Field Evaluation of a Deployable RT-PCR Assay System for Real-Time Identification of Dengue Virus

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ABSTRACT

*Dengue fever and the more severe form of the disease, dengue hemorrhagic fever, occurs in tropical and subtropical regions globally through infection by one or more of four viral serotypes, DEN-1, DEN-2, DEN-3 and DEN-4. Viral transmission to humans is through mosquito vectors, primarily **Aedes aegypti**. Dengue universal and DEN 1-4 serotype specific fluorogenic, real-time RT-PCR assays and positive control nucleic acid were field-formatted by lyophilization and adapted for use on a field-durable, real-time, fluorimetric thermocycler. Nucleic acid extract isolated from DEN1-4 inoculated **Aedes aegypti**, field-captured **Aedes aegypti** and **Culex** spp, and field-captured **Aedes aegypti** and **Culex** spp spiked with total nucleic acid from DEN1-4 inoculated **Aedes aegypti** were used to construct a test panel (n = 64). In blind testing each of the five assays were 100% concordant in **in vitro** sensitivity and 100% concordant in specificity with dengue virus inoculated and spiked field-captured mosquitoes. A field-captured **Aedes aegypti** pool consisting of five females and two males was identified as dengue virus positive by the dengue universal assay; however, serotype identification was inconclusive. This study demonstrates the operational utility of a deployable assay system for rapid, sensitive, and specific screening and serotype identification of dengue virus in mosquito vectors.*

Introduction

Dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) now represent the most significant mosquito-borne viral disease threatening two-fifths of the world's human population (1, 2, 3). Infection with dengue virus causes various degrees of illness ranging from mild febrile discomfort through potentially fatal hemorrhagic disease (1). Vaccines are in development that shows promise (4, 5) however the current method of prevention and treatment is vector avoidance and patient management, respectively (6, 7). Antibody cross-reaction occurs across the *Flaviviridae* family creating ambiguity in immunoassay-based dengue virus surveillance and DF/DHF diagnosis (8, 9). To aid in clinical diagnosis, sensitive and specific dengue virus reverse transcriptase-polymerase chain reaction (RT-PCR) assays have been developed on laboratory-based instrumentation (10, 11, 12, 13, 14, 15, 16).

In areas where laboratory-based diagnostics are not available, deployable surveillance capability is an essential element in achieving timely assessments of transmission risk and time-critical implementation of appropriate

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mosquito control measures and clinical response in a potential outbreak situation (17, 18, 19). Development of pathogen identification assays on field-durable instrumentation fulfils a fundamental requirement in the realization of a complete field-deployable assay platform. While field-durable instrumentation provides deployable assay capability, an omnipotent field-deployable assay platform requires additional components including thermal-stable assay reagents and control nucleic acid, and field-formatted nucleic acid isolation technologies. Surveillance studies conducted in austere environments present logistical and operational constraints that make it impractical to transport, store, and prepare PCR reagents and control nucleic acid. Where resources for cold chain maintenance are unreliable or nonexistent, unknown variables in enzymatic activity and control nucleic acid integrity result. Moreover, sample integrity comes into question where sample collections are made distant from the laboratory or support facilities. Exposure to ambient temperature and freeze-thaw occurrences during transportation often presents an unknown variable in nucleic acid integrity of the sample.

This paper describes a deployable assay platform for rapid, sensitive, and specific screening and serotype identification of dengue virus in mosquito vectors.

Materials and Methods

Primer and probe design

Universal dengue virus assay primer and probe sequences were designed *de novo* by aligning homologous genomic regions of serotypes 1-4 that excluded other clinically significant flaviviruses. Alignments were compared visually using the Clustal algorithm (20) in the MegAlign program of DNA Star software (Perkin Elmer, Norwalk, Conn.)(21). Maximally conserved oligonucleotide sequences were chosen from dengue virus type 1-4 genomes downloaded from Genebank accession numbers U88536, M19197, M93130, AF326825, respectively. Yellow fever, JE, WN, and SLE virus type strain genomic sequences were aligned and visually evaluated to validate heterology with universal primer and probe sequences, Genebank accession numbers X03700/K02749, M18370, M12294/M10103, AF242895 respectively. Universal primer and probe sequences can be requested through the corresponding author. Serotype specific primer and probe sequences were obtained from the literature, [DEN-1 (10); DV2 (11); DEN-3 (10); DEN-4 (10)], and heterology validated as described above. All probes reported here are dual fluorogenic label designed with a 5' reporter dye, 6-carboxyfluorescein (FAM), and 3' quencher dye, 6-carboxytetramethylrhodamine (TAMRA) [22].

