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Duplex Real-Time PCR Assay Distinguishes *Aedes aegypti* From *Ae. albopictus* (Diptera: Culicidae) Using DNA From Sonicated First-Instar Larvae

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Abstract

Aedes aegypti (L.) and *Ae. albopictus* (Skuse) are important arbovirus vectors in the United States, and the recent emergence of Zika virus disease as a public health concern in the Americas has reinforced a need for tools to rapidly distinguish between these species in collections made by vector control agencies. We developed a duplex real-time PCR assay that detects both species and does not cross-amplify in any of the other seven Aedes species tested. The lower limit of detection for our assay is equivalent to ~0.03 of a first-instar larva in a 60-ml sample (0.016ng of DNA per real-time PCR reaction). The assay was sensitive and specific in mixtures of both species that reflected up to a 2,000-fold difference in DNA concentration. In addition, we developed a simple protocol to extract DNA from sonicated first-instar larvae, and used that DNA to test the assay. Because it uses real-time PCR, the assay saves time by not requiring a separate visualization step. This assay can reduce the time needed for vector control agencies to make species identifications, and thus inform decisions about surveillance and control.

Keywords

Aedes aegypti; *Aedes albopictus*; Zika; species identification assay; real-time PCR

The domesticated form of *Aedes aegypti* (L.) is well-known for its close association with human activity and preference for humans as bloodmeal hosts (Powell and Tabachnick 2013). It is a primary vector of the arboviruses that cause diseases such as dengue, yellow fever, and chikungunya. In addition, *Ae. aegypti* has been implicated in the transmission of Zika virus in the New World (Hayes 2009, Messina et al. 2016). The species *Ae. albopictus* (Skuse) is considered a competent vector of the same arboviruses. However, *Ae. albopictus* is more opportunistic in its choice of hosts, and thus is not considered to be as efficient a vector in terms of spreading infectious diseases among humans. The geographic ranges of the two species overlap, and they are generally more common in the southern and eastern

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The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

United States (Hahn et al. 2016). Detailed surveys of where each species currently occurs in the United States are in progress, and range maps will no doubt improve as surveillance proceeds.

Accurate species identification informs and improves vector control and is an important step in developing a plan for surveillance of mosquitoes that transmit diseases. Although *Ae. aegypti* and *Ae. albopictus* are not difficult to distinguish as adults because of distinct patterning on the scutum, clypeus, legs, and abdomen (Savage and Smith 1994), the laboratory resources needed to raise larvae collected from ovitraps to adulthood, as well as the 3–4 d required to do so, can make this practice prohibitive. Farajollahi and Price (2013) developed a key to identify various *Aedes* species from fourth-instar larvae, but again a time lag between collection and identification exists, and resources need to be devoted to specimen rearing. Particularly in outbreak situations, a short turnaround time between the collection of specimens and their identification is desirable. Morphological identification from just-hatched eggs (i.e., early instar larvae) is problematic because of a lack of species-specific characters that can be observed with a dissecting microscope. However, such specimens possess DNA sequence differences which can be used to correctly identify specimens.

Previous work to develop Aedes species identification assays includes work by Beebe et al. (2007) who developed a PCR-RFLP (restriction fragment length polymorphism) assay based on ITS1 (internal transcribed spacer 1 of nuclear ribosomal DNA) sequences to identify several species from in and around northern Australia. Hill et al. (2008) reported on work performed in the same geographic regions, and developed three singleplex real-time PCR assays with primer-probe technology to distinguish ITS1 sequences in three Aedes species. In another assay based on the ITS1 sequence, Higa et al. (2010) developed primers for a multiplex assay run on a regular thermal cycler to distinguish five *Aedes species* in Japan, and visualized the results on an agarose gel. Das et al. (2012) developed a multiplex PCR assay with species-specific primers that amplified ribosomal DNA in three species of *Aedes* mosquitoes from India, and whose results were visualized on agarose gels. A study by Van De Vossenberg et al. (2015) used the primers and probes developed by Hill et al. (2008) and found cross-amplification, i.e., *Ae. albopictus* specimens produced PCR products with the *Ae. aegypti* primer-probe set. Cross-amplification was alleviated by increasing the annealing temperature of the pertinent real-time PCR reaction, resulting in two species-specific annealing temperatures (Van De Vossenberg et al. 2015).

