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Clinical evaluation of a single-reaction real-time RT-PCR for pan-dengue and chikungunya virus detection

Jesse J. Waggoner^a, Gabriela Ballesteros^b, Lionel Gresh^c, Alisha Mohamed-Hadley^d, Yolanda Tellez^b, Malaya K. Sahoo^d, Janaki Abeynayake^{d,1}, Angel Balmaseda^b, Eva Harris^e, and Benjamin A. Pinsky^{a,d,*}

^a Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University School of Medicine, Stanford, CA, USA

^b National Virology Laboratory, Centro Nacional de Diagnóstico y Referencia, Ministry of Health, Managua, Nicaragua

^c Sustainable Sciences Institute, Managua, Nicaragua

^d Department of Pathology, Stanford University School of Medicine, Stanford, CA, USA

^e Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, CA, USA

Abstract

Background—Dengue virus (DENV) and chikungunya virus (CHIKV) now co-circulate throughout tropical regions of the world, with billions of people living at risk of infection. The differentiation of these infections is important for epidemiologic surveillance as well as clinical care, though widely-used molecular diagnostics for DENV and CHIKV require the performance of two to four separate PCR reactions for detection.

Objectives—In the current study, we sought to develop and evaluate a single-reaction, multiplex real-time RT-PCR (rRT-PCR) for the detection and differentiation of DENV and CHIKV (the pan-DENV–CHIKV rRT-PCR).

Study design—From an alignment of all available CHIKV complete genome sequences in GenBank, a new CHIKV rRT-PCR was designed for use in multiplex with a previously described assay for pan-DENV detection. Analytical evaluation was performed in accordance with published recommendations, and the pan-DENV–CHIKV rRT-PCR was clinically compared to reference

* Corresponding author at: 3375 Hillview Ave, Room 2913, Palo Alto, CA 94304, USA. Fax: +1 650 723 6918. bpinsky@stanford.edu (B.A. Pinsky).

¹Present address: Medical Research Institute, Colombo, Sri Lanka.

Author contributions

All authors made substantial contributions to the following: conception and design of the study (J.J.W., L.G., A.B., E.H., B.A.P.); data acquisition, analysis or interpretation (J.J.W., G.B., L.G., A. M-H., Y.L., M.K.S., J.A., A.B., E.H., B.A.P.); the drafting of the article or its critical revision for content (J.J.W., L.G., A.B., E.H., B.A.P.). All the authors have approved the final article.

Competing interests

None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcv.2016.01.007>.

molecular diagnostics for DENV and CHIKV using 182 serum samples from suspected cases in Managua, Nicaragua.

Results—The pan-DENV–CHIKV rRT-PCR had a dynamic range extending from 7.0 to 2.0 log₁₀ copies/μL for each DENV serotype and CHIKV, and the lower limits of 95% detection were 7.9–37.4 copies/μL. The pan-DENV–CHIKV rRT-PCR detected DENV in 81 patients compared to 75 using a reference, hemi-nested DENV RT-PCR, and it demonstrated perfect agreement with a reference CHIKV rRT-PCR (54 positive samples).

Conclusions—The single-reaction, multiplex format of the pan-DENV–CHIKV rRT-PCR, combined with sensitive detection of both viruses, has the potential to improve detection while decreasing testing costs and streamlining molecular workflow.

Keywords

Dengue virus; Chikungunya virus; Molecular diagnostics; Multiplex real-time RT-PCR; Background

1. Background

Over the past decade, chikungunya virus (CHIKV) has emerged from being a relatively rare arbovirus that caused sporadic outbreaks of human disease in Africa and Asia to the cause of a pandemic that has affected millions of people across five continents [1]. As a result, CHIKV has spread to new regions where dengue virus (DENV) is endemic, including, since December 2013, tropical and subtropical regions of the Western Hemisphere [2]. This has created a new challenge for health care systems that care for patients living in or returning from affected areas, as the clinical presentation of dengue and chikungunya overlap significantly [3–6]. The differentiation of infections with DENV and CHIKV is important not only for epidemiologic surveillance but also for clinical care, such as initiating appropriate management and providing prognostic information. While certain clinical and laboratory findings have been associated with dengue (thrombocytopenia, leukopenia) or chikungunya (arthralgia/arthritis), these are not sufficiently accurate to determine the cause of illness in all cases without specific testing [3–6].

