay was challenged with *Ae. aegypti* sample. In all experiments, OSD fluorescence was imaged at endpoint using a cellphone.

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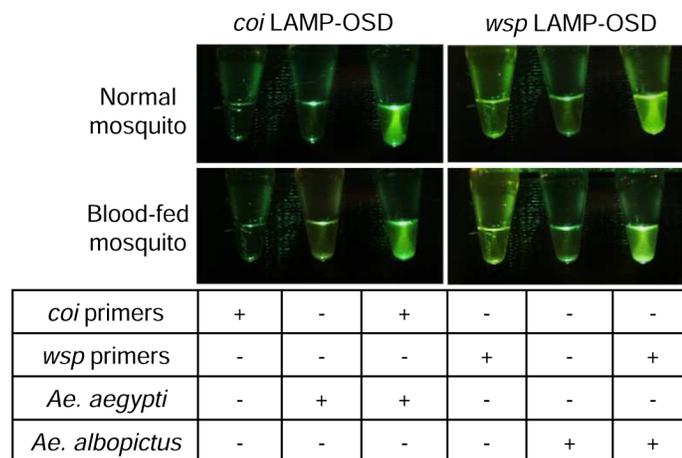
Fig 3. Effect of blood meal on LAMP-OSD analysis of crudely prepared individual mosquitoes. Individual normal or blood-fed *Ae. aegypti* and *Ae. albopictus* mosquitoes were prepared by the in-syringe method. 2 μL of these mosquito samples were analyzed by *coi* and *wsp* LAMP-OSD assays. LAMP-OSD reactions without any macerated mosquito samples served as negative controls. Assays lacking LAMP primers but containing macerated mosquito samples served as controls for monitoring background fluorescence. LAMP-OSD fluorescence was imaged at endpoint using a cellphone.

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Testing blood-fed mosquitoes with LAMP-OSD

Mosquitoes feeding on blood meals have been reported to engorge on 1 nL to as much as 6 μL of blood [55]. It is conceivable that the blood meal might confound visual LAMP-OSD fluorescence analysis by contributing auto-fluorescence. To ascertain compatibility of visually read LAMP-OSD with direct analysis of crudely prepared blood-engorged mosquitoes, we challenged both *coi* and *wsp* LAMP-OSD assays with crude in-syringe preparations of blood engorged *Ae. aegypti* and *Ae. albopictus* mosquitoes. Mosquitoes that had recently consumed a blood meal could be directly analyzed by visual LAMP-OSD without diminution of signal to noise ratio (**Fig 3**).
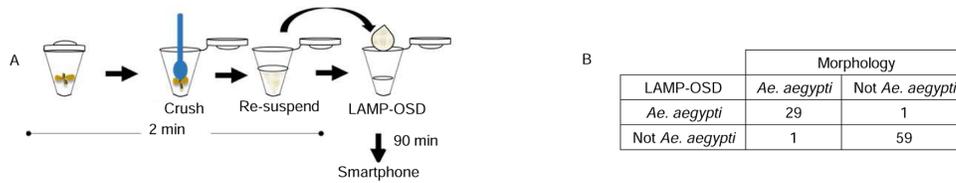
Analysis of blinded field-caught mosquitoes with cellphone-imaged LAMP-OSD assays

To validate assay performance under more rigorous conditions, we challenged the LAMP-OSD system with a blinded set of 90 field-caught mosquitoes comprised of *Ae. aegypti*, *Ae. albopictus*, and *Ochlerotatus* species. The mosquitoes were divided into three groups of 30 individuals that were stored without desiccant at -20˚C, 4˚C, or 37˚C for 1, 2, or 3 weeks prior to testing. To reduce mosquito processing cost, footprint, and time for this large study, we further simplified sample preparation requirements by optimizing the "in-tube" mosquito preparation method wherein each mosquito was crushed with a micropestle directly in a microcentrifuge tube followed by resuspension in water and introduction in a LAMP-OSD reaction.

The visually read *coi* LAMP-OSD assay demonstrated an overall sensitivity (true positive rate) of 97% and specificity (true negative rate) of 98% when compared to morphological typing of field-caught mosquito species (**Figs 4, S5, S6 and S7**). On closer inspection of the data, it is evident that even after three weeks of mosquito collection and storage at temperatures as high as 37˚C the *coi* LAMP-OSD assay was correctly able to identify 29 out of 30 *Ae. aegypti* mosquitoes. The single mosquito that the LAMP-OSD assay failed to identify had been stored at 37˚C for a week prior to testing. We ruled out lack of amplifiable nucleic acids or their incompatibility with *coi* LAMP primers and OSD probe by PCR amplifying the relevant *coi* LAMP target and verifying its sequence.

Of the 60 non-*Ae. aegypti* mosquitoes analyzed by *coi* LAMP-OSD, only one mosquito generated a false positive signal. Sequence analysis of its *coi* gene ruled out mis-firing of the *coi* LAMP-OSD assay. It is possible that this LAMP assay was inadvertently contaminated with a small amount of a pre-formed *Ae. aegypti* amplicon.

The *Wolbachia* *w*AlbB/*w*Pip *wsp* LAMP-OSD assay did not generate a positive signal from any non-*Ae. albopictus* mosquito. This is expected since natural populations of *Ae. aegypti* and most *Ochlerotatus* species are not infected with *Wolbachia* [56, 57]. However, ability of the *wsp* assay to identify *Wolbachia* infection was influenced by the storage temperature of mosquitoes. The *wsp* LAMP assay could readily identify *Wolbachia* infection in 3 out of 4 *Ae. albopictus* mosquitoes stored at -20˚C for as long as 3 weeks. PCR analysis of the *wsp*-negative mosquito using previously described primers (81F and 691R) and protocols [28] did not produce amplicons suggesting that this individual was likely uninfected or had *Wolbachia* levels below the levels detectable by PCR. As the storage temperature was increased the frequency of *Wolbachia* detection dropped. While 40% of *Ae. albopictus* mosquitoes stored at 4˚C gave a positive *wsp* LAMP-OSD signal, none of the *Ae. albopictus* mosquitoes kept at 37˚C for even as little as 1 week were *wsp* positive. All mosquitoes that failed to generate a signal by LAMP also failed to produce *wsp* PCR amplicons. Since, 95–99% of *Ae. albopictus* mosquitoes in the wild are typically found to be infected with *Wolbachia* [58], these results are suggestive of nucleic acid deterioration in mosquitoes upon storage at high temperature.

