Deployable, Field-Sustainable, Reverse Transcription-Polymerase Chain Reaction Assays for Rapid Screening and Serotype Identification of Dengue Virus in Mosquitoes

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Dengue virus universal and serotype 1 to 4 fluorogenic probe hydrolysis, reverse transcription (RT)-polymerase chain reaction (PCR) assays and positive-control RNA template were freeze-dried in a thermally stable, hydrolytic enzyme-resistant format and deployed for testing in a dengue fever-endemic region of Thailand. The study site presented austere testing conditions. Field-collected Aedes aegypti mosquitoes spiked with inoculated A. aegypti mosquitoes and individual and pooled, field-collected, A. aegypti, A. albopictus, and Culex tritaeniorhynchus mosquitoes were used for RT-PCR assay evaluations. For dengue virus-inoculated A. aegypti mosquitoes and spiked samples, in vitro sensitivity and specificity results for all five assays were concordant with indirect fluorescent antibody assay results. A single pool of field-collected, female, A. aegypti mosquitoes was identified as dengue virus positive. Cross-reactivity was not observed across heterologous serotypes, mosquito vectors, or human DNA. The limit of detection was >7 to ≤70 genomic equivalents. Sample processing and analysis required ≤2 hours. These results show promise of field-formatted RT-PCR reagents for rapid, sensitive, specific dengue virus screening and serotype identification in mosquitoes under field-deployed conditions.

Introduction

The globally expanding prevalence of febrile disease causative agents and their vectors and increased frequency of outbreaks place the health of deployed U.S. Armed Forces members and operational capability at risk. Efficacious force health protection at forward locations requires deployable, field-sustainable, real-time, disease agent surveillance capability. Rapid disease risk assessment is paramount in effecting time-critical and focused disease prevention and vector control measures. Over the past two decades, dengue fever (DF) and the potentially fatal forms of the disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome, have become increasingly frequent and pervasive and now represent the most significant mosquito-borne viral disease in tropical and subtropical regions globally.1–5 Vaccines in development show promise; however, approval is not anticipated in the near future.3,4 The current method of prevention is vector avoidance.5,6 Vector surveillance is required in assessment of DF transmission risk, and identification of circulating serotypes, with epidemiological history of causative serotypes in previous outbreak situations, is fundamental to DHF/dengue shock syndrome risk assessment.5

DF is caused by infection with dengue virus. Dengue virus exists as four serologically and genetically distinct serotypes, transmitted to humans by female Aedes aegypti and Aedes albopictus mosquitoes.5–12 Dengue virus surveillance is restricted by limitations in analytical and operational capabilities. Immunoassay-based testing can take ≥1 week, and the results are ambiguous because of cross-reactivity with other flaviviruses.11,12 Highly sensitive and specific dengue virus screening and serotype 1 to 4 reverse transcription (RT)-polymerase chain reaction (PCR) assays currently exist but are for use with laboratory-based instrumentation and require –20°C cold-chain resources for transportation and storage.13–19

Surveillance conducted under austere field conditions presents logistical and operational constraints that make it impractical to transport, store, and prepare cold-chain-maintained reagents. Unknown variations in PCR enzyme activity and positive-control nucleic acid template integrity result when cold-chain resources are inadequate. Commercially available, field-validated, PCR instrumentation and sample-processing reagents fulfill a fundamental requirement in establishing a deployable pathogen identification system. However, development of field-sustainable PCR reagents and positive-control nucleic acid template is essential for the realization of an omnipotent platform. In this work, we describe laboratory and field evaluations of freeze-dried, thermally stable, hydrolytic enzyme-resistant, RT-PCR reagents and positive-control RNA template for rapid dengue virus screening and serotype identification in mosquitoes under austere field conditions.

Methods

Study Site

The study was conducted 358 km north of Bangkok, Thailand, at Kamphaeng Phet Province, Ampur District, in August 2003. Three villages inhabited by patients with recent classic DF symptoms were chosen as collection locations. At the time of the study, villages designated 1 and 642 were inhabited by a febrile child and adult, respectively. A convalescent child diagnosed with DF lived in village 8 (Table I). Reagents were transported at ambient temperatures from our United States-based laboratory facilities to the study site. The field laboratory was an open-air structure where samples were prepared and analyses were conducted under ambient temperature (25–33°C) and humidity...
(80–100%) conditions typical of the rainy season in lower northern Thailand. Refrigeration for reagent and sample storage was unavailable or unreliable.

