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

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20 # Abstract

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

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44 ## Author summary

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

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67 ## Introduction

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

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

87 Unfortunately, mosquitoes are most commonly identified using morphological taxonomic keys.
88 This process can be tedious, and requires highly trained personnel and undamaged mosquitoes.
89 Alternative morphological methods such as the identification of morphometric wing characters
90 [22] are low throughput and require microscopes and complex imaging instruments. Moreover,

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91 traditional morphological-based approaches cannot detect associated symbionts or pathogens.
92 These limitations restrict widespread accessibility and necessitate sample preservation and
93 transport. On the other end of the spectrum, immunoassay-based tools for identifying pathogen-
94 infected mosquitoes, such as VecTest™ dipsticks (Medical Analysis Systems Inc.), are portable
95 and inexpensive. However, these tests have poor sensitivity [23-25] and are not necessarily
96 available for distinguishing mosquito species or identifying *Wolbachia* endosymbionts.

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

108 To mitigate the spurious signals that arise with LAMP, we have previously applied principles that
109 were developed for nucleic acid strand exchange circuits [35-38] to the design of short hemiduplex
110 oligonucleotide strand displacement (OSD) probes for LAMP [39]. The single stranded 'toehold'
111 regions of OSD probes bind to LAMP amplicon loop sequences, and then signal via strand
112 exchange [40] that leads to separation of a fluorophore and quencher [39]. OSDs are the
113 functional equivalents of TaqMan probes and can specifically report single or multiplex LAMP
114 amplicons without interference from non-specific nucleic acids or inhibitors [39, 41]. OSDs
115 significantly enhance the diagnostic applicability of LAMP, allowing it to match the allelic specificity

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116 of real-time PCR. Recently, we engineered these molecular innovations to function fluently in one-
117 pot LAMP-OSD reactions that can directly amplify a few tens to hundreds of copies of DNA and
118 RNA analytes from minimally processed specimens and produce sequence-specific fluorescence
119 signals that are easily observable by the human eye or (more importantly) by unmodified cellphone
120 cameras [42]. The fluorescence endpoints that are produced can be used for yes/no determinations
121 of the presence of an analyte, and also estimation of analyte copies on an order of magnitude scale
122 [42, 43].

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

130 Methods

131 Chemicals and reagents

132 All chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO,
133 U.S.A.) unless otherwise indicated. All enzymes and related buffers were purchased from New
134 England Biolabs (NEB, Ipswich, MA) unless otherwise indicated. All oligonucleotides and gene
135 blocks (summarized in **S1 Table)** were obtained from Integrated DNA Technologies (IDT,
136 Coralville, IA, U.S.A.).

137 Preparation of amplification targets

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

146 **LAMP primer and OSD probe design**

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

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

160 These primer sets were functionally tested in LAMP assays using zero to several hundred copies
161 of purified plasmids as templates. Amplification kinetics were measured in real time using the

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162 fluorogenic intercalating dye Evagreen and the LightCycler 96 real-time PCR machine (Roche).
163 The fastest primer sets that detected the fewest template copies with negligible spurious reactivity
164 in the absence of templates were selected for further assay development.

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

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

175 **LAMP assay with synthetic DNA template**

176 LAMP assays were assembled in a total volume of 25 µl of 1X Isothermal buffer (NEB; 20 mM
177 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
178 buffer was supplemented with 0.4 mM dNTPs, 0.8 M betaine, 2 mM additional $MgCl_2$, 1.6 µM
179 each of FIP and BIP, 0.8 µM of loop primer, 0.4 µM each of F3 and B3 primers, and 16 units of
180 *Bst* 2.0 DNA polymerase. Plasmid DNA templates were serially diluted in TE buffer (10 mM Tris-
181 HCl, pH 7.5:0.1 mM EDTA, pH 8.0) immediately prior to use. Zero to several hundred copies of
182 synthetic plasmid templates were added to the LAMP reaction mixes followed by 90 min of
183 incubation at 65 °C.

184 **Real-time detection of LAMP amplicons using intercalating dyes**

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185 1X EvaGreen (Biotium, Hayward, CA, USA) was included in LAMP assays that were then
186 analyzed using the LightCycler 96 real-time PCR machine (Roche, Basel, Switzerland). Reactions
187 were subjected to 45 cycles of two-step incubations – step 1:150 sec at 65 °C, step 2: 30 sec at
188 65 °C. EvaGreen signal was measured in the FAM channel during step 2 of each cycle.
189 Subsequently, amplicons were subjected to a melt analysis by incubation at 65 °C for 1 min
190 followed by incremental rise in temperature to 97 °C. Amplicon melting was monitored by
191 measuring fluorescence at the rate of 10 readings per °C change in temperature. The resulting
192 data was analyzed using the LightCycler 96 analysis software to measure Cq values for
193 amplification and amplicon melting temperatures.