Our goal was to develop an assay that detected the presence of *Ae. aegypti* and *Ae. albopictus* from mixed pools of first-instar larvae, thereby reducing the time between specimen collection and identification in surveillance efforts involving oviposit traps. Further, we wanted an assay that could be run in duplex instead of singleplex reactions, and chose a real-time PCR format to eliminate a separate visualization step. Finally, we sought to simplify the process of obtaining template DNA from early instar larvae while avoiding the cost of traditional extraction kits, and developed a simple protocol for template DNA preparation.

Materials and Methods

Mosquito Specimens and DNA Extraction

Stored egg papers from colony specimens of *Ae. aegypti* (RexD strain) and *Ae. albopictus* (Lake Charles strain) at the Centers for Disease Control and Prevention (CDC) in Fort Collins were used to develop and test the duplex assay. Individual egg paper strips measuring 3 by 1cm and containing ~50–150 eggs were cut from areas containing high densities of eggs and placed in 1.7-ml microcentrifuge tubes filled with 750 μl of phosphate-buffered saline (PBS). Examples of egg papers with a high density of eggs and a low density of eggs are shown in Supp. Figs. 1 and 2 (online only). Egg papers contained eggs from one species, and a set of several tubes was prepared for each species separately. The tubes containing the egg paper strips were then placed in racks and left on a benchtop at room temperature for a period of time ranging from 6h to overnight. Afterwards, the tubes were visually inspected and if living larvae were present, a P200 pipette was used to transfer one or two 60-μl aliquots containing PBS and first-instar larvae to individual 0.2-ml PCR tubes for template DNA preparation.

PCR tubes containing first-instar larvae were placed in a foam rack, which was placed in an inexpensive jewelry-cleaning sonicator (Branson Ultrasonics, Branson 200, Danbury, CT) that had been filled to a depth of 2.5cm with deionized water. Our sonicator had a nonadjustable cycle time of 4min, 40s. After experimenting with different numbers of cycles, we determined that the highest DNA yields resulted when samples were sonicated four times (~18min total). We used the following steps when sonicating samples: 1) the foam racks holding samples were turned 180 degrees after each cycle, 2) samples were mixed by flicking the tube and briefly centrifuged after the second cycle, and 3) samples were briefly centrifuged again after the fourth cycle. Taking care not to disturb the pellet of debris, the sonicates were used without purification as template for the PCR assays. Sonicates were stored at −20 °C.

In order to estimate the relationship between the concentration of DNA and numbers of first-instar larvae per 60-ml aliquot, 10 samples of each species were prepared as described above (Supp. Figs. 3 and 4 [online only]). Two aliquots were removed from each tube (n=40), and the larvae in each aliquot were counted, transferred to a 0.2-ml PCR tube, and sonicated as described above. The DNA concentration of each aliquot was then quantified using a dsDNA High Sensitivity Assay Kit on a Qubit 3.0 fluorimeter (ThermoFisher).

Real-Time PCR Primer and Probe Design

Noting that *Ae. aegypti* mosquitoes have evolved a distinct preference for humans as hosts, and hypothesizing that this preference may be reflected in sequence differences in odorant receptor genes, several such genes from *Ae. aegypti* and *Ae. albopictus* were screened as potential candidates using sequence data from Vectorbase (http://www.vectorbase.org). The Primer Select program (DNASTAR, Madison, WI) was used to find robust primer and probe sequences, and Seq Man Pro (DNASTAR) was used to compare sequence differences between the two species. We chose a 96bp fragment of the *Ae. aegypti* gene AAEL017129, and an 85-bp fragment of the *Ae. albopictus* gene AALF027084 for further testing and

verification (Table 1). The unique genomic occurrence of each primer-probe set was confirmed using a BLAST search in Vectorbase. Probe sequences were fluorescently 5′ labeled with either FAM (*Ae. aegypti* probe) or HEX (*Ae. albopictus* probe), and both probes were 3′ labeled with black hole quencher 1 (BHQ1). Primers and probes were ordered from Centers for Disease Control and Prevention's Core Facility (Atlanta, GA).