Mosquito Panel
To evaluate RT-PCR assay in vitro sensitivity and specificity, a mosquito nucleic acid extract panel was prepared, samples were labeled by code, and testing was conducted in a blind format. The Mosquito Biology Section, Department of Entomology, U.S. Medical Component Armed Forces Research Institute of Medical Sciences (Bangkok, Thailand), provided dengue virus 1 to 4-inoculated *A. aegypti* mosquitoes for use in field-based assay confirmation testing and mosquito panel construction. Adult, female, *A. aegypti* mosquitoes were inoculated intrathoracically with one of the four dengue virus serotypes and were tested by indirect fluorescent antibody assay.21 One virus-inoculated mosquito of each serotype was placed in an individual, sterile, 1.5-mL Eppendorf tube, and virus inactivation and nucleic acid preservation were accomplished by homogenization in 560 μL of Anschlagpuffer virus lysis (AVL) buffer solution (Qiagen, Valencia, California). Homogenates were transported to the study site at ambient temperature, and nucleic acid extracts were prepared.

Field-collected mosquitoes were captured inside homes, primarily off interior walls, with battery-powered backpack aspirators, transported live to the field laboratory, identified morphologically, pooled according to genus and residence, and preserved in AVL buffer. Field-collected mosquito species in-

### Table I

| Sample No. | Species | Gender | Sample Site | DU-JCM | DEN-1 | DEN-2 | DEN-3 | DEN-4 |
|------------|---------|--------|-------------|--------|-------|-------|-------|-------|
| 1          | *A. aegypti* (DEN-1 inoculated) | 1 F    | AFRIMS      | 20.19  | 30.27 | 0     | 0     | 0     |
| 2          | *A. aegypti* (DEN-2 inoculated) | 1 F    | AFRIMS      | 16.86  | 0     | 28.74 | 0     | 0     |
| 3          | *A. aegypti* (DEN-3 inoculated) | 1 F    | AFRIMS      | 21.63  | 0     | 29.83 | 0     | 0     |
| 4          | *A. aegypti* (DEN-4 inoculated) | 1 F    | AFRIMS      | 20.91  | 0     | 0     | 0     | 26.4  |
| 5          | *A. aegypti*                | 1 F    | Village/house 1/059 | 0     | 0     | 0     | 0     |
| 6          | *Culex tritaeniorhynchus* spiked with DEN-1 | 4 M    | Village/house 1/058 | 19.19  | 17.6  | 0     | 0     |
| 7          | *A. aegypti*                | 1 F    | Village/house 1/058 | 0     | 0     | 0     | 0     |
| 8          | *A. aegypti* spiked with DEN-3 | 1 M    | Village/house 1/058 | 21.16  | 0     | 20.74 | 0     |
| 9          | *A. aegypti* spiked with DEN-4 | 2 M    | Village/house 1/050 | 18.78  | 0     | 0     | 0     | 18.12 |
| 10         | *A. aegypti*                | 6 F    | Village/house 1/049 | 0     | 0     | 0     | 0     |
| 11         | *A. aegypti*                | 1 M    | Village/house 1/049 | 0     | 0     | 0     | 0     |
| 12         | *A. aegypti*                | 1 F    | Village/house 1/054 | 0     | 0     | 0     | 0     |
| 13         | *A. aegypti* spiked with DEN-2 | 5 M    | Village/house 1/054 | 18.32  | 0     | 23.81 | 0     |
| 14         | *A. aegypti*                | 7 F    | Village/house 1/056 | 0     | 0     | 0     | 0     |
| 15         | *A. aegypti*                | 1 M    | Village/house 1/056 | 0     | 0     | 0     | 0     |
| 16         | *A. aegypti*                | 5 F    | Village/house 1/057 | 0     | 0     | 0     | 0     |
| 17         | *A. aegypti*                | 2 F    | Village/house 1/055 | 0     | 0     | 0     | 0     |
| 18         | *A. aegypti*                | 9 F    | Village/house 1/067 | 0     | 0     | 0     | 0     |
| 19         | *A. albopictus*              | 1 F    | Village/house 1/067 | 0     | 0     | 0     | 0     |
| 20         | *A. aegypti*                | 3 F    | Village/house 1/150 | 0     | 0     | 0     | 0     |
| 21         | *A. aegypti*                | 1 F    | Village/house 1/051 | 0     | 0     | 0     | 0     |
| 22         | *A. aegypti*                | 2 F    | Village/house 1/066 | 0     | 0     | 0     | 0     |
| 23         | *A. aegypti*                | 1 F    | Village/house 1/066 | 0     | 0     | 0     | 0     |
| 24         | *A. aegypti*                | 2 F    | Village/house 8/50  | 0     | 0     | 0     | 0     |
| 25         | *A. aegypti*                | 10 M   | Village/house 8/50  | 0     | 0     | 0     | 0     |
| 26         | *A. aegypti*                | 2 F    | Village/house 8/252/11 | 0     | 0     | 0     | 0     |
| 27         | *A. aegypti* (partial blood fed) | 2 F    | Village/house 8/252/12 | 0     | 0     | 0     | 0     |
| 28         | *A. aegypti*                | 3 F/3 M| Village/house 8/32  | 0     | 0     | 0     | 0     |
| 29         | *A. aegypti*                | 1 F/2 M| Village/house 8/45  | 0     | 0     | 0     | 0     |
| 30         | *A. aegypti*                | 2 F/2 M| Village/house 8/19  | 0     | 0     | 0     | 0     |
| 31         | *A. aegypti*                | 5 F/2 M| Village/house 8/25  | 20.55, 21.67 | 0     | 0     |
| 32         | *A. aegypti*                | 2 F    | Village/house 8/51  | 0     | 0     | 0     | 0     |
| 33         | *A. aegypti* (blood fed)    | 1 F/6 M| Village/house 8/22  | 0     | 0     | 0     | 0     |
| 34         | *A. aegypti*                | 1 M    | Village/house 8/21  | 0     | 0     | 0     | 0     |
| 35         | *A. aegypti* (DEN-3 inoculated) | 1 F    | AFRIMS      | 23.05  | 0     | 0     | 28.89 | 0     |
| 36         | *A. aegypti* (DEN-2 inoculated) | 1 F    | AFRIMS      | 18.95  | 0     | 23.81 | 0     |
| 37         | *A. aegypti* (DEN-1 inoculated) | 1 F    | AFRIMS      | 20.07  | 21.34 | 0     | 0     |
| 38         | *A. aegypti* (DEN-4 inoculated) | 1 F    | AFRIMS      | 23.54  | 0     | 0     | 25.69 |
| 39         | *A. aegypti* (DEN-3 inoculated) | 1 F    | AFRIMS      | 18.38  | 0     | 0     | 0     |
| 40         | *A. aegypti*                | 1 F    | Village/house 642/229 | 0     | 0     | 0     |

