Leveraging Arthropod-Borne Disease Surveillance Assays for Clinical Diagnostic Use

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ABSTRACT Researchers at the Walter Reed Army Institute of Research have taken a joint service approach to filling an identified diagnostic capability gap by leveraging a vector surveillance assay. Specifically, the Army took a field-stable real-time polymerase chain reaction assay, developed by the Air Force, for dengue virus surveillance in arthropod vectors and collaborated with Navy researchers for utility in human diagnostics. As current Department of Defense diagnostic PCR assays employ the Joint Biological Agent Identification and Diagnostic System, the dengue assay was tested for use on this platform. The low rates of false negative and false positive dengue samples in clinical matrices demonstrate excellent utility as a human diagnostic assay. Overall, converting an arboviral vector surveillance assay to human diagnostic assay and potentially vice versa is both cost effective and labor reducing. Codevelopment with harmonization of vector surveillance and diagnostics offers monetary and resource advantages to the Department of Defense and should be considered as a path forward in times when downsizing threatens assay development and pathogen discovery.

INTRODUCTION

Dengue fever (DF) is caused by dengue virus, which is a flavivirus that is transmitted by female *Aedes* mosquitoes (mainly *A. aegypti* and *A. albopictus*). Symptoms include but are not limited to high fever, body aches, nausea, vomiting, weakness, and rash.[1] In severe cases, life-threatening dengue hemorrhagic fever or dengue shock syndrome can develop. Dengue is endemic to 100 countries and approximately 2.5 billion people in 69 countries, including some areas of the southern United States and Hawaii, are at risk for dengue infection. An estimated 390 million infections occur worldwide of which 96 million manifest apparently per year.[2] Between 2000 and 2004 alone, the average number of DF and dengue hemorrhagic fever cases reported to the World Health Organization doubled compared with the previous decade.[3] Accordingly, the Department of Defense (DoD) ranks dengue as one of the top five (no. 2 out of 38) infectious disease threats that presents a serious medical risk to military operations throughout the world.[4] Specially, over the past 10 years (2003–2013), there have been over 450 cases (hospitalizations and ambulatory) linked to dengue in active duty and reserve/guard service members.[5]

There are currently no licensed dengue vaccines. For this reason, dengue virus infection must be identified promptly so supportive care can be administered expeditiously. However, the turnaround time for clinical virus isolation and serological examination can take longer than a week.[6] Therefore, over the past 10 years, several dengue virus reverse transcription polymerase chain reaction (RT-PCR) assays have been developed for laboratory use.[7] One of these assays was developed specifically for the DoD by the United States Air Force (USAF) as a field-sustainable quantitative (q)RT-PCR assay for prompt "...dengue virus screening and serotype identification in mosquitoes under austere field conditions."[8] This same group also conducted a study on dengue serotypes 1 to 4, yellow fever, St. Louis encephalitis, and West Nile virus infected mosquitoes showing that this assay is sensitive and specific enough to detection as few as 2 plaque-forming units (pfu) per leg.[9] This assay was the basis for the Joint Biological Agent Identification and Diagnostic System's (JBAIDS) dengue detection qRT-PCR assay.

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The JBAIDS (BioFire Diagnostics, formerly Idaho Technology, Salt Lake City, Utah) is currently the standard qPCR platform (program of record) used by the DoD. It is a portable, reusable biological agent identification and diagnostic system deployed as part of a biothreat detection system for the DoD.[10] JBAIDS development was funded by the DoD to provide health care providers with accurate information for diagnosis and treatment of disease and on which to notify commanders of biological warfare threats and pathogens of operational concern. The JBAIDS is intended for use by medical personnel to quickly identify biological agents in clinical specimens and environmental samples.[11]

A variety of assays were developed for the JBAIDS system and some have been cleared by the FDA for use as in vitro diagnostics for human disease. Some of these FDA-cleared qPCR assays include those for identification of biothreat agents such as *Yersinia pestis* (plague), *Bacillus anthracis* (anthrax), and *Francisella tularensis* (tularemia) as well as infectious diseases such as avian influenza (H5) and influenza A and B (including influenza A subtyping).[12] Development of more infectious disease assays for the JBAIDS has garnered support in recent years after review showed the JBAIDS was underutilized because of its exclusive biowarfare mission. Understandably, the USAF group, who developed qRT-PCR dengue assays for the Ruggedized Advanced Pathogen Identification Device, also developed a Pan-Dengue assay to address detection and surveillance of this arthropod-borne disease before actual human infection.[8]