Real-Time PCR

Primer-probe sets were tested initially as singleplex real-time PCR reactions, and we also tested for cross-amplification by running reactions with the primer-probe set for one species and template DNA of the other. After optimizing annealing temperature and primer-probe concentrations, and confirming that each primer-probe set performed well as a singleplex, duplex real-time PCR reactions were run with the following parameters: reactions were performed in 25 μl using 2X iQ Multiplex Powermix master mix (Bio-Rad, Hercules, CA), 1.25 μl each of two 20X primer-probe mixes (one for each species) with concentrations listed in Table 1, and 2.5 μl of sonicated first-instar larvae per species as template DNA. Reactions were run on a Bio-Rad CFX96 real-time thermal cycler with the following program: 3min at 95 °C, followed by 45 cycles of 15s at 95 °C and 45s at 60 °C. Samples were run in triplicate and Cq (quantification cycle, which is the threshold of detection) values were averaged from data generated using the default baseline and threshold settings on the CFX96. Primer and probe sequences have been deposited with Vectorbase. In addition, the primer-probe sets were tested in duplex with seven other *Aedes* species to check for cross-amplification (Table 2).

Cloning and Sequencing

To verify that amplified sequences were from the target genes, PCR fragments were cloned and sequenced. PCR products were generated in 100-ml reactions using 5 μl of single-species sonicated firstinstar larvae, 5U of HotStar Taq Polymerase (Qiagen, Valencia, CA), 10X PCR buffer, 0.15mM each primer (probes were excluded), and 0.2mM each dNTP on a regular thermal cycler (BioRad DNA Engine) with the following PCR program: 15min at 95 C followed by 35 cycles of 94 °C for 45s, 60 °C for 45s, and 72 °C for 30s, then 10min at 72 °C, and a 4 °C hold. PCR products were purified using Qiaquick PCR purification kits (Qiagen), and samples were eluted with 50 μl of EB Buffer. TOPO-TA cloning kits (ThermoFisher, Waltham, MA) were used to clone purified PCR products according to the manufacturer's instructions using 3 μl of purified PCR product that had been diluted 1:10 with DI water. Plasmid DNA containing the PCR fragment of interest was obtained with Miniprep kits (Qiagen) according to the manufacturer's instructions. Sequencing reactions were carried out in 20-μl volumes using BigDye chemistry and 2 μl of purified plasmid DNA as template, and sequenced on an ABI 3130x Genetic Analyzer sequencer (Applied Biosystems, Waltham, MA). To confirm sequence identity, the resulting sequence traces were compared to GenBank sequences using the blastn search with default parameters (Genbank 2017, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Relative positions of primer and probe sequence differences were visualized using BoxShade version 3.21 (Hoffman and Baron 2017).

Standard Curves and Assay Sensitivity

To assess the efficiency and reproducibility of the assay at various concentrations, as well as to determine the lowest concentration of DNA detectable by the duplex assay, real-time PCR standard curves were generated. Template DNA from sonicated first-instar larvae (two samples per species) was quantified as described above and diluted to 20ng/ml (50ng per reaction). Ten 1:5 serial dilutions were subsequently generated, resulting in a range of DNA concentrations per reaction from 50ng to 25.6 fg. One sample of both species was tested in triplicate in the duplex real-time PCR reaction with reaction conditions as described above using 2.5 μl of each dilution.

