Identification of *Aedes aegypti* and its Respective Life Stages by Real-Time PCR

James C. McAvin[1]*; Major David E. Bowles[2]; Col James A. Swaby[2]; Capt Keith W. Blount[3]; Major Jamie A. Blow[4]; Miguel Quintana[5], PhD; Lt Col John R. Hickman[1]; Lt Col Debra M. Niemeyer[6, 7]; Major Daniel H. Atchley[1]

Epidemiological Surveillance Division, United States Air Force Air Institute for Environment, Safety and Occupational Health Risk Analysis (AFIERA), Brooks, AFB, San Antonio, TX 78235-5237[1] (now designated as Force Air Institute for Operational Health (AFIOH), Brooks City-Base), USAF Force Protection Battlelab, Lackland AFB, San Antonio, TX, 78236-0119[2], USAF School of Aerospace Medicine, Brooks AFB, San Antonio, TX 78235-5237[3], Virology Division, United States Army Medical Research Institute for Infectious Disease (USAMRIID), Fort Detrick, Frederick, MD 21702-5011[4], Environmental Science Division, United States Army Center for Health Promotion and Preventative Medicine-West (USACPPM-W), Fort Lewis, WA 98433-9500[5], USAF Office of the Surgeon General, Bolling Air Force Base, D.C., 20332-0103[6], and Joint Program Executive Office for Chemical and Biological Defense, Falls Church, VA 22041-3203[7]
USA

*Molecular Epidemiology, AFIOH, 2730 Louis Bauer Drive, Building 930, Brooks AFB, San Antonio, TX 78235. Phone: (210) 536-8321. Fax: (210) 536-2638. Email: james.mcavin@brooks.af.mil.

ABSTRACT

*An **Aedes aegypti** specific fluorogenic probe hydrolysis (TaqMan) PCR assay was developed for real-time screening using a field-deployable thermocycler. Laboratory-based testing of **Ae. aegypti, Ae. aegypti** (Trinidad strain); **Culex pipiens; Culex pipiens quinquefasciatus; Anopheles stephensi; Ochlerotatus taeniorhynchus** individual adult mosquitoes and mixed pools (n=10) demonstrated 100% concordance in both **in vitro** sensitivity (6/6) and specificity (10/10). A single adult **Aedes aegypti** was identified in a pool of 100 non-**Aedes aegypti** mosquitoes. The limit of detection of **Aedes aegypti** egg pools was 5 individual eggs. Field-testing was conducted in central Honduras. An **Aedes aegypti** and **Culex spp**. panel of individual and mixed pools (n = 30) of adult mosquitoes, pupae, and larvae demonstrated 100% concordance in sensitivity (22/22) and 97% concordance in specificity (29/30) with one false positive. Field-testing of an **Aedes aegypti** and **Culex spp**. blind panel (n = 16) consisting of individual and mixed pools of adult mosquitoes, pupae, and larvae demonstrated 90% concordance in sensitivity (9/10) and 88% concordance in specificity (14/16).*

Introduction

The anticipation, prediction, identification, prevention, and control of vector-borne disease threats to military personnel are critical in all military operations. Real-time surveillance of mosquitoes and their respective immature stages allows rapid assessment of potential disease transmission risk and timely implementation of appropriate control measures. *Aedes aegypti* is the primary vector of dengue fever and yellow fever viruses therein representing a substantial threat for disease transmission to humans in many subtropical and tropical regions of the world (1). Dengue fever is the most significant mosquito-borne viral disease today. While malarial disease can be prevented by prophylaxis and yellow fever by immunization respectively, dengue

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fever prophylaxis does not exist and an approved vaccine is not anticipated in the near future. Currently, the only method of preventing infection with the dengue virus is vector avoidance.

Ae. aegypti is a peridomestic, diurnally active mosquito that prefers to breed in artificial containers near human habitations. Transmission of viruses to humans is by blood feeding females exclusively since males are non-biting. Vertical and possibly venereal transmission of dengue virus occurs by infected female to progeny (transovarian) (2, 3) and infected male to female during copulation, respectively (4). Therefore, while male mosquitoes do not directly infect humans they must be considered in the transmission cycle. In the absence of viremic hosts, these modes of transmission ensure survival of viruses in nature.

Control of disease transmission in endemic regions has become progressively more challenging as container-breeding mosquito habitat increases with exponentially increasing human populations and diminishing public resources for planning and controlling urban development (1). Depletion of public health resources has resulted in a lack of, or inefficient, mosquito control. Expanding global travel has exacerbated the problem by driving virus circulation in previously non-endemic regions thereby enhancing the potential for epidemics. Moreover, global warming influences local climatic patterns potentially making them more favorable for establishment and development of *Ae. aegypti* (5, 6, 7).