194 **Real-time detection of LAMP amplicons using OSD probes**

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

202 **Endpoint visual readout and smartphone imaging of LAMP-OSD assays**

203 LAMP-OSD assays intended for visual readout and smartphone imaging were assembled in 0.2
204 ml optically clear thin-walled tubes with low auto-fluorescence (Axygen, Union City, CA, USA).
205 Following 90 min of amplification at 65 °C, LAMP-OSD reactions were incubated at 95 °C for 1
206 min followed by immediate transfer to room temperature and fluorescence imaging. Images were
207 acquired using an unmodified iPhone 6 and an UltraSlim-LED transilluminator (Syngene,
208 Frederick, MD, USA). In some experiments, our previously described in-house 3D-printed imaging

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209 device [42] was used for LAMP-OSD fluorescence visualization and smartphone imaging. Briefly,
210 this device uses Super Bright Blue 5 mm light emitting diodes (LED) (Adafruit, New York, NY,
211 USA) to excite OSD fluorescence. Two cut-to-fit layers of inexpensive >500 nm bandpass orange
212 lighting gel sheets (Lee Filters, Burbank, CA, USA) on the observation window filter the OSD
213 fluorescence for observation and imaging.

214 **Rearing and collection of mosquitoes**

215 *Ae. aegypti, Ae. Albopictus, Culex tarsalis, Cu. quinquefasciatus* (Houston), and *Cu.*
216 *quinquefasciatus* (Salvador) mosquitoes were reared under conventional conditions in the
217 insectary at the University of Texas medical Branch, Galveston, TX, USA. Four to seven day old
218 mosquitoes were collected and immediately frozen for shipment, storage and subsequent testing.
219 To obtain blood fed insects, *Aedes* mosquitoes were starved for a period of 24 hours then offered
220 a sheep blood meal (Colorado Serum Company, Denver, CO, USA) using a hemotek membrane
221 system (Hemotek). Unfed mosquitoes were separated and mosquitoes that were engorged were
222 collected 24 hours post feeding and processed in the same manner as unfed mosquitoes. For
223 field collections, female mosquitoes were trapped using Fay prince trap (John W. Hock) baited
224 with CO_2 in Galveston, Texas. 90 mosquitoes were morphologically identified and sorted into
225 three blinded groups that were stored at -20 °C, 4 °C and 37 °C, respectively for up to 3 weeks
226 prior to LAMP-OSD analysis.

227 **LAMP-OSD analysis of mosquitoes**

228 For LAMP-OSD analysis individual mosquitoes were prepared either in 1 cc syringes or in 1.5 ml
229 microcentrifuge tubes as follows. In-syringe preparation: The plunger was removed from a 1 cc
230 syringe and a 0.5 µM pore size 1/8th inch diameter frit (catalog # 59037, Sigma-Aldrich, St. Louis,
231 MO, USA) was placed inside the syringe. A single mosquito was placed on top of the frit and
232 macerated thoroughly using the syringe plunger. 100 µl of water was aspirated into the syringe to

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233 fully re-suspend the macerated mosquito prior to evicting this mosquito-containing water from the
234 syringe into a microcentrifuge collection tube. A 2 µl aliquot of this sample was directly tested by
235 LAMP-OSD assays. In-tube preparation: A single mosquito was placed in a 1.5 ml microcentrifuge
236 tube and manually macerated using a disposable micropestle (Fisherbrand™ RNase-Free
237 Disposable Pellet Pestles, Cat # 12-141-364, Fisher Scientific, Hampton, NH, USA). Each
238 macerated mosquito was resuspended in 100 µl water. A 2 µl 1:10 diluted aliquot of this mosquito
239 sample was directly assessed by LAMP-OSD analysis.

240 Statistical analysis

241 The paired results of morphological identification and LAMP-OSD analysis were compared using
242 2x2 contingency tables. Sensitivity or true positive rate was calculated by using the formula
243 TP/(TP+FN) where TP are true positive samples, and FN are false negative samples. Specificity
244 or true negative rate was calculated using the formula TN/(TN+FP) where TN are true negative
245 samples and FP are false positive samples.

246 Results

247 Development of visually read LAMP-OSD assays to identify mosquito species and 248 *Wolbachia* infection

249 LAMP uses two inner (FIP and BIP) and two outer (F3 and B3) primers specific to six consecutive
250 target sequences (B3, B2, B1, F1c, F2c and F3c) (**S2 Fig**) [47]. *Bst* DNA polymerase extends
251 these primers by strand displacement DNA synthesis to form 10^9 to 10^{10} copies of
252 concatemerized amplicons with loops between the F1 and F2, B1 and B2, F1c and F2c, and B1c
253 and B2c regions. We use an additional fifth primer that binds to one of these loop regions and
254 accelerates amplification [41]. OSD probes, with blocked 3'-ends that prevents spurious signaling
255 from polymerase-mediated extension, hybridize to the second loop region (**S2 Fig**).

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256 To enable molecular identification of *Ae. aegypti* mosquitoes, we designed a smartphone-
257 imaged LAMP-OSD assay to amplify and detect a signature sequence in the mitochondrial
258 cytochrome c oxidase I (*coi*) gene. Each cell has multiple mitochondria and hence several
259 hundred copies of the *coi* gene, which should enable detection from a very small amount of
260 sample. Moreover, mitochondrial *coi* gene sequences are commonly used as barcodes for
261 molecular identification of animal species including distinction of mosquito species; our chosen
262 *coi* signature sequence was assigned to *Ae. aegypti* when queried against the Barcode of Life
263 Data Systems (BOLD; http://www.boldsystems.org/index.php) *coi* signature sequence database
264 [48-51].