Universal and serotype specific primers and probes sequence heterology with genomic sequences of closely related species through diverse genera were validated by BLAST database search (BLAST, Madison, Wisconsin) [23]. Melting temperatures were quantified and the absence of significant primer dimerizations and secondary structure (hairpin) formations were confirmed with PrimerExpress software (PE Applied Biosystems, Foster City, Calif.). Primers and probes were synthesized and quality control conducted commercially (Synthetic Genetics, San Diego, Calif.). The efficacy of assay designs were validated in laboratory-based testing with multiple strains of dengue serotypes 1-4, yellow fever, Japanese encephalitis, West Nile, and St. Louis encephalitis viruses as well as dengue virus infected clinical specimens, vector species and human genomic DNA (McAvin JC, Escamilla EM, Blow JA, et al *Rapid Identification of Dengue Virus by RT-PCR Using Field-Deployable Instrumentation* (Submitted to Military Medicine 2004 Jan.).

Dengue Virus Inoculated Mosquitoes and Assay Test Panel

To field-site validate dengue virus field-formatted RT-PCR assay *in vitro* sensitivity and specificity Den1-4 inoculated *Aedes aegypti* were obtained from the Mosquito Biology Section, Department of Entomology, US

Medical Component Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. One- to 6–day-old adult female *Aedes aegypti* were inoculated intrathoraciclly (24) with one of the following viruses: DEN-1, DEN-2, DEN-3, DEN-4. 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 1-6 legs were removed for analyses. Legs were triturated in grinding diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earle's salts, $NaHCO_3$, and antibiotics) and tested for the presence of virus by plaque assay on LLC-MK-2 cells. One virus-inoculated or an unexposed mosquito body was added to each pool of non-infected mosquitoes. These were placed in sterile 1.5-ml Eppendorf tubes and triturated in 750 µl TrRIzol-LS (Life Technologies, USA).

To evaluate the efficacy of the field-deployable assay system a blind test panel of 64 nucleic acid extracts was prepared from DEN 1-4 inoculated *Aedes aegypti*, field-caught *Aedes aegypti* and *Culex* spp, and field-caught *Aedes aegypti* and *Culex* spp spiked with DEN 1-4 inoculated *Aedes aegypti* total nucleic acid (Table 1). The study site was Kamphaeng District, Thailand, August 2003. Mosquitoes were collected primarily in villages inhabited by patients with classic dengue fever symptoms. Mosquitoes were captured by battery powered hand-held aspiratorswithin village homes, mosquitoes pooled by residence, and transported live to the deployed assay platform.

RNA preparation

Total nucleic acid extracts were prepared with a commercially available, thermo-stable, preformatted viral RNA purification kit, QIAamp viral RNA mini kit (QIAGEN, Valencia, California). Mosquitoes were homogenized in 560 µl AVL Buffer/Carrier RNA component of the kit with sterile, RNase free pestles and 1.5 ml tubes. Homogenate was cleared by centrifugation on a table-top centrifuge at 12,000 rpm for 60 seconds and total nucleic acid extract prepared following the manufacture's spin protocol. Each extract was suspended in 60 µl of elution buffer.

Reaction conditions

Each sample was labelled under code and subjected to blind testing. Assays were conducted on the Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) [Idaho Technology Incorporated, Salt Lake City, Utah; www.idahotech.com] with lyophilized proprietary master mix components (Idaho Technology Incorporated, Salt Lake City, Utah) (25). Master mix reaction solution was prepared by adding 40 µl of PCR grade water to lyophilized master mix reagent and dispensing18 µl volumes into optical capillary tubes. To each capillary, 2 µl of RNA extract added from specimens or 2 µl PCR grade water for no template controls (NTC). Capillaries were placed in tabletop centrifuge and spun for 2-3 seconds at 3000 rpm to drive the reaction mixture to the bottom of the capillary. The dengue virus universal assay lyophilized master mix included thermo-stable, hydrolytic enzyme shielded, dengue virus Armored RNA® control template (Ambion RNA Diagnostics, Austin, Texas) (26) and was prepared by adding 40 µl of PCR grade water.

A standardized RT-PCR thermal cycling protocol was established that consisted of RT at 60°C for 20 minutes followed by an initial cDNA 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. Protocols for all five assays are identical with the exception of DEN-3, extension temperature

was 66°C. 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, Ind.).