Efficacious surveillance of vector species, and their pathogens, is fundamental to the assessment of disease risk and time-critical implementation of appropriate transmission prevention measures and mosquito control. We describe here a real-time polymerase chain reaction (PCR) assay for sensitive and specific identification of *Ae. aegypti* and its respective life stages using field-deployable instrumentation.

Materials and Methods

Primer and probe design

Optimal probe and primer sequences were computed using Primer Express software according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). Primer sequences were identified with Tm values 10 degrees less than that of the probe. The fluorescent reporter molecule at the 5' end of the TaqMan probe was 6-carboxy-fluorescin (FAM) and the quenching molecule was 6-carboxy-tetramethyl-rhodamine (TAMRA). Primers and probe olignucleotides were synthesized commercially (Synthetic Genetics, Rockville MD). Requests for sequences can be submitted through the corresponding author.

Assay optimizations

Preliminary assay optimization was performed on a LightCycler™ (Roche Molecular Biochemicals, Mannheim, Germany) and transferred to the "Ruggedized" Advanced Pathogen Identification Device (R.A.P.I.D.) [Idaho Technology Incorporated, Salt Lake City, UT, www.idahotech.com] using fluorogenic probe hydrolysis (TaqMan) based-PCR (8, 9). Assays were optimized with a proprietary buffer system (Idaho Technology Incorporated, Salt Lake City, Utah) and sensitivity and specificity validation testing completed.

Reaction conditions.

Assay optimizations and cross-reaction testing were conducted on the R.A.P.I.D. prior to sensitivity and specificity validation testing. Master mix reaction solution was prepared and 18 µl volumes dispensed into optical capillary tubes and 2 µl of DNA extract added from specimens and controls or 2 µl PCR grade water

for no template controls (NTC). Capillaries were placed in a tabletop centrifuge and spun for 2-3 seconds at 3000 rpm to drive the assay mixture to the bottom of the tube. Master mix components were 2X Quantitech Probe PCR Master Mix (Qiagen, Valencia, CA). Forward primer concentration was 0.30 µM, reverse primer 0.90 µM, and TaqMan probe 0.10 µM. A standardized RT-PCR thermal cycling protocol was established that consisted of an initial DNA denaturation at 94°C for 2 minutes and PCR for 45 cycles at 94°C for 0 seconds of template denaturation and 60°C for 20 seconds of combined annealing and primer extension. A single data point at the end of each annealing-extension cycle was collected and reported as TaqMan probe fluorescence released by 5'-nuclease activity during primer extension. Fluorimeter gains were set at 8-2-2 on channels 1, 2, 3 respectively. The criterion for a positive result was a significant increase in fluorescence over background levels as defined by an algorithm provided in the R.A.P.I.D analytical software (Roche Molecular Biochemicals, Indianapolis, IN).

Laboratory Evaluations of *Aedes aegypti* PCR Assays

Mosquito panels

Evaluations of the *Aedes* genetic assay for sensitivity and specificity were accomplished under controlled conditions at AFIERA. Lab evaluations were conducted on adult mosquitoes (*Ae. aegypti*, *Anopheles stephensi*, *Culex pipiens*, *Cx. quinquefasciatus*, *Ochlerotatus taeniorhynchus*), various pools of these species, and *Ae. aegypti* eggs provided by the Department of Virology, United States Army Research Institute of Infectious Diseases, Fort Detrick, Maryland (USAMRIID) (Table 1). Species identification and confirmation was accomplished by morphological examination and serological analyses by U.S. Army entomologists.

Mosquitoes were held in cardboard cages, provided a carbohydrate source (either apple slices or a gauze pad soaked in a 10% sucrose solution) and a water-soaked cotton pledget, and held at 26°C for 7 days. Mosquitoes were then killed by exposure to -20°C for 5-10 minutes and these placed into sterile 1.5-ml Eppendorf tubes and triturated in 750 µl TrRIzol-LS (Life Technologies, USA). Panels were established as shown in Table 2 labelled under code at USAMRIID, and shipped on dry ice to Molecular Epidemiology, AFIERA, Brooks AFB, San Antonio, Texas, for nucleic acid extraction and blind PCR analyses.