B

	Morphology	
LAMP-OSD	*Ae. aegypti*	Not *Ae. aegypti*
Ae. aegypti	29	1
Not *Ae. aegypti*	1	59

C

Sample ID	Mosquito morphology	coi LAMP-OSD	wsp LAMP-OSD	Sample ID	Mosquito morphology	coi LAMP-OSD	wsp LAMP-OSD	Sample ID	Mosquito morphology	coi LAMP-OSD	wsp LAMP-OSD	Storage
	(-)20 degrees Celsius				4 degrees Celsius				37 degrees Celsius			
1	*Ochlerotatus sp.*	N	N	61	*Ae. albopictus*	N	Y	31	*Ae. albopictus*	N	N	
2	*Ae. aegypti*	Y	N	62	*Ochlerotatus sp.*	N	N	32	*Ochlerotatus sp.*	N	N	
3	*Ochlerotatus sp.*	N	N	41	*Ae. aegypti*	Y	N	33	*Ae. aegypti*	Y	N	
4	*Ochlerotatus sp.*	N	N	64	*Ochlerotatus sp.*	N	N	34	*Ochlerotatus sp.*	N	N	
5	*Ochlerotatus sp.*	N	N	65	*Ochlerotatus sp.*	N	N	35	*Ae. aegypti*	Y	N	Week 1
21	*Ae. albopictus*	N	Y	66	*Ae. albopictus*	N	N	43	*Ochlerotatus sp.*	N	N	
7	*Ae. aegypti*	Y	N	67	*Ae. aegypti*	Y	N	37	*Ochlerotatus sp.*	N	N	
8	*Ae. aegypti*	Y	N	68	*Ochlerotatus sp.*	N	N	38	*Ae. aegypti*	N	N	
76	*Ochlerotatus sp.*	N	N	81	*Ochlerotatus sp.*	N	N	39	*Ochlerotatus sp.*	N	N	
10	*Ochlerotatus sp.*	N	N	82	*Ochlerotatus sp.*	N	N	51	*Ochlerotatus sp.*	N	N	
	(-)20 degrees Celsius				4 degrees Celsius				37 degrees Celsius			
11	*Ae. aegypti*	Y	N	71	*Ochlerotatus sp.*	Y	N	63	*Ochlerotatus sp.*	N	N	
12	*Ochlerotatus sp.*	N	N	72	*Ae. albopictus*	N	N	42	*Ae. aegypti*	Y	N	
13	*Ochlerotatus sp.*	N	N	73	*Ae. albopictus*	N	Y	36	*Ae. albopictus*	N	N	
14	*Ae. aegypti*	Y	N	74	*Ochlerotatus sp.*	N	N	44	*Ochlerotatus sp.*	N	N	
15	*Ae. aegypti*	Y	N	75	*Ae. aegypti*	Y	N	45	*Ae. aegypti*	Y	N	Week 2
16	*Ochlerotatus sp.*	N	N	9	*Ae. aegypti*	Y	N	46	*Ochlerotatus sp.*	N	N	
17	*Ochlerotatus sp.*	N	N	77	*Ochlerotatus sp.*	N	N	47	*Ae. aegypti*	Y	N	
25	*Ae. albopictus*	N	Y	78	*Ochlerotatus sp.*	N	N	48	*Ae. aegypti*	Y	N	
19	*Ae. aegypti*	Y	N	84	*Ochlerotatus sp.*	N	N	49	*Ochlerotatus sp.*	N	N	
20	*Ae. aegypti*	Y	N	80	*Ae. aegypti*	Y	N	50	*Ochlerotatus sp.*	N	N	
	(-)20 degrees Celsius				4 degrees Celsius				37 degrees Celsius			
6	*Ochlerotatus sp.*	N	N	69	*Ae. albopictus*	N	N	40	*Ae. albopictus*	N	N	
22	*Ae. albopictus*	N	Y	70	*Ae. aegypti*	Y	N	52	*Ae. aegypti*	Y	N	
23	*Ae. aegypti*	Y	N	83	*Ochlerotatus sp.*	N	N	53	*Ae. aegypti*	Y	N	
24	*Ae. albopictus*	N	N	79	*Ae. aegypti*	Y	N	90	*Ochlerotatus sp.*	N	N	
18	*Ae. aegypti*	Y	N	85	*Ochlerotatus sp.*	N	N	55	*Ae. aegypti*	Y	N	Week 3
26	*Ochlerotatus sp.*	N	N	86	*Ochlerotatus sp.*	N	N	56	*Ochlerotatus sp.*	N	N	
27	*Ochlerotatus sp.*	N	N	87	*Ochlerotatus sp.*	N	N	57	*Ochlerotatus sp.*	N	N	
28	*Ochlerotatus sp.*	N	N	88	*Ochlerotatus sp.*	N	N	58	*Ochlerotatus sp.*	N	N	
29	*Ochlerotatus sp.*	N	N	89	*Ochlerotatus sp.*	N	N	59	*Ochlerotatus sp.*	N	N	
30	*Ae. aegypti*	Y	N	54	*Ae. aegypti*	Y	N	60	*Ae. aegypti*	Y	N	

Fig 4. Blinded LAMP-OSD analysis of field-caught mosquitoes. Mosquitoes stored for up to three weeks at the indicated temperatures were prepared by 'in-tube' crude processing. 2 μL of a 1:10 dilution of each mosquito sample was analyzed by *wsp* and *coi* LAMP-OSD assays. OSD fluorescence was imaged at endpoint using a cellphone. **(A)** Schematic depicting 'in-tube' method for rapid preparation of field-caught mosquitoes for LAMP-OSD analysis. **(B)** Comparison of *coi* LAMP-OSD results with morphological identification using 2X2 contingency table. **(C)** Tabulation of *coi* and *wsp* LAMP-OSD readout for each mosquito in the study. *Ae. aegypti*, *Ae. albopictus* and *Ochlerotatus* species are highlighted in blue, orange and white, respectively. 'Y' indicates a positive LAMP-OSD signal (bright fluorescence). 'N' indicates absence of any fluorescence signal in the LAMP-OSD reaction. Red boxes highlight LAMP-OSD tests that generated false positive or false negative outcomes.