Molecular tests are the most sensitive diagnostics for DENV or CHIKV in the acute setting [7,8]. Serologic testing is also frequently performed on paired acute and convalescent serum, but this can only confirm a diagnosis in retrospect. A number of molecular tests have been published for the detection of DENV or CHIKV, but the majority of these assays are run separately [9–14]. This can entail performing up to four amplification reactions for a single patient sample followed by detection using gel electrophoresis or running at least two real-time RT-PCR reactions [9,10]. This results in increased costs and prolonged turn-around times.

The first autochthonous chikungunya cases first appeared in Nicaragua in September of 2014, and since that time, DENV and CHIKV have co-circulated in the country. Our group previously described a real-time RT-PCR (rRT-PCR) for pan-DENV detection (referred to as the pan-DENV assay) that proved more sensitive than reference molecular tests for DENV when evaluated using clinical samples from Nicaragua [12]. In this report, we describe the

design and analytical and clinical evaluation of this multiplex assay, including a clinical comparison with reference molecular testing performed in Nicaragua.

2. Objectives

The objectives of the current study were to (1) develop a new rRT-PCR for CHIKV that could be run in multiplex with the pan-DENV assay and (2) evaluate the analytical and clinical performance of the multiplex pan-DENV–CHIKV rRT-PCR.

3. Study design

3.1. Ethics statement

Protocols for the collection and testing of samples from Nicaraguan pediatric dengue and chikungunya cases were reviewed and approved by the Institutional Review Boards (IRB) of the University of California, Berkeley and the Nicaraguan Ministry of Health. Specific approval was not required from the Stanford IRB for the design and analytical evaluation of the pan-DENV–CHIKV rRT-PCR.

3.2. rRT-PCR design and performance

Design of the pan-DENV assay has been described previously [12]. To design the CHIKV rRT-PCR, all CHIKV complete genome sequences ($n = 130$) available in GenBank (accessed in April, 2013) were aligned using MegAlign software (DNASTar). A consensus sequence was generated that identified bases conserved across 95% of available sequences. Primers and probes were designed from the consensus sequence using Primer3 software. CHIKV primers and probes were tested *in silico* using BLASTn to search the National Center for Biotechnology Information (NCBI) nucleotide database. Following an initial evaluation of three primer sets, primers and probes targeting a region of the nsp2 gene (Table 1) were selected for improved analytical sensitivity (data not shown). To determine optimal concentrations in the final reaction, the CHIKV primers and probe were then tested, in multiplex with the pan-DENV assay, at each combination of 100, 200, and 400 nM primer and 100, 200, and 400 nM probe. The concentration of DENV primers and probes in the final reaction were maintained from the pan-DENV assay [12]. We confirmed that the selected primers and probe matched the contemporary CHIKV strains circulating in the Western Hemisphere by comparing the sequences to a second CHIKV consensus generated using all complete genome sequences deposited as of June 2015.

The pan-DENV–CHIKV rRT-PCR reactions were performed in a total volume of 25 μ L using the SuperScript III Platinum One-Step qRT-PCR kit (Life Technologies) and 5 μ L of eluate. CHIKV primer and probe concentrations in the final reaction mixture are listed in Table 1. Concentrations of DENV primers and probes in the final reaction mixture were the same as those previously reported [12]. Analytical evaluation was performed on a Rotor-Gene Q instrument at Stanford University (RGQ, Qiagen), and the clinical evaluation was performed on a CFX96 instrument (Bio-Rad) at the National Virology Laboratory in Managua, Nicaragua. Cycling conditions were the following: 52 °C for 15 min; 94 °C for 2 min; 45 cycles of 94 °C for 15 s, 55 °C for 40 s, and 68 °C for 20 s. Each run was performed with a negative control (no template) and positive controls for DENV and CHIKV. To

confirm that the pan-DENV–CHIKV assay can detect co-infections, a mixed control, spiked with DENV-4 and CHIKV ssDNA at 10^4 copies/ μ L was also tested as a positive control. Signal was acquired at 55 °C, and analysis was performed on the linear scale. Thresholds were set manually on each instrument and used for analysis of each run (0.025 and 0.1 on the RGQ, 500 and 100 on the CFX96 for DENV and CHIKV, respectively). For both targets, any exponential curve crossing this threshold was considered positive.