Results

In preliminary field-site testing with DEN 1-4 inoculated *Aedes aegypti* control samples, all five field-formatted dengue virus assays had an *in vitro* sensitivity and specificity of 100%. Total nucleic acid extract isolated from DEN1-4 inoculated *Aedes aegypti*, field-captured *Aedes aegypti* and *Culex* spp, and field-captured *Aedes aegypti* and *Culex* spp spiked with extract from DEN 1-4 inoculated *Aedes aegypti* were used to construct a blind test panel (Table 1). In blind testing, the dengue universal assay demonstrated an *in vitro* sensitivity of 100% (14/14) and 100% specificity of (64/64) with DEN1-4 inoculated *Aedes aegypti* and field-captured *Aedes aegypti* and *Culex* spp spiked with DEN 1-4 inoculated *Aedes aegypti*. Field-formatted DEN-1, DV-2, and DEN-4 serotype specific assays demonstrated an *in vitro* sensitivity of 100% (3/3) and specificity of 100% (64/64) with DEN1-4 inoculated *Aedes aegypti* and field-captured *Aedes aegypti* and *Culex* spp spiked with DEN 1-4 inoculated *Aedes aegypti*. The field-formatted DEN-3 assay demonstrated an *in vitro* sensitivity of 100% (4/4) and specificity of 100% (64/64) with DEN1-4 inoculated *Aedes aegypti* and field-captured *Aedes aegypti* and *Culex* spp spiked with DEN 1-4 inoculated *Aedes aegypti*. A field-captured mosquito pool (#47) was identified as dengue virus positive by the dengue virus universal assay. Serotype identification was inconclusive. Sample processing and RT-PCR required less than two hours.

Discussion

The study site was Kamphaeng District, Thailand, August 2003. Ambient temperature and humidity conditions were typical of summer weather in tropical regions, 28-38 degrees centigrade and 80-100%, respectively. Logistical and operational constraints inherent to a developing region made it impractical to transport, store, and prepare conventional PCR reagents and control nucleic acid because cold chain resources were unreliable.

The R.A.P.I.D. provided field-durable, real-time, fluorimetric PCR/RT-PCR instrumentation and commercially available, “off the shelf”, thermo-stable nucleic acid isolation technology facilitated field-collected sample processing. We integrated lyophilized dengue virus RT-PCR assays and thermo-stabilized positive control to construct a fully deployable assay system. The validity of lyophilized dengue virus assays was field demonstrated with thermo-stable, hydrolytic enzyme shielded, Armored RNA® control template (Ambion RNA Diagnostics, Austin, Texas) lyophilized with master mix reagents (Idaho Technology Incorporated, Salt Lake City, Utah). These provided master mix reagents and positive control that were easily transported, field-sustainable, and only required hydration and addition of sample template prior to the RT-PCR. Evaluation of nucleic acid isolation reagents showed promise for field-collected sample processing.

In this field evaluation, a field-captured mosquito pool consisting of five female *Aedes aegypti*, including one blood fed, and two male *Aedes aegypti* was identified as dengue virus positive by the dengue virus universal assay however serotype identification was inconclusive. That the dengue universal and all four serotype specific assays demonstrated 100% concordance in *in vitro* sensitivity and 100% concordance in specificity with DEN1-4 inoculated *Aedes aegypti* and field-captured *Aedes aegypti* and *Culex* spp spiked with DEN 1-4 inoculated *Aedes aegypti* suggests a false negative in serotype identification and the source of error is experimental, most probably due to a sample number recording error. Additional field-testing is planned.

This study demonstrated the operational utility of a deployable assay platform for the surveillance of dengue virus, potential for applications in surveillance of other significant vector-borne pathogens, and promise as an aid in traditional clinical laboratory diagnostics.