Smartphone-imaged LAMP-OSD analysis of pooled mosquitoes

For time- and cost-efficient mosquito surveillance high-throughput analysis of pooled mosquitoes rather than individual insects is often necessary [59, 60]. To determine the utility of our assay platform for analyzing mosquito pools we created four sample pools comprising *Wolbachia*-infected *Ae. albopictus* and un-infected *Ae. aegypti* mosquitoes–Pool A: 99 *Ae. aegypti*, Pool B: 1 *Ae. albopictus* and 99 *Ae. aegypti*, Pool C: 1 *Ae. albopictus* and 49 *Ae. aegypti*, and
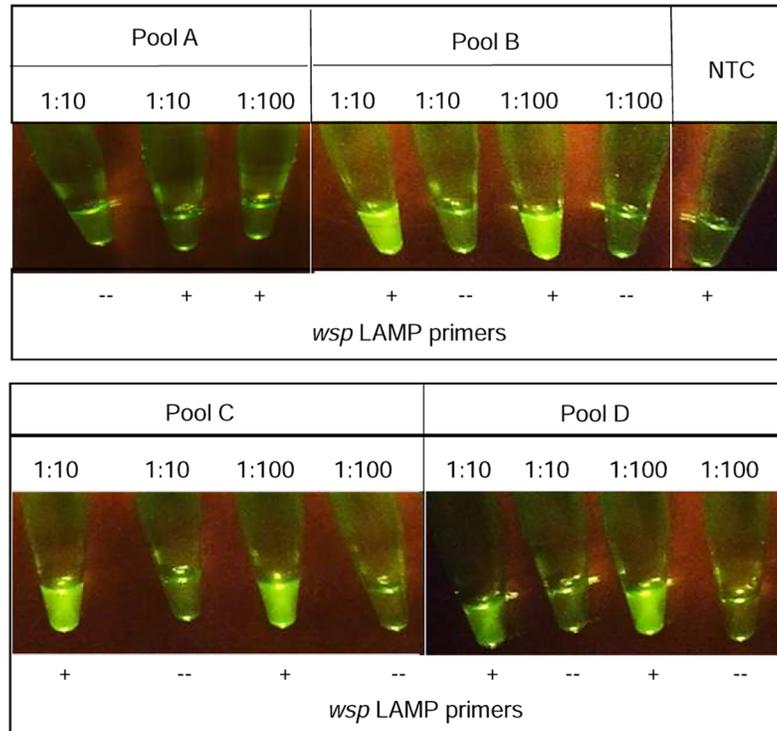
Fig 5. LAMP-OSD analysis of crudely prepared mosquito pools. Pools of mosquitoes crudely prepared by 'in-tube' manual maceration were diluted 1:10 or 1:100 in water and directly analyzed using *wsp* LAMP-OSD assays. Smartphone images were taken at endpoint following 90 min of amplification. Representative results from three biological replicate experiments that included two technical replicates each are depicted. Pool A: 99 un-infected *Ae. aegypti*; Pool B: 1 *Wolbachia*-infected *Ae. albopictus* and 99 uninfected *Ae. aegypti*; Pool C: 1 *Wolbachia*-infected *Ae. albopictus* and 49 un-infected *Ae. aegypti*; and Pool D: 1 *Wolbachia*-infected *Ae. albopictus* and 24 un-infected *Ae. aegypti*. Negative controls include no-template control (NTC), and reactions lacking LAMP primers.

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Pool D: 1 *Ae. albopictus* and 24 *Ae. aegypti*. Entire pools were subjected to 'in-tube' crude sample preparation followed directly by LAMP-OSD analysis for *Wolbachia wsp*. Our data demonstrate that LAMP-OSD could readily detect the presence of a single *Wolbachia*-infected mosquito in pools of 25, 50, and 100 mosquitoes (**Fig 5**). No false signals were produced by pools of 99 *Wolbachia*-free *Ae. aegypti* mosquitoes. These results suggest that our rapid sample preparation method and smartphone-read LAMP-OSD assays can be used for accurate analysis of both individual and pooled mosquitoes.

Discussion

Mosquito control strategies that rely on the introduction of *Wolbachia* are now being deployed around the world [7–9], and the surveillance of efficacy and spread require agile, field-based methods for both mosquito and symbiont detection. Unfortunately, currently available tools for mosquito diagnostics have several shortcomings. Morphological identification methods are inherently low throughput, require extensive technical expertise, and cannot also readily identify pathogens or biocontrol agents such as *Wolbachia*. Spectroscopy, such as near infrared spectroscopy [61] and Fourier transform infrared spectroscopy [62], allow identification of mosquito species, *Wolbachia*, and pathogens, but require expensive instruments and expertise that are generally incompatible with low-resource settings. Immunoassays can detect pathogen-carrying vectors but are insensitive and cannot also identify vector species. Nucleic acid

amplification methods could potentially look at both vector and symbiont sequences, but are heavily reliant on PCR with the ensuing encumbrances of expensive instruments and trained operators, again precluding widespread use.

Isothermal nucleic acid amplification assays would facilitate field-based vector monitoring, but most reported approaches rely on nucleic acid purification and non-specific readout, and thus suffer from laborious setup and the risk of false positives [63–65]. Probe-read isothermal methods such as the recombinase polymerase assay (RPA) are more reliable but still require expensive and proprietary reaction formulations and probes, which limits their flexibility and versatility in assay engineering. Furthermore, most RPA applications for vector diagnostics [66] also depend on extensive sample processing and nucleic acid purification prior to amplification.

These drawbacks led us to develop a simpler, more robust field-deployable assay based on loop-mediated isothermal amplification (LAMP) that can identify both mosquito species and specific *Wolbachia* strains by direct analysis of crudely macerated individual or pooled mosquitoes.

While LAMP assays have previously been developed for *Wolbachia* detection by targeting the 16S rRNA gene for amplification [63], these assays required extraction and purification of DNA prior to assay, and used non-specific readouts that were highly prone to false positive signals. To overcome these barriers to the use of LAMP, we have previously adopted methods that originated in the field of nucleic acid computation: the use of strand exchange reactions that initiate at complementary single-stranded 'toeholds' and progresses via branch migration. The base-pairing predictability and programmability of strand exchange kinetics promotes the construction of exquisitely sequence-specific oligonucleotide strand displacement (OSD) probes for LAMP amplicons (**S1 Fig**), thereby greatly reducing the detection of non-specific amplification background [39, 41, 42]. For instance, we recorded positive *coi* LAMP-OSD signals from field-caught *Ae. aegypti* mosquitos but did not detect signal from closely related *Ae. albopictus* and *Ochlerotatus* species mosquitoes. Similarly, the *wsp* assay detected *w*AlbB and *w*Pip, as expected, but not *w*Mel, *w*Mors, *w*Ana, *w*Aus, or *w*AlbA *Wolbachia*.