265 We developed a second visually read LAMP-OSD assay targeting the *Wolbachia* surface
266 protein (*wsp*) gene to identify *Wolbachia*-infected insects. The *wsp* gene is widely used as a
267 marker for strain typing and screening for infected insect vectors [28, 52]. We engineered our
268 *wsp* LAMP outer and inner primers to be complementary to, and hence amplify, two *Wolbachia*
269 strains, the *w*AlbB and the closely related *w*Pip (**S3 Fig**). We deliberately designed our assay to
270 detect both strains in order to ensure that we could assess field-collected mosquitoes
271 irrespective of temporal and spatial variation in relative abundance of *w*AlbB-infected *Ae.*
272 *albopictus* and *w*Pip-infected *Cu. quinquefasciatus* mosquitoes in our collection area [53, 54].
273 Significant nucleic acid sequence variation should prevent amplification of the *wsp* gene from all
274 other *Wolbachia* groups (**S3 Fig**). To enable transduction of both *w*AlbB and *w*Pip *wsp* LAMP
275 amplicons to visible fluorescence we designed an OSD probe that is specific to an identical loop
276 sequence present in both amplicons.

277 With a single endpoint visual 'yes/no' readout of OSD fluorescence (either directly observed or
278 imaged using cellphone camera), the *Ae. aegypti coi* LAMP-OSD assay could reliably identify
279 the presence of as few as 4 copies of synthetic target DNA (**Fig 1**). Similarly, the cellphone-
280 imaged *wsp* LAMP-OSD assay produced bright visible fluorescence when presented with only

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281 40 copies of its target sequence. In the absence of target DNA, neither assay generated
282 spurious signal.

283

284 **Fig 1. Detection of *Ae. aegypti coi* gene and *Wolbachia w*AlbB *wsp* gene using visually**
285 **read LAMP-OSD assays.** Indicated copies of recombinant plasmids bearing *coi* or *wsp* target
286 sequences were amplified by the *coi*-specific **(A)** or *wsp*-specific **(B)** LAMP-OSD assays,
287 respectively. OSD fluorescence was imaged at endpoint using a cellphone.

288

289 **Direct LAMP-OSD analysis of crudely crushed mosquitoes**

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

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

301 Endpoint visual examination of these assays for the presence or absence of OSD fluorescence
302 revealed that our visually read LAMP-OSD system is compatible with direct analysis of crudely

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303 processed mosquitoes (**Fig 2**). The *coi* LAMP-OSD assay generated bright fluorescence readily
304 distinguishable from sample auto-fluorescence when seeded with crudely prepared *Ae. aegypti*
305 mosquitoes. In contrast, closely related *Ae. albopictus* failed to instigate false positive signal.
306 Similarly, the *wsp* LAMP-OSD assay generated bright fluorescence in response to *Ae.*
307 *albopictus* and *Cu. quinquefasciatus* mosquitoes, which are naturally infected with *w*AlbB and
308 *w*Pip *Wolbachia*, respectively, but remained negative in the presence of unrelated *Wolbachia*
309 *w*Mel and uninfected mosquitoes (**Fig 2 and S4 Fig**).

310

311 **Fig 2. Visually read LAMP-OSD analysis of crudely processed mosquito samples. (A)**
312 Schematic depicting rapid preparation of crude mosquito samples for direct LAMP-OSD
313 analysis by performing in-syringe individual mosquito maceration. **(B)** Analysis of individual *Ae.*
314 *aegypti* and *w*AlbB *Wolbachia*-infected *Ae. albopictus* mosquitoes by *coi* and *wsp* LAMP-OSD
315 assays, respectively. 2 µL of mosquito samples were either added directly to LAMP-OSD
316 assays or subjected to 10-fold serial dilution in water prior to introduction in LAMP-OSD
317 reactions. LAMP-OSD reactions without any macerated mosquito samples served as negative
318 controls. Assays lacking LAMP primers but containing macerated mosquito samples served as
319 controls for monitoring background fluorescence. **(C)** Specificity analysis of *coi* and *wsp* LAMP-
320 OSD assays. The *coi* LAMP-OSD assay was challenged with 2 µL of *Ae. albopictus* in-syringe
321 prepared crude mosquito sample. Similarly, the *wsp* LAMP-OSD assay was challenged with *Ae.*
322 *aegypti* sample. In all experiments, OSD fluorescence was imaged at endpoint using a
323 cellphone.

324

325 These results indicate that the *Wolbachia wsp* and *Ae. aegypti coi* LAMP-OSD assays are able
326 to specifically amplify and signal the presence of their target DNA directly from crudely crushed

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327 mosquito samples without requiring any extraction and purification of nucleic acids.
328 Furthermore, the large burden of non-specific nucleic acids as well as other molecular and
329 macroscopic components present in a crude mosquito sample did not compromise signal
330 accuracy. We also confirmed the absence of significant inhibition of amplification and signaling
331 by recapitulating the detection limit of synthetic DNA targets in a background of crude non-
332 specific mosquito sample. The *coi* and *wsp* LAMP-OSD assays could detect 4 and 40 target
333 copies, respectively, even in the presence of 8% reaction volume of crude mosquito sample (**S5**
334 **Fig)**.