Validation

We examined the ability of the assay to detect DNA from both species in varying ratios. DNA from several sonicates was quantified as described above and diluted to concentrations ranging from 50ng– 0.025ng per reaction, with the extremes representing a 2,000-fold difference in concentrations. Various combinations of concentrations were tested to determine if small quantities of one species' DNA could be detected if there were large quantities of DNA from the other species. Samples were run in triplicate with real-time PCR conditions as described above, using 2.5 μl of both species' DNA.

Results

DNA Extraction and Quantification

We generated template DNA of satisfactory quality using an inexpensive jewelry sonicator. The concentration of DNA from the quantified samples of 60 ml aliquots of sonicated first-instar larvae ranged from 4.3–58.9ng/μl ($\bar{x}$ concentration =29.56±13.6ng/μl) and the number of larvae per aliquot ranged from 6–110 ($\bar{x}$ = 44.1 6±24.9; Supp. Table 1 [online only]). Figure 1 shows the relationship between numbers of larvae and concentration of DNA, and using the average values calculated above indicates a value of 0.6ng/μl per larvae in a 60-ml aliquot. We did not assess the long-term viability of DNA stored at −20°, but sonicates used in the duplex assay after two or three freeze-thaw cycles still produced acceptable results.

PCR Primer-Probe Design and Verification

Figure 2 shows the sequence differences between species for both amplified fragments. There were 24 nucleotide differences in the primer and probe sequences between the 96-bp *Ae. aegypti* fragment and the corresponding sequence in *Ae. albopictus* (Fig. 2, panel A). Similarly, there were 37 nucleotide differences in the primer and probe sequences between the 85-bp *Ae. albopictus* fragment and the orthologous *Ae. aegypti* sequence (Fig. 2, panel B). We did not observe any cross-amplification between species, even when the primer-probe set of one species was used with the other species. The negative template controls showed no primer dimer activity.

Furthermore, we did not observe any cross-amplification between our assay's primer-probe sequences and the other Aedes species tested (Table 2). Because our target sequences were <200bp, they were not submitted to Genbank, which requires sequences to be at least 200bp for deposition. Instead, the consensus for three sonicates is shown as the topmost sequence

in each part of Fig. 2. Comparisons with Genbank sequences using the blastn algorithm confirmed that the fragments amplified by the chosen primer-probe sets were the desired portions of the genes of interest.

Standard Curves and Assay Sensitivity

Figure 3 is a regression plot of Cq values versus the quantity of DNA (per reaction) used to generate the standard curve for each species in the duplex assay. The efficiency value for the *Ae. aegypti* primer-probe set was 91.0% and for *Ae. albopictus* it was 103.5%, indicating that the primer-probe sets in duplex amplified the template DNA in a linear fashion as predicted. The reproducibility for both primer-probe sets was>0.990, suggesting that the duplex assay performs well over four logs of template DNA concentrations.

The lowest four concentrations in the serial dilutions (0.0032ng to 25.6 fg of each species' DNA per reaction, approximately equivalent to 0.001, 0.0002, 0.00005, and 0.00001 first-instar larvae, respectively) produced inconsistent results (data not shown) and were subsequently excluded. Calculations of the amount of DNA that would be theoretically detectable when Cq=40 from the regression lines in Fig. 3 suggest the lowest detectable amounts are 0.0026ng and 0.0015ng per reaction of *Ae. aegypti* and *Ae. albopictus* DNA, respectively. However, the lowest concentration at which consistent results were obtained was 0.016ng per reaction for each species. Thus, we recommend Cq values above 37.6 and 36.6 (averaged over three replicates per sample) for *Ae. aegypti* and *Ae. albopictus*, respectively, be considered negative for that species. Converting the lowest concentration producing consistent results from ng per reaction to ng/μl, the lowest concentration of DNA that consistently amplifies is 0.0064ng/μl, which when divided by the average yield of DNA per larva (0.6ng/μl) indicates that the assay is capable of detecting 0.03 of first-instar larvae.

