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Multiplex Nucleic Acid Amplification Test for Diagnosis of Dengue Fever, Malaria, and Leptospirosis

Jesse J. Waggoner,[a] Janaki Abeynayake,[b]* Ilana Balassiano,[c] Martina Lefterova,[b] Malaya K. Sahoo,[b] Yuanyuan Liu,[b] Juliana Magalhães Vital-Brazil,[c] Lionel Gresh,[d] Angel Balmaseda,[e] Eva Harris,[f] Niaz Banaei,[a,b] Benjamin A. Pinsky[a,b]

Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University School of Medicine, Stanford, California, USA[a]; Department of Pathology, Stanford University School of Medicine, Stanford, California, USA[b]; Laboratório de Zoonoses Bacterianas, Centro de Referência Nacional para Leptospirose, Coleção de Leptospira, WHO/PAHO Centro Colaborador para Leptospirose, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil[c]; Sustainable Sciences Institute, Managua, Nicaragua[d]; National Virology Laboratory, Centro Nacional de Diagnóstico y Referencia, Ministry of Health, Managua, Nicaragua[e]; Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, California, USA[f]

Dengue, leptospirosis, and malaria are among the most common etiologies of systemic undifferentiated febrile illness (UFI) among travelers to the developing world, and these pathogens all have the potential to cause life-threatening illness in returned travelers. The current study describes the development of an internally controlled multiplex nucleic acid amplification test for the detection of dengue virus (DENV) and *Leptospira* and *Plasmodium* species, with a specific callout for *Plasmodium falciparum* (referred to as the UFI assay). During analytical evaluation, the UFI assay displayed a wide dynamic range and a sensitive limit of detection for each target, including all four DENV serotypes. In a clinical evaluation including 210 previously tested samples, the sensitivities of the UFI assay were 98% for DENV (58/59 samples detected) and 100% for *Leptospira* and malaria (65/65 and 20/20 samples, respectively). Malaria samples included all five *Plasmodium* species known to cause human disease. The specificity of the UFI assay was 100% when evaluated with a panel of 66 negative clinical samples. Furthermore, no amplification was observed when extracted nucleic acids from related pathogens were tested. Compared with whole-blood samples, the UFI assay remained positive for *Plasmodium* in 11 plasma samples from patients with malaria (parasitemia levels of 0.0037 to 3.4%). The syndrome-based design of the UFI assay, combined with the sensitivities of the component tests, represents a significant improvement over the individual diagnostic tests available for these pathogens.

Health problems that necessitate medical attention are reported by 8% of travelers to the developing world, amounting to over four million patients annually (1–3). In a study analyzing data from 17,353 patient encounters at GeoSentinel sites, the most common diagnostic syndrome reported was a systemic undifferentiated febrile illness (UFI) that lacked localizing signs or symptoms (1). A specific etiological diagnosis was available for only 59.4% of such patients, with malaria and dengue being identified as the cause for 76.8% of patients who received a diagnosis (1). In a subsequent analysis of acute life-threatening illnesses among 3,655 travelers, *Plasmodium falciparum* was the most common pathogen identified and accounted for 10 of 13 deaths (4). Dengue virus (DENV) infection was the most common viral etiology of life-threatening illness (4). Although diagnosed less often, leptospirosis is also a well-documented cause of severe infections in returned travelers and accounted for 2.4% of life-threatening infections in patients presenting to GeoSentinel sites (4–6).

One limitation of such studies is the lack of gold-standard diagnostic tests for many of the causes of UFIs (1, 7, 8). Traditional microscopic analysis for malaria offers the clearest example of a widely used standard method that provides results in a clinically meaningful time frame. Microscopy can provide morphological species determination and quantitation of the parasite burden (8). However, this approach displays variable performance associated with the experience of the reader, which is of particular concern in regions in which the disease is not endemic (9). Assays used as standard methods for DENV and *Leptospira* detection involve serological testing of acute-phase and convalescent-phase samples; for *Leptospira*, antibody detection using microagglutination testing (MAT) remains the standard (7, 10). MAT requires significant expertise to perform, the maintenance of a local reference panel of bacterial cultures, and acute-phase and convalescent-phase samples to provide confirmed diagnoses (10–12). For either DENV or *Leptospira*, the results from serological testing of a single acute-phase specimen can provide only a presumptive diagnosis.

Rapid diagnostic tests (RDTs) have been developed for all of these pathogens and detect specific antigens, antibodies, or both (9, 10, 13–19). DENV RDTs for the detection of nonstructural protein 1 (NS1) or anti-DENV antibodies are available. While these can provide a diagnosis in as little as 15 min, they are less sensitive in acute infections than are reverse transcription (RT)-PCR assays (17). Rapid serological tests for leptospirosis are available, although they have proved to be less sensitive than MAT (16). The clinical sensitivities and specificities of RDTs for malaria are similar to those of routine microscopy for *P. falciparum* (13, 14). These tests are less sensitive for other *Plasmodium* species, however, in part because of their relatively high analytical detection limits (14).