Strand exchange circuits have the additional advantage that they can be used to embed algorithms and act as 'matter computers' [35–37, 67]. For example, strand exchange transducers can logically integrate multiple analytes; transform nucleic acids to glucose and human chorionic gonadotrophin (hCG); adapt readout to beads, paperfluidics, glucose meters, pregnancy test strips, and cellphones, and allow target copy number estimation using a single endpoint yes/no readout of presence or absence of signal above an adjustable threshold [68–71]. In the current instance, we deliberately designed our *wsp* LAMP-OSD assay to 'compute' the presence of both *w*AlbB and *w*Pip in order to increase our odds of finding infected field-caught mosquitoes. However, the dependence of strand exchange efficiency on toehold binding strength [39] can be exploited to engineer yes/no distinctions between strain-specific single nucleotide polymorphisms [39], and the same dual *wsp* assay could be rendered strain-specific by simply substituting an OSD reporter specific to an alternate polymorphic loop sequence (**S2 Fig**). This might be advantageous for strain discrimination if double infections were released during vector control measures [72, 73].

By using our one-pot LAMP-OSD assay, macerated mosquito homogenates could be directly analyzed and 'yes/no' visual readouts could be quickly ascertained with a cell phone in the field without the requirement for laboratory equipment or technically training. Moreover, since our assays can accurately analyze mosquitoes several days after capture–the *coi* LAMP-OSD assay could for example identify mosquitoes after 3 weeks at 37˚C without desiccant–mosquitoes from remote collection outposts can potentially be analyzed even after delayed retrieval. We are currently automating the assays and workflow on low-cost modular paper

and plastic devices that will not only further streamline diagnostic application, especially for high-throughput analysis, but will also provide biohazard and aerosol containment by restricting mosquito maceration and molecular assay in sealed chambers. The flexibility of assay timing is further accommodated by the fact that lyophilized LAMP-OSD reaction mixes can be stored and deployed without cold chain [42].

Our sample preparation and assay workflow not only simplify application of molecular diagnostics for surveillance but should also reduce operational costs by eliminating the need for nucleic acid extraction and complex instruments for assay incubation and readout. Market cost of LAMP-OSD assay reagents is ~$1.5/reaction. We are in the process of significantly reducing this cost by substituting commercially sourced purified Bst 2.0 DNA polymerase with our recently developed 'cellular reagents' as a cheaper alternative [74]. Open system Taq-Man qPCR assays have been reported to also cost $1.51/reaction [75]. Our re-usable in-house 3D-printed device [42] for fluorescence visualization costs <$5 in parts to build and enables 'instrument-free' readout of visual signal at no additional cost. Fluorescence signal may also be captured for posterity using unmodified standalone camera or any camera cellphone including low cost (<$200) models [42]. In contrast, lab-based qPCR testing is estimated to require ~ $30,000 in startup investment and ~$700 in annual maintenance [75].

These combined features make our assay platform the best tool to date for expanding vector surveillance to resource poor settings [76], especially in that the ease of use should allow minimally trained citizen scientists to participate in otherwise sophisticated public health monitoring operations in the field.

A few caveats must be considered for any nucleic acid based test including qPCR and LAMP-OSD. First, without prior knowledge of integration sites, nucleic acid amplification tests will be unable to differentiate *wsp* target sequences derived from infectious *Wolbachia* bacterial genomes versus *Wolbachia* DNA integrated in nuclear genomes of hosts such as *Drosophila ananassae* [77]. However, other PCR-based assays also face this challenge. Second, nucleic acid amplification may not be significantly diminished by the presence of one or two mismatches between a primer and its target binding site [41, 78]. While, ability to generate a positive signal from very closely related target variants that differ by only one or two nucleotides is desirable, it is important for a nucleic acid test to distinguish polymorphic strains. Unlike two-primer systems such as qPCR, use of at least six primer binding sites during LAMP allows LAMP primers to scan a three times larger target sequence space for distinguishable polymorphisms. As a result, LAMP assays are more likely to distinguish unexpected polymorphic strains due to the likelihood of encountering mismatches in multiple primers that would in turn lead to diminution of amplification and false positive signals.

The development efforts that we have put into LAMP-OSD should now allow it to be generalized to screening other microbes or mosquito phenotypes in field settings. For example, LAMP-based assays have been developed to identify pathogens transmitted by mosquitoes and insecticide resistance alleles [79–82], but these rely on purified nucleic acid as templates and non-specific readout whereas our method functions with mosquito homogenate and ensures accuracy using unique sequence-specific strand exchange probes. In addition, this technology could be used to identify gut microbes and insect-specific viruses associated with mosquitoes, which is of growing interest given that it is becoming clear that the microbiome can shape vector competence for human pathogens [83–85]. Overall, we have demonstrated a versatile nucleic acid diagnostic platform for rapid and accurate analyses of both insect vectors and symbionts, and that can now be further configured for additional applications.

Supporting information

S1 Table. Primers, probes, and target sequences for *A. aegypti coi* and *Wolbachia w*AlbB/*w*Pip *wsp* LAMP-OSD assays.
(PDF)

S1 Fig. LAMP-OSD schematic. LAMP uses 2 inner (FIP and BIP) and 2 outer (F3 and B3) primers specific to 6 blocks of target sequences designated as B3, B2, B1, F1c, F2c and F3c. F2 sequence in FIP (F1c-F2) initiates amplification by Bst DNA polymerase (Stage I). F1c sequence in FIP self-primes subsequent amplification. Similarly, BIP (B1c-B2) initiates DNA synthesis by binding to B2c. F3 and B3 primer-initiated DNA synthesis displaces preceding inner primer-initiated strands, which serve as templates for primer-initiated strand displacement DNA synthesis (Stage II). 3′-ends of the resulting single-stranded, dumbbell-shaped amplicons (Stage III) are extended by Bst polymerase to form hairpins (Stage IV). Inner primers hybridize to the single-stranded loops and initiate another round of strand displacement synthesis that opens the original hairpin to form a concatemerized amplicon containing a self-priming 3′-end hairpin (Stage V). The ensuing continuous amplification (initiated both by new inner primers and by self-priming) generates increasingly complex, double-stranded concatameric amplicons containing self-priming hairpins and single-stranded loops to which the OSD probe hybridizes. "c": denotes complementary target sequences. F and Q on the OSD denote fluorophore and quencher, respectively. OSD probe is denoted in terms of numbered domains, each of which represents a short fragment (usually <12 nt) of DNA sequence in an otherwise continuous oligonucleotide strand. Single stranded toeholds are numbered in red. Complementarity between numbered OSD domains is denoted by a single prime symbol.
(PDF)