AFRIMS, U.S. Armed Forces Research Institute of Medical Sciences.
cluded A. aegypti, A. albopictus, Culex quinquefasciatus, Culex tritaeniorhynchus, Culex gelidus, and Culex vishnui. A blind panel (n = 40) was prepared and labeled under code; it consisted of extracts from 36 field-captured A. aegypti, A. albopictus, and C. tritaeniorhynchus individual mosquitoes and pools, including four dengue virus 1 to 4-spiked pools, and nine dengue virus-inoculated A. aegypti nucleic acid extracts (Table I). Field-collected, female, A. aegypti mosquitoes were present in 22 samples and female A. albopictus mosquitoes in one sample. Samples 27 and 33 were partially blood fed and blood fed, respectively.

RNA Preparation
Individual mosquitoes and pools were homogenized in 560 µL of AVL buffer/carrier RNA solution included in a commercially available, “off-the-shelf,” total nucleic acid extraction kit (QiAamp viral RNA mini-kit; Qiagen). Homogenates were prepared with sterile, RNase-free pestles and 1.5-mL tubes. Each homogenate was cleared by centrifugation on a tabletop centrifuge at 12,000 rpm for 60 seconds, and total nucleic acid extract was prepared by following the manufacturer’s protocol. Each extract was suspended in 60 µL of elution buffer. Twenty-four samples were processed in 45 minutes.