3.3. Control nucleic acids and reference material

Quantitated, positive-sense, single-stranded DNA (ssDNA) oligonucleotides containing the target sequence for each DENV serotype and CHIKV were synthesized (Eurofins MWG Operon) and used in the analytical characterization of the pan-DENV–CHIKV rRT-PCR. The specificity of the pan-DENV–CHIKV rRT-PCR was evaluated by testing genomic RNA from the following viruses: DENV-1Hawaii 1944, DENV-2 New Guinea C strain, DENV-3 strain H87, and DENV-4 strain H241, CHIKV (strain R80422a provided by the CDC Division of Vector Borne Diseases and the S27 Petersfield strain from Vircell Microbiologists, Granada, Spain), West Nile (4 strains), Japanese encephalitis, tick-borne encephalitis, yellow fever (two strains), Saint Louis encephalitis, Zika, o'nyong-nyong (ONNV, strain MP30) [15], Semliki forest, Mayaro, Ross river, Getah, Barmah forest, and Una [12,16]. An additional 50 domestic (USA) samples with detectable hepatitis C virus (HCV) RNA were extracted and tested.

3.4. Analytical characterization

The analytical evaluation of the pan-DENV–CHIKV rRT-PCR was performed according to published recommendations [17] and as previously described [12,16]. Briefly, for each DENV serotype and CHIKV, linearity studies were performed using serial 10-fold dilutions of quantified ssDNA. Four replicates of each dilution from $7.0 \log_{10}$ copies/ μ L to 1 copy/ μ L were tested on a single run. The linear range was established by fitting a best-fit line to the data by regression analysis and included the range where the R^2 value was 0.99. To establish the lower limit of 95% detection (95% LLOD), ten replicates of five 2-fold dilutions from 50 to 3.12 copies/ μ L were tested on a single run. The 95% LLOD was then calculated using probit analysis. Precision of the pan-DENV–CHIKV rRT-PCR was evaluated by testing three concentrations of quantified ssDNA (7.0 , 5.0 , and $3.0 \log_{10}$ copies/ μ L) for each target in triplicate on three separate runs. Dilutions were made on a single day, aliquoted and frozen at -80 °C. Separate aliquots were thawed for the performance of each precision run.

3.5. Clinical samples and nucleic acid extraction

Pre-collected and de-identified serum samples from 182 Nicaraguan children with suspected dengue or chikungunya were used for the clinical evaluation of pan-DENV–CHIKV rRT-PCR. Samples were collected during 2013 and 2014 as part of two ongoing studies: the Nicaraguan Pediatric Dengue Cohort Study and a hospital-based dengue study, both based in Managua. Study design and methods for both of these studies have been described [18–20]. RNA extractions were performed using the QIAamp Viral RNA Mini Kit (Qiagen) with 140 μ L of serum using and an elution volume of 60 μ L.

3.6. Reference testing

All serum samples were tested for DENV RNA using a heminested RT-PCR [10]. Acute and convalescent serum samples were also tested for anti-DENV antibodies by IgM MAC-ELISA and Inhibition ELISA, which were interpreted as previously described [21]. Eighty-one serum samples, collected in 2014, were tested for CHIKV using an rRT-PCR, which maintained a protocol from a published assay [9] but substituted primers and probes designed to detect Asian and East–Central–South African genotypes of CHIKV. This will be referred to as the comparator CHIKV rRT-PCR. Primer and probe sequences for the comparator CHIKV rRT-PCR are as follows: forward primer, CHIKV 3855, GAGCATACGGTTACGCAGATAG; reverse primers, CHIKV 3957c–a, TACTGGTGATACATGGTGGTTTC and CHIKV 3957c–b, TGCTGGTGACACATGGTGGTTTC; probes, CHIKV 3886 FAM-a, FAM-ACGAGTAATCTGCGTACTGGGACGTA-BHQ1 and CHIKV 3886 FAM-b, FAM-ACGAGTCATCTGCGTATTGGGACGCA-BHQ1.

3.7. Statistics

Kappa statistics were calculated with GraphPad software (GraphPad) and used to compare RT-PCR results. C_T values from CHIKV-positive clinical samples were compared by unpaired t test (GraphPad). Probit analysis was performed using SPSS (IBM) to determine the 95% LLOD for the pan-DENV–CHIKV rRT-PCR. Precision was calculated as described [22].