S2 Fig. Comparison of *wsp* sequences from different *Wolbachia* strains. Alignment using *w*AlbB as the reference strain (**A**). "Current *wsp* OSD" refers to the *wsp* OSD probe used in the present study that binds to the loop sequence between the F1 and F2 target regions. It would not distinguish the closely related *w*AlbB and *w*Pip strains. The "*w*AlbB vs *w*Pip OSD", which would bind to the loop region between the B1 and B2 regions of our current *wsp* LAMP assay would allow discrimination of *w*AlbB and *w*Pip strains due to specificity of interaction with the three highlighted polymorphic positions. The *wsp* sequences of the remaining *Wolbachia* strains are significantly different from *w*AlbB *wsp* sequence and are not detected by the *w*AlbB/*w*Pip-specific *wsp* LAMP-OSD assay (**B**). Pooled synthetic DNA representing *w*AlbA, *w*Aus, *w*Mors, and *w*Ana as well as standalone wAlbB *wsp* target sequences were analyzed by *w*AlbB/*w*Pip-specific *wsp* LAMP-OSD assays. Smartphone image was acquired at endpoint following 90 min of amplification.
(PDF)

S3 Fig. Detection of *Wolbachia w*Pip using *wsp* LAMP-OSD assay. *Wolbachia w*Mel-infected *Drosophila melanogaster* (**A**), *Wolbachia* uninfected *Culex tarsalis* (**B**), *Wolbachia w*Pip infected *Culex quinquefasciatus* (Houston) (**C**) and *Culex quinquefasciatus* (Salvador) (**D**) were analyzed using the *wsp* LAMP-OSD assay. 2 μL of crudely crushed individual insect samples (crushed individual fruit flies were resuspended in 20 μL water) were subjected to LAMP amplification for 90 min. OSD fluorescence was imaged at endpoint using a cellphone.
(PDF)

S4 Fig. Effect of crude mosquito sample on the detection limit of visually-read LAMP-OSD. Indicated copies of recombinant plasmids bearing *coi* or *wsp* target sequences were amplified by the *coi*-specific (**A**) or *wsp*-specific (**B**) LAMP-OSD assays, respectively. 8%

volume of all LAMP-OSD reactions was composed of crudely 'in-syringe' prepared non-specific mosquito sample. OSD fluorescence was imaged at endpoint using a cellphone.
(PDF)

S5 Fig. Blinded LAMP-OSD analysis of field-caught mosquitoes. Mosquitoes stored for one week at the indicated temperatures were prepared by 'in-tube' crude processing. 2 µL of a 1:10 dilution of each mosquito sample was analyzed by *coi* LAMP-OSD (A) and by *wsp* LAMP-OSD (B) assays. OSD fluorescence was imaged at endpoint using a smartphone.
(PDF)

S6 Fig. Blinded LAMP-OSD analysis of field-caught mosquitoes. Mosquitoes stored for two weeks at the indicated temperatures were prepared by 'in-tube' crude processing. 2 µL of a 1:10 dilution of each mosquito sample was analyzed by *coi* LAMP-OSD (A) and by *wsp* LAMP-OSD (B) assays. OSD fluorescence was imaged at endpoint using a smartphone.
(PDF)

S7 Fig. Blinded LAMP-OSD analysis of field-caught mosquitoes. Mosquitoes stored for three weeks at the indicated temperatures were prepared by 'in-tube' crude processing. 2 µL of a 1:10 dilution of each mosquito sample was analyzed by *coi* LAMP-OSD (A) and by *wsp* LAMP-OSD (B) assays. OSD fluorescence was imaged at endpoint using a smartphone.
(PDF)

Acknowledgments

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References

1. WHO (2014) A global brief on vector-borne diseases. http://apps.who.int/iris/bitstream/10665/111008/1/WHO_DCO_WHD_2014.1_eng.pdf. Last accessed on March 1st, 2018.

2. Zeller H, Marrama L, Sudre B, Van Bortel W and Warns-Petit E (2013) Mosquito-borne disease surveillance by the European Centre for Disease Prevention and Control. Clin Microbiol Infect 19: 693–698. https://doi.org/10.1111/1469-0691.12230 PMID: 23607415

3. Kraemer MUG, Sinka ME, Duda KA, Mylne AQN, Shearer FM, et al. (2015) The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. Elife 4. https://doi.org/10.7554/eLife.08347 PMID: 26126267

4. Ranson H and Lissenden N (2016) Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. Trends Parasitol 32: 187–196. https://doi.org/10.1016/j.pt.2015.11.010 PMID: 26826784

5. Hay SI, Guerra CA, Tatem AJ, Noor AM and Snow RW (2004) The global distribution and population at risk of malaria: past, present, and future. Lancet Infect Dis 4: 327–336. https://doi.org/10.1016/S1473-3099(04)01043-6 PMID: 15172341

6. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2013) The global distribution and burden of dengue. Nature 496: 504–507. https://doi.org/10.1038/nature12060 PMID: 23563266

7. Iturbe-Ormaetxe I, Walker T and Neill SLO (2011) Wolbachia and the biological control of mosquito-borne disease. EMBO reports 12: 508–518. https://doi.org/10.1038/embor.2011.84 PMID: 21546911

8. Bourtzis K, Dobson SL, Xi ZY, Rasgon JL, Calvitti M, et al. (2014) Harnessing mosquito-Wolbachia symbiosis for vector and disease control. Acta Trop 132: S150–S163. https://doi.org/10.1016/j.actatropica.2013.11.004 PMID: 24252486

9. McGraw EA and O'Neill SL (2013) Beyond insecticides: new thinking on an ancient problem. Nat Rev Microbiol 11: 181–193. https://doi.org/10.1038/nrmicro2968 PMID: 23411863

10. Dutra HLC, Rocha MN, Dias FBS, Mansur SB, Caragata EP, et al. (2016) Wolbachia Blocks Currently Circulating Zika Virus Isolates in Brazilian Aedes aegypti Mosquitoes. Cell Host & Microbe 19: 771–774. https://doi.org/10.1016/j.chom.2016.04.021 PMID: 27156023

11. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu GJ, Pyke AT, et al. (2009) A Wolbachia Symbiont in Aedes aegypti Limits Infection with Dengue, Chikungunya, and Plasmodium. Cell 139: 1268–1278. https://doi.org/10.1016/j.cell.2009.11.042 PMID: 20064373

12. Bian GW, Joshi D, Dong YM, Lu P, Zhou GL, et al. (2013) Wolbachia Invades Anopheles stephensi Populations and Induces Refractoriness to Plasmodium Infection. Science 340: 748–751. https://doi.org/10.1126/science.1236192 PMID: 23661760