Primer and Probe Design
Dengue virus universal assay (DU-JCM) primer and probe sequences were designed de novo by aligning homologous genomic regions of dengue virus serotypes 1 to 4 that excluded sequences previously described.22 Primer and probe design and laboratory-based testing of each assay were previously described.22

Reaction Conditions
Assays were conducted with the “ruggedized” advanced pathogen identification device (R.A.P.I.D.) (Idaho Technology, Salt Lake City, Utah) with field-formatted reagents with thermally stable, freeze-dried, proprietary RT-PCR master mixture components (Idaho Technology). The R.A.P.I.D. has a 32-sample capacity and achieves high-speed thermal cycling through fan-driven air, rather than heat block conduction. The system requires a 110-V power source for operation and can be run by a generator or a 12-V vehicle battery. Data management is automated, consisting of a RT step at 60°C for 2 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 the TaqMan probe fluorescence released by 5’-nuclease activity during primer extension. Fluorimeter gains were set at 8, 2, and 2 on channels 1, 2, and 3, respectively. Protocols for all five assays were identical with the exception of DEN-3; the extension temperature was 66°C.

To quantify the dengue universal assay (DU-JCM) limit of detection (LOD), a standard curve was generated with 6 logarithmic units of double-replicate samples of Armored RNA-dengue virus (7.0 × 10^4 to 7 × 10^-1 copies per reaction). Exclusivity of human DNA was evaluated with double-replicate samples of 1.0 ng of TaqMan control human genomic DNA (Applied Biosystems, Foster City, California).

Results
The LOD of the dengue virus universal assay (DU-JCM) was >7 to ≤70 genomic equivalents in linear regression analysis (slope, ΔΔCt cycle number/ΔGE, was ~4.401; error, 0.362%; correlation coefficient, −0.98) (data not shown). Mean C, and SD were as follows: 7 × 10^4, mean, 20.21; SD, 0.96; 7.0 × 10^3, mean, 25.02; SD, 0.92; 7.0 × 10^2, mean, 28.46; SD, 0.29; 7.0 × 10^1, mean, 31.99; SD, 0.33; 7.0 × 10^0, mean, 39.77; SD, 0.00; 7.0 × 10^-1, mean, 0.00; SD, 0.00. Fluorescence above background was not observed in testing with human genomic DNA.

In field testing, RT-PCR assays were evaluated with a blind panel (n = 40) consisting of nine dengue 1 to 4-inoculated A. aegypti mosquitoes, four field-collected mosquito samples spiked with dengue virus 1 to 4-inoculated A. aegypti mosquitoes, and 27 samples of individual mosquitoes and pools of field-collected A. aegypti, A. albopictus, and C. tritaeniorhynchus mosquitoes (Table I). The in vitro sensitivity of the dengue virus RT-PCR universal assay and each serotype-specific assay with each master mixture vial and dispensing 18-µL volumes into optical capillary tubes. To each capillary tube, 2 µL of nucleic acid extract was added from each sample (or 2 µL of PCR-grade water for Armored RNA-dengue virus positive-control and nontemplate control assays). Capillary tubes were placed in a tabletop centrifuge and centrifuged for 2 to 3 seconds at 3,000 rpm, to drive the reaction mixture to the bottom of the capillary. Master mixture components consisted of forward and reverse primers, TaqMan probe,25 26 dATP, dTTP, dGTP, and dCTP, Mn(A0c), Tth polymerase, and proprietary 5x RT buffer (Idaho Technology). The master mixture composition of each assay was previously described.22

A standardized, RT-PCR, thermal cycling protocol was established, consisting of a RT step 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 the TaqMan probe fluorescence released by 5’-nuclease activity during primer extension. Fluorimeter gains were set at 8, 2, and 2 on channels 1, 2, and 3, respectively. Protocols for all five assays were identical with the exception of DEN-3; the extension temperature was 66°C.

Sample identification and specifications were entered electronically in the R.A.P.I.D. operating system run protocol for dengue virus RT-PCR before analyses, and results were automatically archived. The criterion for a positive result was a significant increase in fluorescence over background levels, i.e., critical threshold (Ct), defined by an algorithm provided in the R.A.P.I.D analytical software (Roche Molecular Biochemicals, Indianapolis, Indiana). The Ct is defined as the first PCR cycle with significant fluorescence when normalized against background fluorescence.

To quantify the dengue universal assay (DU-JCM) limit of detection (LOD), a standard curve was generated with 6 logarithmic units of double-replicate samples of Armored RNA-dengue virus (7.0 × 10^4 to 7 × 10^-1 copies per reaction). Exclusivity of human DNA was evaluated with double-replicate samples of 1.0 ng of TaqMan control human genomic DNA (Applied Biosystems, Foster City, California).