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Address correspondence to Benjamin A. Pinsky, bpinsky@stanford.edu.
* Present address: Janaki Abeynayake, Division of Clinical Virology, Teaching Hospital Karapitiya, Galle, Sri Lanka.

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TABLE 1 *Leptospira* 16S rRNA, *P. falciparum* Pfr364, and *Plasmodium* 18S rRNA primers included in the UFI assay

Name	Primer sequence (5′ to 3′)	Concentration (nM)[a]
Leptospira		
16S forward	CGGGAGGCAGCAGTTAAGAA	400
16S reverse	AACAACGCTTGCACCATACG	400
P. falciparum		
Pfr364 forward	GCGACTAGTTCATTCGACTCAGA	200
Pfr364 reverse	GGAATTGCCACAAGGTTGGAAA	200
Plasmodium 18S		
18S forward	TCGCGCAAGCGAGAAAGTT	500
18S reverse	CATGCATCACCATCCAAGAA	500

[a] The concentration provided is the concentration of each primer in the final reaction mixture. Predicted amplicon sizes are as follows: Pfr364, 200 bp; *Plasmodium* 18S rRNA, 160 bp; *Leptospira* 16S rRNA, 197 bp. DENV and RNase P primer sequences have been published elsewhere (24).

Many separate nucleic acid amplification tests (NAATs) have been described for the detection of DENV, *Leptospira*, and *Plasmodium* species. While the reported sensitivities of individual assays have varied, NAATs have proved to be the most sensitive diagnostic tests for these pathogens in the setting of acute infection (8, 17, 20–23). Recently, our group reported the development of an internally controlled, real-time RT-PCR (rRT-PCR) assay for pan-DENV detection (24). This pan-DENV assay detects all four DENV serotypes using hydrolysis probes labeled with a single fluor and targets RNase P as the internal control (IC). In a study of 199 clinical samples, the pan-DENV assay proved more sensitive than three comparator assays (25), i.e., a widely used, seminested RT-PCR assay originally reported by Lanciotti et al. (26, 27); a commercially produced, internally controlled, rRT-PCR assay; and the recently FDA-cleared CDC DENV-1-4 real-time RT-PCR assay (28).

In this study, we describe the design and evaluation of the UFI assay, which is a single-reaction, internally controlled, multiplex NAAT for the detection of DENV-1 to -4 (based on the pan-DENV assay), all species of *Leptospira*, and the five *Plasmodium* species known to be pathogenic in humans, with a specific callout for *P. falciparum*. The goals of assay design were to maintain the sensitivity of the pan-DENV assay for DENV detection while adding the ability to detect other pathogens in the differential diagnosis. DENV, *Leptospira*, and *Plasmodium* were selected for inclusion in the UFI assay given their widespread nature, their potential to cause severe disease, and the possibility of improving patient outcomes with early diagnosis and appropriate management.

MATERIALS AND METHODS

Ethics. Protocols for the collection and testing of samples from Nicaraguan pediatric dengue cases were reviewed and approved by the institutional review boards of the University of California, Berkeley, the Nicaraguan Ministry of Health, and Stanford University. Parents or legal guardians of all subjects provided written informed consent; subjects ≥6 years of age provided assent. Specific institutional review board approval for the development of the UFI assay was not required, as all samples were precollected and deidentified.

Assay design. The DENV and RNase P primer and probe sequences were previously published (24). The UFI assay incorporated the following modifications: the DENV probe concentration in the final reaction mixture was decreased to 100 nM, the RNase P primer concentration was decreased to 50 nM, and the RNase P probe concentration was increased to 100 nM. No changes in the concentrations of DENV primers were made.

The 16S rRNA (*rrs*) gene was selected for use in the assay to detect *Leptospira* spp. We aligned 704 publicly available *Leptospira* 16S rRNA gene sequences from the National Center for Biotechnology Information (NCBI) nucleotide database using MegAlign software (DNASTAR, Madison, WI). This alignment included both pathogenic and saprophytic species of *Leptospira*. Regions where the consensus nucleic acid sequence was conserved across ≥95% of the aligned sequences were identified as potential targets for primers and probes. By using BLAST to compare sequences, regions with significant homology to *Staphylococcus*, *Listeria*, *Treponema*, or *Borrelia* sequences were eliminated from consideration. Primers and probes were then designed using Primer-BLAST software (29).

The primers and probe for *P. falciparum* detection were designed against the Pfr364 repetitive element using RealTimeDesign software (Biosearch Technologies, Novato, CA). The sequence of Pfr364 has been published previously (30). A pan-*Plasmodium* assay targeting the 18S rRNA gene was initially designed using RealTimeDesign and then refined using 163 *Plasmodium* sequences, including sequences from *P. falciparum* ($n = 25$), *Plasmodium knowlesi* ($n = 86$), *Plasmodium malariae* ($n = 4$), *Plasmodium ovale* ($n = 23$), and *Plasmodium vivax* ($n = 25$). Sequence alignment and target selection were performed as described for *Leptospira*, and final primer and probe sequences were selected using Primer3 (31). The primer and probe sequences for the *Leptospira*, Pfr364, and *Plasmodium* 18S rRNA gene assays are shown in Tables 1 and 2.