In this study, the USAF Pan-Dengue qRT-PCR assay (JBAIDS Pan-Dengue detection PCR assay) was tested for clinical use on JBAIDS. In particular, JBAIDS Pan-Dengue detection PCR assay's sensitivity was compared with a second, independent, commercially available qRT-PCR assay, the Tetracore Dengue Group RT-PCR assay (Tetracore, Rockville, Maryland). The Tetracore Dengue Group RT-PCR assay was previously tested by the U. S. Navy and found to effectively detect dengue virus in clinical specimens with high sensitivity and specificity.[13] Moreover, the clinical performance of the JBAIDS Pan-Dengue detection PCR assay was evaluated with viral isolation positive dengue virus archived serum specimens. Overall, we show that a vector surveillance assay can effectively be used as a clinical diagnostic assay, and, consequently, future efforts should focus on codevelopment of diagnostic and detection assays.

METHODS

Human Use Statement

The procedures applied in this study were conducted in accordance with the ethical standards of the Naval Medical Research Center Institutional Review Board and with the Declaration of Helsinki 1975, as revised in 1983. Study protocols were approved by the Naval Medical Research Center Institutional Review Board (NMRCD.2000.0006 and NMRC.2005.0007) in compliance with all applicable federal regulations governing the protection of human subjects. Study protocols were also reviewed by public health authorities in Peru (Dirección General de Epidemiología and the Instituto Nacional de Salud). Informed consent was obtained for all participants ≥18 years of age, and for children <18 years of age written consent was obtained from a parent or legal guardian. Written assent was obtained from all minors aged 8 to 17 years.

Clinical Samples

Samples for this study were collected during 2009–2010 from numerous sites in Peru. Subjects were recruited from clinic-based passive and community-based febrile surveillance studies carried out by the Naval Medical Research Unit 6 (NAMRU-6). The passive surveillance study recruited patients presenting at 13 health centers or hospitals.[14] In the second study, participants with acute febrile illness were identified through home visits in a cohort of approximately 5,000 Iquitos City residents. Inclusion in the study required an oral fever >38°C of 7 days or less in duration plus one or more of the following symptoms: headache, muscle, ocular and/or joint pain, generalized fatigue, cough, nausea/vomiting, sore throat, rhinorrhea, difficulty breathing, diarrhea, bloody stools, jaundice, dizziness, disorientation, stiff neck, petechiae, ecchymoses, bleeding gums and/or epistaxis, and no other identifiable focus of infection.

Confirmation of Dengue Virus in Archived Clinical Specimens

Presence or absence of dengue virus in the archived clinical specimens collected from acute febrile individuals was determined by viral isolation. The following work was performed by the NAMRU-6 in Peru. Each clinical specimen was inoculated in *A. albopictus* C6/36 and Vero cells for 10 days with Eagle's Minimum Essential medium with 2% fetal bovine serum. Samples were observed daily for cytopathic effects. After the incubation period, slides were prepared for indirect fluorescent antibody and first screened with polyclonal and then with monoclonal antibodies specific to dengue.[15,16]

Sample Preparation: Spiked Serum Samples and Archived Clinical Specimens

A 10-fold serial dilution was prepared using dengue virus (DEN-1 FST 2407; 10^7 pfu/mL) and human serum (Bioreclamation, LLC, Westbury, New York) obtained from healthy volunteers to produce the following range of concentrations: 10^4 to 10^1 pfu/mL. Specimens were stored on ice until nucleic acid extraction could be initiated. Specimens were not stored longer than 5 minutes.

Archived patient serum specimens from Iquitos, Peru, were used for this study. Positive and negative patient serum specimens were randomized and labeled with study code numbers such that operators were blinded to the expected test result. Each sample was stored on ice until nucleic acid extraction could be initiated. Samples were not stored longer than 5 minutes.

Nucleic Acid Extraction

Before extraction, each specimen was split and realiquoted for extraction in parallel with the ITI 1-2-3 Platinum Path Sample Purification Kit (BioFire Diagnostics) and the QIAamp Viral RNA Mini Kit (Qiagen Sciences, Germantown, Maryland). Nucleic acid was extracted using the manufacturers' protocols (Qiagen and BioFire). For evaluation on JBAIDS with the Pan-Dengue detection PCR assay, nucleic acid was extracted with the Platinum Path kit (BioFire). Nucleic acid used in the Tetracore Dengue Group RT-PCR assay was extracted with Qiagen's viral RNA kit. A negative extraction control (molecular grade water) was extracted with each batch of archived patient sera to monitor for contamination.