13. Kambris Z, Cook PE, Phuc HK and Sinkins SP (2009) Immune Activation by Life-Shortening Wolbachia and Reduced Filarial Competence in Mosquitoes. Science 326: 134–136. https://doi.org/10.1126/science.1177531 PMID: 19797660

14. Hughes GL, Koga R, Xue P, Fukatsu T and Rasgon JL (2011) Wolbachia Infections Are Virulent and Inhibit the Human Malaria Parasite Plasmodium Falciparum in Anopheles Gambiae. PLoS Path 7. https://doi.org/10.1371/journal.ppat.1002043 PMID: 21625582

15. van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, et al. (2012) Impact of Wolbachia on Infection with Chikungunya and Yellow Fever Viruses in the Mosquito Vector Aedes aegypti. Plos Neglected Tropical Diseases 6. https://doi.org/10.1371/journal.pntd.0001892 PMID: 23133693

16. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. Nature 476: 454–U107. https://doi.org/10.1038/nature10356 PMID: 21866160

17. O'Connor L, Plichart C, Sang AC, Brelsfoard CL, Bossin HC, et al. (2012) Open Release of Male Mosquitoes Infected with a Wolbachia Biopesticide: Field Performance and Infection Containment. Plos Neglected Tropical Diseases 6. https://doi.org/10.1371/journal.pntd.0001797 PMID: 23166845

18. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, et al. (2011) The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. Nature 476: 450–453. https://doi.org/10.1038/nature10355 PMID: 21866159

19. Day JF and Shaman J (2011) Mosquito-Borne Arboviral Surveillance and the Prediction of Disease Outbreaks. In: Ruzek D., editor editors. Flavivirus Encephalitis. InTech. pp. 105–130. 110.5772/24068.

20. Ferraguti M, Martinez-de la Puente J, Roiz D, Ruiz S, Soriguer R, et al. (2016) Effects of landscape anthropization on mosquito community composition and abundance. Sci Rep 6: 29002. https://doi.org/10.1038/srep29002 PMID: 27373794

21. Vazquez-Prokopec GM, Chaves LF, Ritchie SA, Davis J and Kitron U (2010) Unforeseen Costs of Cutting Mosquito Surveillance Budgets. Plos Neglected Tropical Diseases 4. https://doi.org/10.1371/journal.pntd.0000858 PMID: 21049010

22. Wilke ABB, Christe RD, Multini LC, Vidal PO, Wilk-da-Silva R, et al. (2016) Morphometric Wing Characters as a Tool for Mosquito Identification. Plos One 11. https://doi.org/10.1371/journal.pone.0161643 PMID: 27551777

23. Beier MS, Schwartz IK, Beier JC, Perkins PV, Onyango F, et al. (1988) Identification of Malaria Species by Elisa in Sporozoite and Oocyst Infected Anopheles from Western Kenya. Am J Trop Med Hyg 39: 323–327. PMID: 3056055

24. Burkhalter KL, Lindsay R, Anderson R, Dibernardo A, Fong W, et al. (2006) Evaluation of commercial assays for detecting West Nile virus antigen. J Am Mosq Control Assoc 22: 64–69. https://doi.org/10.2987/8756-971X(2006)22[64:EOCAFD]2.0.CO;2 PMID: 16646324

25. Appawu MA, Bosompem KM, Dadzie S, McKakpo US, Anim-Baidoo I, et al. (2003) Detection of malaria sporozoites by standard ELISA and VecTest dipstick assay in field-collected anopheline mosquitoes from a malaria endemic site in Ghana. Trop Med Int Health 8: 1012–1017. PMID: 14629768

26. Lee SF, White VL, Weeks AR, Hoffmann AA and Endersby NM (2012) High-Throughput PCR Assays To Monitor Wolbachia Infection in the Dengue Mosquito (Aedes aegypti) and Drosophila simulans. Appl Environ Microbiol 78: 4740–4743. https://doi.org/10.1128/AEM.00069-12 PMID: 22522691

27. de Oliveira CD, Goncalves DS, Baton LA, Shimabukuro PHF, Carvalho FD, et al. (2015) Broader prevalence of Wolbachia in insects including potential human disease vectors. Bull Entomol Res 105: 305–315. https://doi.org/10.1017/S0007485315000085 PMID: 25772521

28. Zhou WG, Rousset F and O'Neill S (1998) Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. Proceedings of the Royal Society B-Biological Sciences 265: 509–515. https://doi.org/10.1098/rspb.1998.0324 PMID: 9569669

29. de Paz HD, Brotons P and Munoz-Almagro C (2014) Molecular isothermal techniques for combating infectious diseases: towards low-cost point-of-care diagnostics. Expert Rev Mol Diagn 14: 827–843. https://doi.org/10.1586/14737159.2014.940319 PMID: 25052202

30. Boyle DS, Lehman DA, Lillis L, Peterson D, Singhal M, et al. (2013) Rapid Detection of HIV-1 Proviral DNA for Early Infant Diagnosis Using Recombinase Polymerase Amplification. Mbio 4: pii: e00135-00113. https://doi.org/10.1128/mBio.00135-13 PMID: 23549916

31. Rohrman BA and Richards-Kortum RR (2012) A paper and plastic device for performing recombinase polymerase amplification of HIV DNA. Lab Chip 12: 3082–3088. https://doi.org/10.1039/c2lc40423k PMID: 22733333

32. Tomita N, Mori Y, Kanda H and Notomi T (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nature Protocols 3: 877–882. https://doi.org/10.1038/nprot.2008.57 PMID: 18451795

33. Kuboki N, Sakurai T, Di Cello F, Grab DJ, Suzuki H, et al. (2003) Loop-mediated isothermal amplification for detection of African trypanosomes. J Clin Microbiol 41: 5517–5524. https://doi.org/10.1128/JCM.41.12.5517-5524.2003 PMID: 14662933

34. Abbasi I, Kirstein OD, Hailu A and Warburg A (2016) Optimization of loop-mediated isothermal amplification (LAMP) assays for the detection of Leishmania DNA in human blood samples. Acta Trop 162: 20–26. https://doi.org/10.1016/j.actatropica.2016.06.009 PMID: 27288706

35. Zhang DY, Turberfield AJ, Yurke B and Winfree E (2007) Engineering entropy-driven reactions and networks catalyzed by DNA. Science 318: 1121–1125. https://doi.org/10.1126/science.1148532 PMID: 18006742

36. Seelig G, Soloveichik D, Zhang DY and Winfree E (2006) Enzyme-free nucleic acid logic circuits. Science 314: 1585–1588. https://doi.org/10.1126/science.1132493 PMID: 17158324