DNA preparation

Single adult mosquitoes and mosquito pools were placed in sterile 1.5 ml eppendorf tubes, homogenized with a sterile, blunted 1000 µl pipette tip in 200 µl of sterile water. Sample homogenates were spun for 60 seconds at 13,500 rpm on a tabletop centrifuge, and approximately 200 µl of supernatant were pipetted into the MagNAPure LC sample cartridge for processing. Nucleic acid was isolated using the MagNAPure LC System and MagNAPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany) (10, 11). All post loading processing was completed in a closed system by automated robotics with preformatted reagents and a nucleic acid isolation matrix. Cell lysis and nucleic acid stabilization was completed with buffer containing guanidinium thiocyanate and proteinase K. Nucleic acid bound to the surface of magnetic glass particles was isolated from other cellular components by washing and eluting with a low-salt buffer. Nucleic acid extraction of mosquito eggs was by Trizol (Life Technologies, Rockville, MD) according to the manufacturer's instructions with the exception that sample homogenate was spun for 60 seconds at 13,500 rpm on a table-top centrifuge and 500 µl of supernatant exposed to the extraction process.

Field Evaluations of *Aedes aegypti* PCR Assays

Mosquito panels

The field site was central Honduras, 17-25 September 2002. Sampling was conducted in Comayagua and Tegucigalpa. Two teams of approximately 3-4 people each consisting of entomologists, physicians, public health professionals and technicians used battery-powered, hand-held aspirators to collect mosquitoes from the homes of consenting individuals, discarded tires and other structures. Immature mosquitoes were collected from various natural and man-made containers when present. For the field evaluations, all life stages, exclusive of eggs, were evaluated. Captured adult mosquitoes were temporarily held in storage tubes placed on dry ice (3 hours or less) and immature stages where held in "mosquito breeders" and returned to the field laboratory for processing. Additional specimens of pupae and larvae were collected and preserved in 95% ethyl alcohol for later identification and verification. United States Air Force entomologists identified and pooled captured and reared live adult mosquitoes, pupae, and larvae.

Specimens were pooled as *Ae. aegypti* alone, and in various combinations with *Culex* spp. Adult mosquitoes were placed in a freezer until they were rendered moribund, immediately transferred into 500 µl of Trizol, and then nucleic acid was extracted as described above. Larvae and pupae were placed directly into Trizol reagent prior to the extraction process. Optimized PCR assays described above were conducted using the R.A.P.I.D. Two experiments were conducted on the field-collected mosquitoes. In the first experiment, the R.A.P.I.D. operator had prior knowledge of the species composition in each prepared pool (Table 1). In the second experiment, the operator was provided mosquito pools as blind samples of unknown identity and composition (Table 2).

Results

Laboratory Evaluations

Sensitivity and specificity testing in laboratory evaluations showed the assay to be highly efficacious with excellent levels of detection for this species (Table 1). Laboratory testing of individual adult mosquitoes and mixed mosquito pools demonstrated 100% concordance in both *in vitro* sensitivity (6/6) and specificity (10/10) testing. Single adult *Ae. aegypti* were identified in pools of 100 non-*Aedes aegypti* mosquitoes, and the limit of detection of *Ae. aegypti* egg pools was five eggs. Because large mosquito pools are not technically practical with our current method of nucleic acid extraction, pools of greater than 100 were not evaluated in this study. Moreover, egg pool sizes of 5-10 exceed surveillance requirements therefore assay sensitivity was not optimized to a limit of detection of a single egg. Inhibition of PCR did not occur with Ae. aegypti-spiked pools of non-*Ae. aegypti* species.

Field Evaluations

Field-testing of the assay with a known panel consisting of individual and mixed pools of adult mosquitoes, pupae, and larvae demonstrated 100% *in vitro* sensitivity (22/22) and 97% specificity (29/30) with one false positive (Table 2). A single female *Culex* appeared to test positive after 40 PCR cycles (Ct = 40.52), but this likely was due to cross contamination in the laboratory since this specimen may have picked up some *Ae. aegypti* DNA when it was in combined storage before separation. Field-testing of the assay with a blind panel consisting of individual and mixed pools of adult mosquitoes, pupae, and larvae demonstrated 90% *in vitro* sensitivity (9/10) and 88% specificity (14/16) [Table 2]. One *Ae. aegypti* in a pool of 12 *Culex* produced a

negative result and a single *Culex* larva registered as a false positive. The *Culex* false positive (Ct = 39.03) error was likely due to species cross-contamination that occurred when the specimens were in storage. Whereas, multiple field-collected specimens at various stages of development would be tested *in situ*, sensitivity and specificity performance meets vector surveillance requirements.