Assay Conditions

For the Tetracore Dengue Group RT-PCR assay, the qRT-PCR master mix was prepared according to manufacturer's instructions. The qRT-PCR reactions consisted of 20 μL master mix and 5 μL extracted sample nucleic acid, which were aliquoted into a SmartCycler (Cepheid, Sunnyvale, California) reaction tube. An inhibition control as well as positive and negative controls (included with the assay) were included with each test run. The Tetracore Dengue Group RT-PCR assay was run on the SmartCycler using the 1.7b DX software with the following conditions: 50°C for 30 minutes, 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. Data were collected during the extension/annealing stage.

JBAIDS lyophilized reagents were prepared according to manufacturer's instructions. Briefly, freeze-dried reagent vials were reconstituted with 20 μL of purified nucleic acid along with 20 μL of reconstitution buffer. Following assay reconstitution, the qRT-PCR mix was split (≈19 μL in each) between duplicate capillary reaction tubes (Roche, Indianapolis, Indiana). The qRT-PCR amplification was performed on the JBAIDS instrument (software version 3.5.0.72) using the standard JBAIDS RNA protocol: 40°C for 30 minutes, 94°C for 2 minutes, followed by 45 cycles of 94°C for 0 seconds, and 60°C for 20 seconds.

Calculations

A 95% binomial confidence interval (CI) was calculated for the JBAIDS Pan-Dengue detection PCR assay and the Tetracore Dengue Group RT-PCR assay (Tables I and II) using a web-based program available from StatPages.org (http://statpages.org/confint.html) to show the reliability of our data for the sample size we used.

TABLE I. JBAIDS Pan-Dengue Detection PCR Assay and Viral Isolation Comparison

JBAIDS	Viral Isolation Positive[a]	Viral Isolation Negative[b]	JBAIDS Performance	95% CI of Performance
Positive	40	2	40/40 (100%)	91.2–100
Negative	0	18	18/20 (90%)	70.8–98.8
Total	40	20		

[a]Assay sensitivity (true positives/[true positives + false negatives]) was determined to be 100% with a 95% CI of 91.2 to 100. [b]Assay specificity (true negatives/[true negatives + false positives]) was determined to be 90% with a 95% CI of 70.8 to 98.9.

TABLE II. Tetracore Dengue Group RT-PCR Assay and Viral Isolation Comparison

Tetracore	Viral Isolation Positive[a]	Viral Isolation Negative[b]	Tetracore Performance	95% CI of Performance
Positive	40	1	40/40 (100%)	91.2–100
Negative	0	19	19/20 (95%)	75.1–99.9
Total	40	20		

[a]Assay sensitivity (true positives/[true positives + false negatives]) was determined to be 100% with a 95% CI of 91.2 to 100. [b]Assay specificity (true negatives/[true negatives + false positives]) was determined to be 95% with a 95% CI of 76.2 to 99.9.

RESULTS

Comparison Study (Spiked Serum Samples)

Overall, the Tetracore Dengue Group RT-PCR assay exhibited similar or greater sensitivity than the JBAIDS Pan-Dengue detection PCR assay (Table III). The extinction point for the

TABLE III. Dengue Virus Detection Comparison Study

JBAIDS Pan-Dengue Detection PCR		Tetracore Dengue Group RT-PCR	
Titer (pfu/mL)[a]	PCR Positive Replicates[b]	Titer (pfu/mL)[a]	PCR Positive Replicates[b]
Run No. 1			
10^4	6/6	10^4	3/3
10^3	6/6	10^3	3/3
10^2	6/6	10^2	3/3
10^1	6/6	10^1	3/3
Run No. 2			
10^2	6/6	10^2	3/3
10^1	6/6	10^1	3/3
10^0	5/6	10^0	3/3
10^{-1}	0/6	10^{-1}	2/3

[a]Determined by standard dengue virus plaque assay. [b]Denotes PCR positive out of total number of samples assayed.

Tetracore assay was 10^{-1} pfu/mL, a concentration where 2 out of the 3 replicates gave positive results. The extinction point for the JBAIDS assay was 10^{0} pfu/mL where 5 out of the 6 replicates were positive. The JBAIDS assay did not detect any of the 6 replicates for the 10^{-1} pfu/mL dilutions.