335 Testing blood-fed mosquitoes with LAMP-OSD

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

344

345 **Fig 3. Effect of blood meal on LAMP-OSD analysis of crudely prepared individual**
346 **mosquitoes.** Individual normal or blood-fed *Ae. aegypti* and *Ae. albopictus* mosquitoes were
347 prepared by the in-syringe method. 2 μL of these mosquito samples were analyzed by *coi* and
348 *wsp* LAMP-OSD assays. LAMP-OSD reactions without any macerated mosquito samples served
349 as negative controls. Assays lacking LAMP primers but containing macerated mosquito samples

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350 served as controls for monitoring background fluorescence. LAMP-OSD fluorescence was
351 imaged at endpoint using a cellphone.

352

353 **Analysis of blinded field-caught mosquitoes with cellphone-imaged LAMP-OSD assays**

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

363 The visually read *coi* LAMP-OSD assay demonstrated an overall sensitivity (true positive rate)
364 of 97% and specificity (true negative rate) of 98% when compared to morphological typing of
365 field-caught mosquito species (**Fig 4**, **S6, S7, S8**). On closer inspection of the data, it is evident
366 that even after three weeks of mosquito collection and storage at temperatures as high as 37 °C
367 the *coi* LAMP-OSD assay was correctly able to identify 29 out of 30 *Ae. aegypti* mosquitoes.
368 The single mosquito that the LAMP-OSD assay failed to identify had been stored at 37 °C for a
369 week prior to testing. We ruled out lack of amplifiable nucleic acids or their incompatibility with
370 *coi* LAMP primers and OSD probe by PCR amplifying the relevant *coi* LAMP target and verifying
371 its sequence.

372

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

384

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

389 The *Wolbachia w*AlbB/*w*Pip *wsp* LAMP-OSD assay did not generate a positive signal from any
390 non-*Ae. albopictus* mosquito. This is expected since natural populations of *Ae. aegypti* and most
391 *Ochlerotatus* species are not infected with *Wolbachia* [56, 57]. However, ability of the *wsp* assay
392 to identify *Wolbachia* infection was influenced by the storage temperature of mosquitoes. The
393 *wsp* LAMP assay could readily identify *Wolbachia* infection in 3 out of 4 *Ae. albopictus*
394 mosquitoes stored at -20 °C for as long as 3 weeks. PCR analysis of the *wsp*-negative mosquito
395 using previously described primers (81F and 691R) and protocols [28] did not produce
396 amplicons suggesting that this individual was likely uninfected or had *Wolbachia* levels below

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397 the levels detectable by PCR. As the storage temperature was increased the frequency of
398 *Wolbachia* detection dropped. While 40% of *Ae. albopictus* mosquitoes stored at 4 °C gave a
399 positive *wsp* LAMP-OSD signal, none of the *Ae. albopictus* mosquitoes kept at 37 °C for even
400 as little as 1 week were *wsp* positive. All mosquitoes that failed to generate a signal by LAMP
401 also failed to produce *wsp* PCR amplicons. Since, 95-99% of *Ae. albopictus* mosquitoes in the
402 wild are typically found to be infected with *Wolbachia* [58], these results are suggestive of
403 nucleic acid deterioration in mosquitoes upon storage at high temperature.

404 ## Discussion

405 Mosquito control strategies that rely on the introduction of *Wolbachia* are now being deployed
406 around the world [7-9], and the surveillance of efficacy and spread require agile, field-based
407 methods for both mosquito and symbiont detection. Unfortunately, currently available tools for
408 mosquito diagnostics have several shortcomings. Morphological identification methods are
409 inherently low throughput, require extensive technical expertise, and cannot also readily identify
410 pathogens or biocontrol agents such as *Wolbachia*. Spectroscopy, such as near infrared
411 spectroscopy [59] and Fourier transform infrared spectroscopy [60], allow identification of
412 mosquito species, *Wolbachia,* and pathogens, but require expensive instruments and expertise that
413 are generally incompatible with low-resource settings. Immunoassays can detect pathogen-
414 carrying vectors but are insensitive and cannot also identify vector species. Nucleic acid
415 amplification methods could potentially look at both vector and sybmiont sequences, but are heavily
416 reliant on PCR with the ensuing encumbrances of expensive instruments and trained operators,
417 again precluding widespread use.

418 Isothermal nucleic acid amplification assays would facilitate field-based vector monitoring, but most
419 reported approaches rely on nucleic acid purification and non-specific readout, and thus suffer from
420 laborious setup and the risk of false positives [61-63]. Probe-read isothermal methods such as the

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421 recombinase polymerase assay (RPA) are more reliable but still require expensive and proprietary
422 reaction formulations and probes, which limits their flexibility and versatility in assay engineering.
423 Furthermore, most RPA applications for vector diagnostics [64] also depend on extensive sample
424 processing and nucleic acid purification prior to amplification.