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Table 1 RT-PCR Results

#	Genus	*species*	Sex	Collection location	DU-JCM	DEN-1	DV-2	DEN-3	DEN-4
1	*Aedes*	*aegypti*		DEN-1 Inoculated	+	+			
2	*Aedes*	*aegypti*		DEN-2 Inoculated	+		+		
3	*Aedes*	*aegypti*		DEN-3 Inoculated	+			+	
4	*Aedes*	*aegypti*		DEN-4 Inoculated	+				+
5	*Culex*	*quinquefasciatus/tritaeniorhynchus*	3 F	Village 1/House 053 (1/053)					
6	*Aedes*	*aegypti*	1 F	1/059					
7	*Culex*	*tritaeniorhynchus*	1 F	1/059					
8	*Culex*	*tritaeniorhynchus*	5 F	1/058					
9	*Culex*	*tritaeniorhynchus* spiked with DEN-1	4 M	1/058	+	+			
10	*Aedes*	*aegypti*	1 F	1/058					
11	*Aedes*	*aegypti* spiked with DEN-3	1 M	1/058	+			+	
12	*Aedes*	*aegypti* spiked with DEN-4	2 M	1/050	+				+
13	*Aedes*	*aegypti*	6 F	1/049					
14	*Aedes*	*aegypti*	1 M	1/049					
15	*Culex*	*tritaeniorhynchus*	1 F	1/049					
16	*Culex*	*gelidus*	2 F	1/054					
17	*Culex*	*tritaeniorhynchus*	8 F	1/054					
18	*Aedes*	*aegypti*	1 F	1/054					

#	Genus	*species*	Sex	Collection location	DU-JCM	DEN-1	DV-2	DEN-3	DEN-4
19	*Aedes*	*aegypti* spiked with DEN-2	5 M	1/054	+		+		
20	*Aedes*	*aegypti*	7 F	1/056					
21	*Aedes*	*aegypti*	1 M	1/056					
22	*Culex*	*tritaeniorhynchus*	1 F	1/056					
23	*Culex*	*tritaeniorhynchus*	10 F	1/057					
24	*Culex*	*tritaeniorhynchus*	10 F	1/057					
25	*Culex*	*tritaeniorhynchus*	12 F	1/057					
26	*Aedes*	*aegypti*	5 F	1/057					
28	*Aedes*	*aegypti*	2 F	1/055					
29	*Aedes*	*aegypti*	9 F	1/067					
30	*Aedes*	*albopictus*	1 F	1/067					
31	*Culex*	*tritaeniorhynchus/geldidus*	9 F/ 2F	1/060					
32	*Culex*	*tritaeniorhynchus/vishnui*	2 F/2M	1/150					
33	*Aedes*	*aegypti*	3 F	1/150					
34	*Aedes*	*aegypti*	1 F	1/051					
35	*Culex*	*quinquefasciatus/tritaeniorhynchus*	2F/2F	1/062					
36	*Aedes*	*aegypti*	2 F	1/066					
37	*Aedes*	*aegypti*	1 F	1/066					
38	*Culex*	*quinquefasciatus/tritaeniorhynchus*	2F/3F	Village 8 House 50 (8/50)					
39	*Culex*	*quinquefasciatus*	10 F	Village 8 House 50 (8/50)					
40	*Aedes*	*aegypti*	2 F	8/50					
41	*Aedes*	*aegypti*	10 M	8/50					
42	*Aedes*	*aegypti*	2 F	8/252/11					
43	*Aedes*	*aegypti*	2 F (PBM)	8/252/12					
44	*Aedes*	*aegypti*	3F/3M	8/32					
45	*Aedes*	*aegypti*	1F/2M	8/45					

#	Genus	*species*	Sex	Collection location	DU-JCM	DEN-1	DV-2	DEN-3	DEN-4
46	*Aedes*	*aegypti*	2F/2M	8/19					
47	*Aedes*	*aegypti*	5F(1FBM)/2M	8/25	+				
48	*Aedes*	*aegypti*	2 F	8/51					
49	*Aedes*	*aegypti*	1F (BF)/6M	8/22					
50	*Aedes*	*aegypti*	1 M	8/21					
51	*Aedes*	*aegypti*	1 F	AFRMIS- Inoculated Dengue 3	+			+	
52	*Aedes*	*aegypti*	1 F	AFRMIS- Inoculated Dengue 2	+		+		
53	*Aedes*	*aegypti*	1 F	AFRMIS- Inoculated Dengue 1	+	+			
56	*Aedes*	*aegypti*	1 F	Village 642 House 229 (642/229)					
57	*Culex*	*quinquefasciatus*	4 F	642/231					
58	*Culex*	*quinquefasciatus*	12 M	642/231					
59	*Culex*	*quinquefasciatus*	1 F (BF)	642/231					
60	*Culex*	*quinquefasciatus*	1 F (PBM)	642/231					
61	*Culex*	*quinquefasciatus*	1 F (PBM)	642/231					
62	*Culex*	*quinquefasciatus*	1 F (BF)	642/231					
63	*Culex*	*quinquefasciatus*	1 F (PBM)	642/231					
64	*Culex*	*quinquefasciatus*	1 F	642/231					

F=Female

M= Male

BM= Bloodmeal

PBM = Partial Bloodmeal

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