Validation With Mixed DNA

We tested mixtures of various amounts of *Ae. aegypti* and *Ae. albopictus* DNA ranging from 0.025ng to 50ng per reaction with our duplex real-time PCR assay. All combinations of DNA tested produced satisfactory amplification for both species. Figure 4 shows the Cq values for each species for all combinations tested.

Discussion

Mosquito-borne arboviruses represent an ongoing public health issue. Agencies tasked with vector control need efficient ways to determine species composition in the areas they serve. PCR-based assays are a valuable tool to quickly and accurately identify vector mosquito species during routine surveillance and outbreak investigations. We developed an assay that distinguishes *Ae. aegypti* from *Ae. albopictus* using sonicated pools of first-instar larvae. To our knowledge, it is the first duplexed real-time PCR assay that distinguishes between these species. The assay consists of one step and unlike several previous species detection assays, saves time by not requiring a restriction enzyme digest or a separate visualization step.

The method we developed for DNA extraction is simple and results in DNA of sufficient quality to run in the assay. Sonicating jewelry cleaners are widely available, and can be less expensive than prepackaged kits to extract DNA. Since the assay takes advantage of DNA

sequence differences, it will work with genomic DNA extracted by whatever method the user chooses, and furthermore can be used on specimens of any life stage.

Because the density of eggs deposited on egg papers can vary widely, we presented only one method of obtaining template DNA, namely, using strips cut from egg papers with a high density of eggs. If eggs are sparsely distributed on a paper, we suggest pipetting 750 μl of PBS into a reagent reservoir (Fisher Scientific, Waltham, MA), tilting the reservoir to collect the liquid, and carefully scraping 25–150 eggs into the liquid with a pipette tip. If the egg density on papers is particularly sparse, the amount of PBS can be adjusted downward. After the eggs are in PBS, a P1000 pipette and pipette tip with the end cut off can be used to transfer the eggs and PBS into a 1.7-ml microcentrifuge tube for incubation followed by sonication as described above. Another possible outcome of collecting eggs with ovitraps is that the eggs show low viability and few, if any, hatch. In this case, we recommend repeating collection efforts because our preliminary testing with sonicated unhatched eggs did not yield sufficient quantities of DNA to run the assay. In the event that the density of eggs is so high that cutting and incubating small strips is prohibitively time consuming, the user could scrape the eggs from larger portions of the egg paper (for example, dividing it into eighths, or tenths) into a reagent reservoir filled with 1.5 ml of PBS, followed by incubation and sonication as described above. Samples with high concentrations of DNA should be diluted (see below).

We tested mixtures of template DNA from each species that at their most extreme represented a 2,000-fold difference in the amount of DNA. In all cases, there was acceptable amplification for both species. Although these results and the results of the standard curve indicate there is a linear relationship between the amount of starting DNA and the resulting Cq values, the ability of this assay to perform as a strictly quantitative assay was not tested.

When equal amounts of DNA for each species were used to construct a standard curve from 1:5 serial dilutions of 20ng/μl samples, we observed very good patterns of amplification across the first six concentrations. We constructed an earlier standard curve starting with 50ng/μl samples and found that the highest concentration produced a shallow amplification curve without the characteristic S-shape (data not shown). Using too high a concentration of template (100ng per reaction or 70 or more first-instar larvae per 60 μl sonicate) can cause such abnormal curves (Life Technologies 2012). Because of this observation, we recommend diluting aliquots of the sonicates if small or shallow amplification curves are observed when using high density egg paper strips. Dilution of sonicates from high numbers of eggs is particularly important if it is suspected that other *Aedes* species are present, to ensure that the DNA of the target species will be amplified if present.