37. Yin P, Choi HM, Calvert CR and Pierce NA (2008) Programming biomolecular self-assembly pathways. Nature 451: 318–322. https://doi.org/10.1038/nature06451 PMID: 18202654

38. Chakraborty K, Veetil AT, Jaffrey SR and Krishnan Y (2016) Nucleic Acid-Based Nanodevices in Biological Imaging. Annual Review of Biochemistry, Vol 85 85: 349–373. https://doi.org/10.1146/annurev-biochem-060815-014244 PMID: 27294440

39. Jiang YS, Bhadra S, Li B, Wu YR, Milligan JN, et al. (2015) Robust strand exchange reactions for the sequence-specific, real-time detection of nucleic acid amplicons. Anal Chem 87: 3314–3320. https://doi.org/10.1021/ac504387c PMID: 25708458

40. Zhang DY and Winfree E (2009) Control of DNA strand displacement kinetics using toehold exchange. J Am Chem Soc 131: 17303–17314. https://doi.org/10.1021/ja906987s PMID: 19894722

41. Bhadra S, Jiang YS, Kumar MR, Johnson RF, Hensley LE, et al. (2015) Real-time sequence-validated loop-mediated isothermal amplification assays for detection of Middle East respiratory syndrome

coronavirus (MERS-CoV). PLoS One 10: e0123126. https://doi.org/10.1371/journal.pone.0123126 PMID: 25856093

42. Jiang YS, Riedel TE, Popoola JA, Morrow BR, Cai S, et al. (2017) Portable platform for rapid in-field identification of human fecal pollution in water. Water Res 131: 186–195. https://doi.org/10.1016/j. watres.2017.12.023 PMID: 29278789

43. Jiang YS, Stacy A, Whiteley M, Ellington AD and Bhadra S (2017) Amplicon Competition Enables End-Point Quantitation of Nucleic Acids Following Isothermal Amplification. Chembiochem: https://doi.org/10.1002/cbic.201700317 PMID: 28628741

44. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6: 343–345. https://doi.org/10.1038/nmeth.1318 PMID: 19363495

45. Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2 PMID: 2231712

46. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402. PMID: 9254694

47. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, et al. (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28: E63. PMID: 10871386

48. Batovska J, Blacket MJ, Brown K and Lynch SE (2016) Molecular identification of mosquitoes (Diptera: Culicidae) in southeastern Australia. Ecology and Evolution 6: 3001–3011. https://doi.org/10.1002/ece3.2095 PMID: 27217948

49. Ratnasingham S and Hebert PDN (2007) BOLD: The Barcode of Life Data System (www.barcodinglife.org). Mol Ecol Notes 7: 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x PMID: 18784790

50. Cywinska A, Hunter FF and Hebert PDN (2006) Identifying Canadian mosquito species through DNA barcodes. Med Vet Entomol 20: 413–424. https://doi.org/10.1111/j.1365-2915.2006.00653.x PMID: 17199753

51. Wang G, Li CX, Guo XX, Xing D, Dong YD, et al. (2012) Identifying the Main Mosquito Species in China Based on DNA Barcoding. Plos One 7. https://doi.org/10.1371/journal.pone.0047051 PMID: 23071708

52. Parvizi P, Bordbar A and Najafzadeh N (2013) Detection of Wolbachia pipientis, including a new strain containing the wsp gene, in two sister species of Paraphlebotomus sandflies, potential vectors of zoonotic cutaneous leishmaniasis. Mem Inst Oswaldo Cruz 108: 414–420. https://doi.org/10.1590/S0074-0276108042013004 PMID: 23828002

53. de Valdez MRW (2017) Mosquito species distribution across urban, suburban, and semi-rural residences in San Antonio, Texas. J Vector Ecol 42: 184–188. https://doi.org/10.1111/jvec.12254 PMID: 28504436

54. Leisnham PT, LaDeau SL and Juliano SA (2014) Spatial and temporal habitat segregation of mosquitoes in urban Florida. PLoS One 9: e91655. https://doi.org/10.1371/journal.pone.0091655 PMID: 24621592

55. Konishi E (1989) Size of Blood Meals of Aedes-Albopictus and Culex-Tritaeniorhynchus (Diptera, Culicidae) Feeding on an Unrestrained Dog Infected with Dirofilaria-Immitis (Spirurida, Filariidae). J Med Entomol 26: 535–538. PMID: 2585448

56. Rasgon JL and Scott TW (2004) An initial survey for Wolbachia (Rickettsiales: Rickettsiaceae) infections in selected california mosquitoes (Diptera: Culicidae). J Med Entomol 41: 255–257. PMID: 15061286

57. Kittayapong P, Baisley KJ, Baimai V and O'Neill SL (2000) Distribution and diversity of Wolbachia infections in southeast Asian mosquitoes (Diptera: Culicidea). J Med Entomol 37: 340–345. PMID: 15535575

58. Ahmad NA, Vythilingam I, Lim YAL, Zabari NZAM HL (2017) Detection of Wolbachia in Aedes albopictus and Their Effects on Chikungunya Virus. Am J Trop Med Hyg 96: 148–156. https://doi.org/10.4269/ajtmh.16-0516 PMID: 27920393

59. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, et al. (2000) Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 38: 4066–4071. PMID: 11060069

60. Chisenhall DM, Vitek CJ, Richards SL and Mores CN (2008) A method to increase efficiency in testing pooled field-collected mosquitoes. J Am Mosq Control Assoc 24: 311–314. https://doi.org/10.2987/5671.1 PMID: 18666541

61. Sikulu-Lord MT, Maia MF, Milali MP, Henry M, Mkandawile G, et al. (2016) Rapid and Non-destructive Detection and Identification of Two Strains of Wolbachia in Aedes aegypti by Near-Infrared Spectroscopy. PLoS Negl Trop Dis 10: e0004759. https://doi.org/10.1371/journal.pntd.0004759 PMID: 27362709

62. Khoshmanesh A, Christensen D, Perez-Guaita D, Iturbe-Ormaetxe I, O'Neill SL, et al. (2017) Screening of Wolbachia Endosymbiont Infection in Aedes aegypti Mosquitoes Using Attenuated Total Reflection Mid-Infrared Spectroscopy. Anal Chem 89: 5285–5293. https://doi.org/10.1021/acs.analchem.6b04827 PMID: 28332822