UFI assay performance. All reactions were performed with a Rotor-Gene Q instrument (Qiagen, Germantown, MD), using 25-μl reaction volumes and the SuperScript III Platinum One-Step quantitative RT-PCR kit (Invitrogen, Carlsbad, CA). Primer and probe concentrations were initially optimized in monoplex reactions using positive-control samples. Assays were then combined sequentially, and primer and probe concentrations were adjusted to maintain sensitivity and to minimize bleed-through between channels. Hydrolysis probes (Biosearch Technologies, Novato, CA) were labeled to provide DENV detection in the green channel (FAM), *Leptospira* in yellow (Cal Fluor 560), *P. falciparum* in orange (Cal Fluor 610), *Plasmodium* 18S rRNA in red (Quasar 670), and RNase P in crimson (Quasar 705).

For the UFI assay, each reaction mixture contained 5 μl of nucleic acid template and the final primer and probe concentrations shown in Tables 1 and 2. Cycling conditions were as follows: 52°C for 15 min (RT step for DENV); 94°C for 2 min; and 45 cycles at 94°C for 15 s, 55°C for 40 s, and 68°C for 20 s. Detection was performed at 55°C in all channels; the gain for each channel was set following optimization during initial testing (5.33 for green and 10 for all other channels). During analysis, the first five cycles were cropped from each channel to improve baseline normalization. Results were evaluated on a linear scale with slope correction. The crossing

TABLE 2 *Leptospira* 16S rRNA, *P. falciparum* Pfr364, and *Plasmodium* 18S rRNA probe sequences for the UFI assay

Probe	5′ Fluor	Probe sequence (5′ to 3′)	3′ Quencher	Concentration (nM)[a]
Leptospira 16S rRNA	Cal Fluor 560	TGAAGCAGCGACGCCGCGTG	BHQ-1	100
Pfr364	Cal Fluor 610	ATACCGCTGCATCGCGGTGCA	BHQ-2	400
Plasmodium 18S rRNA	Quasar 670	CGTGGAGCTTGCGGCTTAAT	BHQ-2	200

[a] The concentration provided is the concentration for each probe in the final reaction mixture. DENV and RNase P probe sequences have been published elsewhere (24).

threshold was set at 0.05 for all channels. Any exponential curve crossing this threshold prior to cycle 45 was considered a positive result in the green, yellow, orange, and red channels. Curves crossing after cycle 40 in the crimson channel (RNase P detection) and negative in all other channels were considered IC failures.

Control nucleic acids and reference material. Quantitated, positive-sense, single-stranded DNA (ssDNA) oligonucleotides containing the target sequence for each DENV serotype were synthesized (Eurofins MWG Operon, Huntsville, AL) and used in the analytical characterization of the UFI assay. For *Leptospira* 16S rRNA, Pfr364, and *Plasmodium* 18S rRNA, quantitated plasmid DNA was utilized. Amplicons generated from monoplex reactions for *Leptospira*, *P. falciparum* (Pfr364 target), and *P. vivax* (18S rRNA gene target) were cloned and sequenced using M13 primers as described previously (24, 32). *Leptospira interrogans* serovar Copenhageni, strain Fiocruz L1-130 (ATCC BAA-1198D-5; ATCC, Manassas, VA), template genomic DNA was kindly provided by Alexandria Boehm (Stanford University). This was used as the template for *Leptospira* amplicon production. Extracted nucleic acids from clinical samples obtained from separate patients with microscopy-confirmed *P. falciparum* and *P. vivax* were used as templates for amplicon production. Extracted genomic DNA from 39 cultured isolates of *Leptospira* was obtained for testing in the UFI assay. These included strains from 7 species and 23 different serovars of *Leptospira* (Table 3).

UFI assay analytical characterization. The UFI assay was analytically characterized according to published recommendations (33). For these experiments, total nucleic acids were extracted and pooled from serum samples sent to the Stanford Clinical Virology Laboratory for hepatitis C virus (HCV) testing. Pooled extract was spiked into the reaction mixture to mimic the IC in clinical samples (5 μl of extract per reaction). To establish the dynamic range, four replicates of serial 10-fold dilutions of ssDNA or plasmid DNA from 7.0 $\log_{10}$ copies/μl to 1 copy/μl were tested in a single run. The highest concentration available for the *P. falciparum* plasmid was 6.5 $\log_{10}$ copies/μl. The dynamic range was established by fitting a best-fit line to the data by regression analysis and included the range where the R^2 value for this line was ≥0.99.

To establish the 95% lower limit of detection (LLOD), 10 replicates of serial 2-fold dilutions were tested in a single run. Four dilutions were tested for each target, beginning with a concentration 2-fold higher than the lowest concentration at which all replicates were detectable during the dynamic range study. The 95% LLOD was then calculated using probit analysis. To compare the analytical sensitivity of DENV detection in the UFI assay versus the original pan-DENV assay, the 95% LLOD of the pan-DENV assay was also evaluated using the same quantitated ssDNA standards. The pan-DENV assay was performed as previously described (24).