The conservative lower limit of sensitivity for this assay is ~0.016ng per reaction (equal to 0.0064ng/ml, or 0.03 of a first-instar larva), and tests with mixtures indicate that large differences in starting concentrations do not prevent small quantities of one or the other species' DNA from being detected. As an example, we were able to detect each species when present in amounts equal to a 2,000-fold difference (50ng vs. 0.025ng per reaction). The sensitivity of the assay, therefore, should be sufficient to detect small numbers of one species' larvae when mixed with the other. As an additional confirmation of the sensitivity

of our assay, the results of the experiment where first-instar larvae were counted, followed by sonication and DNA quantification indicate the amount of DNA per larvae in a 60-µl sample is above the limit of detection, and thus samples with small numbers of larvae should still yield accurate species identification results.

Finally, we note that although other Aedes species could be collected when surveillance is conducted on *Ae. aegypti* and *Ae. albopictus* in the United States, the DNA from the seven additional Aedes species tested was shown not to cross-amplify with the primer-probe sets in our assay. Since these other species have not had their genomes sequenced, data on whether orthologs to the genes in the present assay exist and the degree to which they are similar are unknown.

An accurate ascertainment of species composition is important for effective vector control. Although morphology can be used to distinguish species at certain life stages, rearing mosquitoes in controlled conditions requires time, space, and laboratory resources. PCR-based identification methods reduce the amount of time needed to identify species, with real-time assays eliminating the need for a separate visualization step. Our assay identifies *Ae. aegypti* and *Ae. albopictus* from sonicated first-instar larvae, and can serve as a tool to inform vector control agencies while they conduct surveillance and work to limit the spread of mosquito-borne pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We used egg papers from the *Aedes aegypti* (RexD) and Aedes albopictus (Lake Charles) colonies maintained by Andrea Sherman and Sean Masters of the Animal Care group at the CDC in Fort Collins. Janet McAllister's laboratory kindly lent us their sonicator to develop the assay.

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Vectorbase 2017b Aedes albopictus. (https://www.vectorbase.org/organisms/aedes-albopictus)

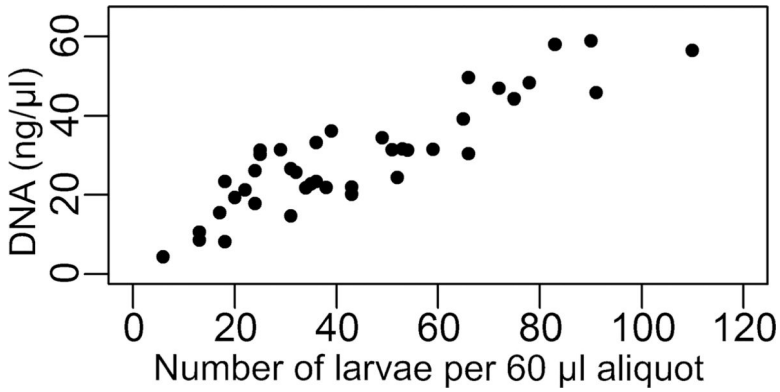

Fig. 1.

Relationship between number of first-instar larvae and concentration of DNA (ng/µl). Ten samples of each species were prepared using strips of egg papers (Supp. Figs. 3 and 4 [online only]). Two 60-µl aliquots were removed from each tube and the larvae in each aliquot were counted. The aliquots were subsequently sonicated as described in the Methods, and the concentration of DNA in the aliquots was quantified.