425 These drawbacks led us to develop a simpler, more robust field-deployable assay based on loop-
426 mediated isothermal amplification (LAMP) that can identify both mosquito species and specific
427 *Wolbachia* strains in mosquitoes. While LAMP assays have previously been developed for
428 *Wolbachia* detection by targeting the 16S rRNA gene for amplification [61], these assays required
429 extraction and purification of DNA prior to assay, and used non-specific readouts that were highly
430 prone to false positive signals.

431 To overcome these barriers to the use of LAMP, we have previously adopted methods that
432 originated in the field of nucleic acid computation: the use of strand exchange reactions that
433 initiate at complementary single-stranded 'toeholds' and progresses via branch migration. The
434 base-pairing predictability and programmability of strand exchange kinetics promotes the
435 construction of exquisitely sequence-specific oligonucleotide strand displacement (OSD) probes
436 for LAMP amplicons (**S2 Fig**), thereby greatly reducing the detection of non-specific amplification
437 background [39, 41, 42]. For instance, we recorded positive *coi* LAMP-OSD signals from field-
438 caught *Ae. aegypti* mosquitos but did not detect signal from closely related *Ae. albopictus* and
439 *Ochlerotatus* species mosquitoes. Similarly, the *wsp* assay detected *w*AlbB and *w*Pip, as
440 expected, but not *w*Mel *Wolbachia*.

441 Strand exchange circuits have the additional advantage that they can be used to embed
442 algorithms and act as 'matter computers' [35-37, 65]. For example, strand exchange transducers
443 can logically integrate multiple analytes; transform nucleic acids to glucose and human chorionic
444 gonadotrophin (hCG); adapt readout to beads, paperfluidics, glucose meters, pregnancy test

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445 strips, and cellphones, and allow target copy number estimation using a single endpoint yes/no
446 readout of presence or absence of signal above an adjustable threshold [66-69]. In the current
447 instance, we deliberately designed our *wsp* LAMP-OSD assay to 'compute' the presence of both
448 *w*AlbB and *w*Pip in order to increase our odds of finding infected field-caught mosquitoes.
449 However, the dependence of strand exchange efficiency on toehold binding strength [39] can be
450 exploited to engineer yes/no distinctions between strain-specific single nucleotide polymorphisms
451 [39], and the same dual *wsp* assay could be rendered strain-specific by simply substituting an
452 OSD reporter specific to an alternate polymorphic loop sequence (**S3 Fig**). This might be
453 advantageous for strain discrimination if double infections were released during vector control
454 measures [70, 71].

455 By using our one-pot LAMP-OSD assay, macerated mosquito homogenates could be directly
456 analyzed and 'yes/no' visual readouts could be quickly ascertained with a cell phone in the field
457 without the requirement for laboratory equipment or technically training. Moreover, since our
458 assays can accurately analyze mosquitoes several days after capture – the *coi* LAMP-OSD assay
459 could for example identify mosquitoes after 3 weeks at 37 °C without desiccant – mosquitoes from
460 remote collection outposts can potentially be analyzed even after delayed retrieval. The flexibility
461 of assay timing is further accommodated by the fact that lyophilized LAMP-OSD reaction mixes
462 that can be stored and deployed without cold chain [42]. These combined features make our
463 assay platform the best tool to date for expanding vector surveillance to resource poor settings
464 [72], especially in that the ease of use should allow minimally trained citizen scientists to
465 participate in otherwise sophisticated public health monitoring operations in the field.

466 The development efforts that we have put into LAMP-OSD should now allow it to be generalized
467 to screening other microbes or mosquito phenotypes in field settings. For example, LAMP-based
468 assays have been developed to identify pathogens transmitted by mosquitoes and insecticide
469 resistance alleles [73-76], but these rely on purified nucleic acid as templates and non-specific

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470 readout whereas our method functions with mosquito homogenate and ensures accuracy using
471 unique sequence-specific strand exchange probes. In addition, this technology could be used to
472 identify gut microbes and insect-specific viruses associated with mosquitoes, which is of growing
473 interest given that it is becoming clear that the microbiome can shape vector competence for
474 human pathogens [77-79]. Overall, we have demonstrated a versatile nucleic acid diagnostic
475 platform for rapid and accurate analyses of both insect vectors and symbionts, and that can now
476 be further configured for additional applications.

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693

694 Supporting information

695 **S1 Table. Primers, probes, and target sequences for A. aegypti coi and Wolbachia**
696 **wAlbB/wPip wsp LAMP-OSD assays.**

697 **S2 Fig. LAMP-OSD schematic.** LAMP uses 2 inner (FIP and BIP) and 2 outer (F3 and B3)
698 primers specific to 6 blocks of target sequences designated as B3, B2, B1, F1c, F2c and F3c. F2
699 sequence in FIP (F1c-F2) initiates amplification by Bst DNA polymerase (Stage I).
700 F1c sequence in FIP self-primes subsequent amplification. Similarly, BIP (B1c-B2) initiates DNA
701 synthesis by binding to B2c. F3 and B3 primer-initiated DNA synthesis displaces preceding inner
702 primer-initiated strands, which serve as templates for primer-initiated strand displacement DNA
703 synthesis (Stage II). 3′-ends of the resulting single-stranded, dumbbell-shaped amplicons (Stage
704 III) are extended by Bst polymerase to form hairpins (Stage IV). Inner primers hybridize to the
705 single-stranded loops and initiate another round of strand displacement synthesis that opens the
706 original hairpin to form a concatemerized amplicon containing a self-priming 3′-end hairpin (Stage
707 V). The ensuing continuous amplification (initiated both by new inner primers and by self-priming)
708 generates increasingly complex, double-stranded concatameric amplicons containing self-
709 priming hairpins and single-stranded loops to which the OSD probe hybridizes. "c": denotes
710 complementary target sequences. F and Q on the OSD denote fluorophore and quencher,
711 respectively. OSD probe is denoted in terms of numbered domains, each of which represents a
712 short fragment (usually <12 nt) of DNA sequence in an otherwise continuous oligonucleotide