Specificity. The specificity of the UFI assay was evaluated by testing genomic RNA from the following viral strains: yellow fever virus (YFV) 17D and chikungunya virus (one strain each) obtained from Vircell Microbiologists (Granada, Spain) and inactivated cultured isolates of YFV (Asibi strain), chikungunya virus, West Nile virus (WNV) (NY 1999 strain), St. Louis encephalitis virus (SLEV), and Zika virus kindly provided by the CDC Vector Borne Diseases Branch (Fort Collins, CO). Extracted DNA from cultured strains of *Salmonella enterica* subsp. *arizonae*, *S. enterica* serovar Typhi, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (one strain each) and *Mycobacterium tuberculosis* (two strains) was tested to evaluate the specificity of *Leptospira* detection. Two clinical samples (EDTA-treated whole blood) that tested positive for *Babesia microti* also underwent nucleic acid extraction and were tested in the UFI assay. In addition, specificity was evaluated by testing 50 serum samples that had been obtained from hepatitis C virus (HCV)-positive patients and sent to the Stanford Clinical Virology Laboratory for HCV viral load testing.

DENV, *Leptospira*, and *Plasmodium* samples. DENV detection was evaluated by using extracted RNA from a set of 60 serum samples collected from pediatric dengue cases in Nicaragua. For serum collection, whole blood was allowed to clot in the collection tubes; serum was then stored at −80°C until use. These samples tested positive by at least two different molecular assays and have been described previously (24).

Sixty-five samples (63 serum and 2 plasma samples) from patients in Brazil with suspected leptospirosis were tested using the UFI assay. Serum was collected in serum-separator tubes, and plasma was obtained from EDTA-containing tubes. The samples, which were collected between April 2009 and November 2013, were originally sent to the Laboratório de Zoonoses Bacterianas, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, and were stored at −20°C until use. All samples were tested with PCRs for *flaB* and *lipL41*, as described previously (34, 35). Amplification products were detected by polyacrylamide gel electrophoresis. Samples were considered positive by conventional *Leptospira* PCR if the expected products for *flaB*, *lipL41*, or both were detected by PCRs. Samples were considered negative if no product was detectable from either PCR. Fifty-five acute-phase specimens were also evaluated by MAT using a regionally optimized reference panel. Samples with detectable antibodies against any serovar (titers of 400 to 1,600) were considered positive by MAT. Sixteen samples had detectable *Leptospira* DNA in PCRs for *flaB*, *lipL41*, or both. Eight

TABLE 3 Reference *Leptospira* isolates tested in the UFI assay

Origin and species[a]	Serovar	Strain	CLEP code
Obtained from CLEP (Rio de Janeiro, Brazil)			
L. biflexa	Semaranga	Patoc 1	00015
L. biflexa	Andamana	CH11	00021
L. borgpetersenii	Tarassovi	Perepelitsin	00016
L. borgpetersenii	Javanica	Veldrat Batavia 46	00010
L. borgpetersenii	Castellonis	Castellon 3	00008
L. fainei	Hurstbridge	But 6	00026
L. interrogans	Icterohaemorrhagiae	RGA	00001
L. interrogans	Copenhageni	M20	00002
L. interrogans	Canicola	Hond Utrecht IV	00003
L. interrogans	Pomona	Pomona	00005
L. interrogans	Australis	Ballico	00006
L. interrogans	Autumnalis	Akiyami A	00017
L. interrogans	Pyrogenes	Salinem	00012
L. interrogans	Lai	Lai	00028
L. kirschneri	Grippotyphosa	Moskva V	00004
L. kirschneri	Mozdok	5621	00091
L. noguchii	Panama	CZ214K	00011
L. weilii	Vughia	LT 89-68	00040
Obtained from Queensland Health (Queensland, Australia)			
L. borgpetersenii	Arborea		
L. borgpetersenii	Hardjo (Bovis)		
L. borgpetersenii	Tarassovi		
L. interrogans	Australis		
L. interrogans	Bindjei		
L. interrogans	Broomi		
L. interrogans	Canicola		
L. interrogans	Copenhageni		
L. interrogans	Hardjo		
L. interrogans	Kremastos		
L. interrogans	Mankarso		
L. interrogans	Medanensis		
L. interrogans	Pomona		
L. interrogans	Robinsoni		
L. interrogans	Szwajizak		
L. interrogans	Valbuzzi		
L. interrogans	Zanoni		
L. kirschneri	Bulgarica		
L. kirschneri	Grippotyphosa		
L. weilii	Celledoni		
L. weilii	Topaz		

[a] CLEP, Fiocruz *Leptospira* Collection. Strain designations were not provided for isolates from Queensland Health.