63. Goncalves Dda S, Cassimiro AP, de Oliveira CD, Rodrigues NB and Moreira LA (2014) Wolbachia detection in insects through LAMP: loop mediated isothermal amplification. Parasit Vectors 7: 228. https://doi.org/10.1186/1756-3305-7-228 PMID: 24885509

64. Aonuma H, Yoshimura A, Perera N, Shinzawa N, Bando H, et al. (2009) Loop-mediated isothermal amplification applied to filarial parasites detection in the mosquito vectors: Dirofilaria immitis as a study model. Parasit Vectors 2: 15. https://doi.org/10.1186/1756-3305-2-15 PMID: 19284882

65. Bonizzoni M, Afrane Y and Yan G (2009) Loop-mediated isothermal amplification (LAMP) for rapid identification of Anopheles gambiae and Anopheles arabiensis mosquitoes. Am J Trop Med Hyg 81: 1030–1034. https://doi.org/10.4269/ajtmh.2009.09-0333 PMID: 19996433

66. Escadafal C, Faye O, Sall AA, Faye O, Weidmann M, et al. (2014) Rapid molecular assays for the detection of yellow fever virus in low-resource settings. PLoS Negl Trop Dis 8: e2730. https://doi.org/10.1371/journal.pntd.0002730 PMID: 24603874

67. de Silva AP and Uchiyama S (2007) Molecular logic and computing. Nature nanotechnology 2: 399–410. https://doi.org/10.1038/nnano.2007.188 PMID: 18654323

68. Jung C and Ellington AD (2014) Diagnostic applications of nucleic acid circuits. Acc Chem Res 47: 1825–1835. https://doi.org/10.1021/ar500059c PMID: 24828239

69. Du Y, Hughes RA, Bhadra S, Jiang YS, Ellington AD, et al. (2015) A Sweet Spot for Molecular Diagnostics: Coupling Isothermal Amplification and Strand Exchange Circuits to Glucometers. Sci Rep 5: 11039. https://doi.org/10.1038/srep11039 PMID: 26050646

70. Li B, Ellington AD and Chen X (2011) Rational, modular adaptation of enzyme-free DNA circuits to multiple detection methods. Nucleic Acids Res 39: e110. https://doi.org/10.1093/nar/gkr504 PMID: 21693555

71. Allen PB, Arshad SA, Li B, Chen X and Ellington AD (2012) DNA circuits as amplifiers for the detection of nucleic acids on a paperfluidic platform. Lab Chip 12: 2951–2958. https://doi.org/10.1039/c2lc40373k PMID: 22729075

72. Joubert DA, Walker T, Carrington LB, De Bruyne JT, Kien DHT, et al. (2016) Establishment of a Wolbachia Superinfection in Aedes aegypti Mosquitoes as a Potential Approach for Future Resistance Management. PLoS Path 12. https://doi.org/10.1371/journal.ppat.1005434 PMID: 26891349

73. Ant TH, Herd CS, Geoghegan V, Hoffmann AA and Sinkins SP (2018) The Wolbachia strain wAu provides highly efficient virus transmission blocking in Aedes aegypti. PLoS Path 14. https://doi.org/10.1371/journal.ppat.1006815 PMID: 29370307

74. Bhadra S, Pothukuchy A, Shroff R, Byrom M, Ellefson JW, et al. (2018) Cellular reagents for diagnostics and synthetic biology. PLoS One: Manuscript accepted for publication in PLoS One.

75. Riedel TE, Zimmer-Faust AG, Thulsiraj V, Madi T, Hanley KT, et al. (2014) Detection limits and cost comparisons of human- and gull-associated conventional and quantitative PCR assays in artificial and environmental waters. J Environ Manage 136: 112–120. https://doi.org/10.1016/j.jenvman.2014.01.029 PMID: 24583609

76. Bordenstein SR, Brothers C, Wolfe G, Bahr M, Minckley RL, et al. (2010) Using the Wolbachia Bacterial Symbiont to Teach Inquiry-Based Science: A High School Laboratory Series. American Biology Teacher 72: 478–483. https://doi.org/10.1525/abt.2010.72.8.3

77. Choi JY, Bubnell JE and Aquadro CF (2015) Population Genomics of Infectious and Integrated Wolbachia pipientis Genomes in Drosophila ananassae. Genome Biology and Evolution 7: 2362–2382. https://doi.org/10.1093/gbe/evv158 PMID: 26254486

78. Byrom M, Bhadra S, Jiang YS and Ellington AD (2014) Exquisite allele discrimination by toehold hairpin primers. Nucleic Acids Res 42: e120. https://doi.org/10.1093/nar/gku558 PMID: 24990378

79. Aonuma H, Yoshimura A, Perera N, Shinzawa N, Bando H, et al. (2009) Loop-mediated isothermal amplification applied to filarial parasites detection in the mosquito vectors: Dirofilaria immitis as a study model. Parasites & Vectors 2. https://doi.org/10.1186/1756-3305-2-15 PMID: 19284882

80. Aonuma H, Suzuki M, Iseki H, Perera N, Nelson B, et al. (2008) Rapid identification of Plasmodium-carrying mosquitoes using loop-mediated isothermal amplification. Biochem Biophys Res Commun 376: 671–676. https://doi.org/10.1016/j.bbrc.2008.09.061 PMID: 18809384

81. Mao R, Ge G, Wang Z, Hao RZ, Zhang GH, et al. (2018) A multiplex microfluidic loop-mediated isothermal amplification array for detection of malaria-related parasites and vectors. Acta Trop 178: 86–92. https://doi.org/10.1016/j.actatropica.2017.10.025 PMID: 29102457

82. Aonuma H, Badolo A, Okado K and Kanuka H (2013) Detection of mutation by allele-specific loop-mediated isothermal amplification (AS-LAMP). Methods Mol Biol 1039: 121–127. https://doi.org/10.1007/978-1-62703-535-4_10 PMID: 24026691

83. Hegde S, Rasgon JL and Hughes GL (2015) The microbiome modulates arbovirus transmission in mosquitoes. Current Opinion in Virology 15: 97–102. https://doi.org/10.1016/j.coviro.2015.08.011 PMID: 26363996

84. Saraiva RG, Kang S, Simoes ML, Anglero-Rodriguez YI and Dimopoulos G (2016) Mosquito gut anti-parasitic and antiviral immunity. Dev Comp Immunol 64: 53–64. https://doi.org/10.1016/j.dci.2016.01.015 PMID: 26827888

85. Vasilakis N and Tesh RB (2015) Insect-specific viruses and their potential impact on arbovirus transmission. Current Opinion in Virology 15: 69–74. https://doi.org/10.1016/j.coviro.2015.08.007 PMID: 26322695