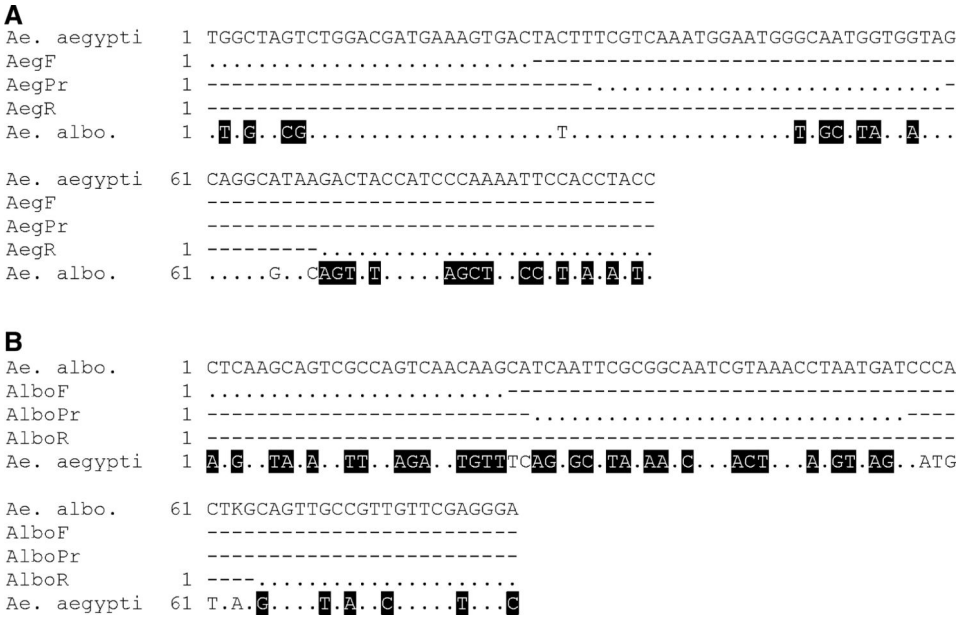

Fig. 2.

Sequence alignments of (**A**) 96-bp *Ae. aegypti* and (**B**) 85-bp *Ae. albopictus* PCR products. The first line of each panel is the consensus sequence of three separate sonicates of an odorant receptor gene, Vectorbase gene IDs AAEL017129 (*Ae. aegypti*) and AALF027084 (*Ae. albopictus*). The second through fourth lines show the name, sequence, and position of the forward primer (AegF, AlboF), probe (AegPr, AlboPr), and reverse primer (AegR, AlboR), respectively. The fifth line shows the sequence of the ortholog in the other species. Dots indicate sequence is the same as reference in top row. Dashes indicate no sequence is present. Shaded letters indicate differences between *Ae. aegypti* and *Ae. albopictus* in primer and probe sequences. Unshaded letters in the bottom row of each section show additional sequence differences.

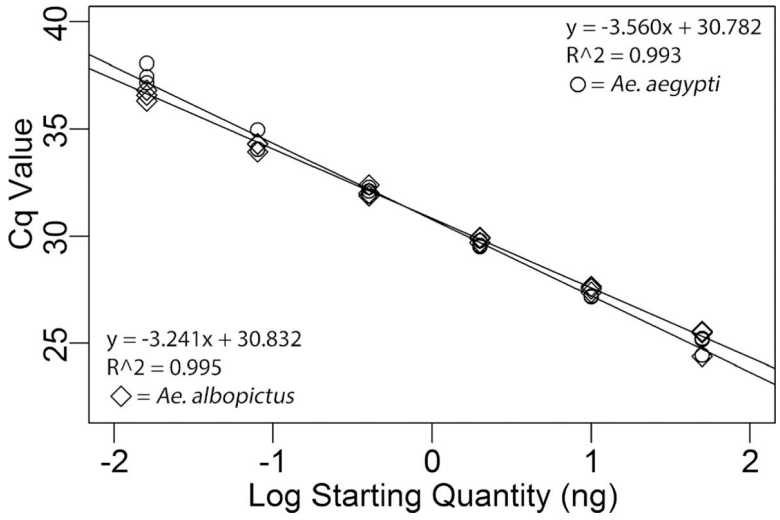

Fig. 3.

Regression plot from standard curve for duplex assay to identify *Ae. aegypti* (open circles) and *Ae. albopictus* (open diamonds) from sonicated first-instar larvae. Six 1:5 serial dilutions were made from a 20ng/μl sample of sonicated DNA per species, and each dilution was run in triplicate. Cq is the quantification cycle, which is the threshold of detection.