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713 strand. Single stranded toeholds are numbered in red. Complementarity between numbered OSD
714 domains is denoted by a single prime symbol.

715 **S3 Fig. Alignment of *wsp* sequences from different *Wolbachia* strains.** "Current *wsp* OSD"
716 refers to the *wsp* OSD probe used in the present study that binds to the loop sequence between
717 the F1 and F2 target regions. It would not distinguish the closely related *w*AlbB and *w*Pip strains.
718 The "*w*AlbB vs *w*Pip OSD", which would bind to the loop region between the B1 and B2 regions
719 of our current *wsp* LAMP assay would allow discrimination of *w*AlbB and *w*Pip strains due to
720 specificity of interaction with the three highlighted polymorphic positions. The *wsp* sequences of
721 the remaining *Wolbachia* strains are significantly different from *w*AlbB *wsp* sequence. Hence, the
722 *wsp* LAMP-OSD assay described here will not detect these strains.

723 **S4 Fig. Detection of *Wolbachia w*Pip using *wsp* LAMP-OSD assay.** *Wolbachia w*Mel-infected
724 *Drosophila melanogaster* **(A)**, *Wolbachia* uninfected *Culex tarsalis* **(B)**, *Wolbachia w*Pip infected
725 *Culex quinquefasciatus* (Houston) **(C)** and *Culex quinquefasciatus* (Salvador) **(D)** were analyzed
726 using the *wsp* LAMP-OSD assay. 2 µL of crudely crushed individual insect samples (crushed
727 individual fruit flies were resuspended in 20 µL water) were subjected to LAMP amplification for
728 90 min. OSD fluorescence was imaged at endpoint using a cellphone.

729 **S5 Fig. Effect of crude mosquito sample on the detection limit of visually-read LAMP-OSD.**
730 Indicated copies of recombinant plasmids bearing *coi* or *wsp* target sequences were amplified by
731 the *coi*-specific **(A)** or *wsp*-specific **(B)** LAMP-OSD assays, respectively. 8% volume of all LAMP-
732 OSD reactions was composed of crudely 'in-syringe' prepared non-specific mosquito sample.
733 OSD fluorescence was imaged at endpoint using a cellphone.

734 **S6 Fig. Blinded LAMP-OSD analysis of field-caught mosquitoes.** Mosquitoes stored for one
735 week at the indicated temperatures were prepared by 'in-tube' crude processing. 2 µL of a 1:10

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736 dilution of each mosquito sample was analyzed by *coi* LAMP-OSD (A) and by *wsp* LAMP-OSD
737 (B) assays. OSD fluorescence was imaged at endpoint using a smartphone.

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

742 **S8 Fig. Blinded LAMP-OSD analysis of field-caught mosquitoes.** Mosquitoes stored for
743 three weeks at the indicated temperatures were prepared by 'in-tube' crude processing. 2 µL of
744 a 1:10 dilution of each mosquito sample was analyzed by *coi* LAMP-OSD (A) and by *wsp*
745 LAMP-OSD (B) assays. OSD fluorescence was imaged at endpoint using a smartphone.

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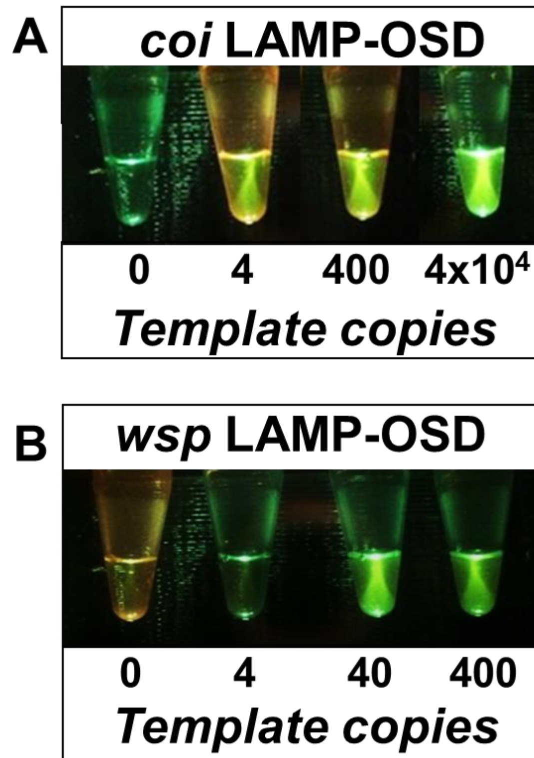

Fig 1. Detection of *Ae. aegypti coi* gene and *Wolbachia w*AlbB *wsp* gene using visually read LAMP-OSD assays. Indicated copies of recombinant plasmids bearing *coi* or *wsp* target sequences were amplified by the *coi*-specific **(A)** or *wsp*-specific **(B)** LAMP-OSD assays, respectively. OSD fluorescence was imaged at endpoint using a cellphone.