Discussion

Rapid identification of both pathogens and their arthropod vectors is paramount for protecting military personnel (12). Likewise, surveillance of mosquitoes and their respective immature stages allows continued assessment of potential transmission risk and timely implementation of appropriate mosquito control measures. However, many military entomologists lack the taxonomic skills necessary to accurately identify vectors beyond the genus level. Public health personnel who are often tasked with conducting entomological surveillance generally are less experienced in species identification.

In the United States Air Force (USAF), arthropods, primarily mosquitoes and ticks, collected during routine surveillance are packaged and shipped to an out-of-area laboratory for identification by an entomologist with taxonomic skills. Although this approach is largely successful for getting specific identifications of potential vectors, the time involved for this process often conflicts with the requirement for rapid and specific identification to help in the prediction and prevention of vector-borne disease outbreaks. For example, the USAF primarily uses ovitraps to conduct base-level surveillance for *Aedes* (*Stegomyia*) mosquitoes and then rears the collected eggs to obtain adults for positive identification (13, 14, 15, 16, 17). However, under field conditions, especially in areas where disease transmission is active or where environmental conditions prohibit use of ovitraps (18), this method may not be practical. Identifying *Ae. (Stegomyia)* mosquitoes under field conditions also may not be practical when adults are not present, and identification of immature stages can prove challenging for untrained personnel. Moreover, there is an occasional requirement to conduct mosquito surveillance over a large geographical area or from a large number of locations that may involve the separation of the immature stages of *Ae. aegypti* and related species and/or the laboratory rearing of mosquitoes from positive ovitraps (19). Because of space and time requirements, substantial logistical problems can arise for such large-scale studies (20).

The *Ae. aegypti* assay described in this work clearly shows that both adult and immature specimens of this species can be accurately and rapidly identified by untrained personnel using the R.A.P.I.D. from both pure culture and mixed species pools. Our efforts have demonstrated the field utility and practicality of a rapid and accurate genomics-based vector identification capability. This methodology may offer a faster and more direct approach to identifying container-breeding *Aedes* species by eliminating the time consuming requirements or rearing adults from eggs collected in ovitraps. However, we have not yet fully evaluated the specificity of our assay on other mosquito taxa, and until this data is obtained we consider these data preliminary. Validation testing of assay specificity will remain an ongoing process as additional species of *Aedes* (*Stegomyia*) and other mosquito taxa become a part of our continually-expanding nucleic acid archive. We ultimately envision expanding this detection capability to include all of the principal vector species and pathogens of military importance.

PCR-based genetic assays are relatively simple and inexpensive to develop and use in both laboratory and field environments. Ultimately, they may offer a powerful tool for conducting surveillance of important vectors species without the requirement of basing identification on adult stages. Identifying mosquitoes can prove challenging for the untrained observer even with simplified diagnostic information (21). We believe that

our findings may have application for mosquito researchers and public health organizations requiring rapid identification of large numbers of samples, or diverse samples that may contain multiple vector species rather than using traditional time-consuming sorting and identification methods. Our assay system allows rapid, field identification of adult, larval, pupal and egg stages of *Aedes aegypti*.

Reference to trade name, vendor, proprietary product or specific equipment is not an endorsement, a guarantee or a warranty by the Department of the Defense, Army or Air Force, and does not imply an approval to the exclusion of other products or vendors that also may be suitable. The manuscript was cleared through the Technical Publication/Presentation Control Record, Brooks City-Base, TX for Open Publication, January, 2004. The conclusions and opinions expressed in this document are those of the authors. They do not reflect the official position of the U.S. Government, Department of Defense, Joint Program Office for Chemical and Biological Defense, U.S. Air Force, U.S. Army, or the Association of Military Surgeons of the United States.

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Table 1 Laboratory Evaluation of *Aedes aegypti* PCR assay using the LightCycler and R.A.P.I.D.

Sample Composition	PCR Results	Cycles (Ct)
1 Ae. aegypti	Positive	20.15
1 Ae. aegypti	Positive	20.88
1 Ochlerotatus taeniorhynchus	-	
1 Ochlerotatus taeniorhynchus	-	
2 Culex pipiens	-	
2 Culex pipiens	-	
2 Ae. aegypti (Trinidad strain)	Positive	26.93
2 Ae. aegypti (Trinidad strain)	Positive	26.39
2 Culex pipiens quinquefasciatus	-	
2 Culex pipiens quinquefasciatus	-	
2 Anopheles stephensi	-	
2 Anopheles stephensi	-	
24 Ae. aegypti (Trinidad strain) / 1 Ae. aegypti	Positive	25.73
24 Ae. aegypti (Trinidad strain) / 1 Ae. aegypti	Positive	26.43
24 Culex pipiens / 1 Ae. aegypti	Positive	34.18
24 Culex pipiens / 1 Ae. aegypti	Positive	33.61
24 Culex pipiens quinquefasciatus / 1 Ae.aegypti	Positive	33.95
24 Culex pipiens quinquefasciatus / 1 Ae.aegypti	Positive	34.52