4. Results

4.1. Analytical evaluation

Using serial 10-fold dilutions of ssDNA, the linear range of the pan-DENV–CHIKV rRT-PCR for each DENV serotype and CHIKV extended from 7.0 to 2.0 $\log_{10}$ copies/ μ L (Fig. S1). The 95% LLOD was calculated to be 12.0 copies/ μ L for DENV-1, 7.9 for DENV-2, 12.9 for DENV-3, 37.4 for DENV-4, and 15.3 for CHIKV. The results of precision experiments for each target (DENV-1-4 and CHIKV) are shown in Table 2.

4.2. Specificity

Amplification of genomic RNA from ONNV was observed in the CHIKV channel of the pan-DENV–CHIKV rRT-PCR. This generated a signal with less than one-half of the normalized fluorescence produced by CHIKV genomic RNA controls (Supplemental Fig. S1). Amplification of separate DENV and CHIKV controls was detected in the appropriate channels without any cross-reaction, and both targets were consistently co-detected using a single co-infection control containing both DENV and CHIKV target nucleic acids. No amplification was observed when the pan-DENV–CHIKV rRT-PCR was performed using genomic RNA from the aforementioned viruses (see material and methods) or extracted nucleic acids from 50 patients with HCV (mean HCV viral load, 6.10 $\log_{10}$ IU/mL; standard deviation 0.76).

4.3. Clinical samples

Sera from 182 Nicaraguan children with suspected dengue or chikungunya were tested for DENV using the pan-DENV-CHIKV rRT-PCR and hemi-nested DENV RT-PCR. Results of this comparison are shown in Table 3. The two assays showed very good agreement (kappa statistic, 0.91). Seventy-five patients tested positive for DENV using the hemi-nested RT-PCR, including patients with DENV-1 ($n = 44$), DENV-2 ($n = 19$), and DENV-3 ($n = 12$). Seventy-four (98.7%) patients had detectable DENV RNA using the pan-DENV-CHIKV rRT-PCR. A single patient tested positive for DENV-1 in the hemi-nested RT-PCR but was not detected in the pan-DENV-CHIKV rRT-PCR. For an additional seven patients, DENV RNA could only be detected using the pan-DENV-CHIKV rRT-PCR (average C_T 41.86, standard deviation 2.11). Five of these seven patients also tested positive for DENV by IgM MAC-ELISA ($n = 4$) or Inhibition ELISA ($n = 1$).

Serum samples from 81 children, collected in 2014, were also tested for CHIKV using the comparator CHIKV rRT-PCR (Table 4). CHIKV test results demonstrated complete agreement between the pan-DENV-CHIKV and comparator CHIKV rRT-PCRs (kappa statistic, 1.0). C_T values for the 57 CHIKV-positive samples did not differ significantly between the comparator CHIKV rRT-PCR (mean 25.05, standard deviation 5.62) and pan-DENV-CHIKV rRT-PCR (mean 26.17, standard deviation 4.97; $p = 0.27$). CHIKV RNA was not detected in any of the remaining 101 serum samples (collected in 2013) using the pan-DENV-CHIKV rRT-PCR, and no co-infections were identified.

5. Discussion

In the current study, we describe the development and analytical and clinical evaluation of the pan-DENV-CHIKV rRT-PCR. This assay demonstrated excellent agreement with separate molecular comparators for DENV and CHIKV. In addition, the pan-DENV-CHIKV rRT-PCR requires only one reaction per sample instead of the three reactions and gel electrophoresis that are needed for the comparator assays. In this study, the pan-DENV-CHIKV rRT-PCR did not significantly improve DENV detection compared to the hemi-nested RT-PCR, which has been shown for the pan-DENV assay previously [12]. However, the current study was not powered to demonstrate superiority. Of the 97 patients in this study who tested negative for DENV using the hemi-nested RT-PCR and had available serological results, only 12 patients (12.4%) had results consistent with a recent DENV infection. Five of these patients were detected using the pan-DENV CHIKV assay.