(50%) of these patients had corresponding MAT results, and all were nonreactive. Forty-seven (95.9%) of the 49 patients with negative PCR test results for *flaB* and *lipL41* had corresponding MAT results, which were positive in seven cases (14.9%). For 52 samples, PCR products from the UFI assay were purified using a GeneJET PCR purification kit (Thermo Scientific, Waltham, MA), according to the manufacturer's recommendations. Purified amplicons were sequenced by bidirectional dideoxynucleotide termination sequencing using the *Leptospira* 16S rRNA forward and reverse primers (Elim Biopharmaceuticals, Inc., Hayward, CA).

A panel of 26 samples was used to evaluate *Plasmodium* detection in the UFI assay. The panel included extracted DNA from 25 samples sent to or obtained by the Stanford Clinical Microbiology Laboratory for malaria testing with smear microscopy and the BinaxNOW Malaria rapid diagnostic test (Alere, Waltham, MA). This set contained 10 samples negative for malaria and 15 samples positive for *P. falciparum* ($n = 5$), *P. vivax* ($n = 6$), *P. malariae* ($n = 2$), or *P. ovale* ($n = 2$). The panel also included a single reference sample of *P. knowlesi* provided by the CDC (Atlanta, GA). Ten whole-blood samples that were sent to the clinical laboratory at the Mayo Clinic for malaria testing by thin-smear analysis were evaluated using the UFI assay. Whole blood was collected in EDTA-containing tubes and stored at 4°C for up to 8 days prior to shipment. Samples were obtained from four patients without malaria as well as patients infected with *B. microti* ($n = 2$), *P. falciparum* ($n = 2$), *P. ovale* ($n = 1$), or *P. malariae* ($n = 1$).

Nucleic acid extraction. The extraction of RNA from samples used for the evaluation of DENV detection was described previously (24). The extraction of total nucleic acids from serum or plasma was performed using an easyMAG instrument (bioMérieux, Durham, NC), according to the manufacturer's recommendations. Extractions from whole blood were also performed using the easyMAG instrument with the Specific B protocol. All easyMAG extractions were performed using 140 μl of starting material and final elution volumes of 60 μl for serum or plasma and 50 μl for whole blood (instrument settings for the Specific B protocol). The extraction of DNA from suspected leptospirosis cases was performed onsite in Brazil prior to the shipment of samples for testing in the UFI assay. DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen, Germantown, MD), according to the manufacturer's recommendations. All extracted nucleic acids were stored at −80°C until use.

Statistics. Two-tailed Fisher's exact tests were used in comparisons of PCR test results, and *t* tests were used for the comparison of two means. Fisher's exact tests and *t* tests were performed using GraphPad software (GraphPad, La Jolla, CA). Probit analysis was performed using SPSS (IBM, Armonk, NY).

RESULTS

UFI assay analytical evaluation. The dynamic range of the UFI assay extended from 7.0 to 1.0 $\log_{10}$ copies/μl for DENV-1 and DENV-3 and from 7.0 to 2.0 $\log_{10}$ copies/μl for DENV-2 and DENV-4. The dynamic range for *Leptospira* was 7.0 to 1.0 $\log_{10}$ copies/μl. For *P. falciparum* with the Pfr364 assay, the dynamic range extended from 6.5 to 2.0 $\log_{10}$ copies/μl. Lastly, using a plasmid containing the cloned amplicon from a *P. vivax* isolate, the dynamic range of the *Plasmodium* 18S rRNA assay extended from 7.0 to 1.0 $\log_{10}$ copies/μl. Results of the dynamic range studies are displayed in Fig. 1.

The 95% LLOD was determined for each target in the UFI assay by probit analysis using dilutions of quantitated standards. The 95% LLOD for each target was as follows: DENV-1, 32.6 copies/μl of eluate; DENV-2, 19.6 copies/μl; DENV-3, 11.2 copies/μl; DENV-4, 64.3 copies/μl; *Leptospira*, 8.0 copies/μl; *P. falciparum*, 64.5 copies/μl; *Plasmodium*, 2.9 copies/μl. For direct comparison of the analytical sensitivity of DENV detection in the UFI and pan-DENV assays, the 95% LLOD was determined for the pan-DENV assay using the same quantitated ssDNA standards. The 95% LLOD for each serotype in the pan-DENV assay was 19.4 copies/μl of eluate for DENV-1, 8.9 copies/μl for DENV-2, 9.2 copies/μl for DENV-3, and 87.9 copies/μl for DENV-4.

Specificity. The specificity of the UFI assay was evaluated by testing extracted nucleic acids from a set of viral, bacterial, and parasitic samples. No amplification was observed when the UFI assay was performed using nucleic acids from YFV (2 strains), chikungunya virus (2 strains), and a single strain each of WNV, SLEV, and Zika virus. Nucleic acids from cultured isolates of *S. enterica* subsp. *arizonae*, *S. enterica* serovar Typhi, *S. aureus*, *E. coli*, *P. aeruginosa*, and *M. tuberculosis* (2 strains) were tested and yielded no amplification. Also, total nucleic acids were extracted from two whole-blood samples from patients with *B. microti* detected by peripheral blood smears. Both samples had detectable IC levels but otherwise showed no amplification in the UFI assay. Fifty HCV-positive clinical samples were also tested in the UFI assay; these samples had a median HCV viral load of 5.9 $\log_{10}$ IU/ml of patient plasma (range, 3.54 to 7.47 $\log_{10}$ IU/ml). All samples had detectable RNase P (mean threshold cycle [C_T] in the crimson channel, 27.18; standard deviation, 1.89). No amplification from these samples was observed in the remaining channels.