24 Ochlerotatus taeniorhynchus / 1 Ae. aegypti	Positive	32.37
24 Ochlerotatus taeniorhynchus / 1 Ae. aegypti	Positive	32.29

Mosquito Pools: *Ae. aegypti* / Non-*Ae. aegypti*

1/50	Positive	28.94
1/50	Positive	28.72
1/50	Positive	30.96
1/75	Positive	33.64
1/75	Positive	33.21
1/100	Positive	33.51
1/100	Positive	33.91

***Ae. aegypti* Egg Pools**

100	Positive	30.15
50	Positive	32.96
10	Positive	34.7
5	Positive	30.68
1	Negative	

Table 2 Field Evaluation of *Aedes aegypti* PCR assay using the R.A.P.I.D.

Sample Composition: Known Panel	PCR Results	Cycles (Ct)
1 *Aedes aegypti* female	Positive	32.8
1 *Aedes aegypti* female	Positive	32.7
1 *Culex* female	-	
1 *Culex* female	Positive	40.52
2 *Aedes aegypti* females	Positive	29.88
2 *Aedes aegypti* females	Positive	34.72
2 *Aedes aegypti* males	Positive	32.05
15 *Aedes aegypti* male/female	Positive	29.7
1 *Aedes aegypti* female/12 *Culex*	Positive	30.91
1 *Aedes aegypti* female/12 *Culex*	Positive	32.48
1 Aedes aegypti	Positive	28.37
1 Aedes aegypti	Positive	28.97
1 *Aedes* larva	Positive	26.01
1 *Aedes* larva	Positive	27.31
1 *Aedes* larva	Positive	26.57
1 *Aedes* larva	Positive	25.97
1 *Aedes* pupa	Positive	25.85
1 *Aedes* pupa	Positive	25.94
1 *Aedes* pupa	Positive	25.92
1 *Aedes* pupa	Positive	26.48
1 *Culex* larva	-	
1 *Culex* larva	-	

Sample Composition: Known Panel	PCR Results	Cycles (Ct)
1 *Culex* larva	-	
1 *Culex* pupa	-	
1 *Culex* pupa	-	
1 *Aedes aegypti* larva/12 *Culex* larvae	Positive	33.00
1 *Aedes aegypti* larva/12 *Culex* larvae	Positive	38.21
1 *Aedes aegypti* larva/12 *Culex* larvae	Positive	30.60
1 *Aedes aegypti* larva/12 *Culex* larvae	Positive	31.35

Sample Composition: Blind Panel	PCR Results	Cycles (Ct)
1 Aedes aegypti	Positive	20.71
1 Aedes aegypti	Positive	24.24
1 *Aedes aegypti* larva	Positive	28.32
1 *Aedes aegypti* larva	Positive	26.91
1 *Aedes aegypti* larva	Positive	28.19
1 *Culex* larva	-	
1 *Culex* larva	-	
1 *Culex* larva	Positive	39.03
1 *Aedes aegypti* larva/3 *Culex* larvae	Positive	30.75
1 pupa unknown (presumed *Culex*)	-	
1 pupa unknown (presumed *Culex*)	-	
1 *Aedes aegypti* larva/12 *Culex* larvae w/debris	Positive	33.81
1 *Aedes aegypti* larva/12 *Culex* larvae w/debris	-	
1 *Aedes aegypti* larva/12 *Culex* larvae w/debris	Positive	38.60

Sample Composition: Blind Panel	**PCR Results**	**Cycles (Ct)**
12 *Culex* larvae w/ debris	-	
Debris only *Culex* container	-	
Debris only *Aedes aegypti* container	-	

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SYMPOSIA DISCUSSION - PAPER 22

Authors Name: Mr McAvin (US)

Discussor's Name: Dr Rios-Tejada (SP)

Question:
Can studies performed in Honduras can be extrapolated to other environments or sites?

Author's Reply:
Definitely not directly on site testing should be performed.

Authors Name: Mr McAvin (US)

Discussor's Name: Dr Rios-Tejada (SP)

Question:
When will serotype identification be ready and available on site?

Author's Reply:
It is ready and on the way to be adopted in a commercial way.