

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 far-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.^{1,2} 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 identi-

fication 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.^{5,7-10} 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 ($25\text{--}33^{\circ}\text{C}$) and humidity

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This manuscript was received for review in November 2005. The revised manuscript was accepted for publication in July 2006.

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TABLE I
DENGUE VIRUS SCREENING AND SEROTYPE 1 TO 4 RT-PCR ASSAY RESULTS

Sample	Species	No. of Gender	Sample Site	Ct				
				DU-JCM	DEN-1	DEN-2	DEN-3	DEN-4
1	<i>A. aegypti</i> (DEN-1 inoculated)	1 F	AFRIMS	20.19	30.27	0	0	0
2	<i>A. aegypti</i> (DEN-2 inoculated)	1 F	AFRIMS	16.86	0	28.74	0	0
3	<i>A. aegypti</i> (DEN-3 inoculated)	1 F	AFRIMS	21.63	0	0	29.83	0
4	<i>A. aegypti</i> (DEN-4 inoculated)	1 F	AFRIMS	20.91	0	0	0	26.4
5	<i>A. aegypti</i>	1 F	Village/house 1/059	0	0	0	0	0
6	<i>Culex tritaeniorhynchus</i> spiked with DEN-1	4 M	Village/house 1/058	19.19	17.6	0	0	0
7	<i>A. aegypti</i>	1 F	Village/house 1/058	0	0	0	0	0
8	<i>A. aegypti</i> spiked with DEN-3	1 M	Village/house 1/058	21.16	0	0	20.74	0
9	<i>A. aegypti</i> spiked with DEN-4	2 M	Village/house 1/050	18.78	0	0	0	18.12
10	<i>A. aegypti</i>	6 F	Village/house 1/049	0	0	0	0	0
11	<i>A. aegypti</i>	1 M	Village/house 1/049	0	0	0	0	0
12	<i>A. aegypti</i>	1 F	Village/house 1/054	0	0	0	0	0
13	<i>A. aegypti</i> spiked with DEN-2	5 M	Village/house 1/054	18.32	0	23.81	0	0
14	<i>A. aegypti</i>	7 F	Village/house 1/056	0	0	0	0	0
15	<i>A. aegypti</i>	1 M	Village/house 1/056	0	0	0	0	0
16	<i>A. aegypti</i>	5 F	Village/house 1/057	0	0	0	0	0
17	<i>A. aegypti</i>	2 F	Village/house 1/055	0	0	0	0	0
18	<i>A. aegypti</i>	9 F	Village/house 1/067	0	0	0	0	0
19	<i>A. albopictus</i>	1 F	Village/house 1/067	0	0	0	0	0
20	<i>A. aegypti</i>	3 F	Village/house 1/150	0	0	0	0	0
21	<i>A. aegypti</i>	1 F	Village/house 1/051	0	0	0	0	0
22	<i>A. aegypti</i>	2 F	Village/house 1/066	0	0	0	0	0
23	<i>A. aegypti</i>	1 F	Village/house 1/066	0	0	0	0	0
24	<i>A. aegypti</i>	2 F	Village/house 8/50	0	0	0	0	0
25	<i>A. aegypti</i>	10 M	Village/house 8/50	0	0	0	0	0
26	<i>A. aegypti</i>	2 F	Village/house 8/252/11	0	0	0	0	0
27	<i>A. aegypti</i> (partial blood fed)	2 F	Village/house 8/252/12	0	0	0	0	0
28	<i>A. aegypti</i>	3 F/3 M	Village/house 8/32	0	0	0	0	0
29	<i>A. aegypti</i>	1 F/2 M	Village/house 8/45	0	0	0	0	0
30	<i>A. aegypti</i>	2 F/2 M	Village/house 8/19	0	0	0	0	0
31	<i>A. aegypti</i>	5 F/2 M	Village/house 8/25	20.55, 21.67	0	0	0	0
32	<i>A. aegypti</i>	2 F	Village/house 8/51	0	0	0	0	0
33	<i>A. aegypti</i> (blood fed)	1 F/6 M	Village/house 8/22	0	0	0	0	0
34	<i>A. aegypti</i>	1 M	Village/house 8/21	0	0	0	0	0
35	<i>A. aegypti</i> (DEN-3 inoculated)	1 F	AFRIMS	23.05	0	0	28.89	0
36	<i>A. aegypti</i> (DEN-2 inoculated)	1 F	AFRIMS	18.95	0	23.81	0	0
37	<i>A. aegypti</i> (DEN-1 inoculated)	1 F	AFRIMS	20.07	21.34	0	0	0
38	<i>A. aegypti</i> (DEN-4 inoculated)	1 F	AFRIMS	23.54	0	0	0	25.69
39	<i>A. aegypti</i> (DEN-3 inoculated)	1 F	AFRIMS	18.58	0	0	0	0
40	<i>A. aegypti</i>	1 F	Village/house 642/229	0	0	0	0	0

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(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. Military 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 intrathoraci-

cally²⁰ 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 μ L 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-

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 other clinically significant flaviviruses. Serotype-specific primer and probe sequences were adopted from the literature (i.e., DEN-1,¹³ DEN-2,¹⁴ DEN-3,¹³ and DEN-4¹³). 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. Sample processing and RT-PCR require <2 hours.

Thermally stable, hydrolytic enzyme-shielded, Armored RNA-dengue virus was used as a positive-control template for the DU-JCM assay (Ambion Diagnostics, Austin, Texas).^{23,24} The concentration of Armored RNA-dengue virus positive-control template was 6.4×10^4 target templates per 20 μL of reaction mixture. Armored RNA-dengue virus preparations were homogenous and noninfectious.