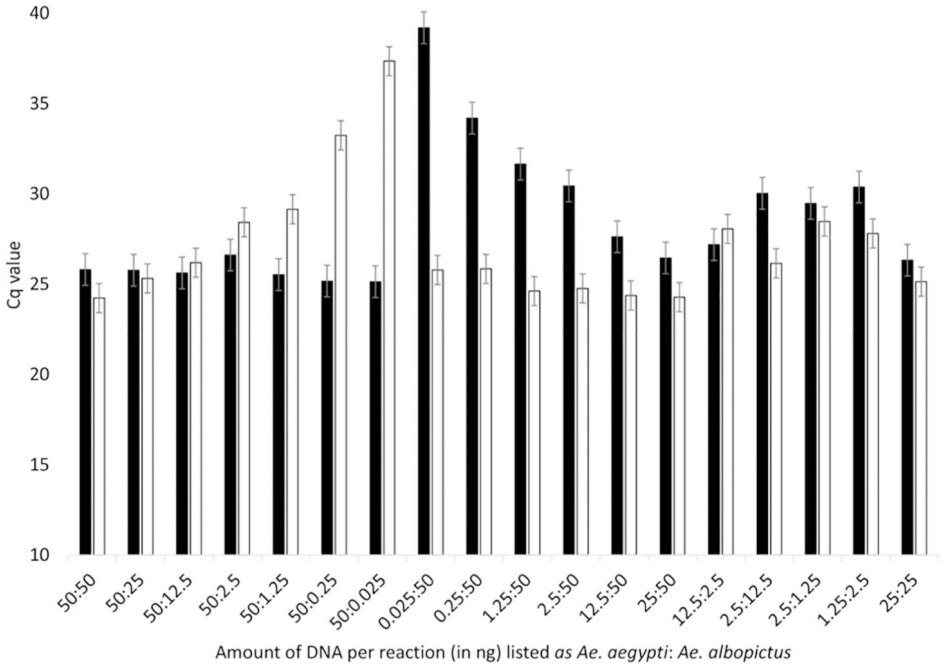

Fig. 4.

Bar graph of Cq values of various mixtures of *Ae. aegypti* (black bars) and *Ae. albopictus* (white bars) DNA. Concentrations of DNA (x axis) ranged from 0.025ng to 50ng per reaction.

Table 1.

Primer and probe sequences for *Aedes* species identification duplex assay

Name[a]	5′–3′ Sequence	5′ Dye–3′ Dye	Amount per reaction (nM)	Tm (°C)[b]
AegF	TGGCTAGTCTGGACGATGAAAGTGAC	–	100	59.6
AegR	GGTAGGTGGAATTTTGGGATGGTAGTC	–	100	60.0
AegProbe	TCGTCAAATGGAATGGGCAATGGTGGTA	FAM-BHQ1	200	67.8
AlboF	CTCAAGCAGTCGCCAGTCAACAAG	–	250	59.9
AlboR	TCCCTCGAACAACGGCAACTG	–	250	59.6
AlboProbe	TCAATTCGCGGCAATCGTAAACCTAATGAT	HEX-BHQ1	250	66.2

[a] Aeg—*Ae. aegypti* primer-probe set; Albo—*Ae. albopictus* primer-probe set.

[b] Tm—melting temperature (°C).

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Table 2.

Collection information for additional *Aedes* species tested in the development of this assay

Species	Collection location	Year collected
Ae. atropalpus (Coquillett)	Rabun County, GA	2012
Ae. bahamensis Berlin	Miami-Dade County, FL	2004
Ae. brelandi Zavortink	Brewster County, TX	2014
Ae. hendersoni Cockerell	Midland County, MI	2013
Ae. japonicus japonicas (Theobald)	Jackson County, NC	2013
Ae. Mediovittatus (Coquillett)	San Juan, Puerto Rico (colony)	2006
Ae. triseriatus (Say)	Hampshire County, MD	2008