DENV samples. A set of 60 samples collected from pediatric dengue cases in Nicaragua was tested using the UFI assay. All samples had previously tested positive in at least two different molecular tests, although RNA extracts had undergone 5 or 6 freeze-thaw cycles prior to this evaluation (24). These samples included samples from patients infected with DENV-1 ($n = 26$), DENV-2 ($n = 11$), and DENV-3 ($n = 23$), which represent the prevalent DENV serotypes in Nicaragua (36, 37). A single sample tested negative for both DENV and IC and was excluded from further analysis. Fifty-eight of 59 samples tested positive for DENV in the UFI assay (98.3%; $P = 1.0$). The mean C_T value for samples with detectable DENV RNA in both assays was 0.73 cycles later in the UFI assay than in the pan-DENV assay (95% confidence interval [CI], −2.10 to 0.65; $P = 0.30$). The sample that was missed in the UFI assay tested positive for DENV-1, with a C_T of 34.25, in the original pan-DENV assay.

***Leptospira* samples.** The *Leptospira* 16S rRNA gene portion of the UFI assay was evaluated by testing DNA extracted from 39 cultured *Leptospira* isolates, including samples from 7 different species and 23 serovars (Table 3). All isolates were detected using the UFI assay. All 65 clinical samples from suspected leptospirosis cases tested positive for *Leptospira* in the UFI assay. The rate of *Leptospira* detection in the UFI assay was significantly greater than that in the reference PCRs for *flaB* and *lipL41* (24.6%; $P < 0.0001$). C_T values in the UFI assay ranged from 19.65 to 39.59; there was no significant difference in the mean C_T values for samples that tested positive or negative by *flaB* and *lipL41* PCRs (30.38 versus 31.33; $P = 0.38$). Amplicons from 52 clinical samples were sequenced using the *Leptospira* forward and reverse primers. Bidirectional sequences were obtained for 50 samples, including 4 samples that tested positive in both the reference and UFI assays and 46 samples that tested positive in only the UFI assay. Amplicon sequences were entered into a BLAST search of the NCBI Nucleotide database, and hits with the maximum alignment score for all samples included only sequences of the *Leptospira* 16S rRNA gene. Given the highly conserved nature of this region, a final species determination could not be made. However, five distinct sequences that matched pathogenic species of *Leptospira*