Assay preparation required only hydration and addition of sample template before analyses. Two freeze-dried RT-PCRs per 1.5-mL amber, plastic, screwtop vial were preformatted in kits with 1.5-mL vials of PCR-grade water and proprietary 2 \times reconstitution solution (Idaho Technology). Master mixture reaction solution was prepared by adding 36 μL of hydration solution (1:1, v/v, PCR-grade water and 2 \times reconstitution solution) to

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 no-template 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(AOc), Tth polymerase, and proprietary 5 \times 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 (C_t), defined by an algorithm provided in the R.A.P.I.D. analytical software (Roche Molecular Biochemicals, Indianapolis, Indiana). The C_t 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).

Results

The LOD of the dengue virus universal assay (DU-JCM) was >7 to ≤ 70 genomic equivalents in linear regression analysis (slope, $\Delta\text{PCR cycle number}/\Delta\text{GE}$, was -4.401 ; error, 0.362%; correlation coefficient, -0.98) (data not shown). Mean C_t 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, 00.00; 7.0×10^{-1} , mean, 00.00; SD, 00.00. 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

nucleic acid extract from dengue 1 to 4-inoculated *A. aegypti* mosquitoes was 100% concordant with laboratory-based, indirect fluorescent antibody assay results, with the exception of DEN-3 (67% concordant); the assay failed to identify a single dengue 3-inoculated specimen (sample 39) (DU-JCM, nine of nine samples; DEN-1, two of two samples; DEN-2, two of two samples; DEN-3, two of three samples; DEN-4, two of two samples). Spiked sample in vitro sensitivity was 100% concordant (DU-JCM, four of four samples; DEN-1, one of one sample; DEN-2, one of one sample; DEN-3, one of one sample; DEN-4, one of one sample). Of 23 nonspiked samples with one or more field-collected *A. aegypti* female mosquito and an individual *A. albopictus* female mosquito, a single pool (sample 31, village 8), consisting of five female and two male *Aedes* mosquitoes, was reported as a strong positive with the dengue virus universal assay ($C_t = 20.55$; when repeated, $C_t = 21.67$) (Table I). Serotype identification was inconclusive. Mean C_t and SD were as follows: DU-JCM, mean, 20.23; SD, 1.83; DEN-1, mean, 23.07; SD, 6.51; DEN-2, mean, 25.45; SD, 2.85; DEN-3, mean, 26.49; SD, 5.00; DEN-4, mean, 23.40; SD, 4.59. The dengue universal assay with Armored RNA-dengue virus template consistently reported fluorescence (mean C_t , 28.64; SD, 0.62; $n = 14$) (data not shown).

The specificity of each dengue virus serotype assay was 100% concordant with dengue virus 1 to 4-inoculated *A. aegypti* and spiked samples; no cross-reactivity was observed across heterologous serotypes (Table I). Cross-reactivity with mosquito genomic DNA was not observed with the dengue universal assay and each serotype-specific assay. Inhibition of the RT-PCR by mosquito extract was not observed in field-collected samples of male *Aedes* and *Culex* spp. spiked with dengue virus 1 to 4-inoculated *A. aegypti* mosquito extract (samples 6, 13, 8, and 9, respectively). No fluorescence above background was observed with no-template control samples. The observed infection rate was 1.64% (1 of 61 samples); one infected pool of 23 pools represented a total of 60 female *A. aegypti* mosquitoes and one female *A. albopictus* mosquito.

Discussion

This article describes freeze-dried, dengue virus universal and serotype 1 to 4 RT-PCR assays and positive-control RNA template that, when integrated with field-durable instrumentation and preformatted sample preparation materials, constituted a portable, field-sustainable, sensitive, specific, real-time, pathogen identification system. Field-deployed analytic capability has profound implications in force health protection, by allowing real-time disease transmission risk assessment and implementation of time-critical and focused prevention and control measures.

Dengue virus universal and serotype 1 to 4 assay results were concordant when tested against dengue virus 1 to 4-inoculated *A. aegypti* and spiked field-collected specimens. The finding that the DEN-3 assay failed to report a single dengue 3 virus-inoculated *A. aegypti* mosquito does not imply degradation of field-formatted reagents but indicates experimental error, because the performance of the assay system was highly sensitive and specific, based on overall test panel results. In the only village with a DF-diagnosed individual of the three collections sites, a single pool of female *A. aegypti* mosquitoes was identified as

dengue virus positive with the dengue virus universal assay. The observed infection rate was consistent with published results.²⁷⁻³¹ 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.¹³ In this work, Armored RNA-dengue virus was used as the quantitative standard for LOD determination. As in nature, a protein coat (synthetic MS2 bacteriophage coat protein) protects RNA from degradation.²³ 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.²⁴ 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.³²⁻³⁵

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.³⁶ 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.³⁷ 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.

Acknowledgments


Thanks go to James W. Jones and Ratana Sithiprasasna, Armed Forces Research Institute of Medical Sciences, Walter Reed Army Institute of Health, U.S. Army Medical Research and Materiel Command (Bangkok, Thailand), for their efforts in coordinating approval of the study through the Ministry of Public Health, facilitating country clearances, and providing access to the field site, entomological expertise, and technical and logistical support. We thank Russell E. Coleman, Division of Entomology, Walter Reed Army Institute of Health (Silver Spring, Maryland), for providing laboratory facilities and resources.

This work was funded by U.S. Air Force Force Protection BattleLab (Lackland Air Force Base, Texas) and conducted through an Air Force Institute for Operational Health/Idaho Technology Cooperative Research and Development Agreement.

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