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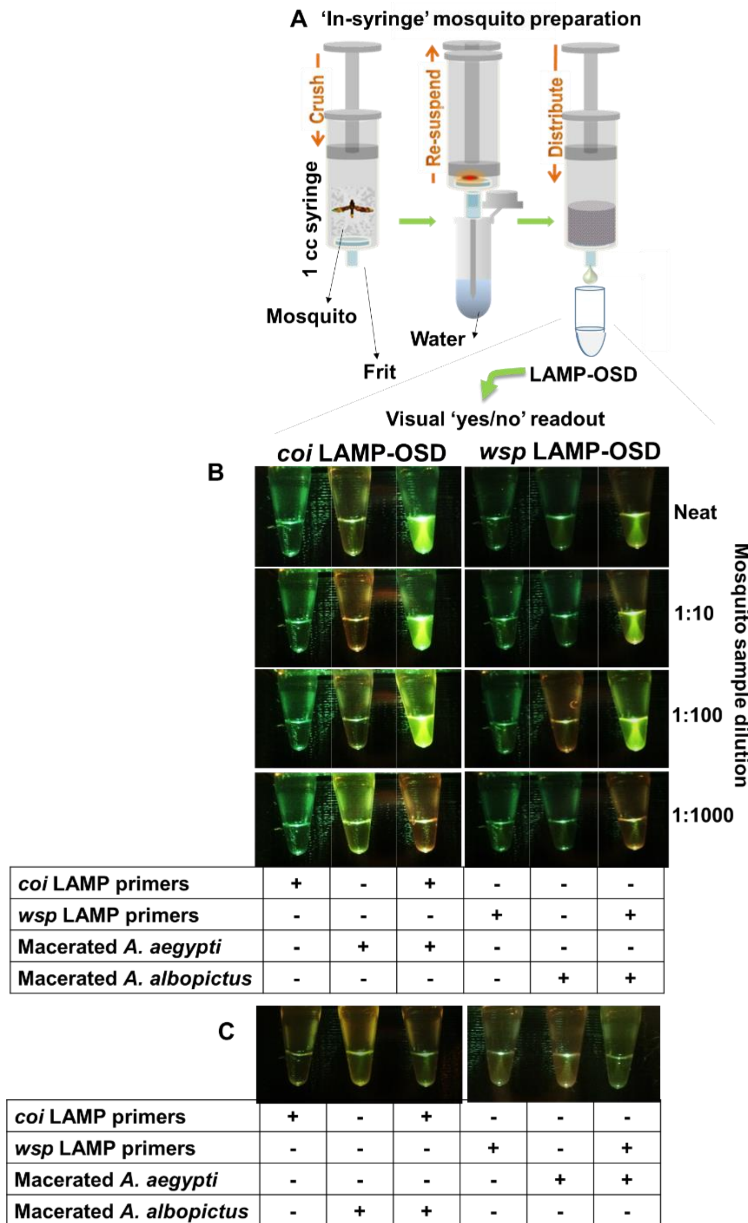

Fig 2. Visually read LAMP-OSD analysis of crudely processed mosquito samples. (A) Schematic depicting rapid preparation of crude mosquito samples for direct LAMP-OSD analysis by performing in-syringe individual mosquito maceration. **(B)** Analysis of individual *Ae. aegypti* and *w*AlbB *Wolbachia*-infected *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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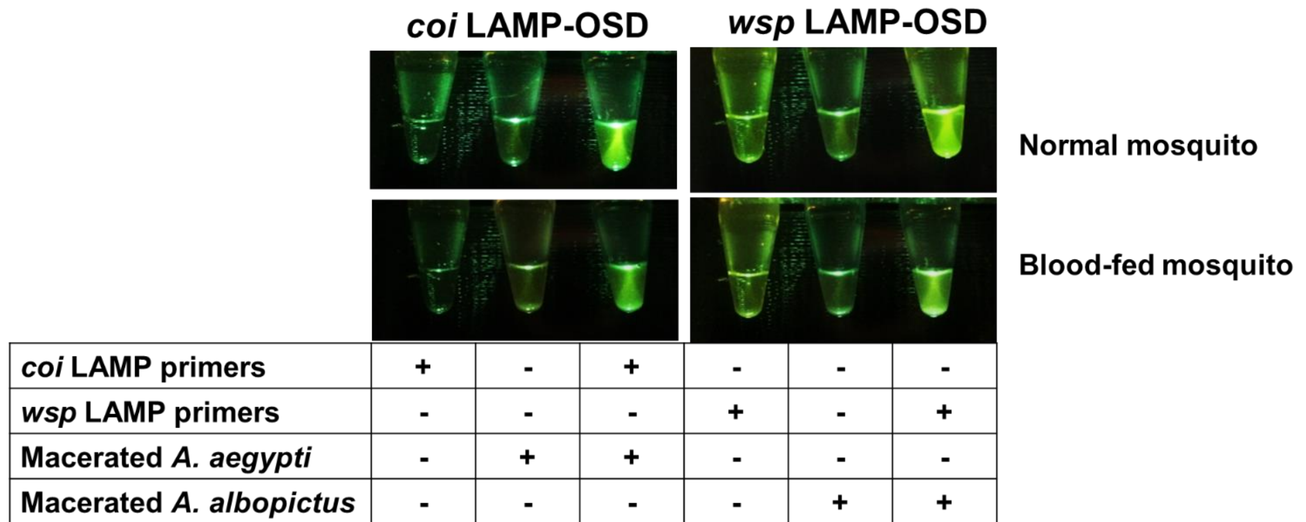