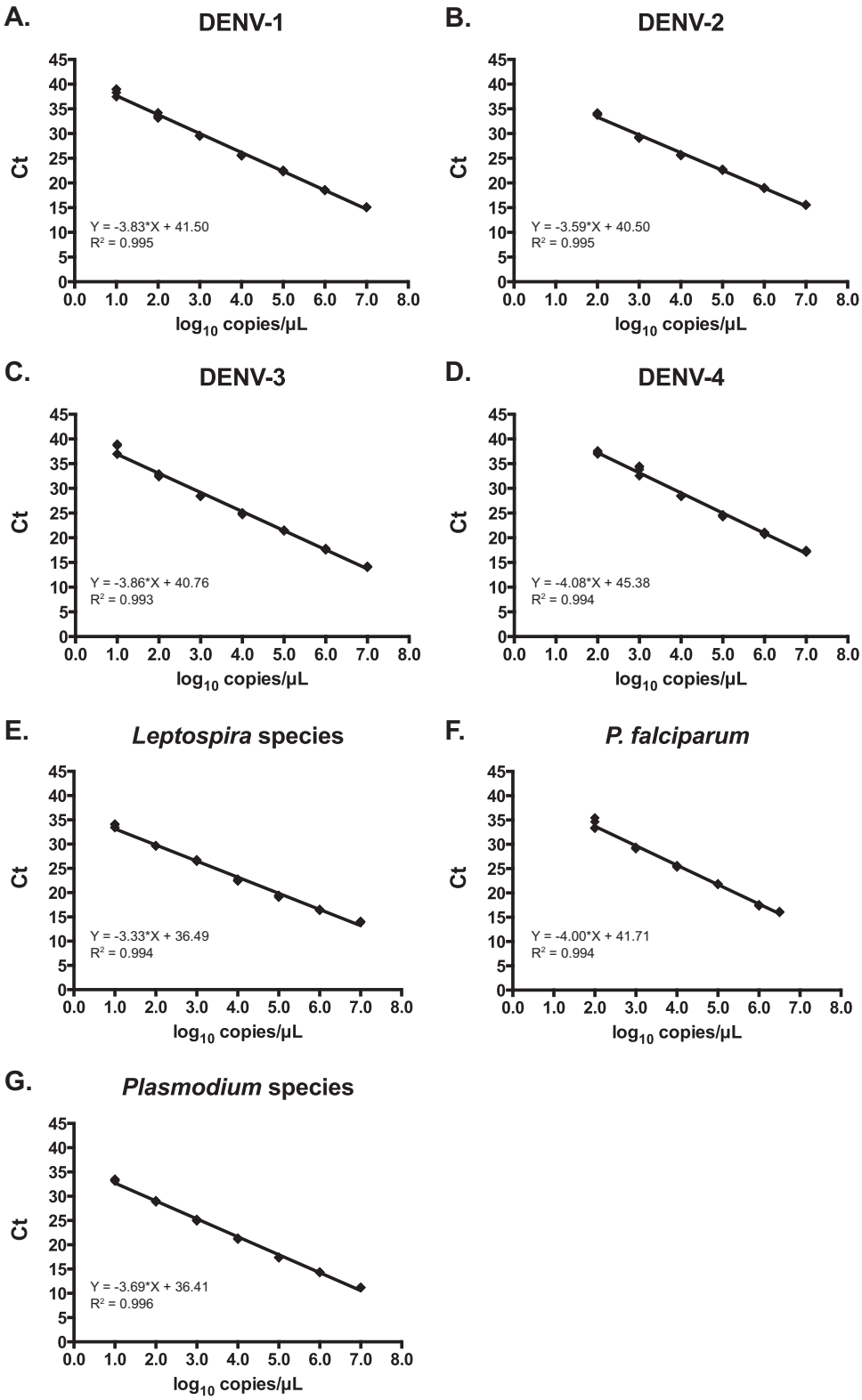

FIG 1 Dynamic range of the UFI assay for the detection of DENV-1 (A), DENV-2 (B), DENV-3 (C), and DENV-4 (D) and *Leptospira* (E), *P. falciparum* (F), and *Plasmodium* (G) species. Four replicates of serial 10-fold dilutions were analyzed in a single run. C_T values for each replicate at the given concentrations are shown.

TABLE 4 C_T values in the Pfr364 and *Plasmodium* 18S rRNA portions of the UFI assay for *Plasmodium* samples following nucleic acid extraction from whole-blood or plasma samples[a]

Sample	Parasitemia (%)	Dilution	C_T values for:			
			Pfr364		*Plasmodium* 18S rRNA	
			Whole blood	Plasma	Whole blood	Plasma
P. falciparum, sample 1	0.37		16.14	22.89	13.87	21.81
		1:2	16.70	23.82	14.10	22.74
		1:5	18.00	25.24	15.29	24.04
		1:10	18.83	25.13	16.78	24.11
		1:100	22.54	29.19	20.38	28.15
P. falciparum, sample 2	3.4		13.24	15.97	9.87	13.02
		1:10	16.63	19.91	13.53	17.55
		1:100	20.11	25.52	17.23	23.30
P. ovale	0.2				15.61	20.45
		1:10			19.62	22.61
P. malariae	0.02				19.09	30.58

[a] *P. falciparum* and *P. ovale* whole-blood samples were diluted with negative whole blood. Parasitemia levels were determined in the original samples by light microscopy.

were detected in 49 samples. Forty-five samples matched sequences for *L. interrogans*, *Leptospira kirschneri*, or *Leptospira noguchii* (99.5 to 100% identity), and three samples matched sequences for *Leptospira borgpetersenii*, *Leptospira santarosai*, or *Leptospira weilii* (100% identity). These two sets of sequences differed at two base pairs, as expected from the alignment of 16S rRNA gene sequences performed during the assay design. A single amplicon displayed a mixed pattern for these bases and therefore matched both sets with 99.5% identity. One sequence matched the 16S rRNA gene from nonpathogenic *Leptospira* spp. (*Leptospira biflexa*, *Leptospira meyeri*, or *Leptospira wolbachii*; 99.0% identity). This last sample was positive in both the reference and UFI assays, and it had been obtained from a patient who presented with fever, conjunctival congestion, vomiting, and jaundice.

Plasmodium samples. To evaluate *Plasmodium* detection with the UFI assay, a panel of 26 reference samples was tested. The UFI assay correctly identified all 16 cases of malaria as either *P. falciparum* (signals in the orange and red channels; $n = 5$) or *Plasmodium*, not *falciparum* (signal in the red channel; $n = 11$). None of the patients without malaria had detectable amplification in the UFI assay, although all samples were positive for the IC. No signal was observed in the Pfr364 assay for patients with non-*falciparum* malaria.

Eleven whole-blood samples collected from four patients were used to evaluate the sensitivity of the UFI assay for *Plasmodium* detection when nucleic acids were extracted from whole blood versus plasma (Table 4). Patients were infected with *P. falciparum* (2 patients), *P. ovale* (1 patient), or *P. malariae* (1 patient). Dilutions of the *P. falciparum* and *P. ovale* samples were made using negative whole blood prior to plasma separation and nucleic acid extraction. The levels of parasitemia in these samples ranged from 3.4% (*P. falciparum*, sample 2, undiluted) to an estimated 0.0037% (*P. falciparum*, sample 1, 100-fold dilution). Although C_T values occurred later for plasma samples than for whole-blood samples (mean values of 22.95 versus 16.71; $P < 0.0001$), all samples and dilutions were detected using both specimen types.