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A. albopictus represented a total of 60 female mosquitoes with no-template control samples. The observed infection rate was consistent with published results. The Armored RNA-dengue virus positive-control samples consistently demonstrated fluorescence. The LOD of the dengue universal assay was ≤ 1 log unit of target template. The mean C\_t of samples tested with the screening assay showed that the LOD was 3 log units more sensitive than required for identification of dengue virus-infected mosquitoes. We cannot relate the LOD to potential efficacy in human diagnostic tests, because the concentration of dengue RNA in DF/DHF patient samples and the correlation between copy number and plaque-forming units have not been established. We have a study underway to quantify in vitro sensitivity with dengue virus-infected serum samples. Specificity testing with genotypically similar and clinically relevant species and strains remains an ongoing process as we expand our nucleic acid archive.

Analysis of pure RNA target template is required to express RT-PCR assay sensitivity in genomic equivalents. Purified RNA extracts from cultures or in vitro transcripts are typically used but must be maintained and handled meticulously, because of the inherent fragility of single-stranded template. Challenges in RT-PCR assay sensitivity quantification have been previously discussed. In this work, Armored RNA-dengue virus was used as the quantitative standard for LOD determination. As nature, a protein coat (synthetic MS2 bacteriophage coat protein) protects RNA from degradation. Upon heating in the RT step of the RT-PCR, the protein “armor” is removed and RNA is immediately synthesized into cDNA, providing a stable template from RT through PCR. Armored RNA stability provides promise for the development of real-time quantitative assays. The ability to temporally monitor virus titers has profound implications in vaccine testing. Armored RNA currently has a quantitative application as an internal control. Studies have shown that, by adding Armored RNA to mosquito vector homogenates and patient body fluid and stool samples, the efficiency of the nucleic acid preparation process and each step of the RT-PCR can be monitored quantitatively.

Sample processing was simplified by using a commercially available nucleic acid extraction kit. We chose the kit because of its preformatted composition and uncomplicated protocol, but we found additional advantages. The AVL buffer has been shown to inactivate virus. The significance of rendering specimens noninfectious during handling and processing under austere field conditions and low-level containment cannot be overstated. Moreover, preliminary testing indicates that dengue virus genomic RNA remains stable in AVL buffer at ambient temperatures (25–37°C) for days to weeks. Once fully validated in stability studies, this should facilitate sample handling, in terms of cold-chain requirements. With respect to technology transfer in the context of logistics and training, a single protocol is applicable for RNA and DNA genomes, because total nucleic acid is extracted.

In our work, the concept was reinforced that human error attributable to environmental factor-induced fatigue must be addressed in experiment planning for studies conducted under extreme or hazardous conditions. Established experiment protocols that are simple to follow and well illustrated with cue cards and checklists, preformatted kits, color coding, predeployment training including simulations, cross-train-
ing, and readily accessible reach-back support must be implemented in the work plan, to minimize the risk of experimental error.

The expense of freeze-dried RT-PCR reagents and Armored RNA-dengue virus as well as sample-processing kits and instrumentation, is cost-effective, compared with the expense and practicality of establishing far-forward, fixed-site laboratories and using classic analytic methods, including virus isolation and immunoassay. Moreover, real-time intervention in DF outbreaks through expeditious and focused vector control represents incalculable cost savings in terms of patient care.

The Joint Program Executive Office for Chemical and Biological Defense has designated dengue virus as a threat agent (block 1, tier 2) and established a requirement for identification technologies.27 To meet this requirement, testing is underway on the Joint Program Executive Office/Department of Defense-accepted PCR thermocycler, the Joint Biological Agent Identification and Diagnostic System (J.B.A.I.D.S.). Upon validation, dengue virus freeze-dried assay technologies will be transferred to the Joint Program Executive Office, as stipulated in the Critical Reagents Program multilaboratory agreement.

Conclusions

Our study demonstrates the utility of freeze-dried, thermally stable, hydrolytic enzyme-resistant, dengue virus RT-PCR assays and Armored RNA-dengue virus for rapid, sensitive, and specific screening and serotype identification in mosquitoes under austere field conditions. This system shows promise as a fully deployable package for surveillance of dengue virus in mosquitoes.

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