coi LAMP primers	+	-	+	-	-	-
wsp LAMP primers	-	-	-	+	-	+
Macerated *A. aegypti*	-	+	+	-	-	-
Macerated *A. albopictus*	-	-	-	-	+	+

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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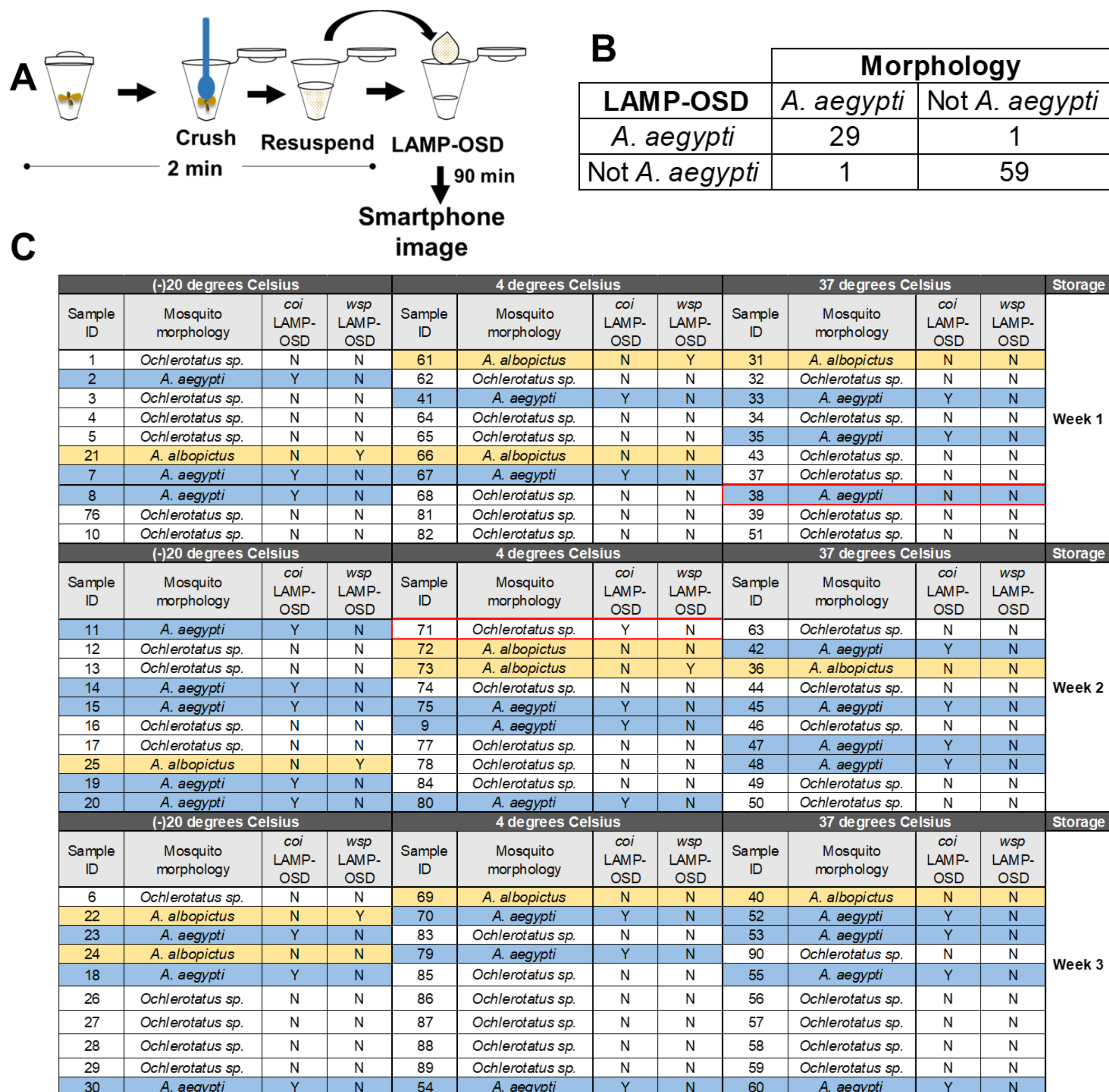

LAMP-OSD	Morphology: *A. aegypti*	Morphology: Not *A. aegypti*
A. aegypti	29	1
Not *A. aegypti*	1	59

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