DISCUSSION

In the current study, we describe the design and evaluation of the UFI assay, an internally controlled, multiplex assay for the detection of DENV, *Leptospira*, and *Plasmodium* species from serum or plasma. One of the design goals for the UFI assay was to maintain the sensitivity for DENV detection of the original pan-DENV assay. In the current evaluation, the UFI assay demonstrated analytical and clinical sensitivities equivalent to those of the pan-DENV assay. DENV RNA was detected in 58 of 59 clinical samples that had previously tested positive in the pan-DENV assay. The discrepant sample came from a patient infected with DENV-1, and it originally tested negative in the reference seminested RT-PCR assay.

Molecular diagnostic tests for leptospirosis have often been designed to preferentially detect or differentiate pathogenic species from saprophytic species of *Leptospira* (12, 38–40). The evolving taxonomy of this genus has complicated such efforts, and assays targeting common regions of the *Leptospira* genome have not shown lower clinical specificity due to the amplification of saprophytic species (12, 23, 41). For example, in a comparison of PCR assays targeting the 16S rRNA gene and a gene specific to pathogenic *Leptospira*, i.e., *lipL32*, the 16S rRNA gene assay proved to be more clinically sensitive without a significant loss in specificity (41). Furthermore, it has not been shown that the detection of saprophytic *Leptospira* DNA in the plasma or serum of an acutely ill patient can be safely interpreted as a contaminant and disregarded. Consistent with this reasoning, the 16S rRNA target in the UFI assay was designed to sensitively detect all *Leptospira* species, regardless of whether they are currently identified as pathogenic or saprophytic.

The UFI assay detected *Leptospira* DNA in every sample from a set of 65 Brazilian patients for whom leptospirosis was clinically suspected. Concerns that arise from this finding include the nonspecific amplification of sequences found in patient samples or the contamination of laboratory reagents. There was no evidence of nonspecific amplification in the *Leptospira* assay during the analytical evaluation of the UFI assay or the testing of clinical samples from patients with HCV, dengue, or malaria. The sequencing of amplicons from 50 samples was also consistent with the detection of *Leptospira* spp. The finding that six sequences, matching at least three different species, were detected argues against laboratory contamination. Taken together, these findings indicate that the UFI assay has greater clinical sensitivity than the *Leptospira* molecular assays used as reference. This is likely due, in part, to the

fact that the assays for *flaB* and *lipL41* are conventional PCRs that generate large amplicons (793 bp and 520 bp, respectively). Future studies will be required to confirm these results, to provide a comparison with MAT using paired acute-phase and convalescent-phase samples, and to investigate the clinical utility of more-sensitive *Leptospira* detection.

While molecular testing for DENV and *Leptospira* is often performed on serum or plasma samples, assays for malaria typically utilize whole-blood samples (21, 42–44). Lamikanra et al. performed a direct comparison of *P. falciparum* detection by 18S rRNA PCR assay using paired whole-blood and plasma samples from children. *P. falciparum* detection was shown to be greater in whole blood than in plasma, which resulted from improved detection of low levels of parasitemia (21). In the current study, we were able to detect *Plasmodium* in 11 paired whole-blood and plasma samples from patients infected with *P. falciparum*, *P. malariae*, or *P. ovale*. This evaluation included samples with parasitemia levels of 0.02% to 0.0037%, that is, at least 10-fold lower than the sensitivity obtainable with thin smears if a single trophozoite is detected among 500 counted red blood cells (parasitemia level of 0.2%). While these results support the use of plasma for the diagnosis of malaria using the UFI assay, these findings will need to be confirmed with a larger sample set including all five *Plasmodium* species.

Despite the testing of 210 clinical samples and a range of reference samples, the evaluation of the UFI assay in the current study was limited by the availability of positive samples for DENV-4. DENV-4 has not been detected recently in Nicaragua (36, 37), and this will require evaluation in future studies. Limitations of this assay also involve concerns common to all NAATs regarding the emergence of divergent strains of viruses, bacteria, or parasites with mutations in the sequences targeted by the primers and probes of a given assay. We have attempted to address these limitations by targeting highly conserved sequences for all pathogens, but this concern cannot be eliminated. Finally, the UFI assay requires the extraction of total nucleic acids from clinical samples. While a number of platforms and extraction protocols exist for the extraction of RNA and DNA, this may require adjustments to the protocols used to detect any one of these pathogens individually.

In summary, we present the development of the UFI assay, which is a single-reaction, internally controlled assay for the detection of DENV, *Leptospira*, and *Plasmodium* species with a specific callout for *P. falciparum*. To our knowledge, this represents the first description of a syndrome-based NAAT for the detection of any combination of these common and potentially fatal causes of a UFI. We feel that this design, combined with the sensitivity of the component assays, represents a significant improvement over the individual available diagnostic tests for these pathogens.

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