Archived Clinical Specimens

The sample set consisted of a total of 40 dengue positive samples and 20 negative samples, as determined by viral isolation. The archived clinical specimen results obtained from the JBAIDS and Tetracore assays were compared with the results obtained from the viral isolation (Tables I and II). Tetracore and JBAIDS assays successfully detected each of the 40 positive samples signifying 100% sensitivity of this assay in regards to clinical specimens. When testing viral isolation negative specimens (considered true negatives), both PCR assays produced a positive result for sample IQA0490. A second viral isolation negative sample, IQA0550, produced a positive result with the JBAIDS Pan-Dengue detection PCR assay only. The positive JBAIDS results for this sample could not be confirmed by the Tetracore Dengue Group RT-PCR assay. These results indicate that specificity for the Tetracore Dengue Group RT-PCR assay is 95% and 90% for the JBAIDS Pan-Dengue detection PCR assay.

DISCUSSION

Because of similarities in detection technology, arthropod-borne pathogen surveillance assays should be considered for human diagnostic use. This tactic transmutes a single purpose assay into a multipurpose one. Additionally, as diagnostic assays require FDA clearance and vector-borne assays do not, it is much more economical to verify current vector surveillance PCR assay performance data rather than developing a clinical diagnostic assay outright. When developing future assays, it is prudent to take both diagnostic (human) and detection (vector) missions into consideration. In doing so, the following variables must be considered for potential assay modifications: initial sample volume required for nucleic acid extraction, potential inhibitors present in the sample, and the differences in efficiency of nucleic acid extraction kits.[13]

On the basis of McAvin et al[8,9] results, we hypothesized the JBAIDS Pan-Dengue detection PCR assay would produce equivocal detection performance in clinically relevant samples. We were able to show clinical sensitivity of 100% (95% CI: 91.2–100) and specificity of 90% (95% CI: 68.3–98.9) relative to viral isolation (Table I), which verified the JBAIDS Pan-Dengue detection PCR assay's ability to produce appropriate results in diagnostic samples. These results were comparable with the Tetracore Dengue Group RT-PCR assay with respect to assay sensitivity. However, the sensitivity of the Tetracore Dengue Group RT-PCR assay was higher than the JBAIDS Pan-Dengue detection PCR assay (95%:90%, respectively). This difference may have been a result from the different nucleic acid extraction methods used and/or the different volumes of nucleic acid that was added to the qRT-PCR reaction mix. Additionally, it is possible that the 2 virus isolation negative samples (IQA0490 and IQA0550) had nonviable virus in them and should be considered isolation negative and qRT-PCR positive.

Although traditional immunological techniques have been used extensively for viral detection in environmental samples, they are used less frequently in clinical diagnostics for the following reasons described by Wu et al[13]: trained personnel are required, are labor intensive, and are time consuming. Our data mirror this concept and suggest that qRT-PCR is more sensitive and, by nature of the technology, more rapid. Specifically, the JBAIDS Pan-Dengue detection PCR assay, originally developed to detect virus in insect vectors, operates with smaller sample volumes than traditional clinical sample volumes and, consequently, offers excellent sensitivity with rapid results to the diagnostics realm.

To meet the JBAIDS capability gaps, the Next Generation Diagnostics System (NGDS) is currently being developed. Unlike the JBAIDS, the NGDS will focus on both biological warfare agent and clinically relevant disease detection. Until the NGDS is available for use in the field by all U.S. military services (fiscal year 2018), the JBAIDS continues to be the qPCR platform program of record for the DoD. Thus, there is a need to develop detection assays for infectious agents that are of operational concern to the military. It is our belief that once a toolbox of reagents is assembled with proven primers and probes, these chemistries can be optimized for use on other platforms. Therefore, developing well-characterized JBAIDS qPCR assays allows for these assays to be transitioned over to the NGDS platform once it becomes available.

Overall, in time of fiscal constraints, we feel it necessary to develop assays that can serve multiple missions, such as clinical diagnostics and vector surveillance. The JBAIDS Pan-Dengue detection PCR assay is an example of using an existing arthropod-borne pathogen surveillance assay as a clinical diagnostic assay. Leveraging assays for either diagnostic use or surveillance detection is a practical approach to assay development not only when the need for a new assay arises, but definitely in time of budgetary shortfalls.

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