Multiplex RT-PCRs for the detection of DENV and CHIKV have been reported in the literature previously [23–28]. However, only three groups have described single-reaction real-time assays for DENV and CHIKV [23,26,27], whereas others reported modified conventional RT-PCRs that require gel electrophoresis for detection [24,25,28]. All multiplex rRT-PCRs appear to perform well, as reported, though most primer and probe sequences contain mismatches when aligned to consensus DENV and CHIKV sequences that include contemporary viruses obtained globally (see materials and methods). Furthermore, in these studies less common comparator assays were used as reference or the exact molecular comparator was unclear. These factors complicated the evaluation of the different multiplex rRT-PCRs reported in the literature, and made them unsuitable for use as

reference molecular assays for these viruses. The goal of our study was, therefore, to design an rRT-PCR for CHIKV that would be compatible with the pan-DENV assay, an assay that has excellent clinical and analytical performance characteristics and that has been extensively evaluated in single- and multiplex formats [12,16,29]. The resulting pan-DENV–CHIKV rRT-PCR was then compared to widely used, reference assays for DENV and CHIKV in Nicaragua, which is experiencing the transmission of both viruses.

Although the pan-DENV–CHIKV rRT-PCR demonstrated very good analytical performance against all DENV serotypes and CHIKV, our study was limited by the absence of DENV-4 in clinical samples. DENV-4 was not circulating during the 2013 and 2014 DENV seasons in Managua, and it has rarely been identified in clinical dengue cases in Managua over the past 10 years [30]. During the analytical evaluation, the CHIKV rRT-PCR amplified genomic RNA from a reference ONNV isolate. This isolate produced a signal with reduced normalized fluorescence and could be distinguished from CHIKV controls (Supplemental Fig. S1). The *in silico* evaluation of our primers and probe predicted two to four mismatches with ONNV, though only four complete ONNV genomes were available for analysis. However, the significance of this cross-reaction remains unclear given the rarity of ONNV infections. Finally, the CHIKV rRT-PCR was designed from an alignment performed prior to the emergence of CHIKV in the Western Hemisphere. The alignment was re-run using all complete genomes available as of June, 2015, and no new strains were identified with mutations in the primer and probe sequences.

In conclusion, we report the development and evaluation of the pan-DENV–CHIKV rRT-PCR. This assay demonstrated very good agreement with individual molecular comparators for DENV and CHIKV. The single-reaction, multiplex format of the pan-DENV–CHIKV rRT-PCR, combined with sensitive detection of both viruses, has the potential to improve detection while decreasing test costs and streamlining molecular workflow.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- DENV and CHIKV co-circulate and result in overlapping clinical presentations.
- A single-reaction pan-DENV–CHIKV rRT-PCR was designed.
- The pan-DENV–CHIKV rRT-PCR was evaluated using serum samples from Nicaragua.
- The pan-DENV–CHIKV rRT-PCR proved as sensitive as individual reference tests.
- This DENV–CHIKV multiplex test may reduce costs and improve molecular workflow.

Table 1

CHIKV primer and probe sequences included in the pan-DENV-CHIKV rRT-PCR.

Name	Sequence (5' → 3')	Concentration ^a	Location ^a
CHIKV forward	CATCTGCACYCAAGTGTACCA	200 nM	2578-2598
CHIKV reverse	GCGCATTTTGCCTTCGTAATG	200 nM	2654-2674
CHIKV probe ^b	GCGGTGTACTGCCTGTGACYGC	100nM	2614-2637

^aThe concentration of each oligonucleotide in the final reaction mixture is provided.

^bGenomic locations are provided based on the reference sequence Chikungunya virus strain S27-African prototype (Genbank: AF369024.2).

^cThe 5' fluor and 3' quencher on the CHIKV probe were Cal Fluor 610 and BHQ-2, respectively.

Table 2

Within-run, between-run, and total (within-laboratory) imprecision of the pan-DENV-CHIKV rRT-PCR determined using quantitated ssDNA controls.

	Concentration ^a	Mean		Within-run		Between-run		Total	
		Observed C _T		SD	%CV	SD	%CV	SD	%CV
DENV-1	7.0	17.47		0.06	0.37	0.12	0.71	0.13	0.77
	5.0	24.39		0.54	2.20	0.65	2.66	0.78	3.21
	3.0	33.81		0.32	0.94	1.81	5.37	1.83	5.42
DENV-2	7.0	17.19		0.11	0.61	0.27	1.59	0.29	1.67
	5.0	25.31		0.34	1.33	0.91	3.61	0.95	3.77
	3.0	35.56		0.95	2.66	2.75	7.72	2.85	8.02
DENV-3	7.0	17.78		0.14	0.77	0.49	2.73	0.50	2.80
	5.0	24.60		0.97	3.95	0.77	3.13	1.11	4.50
	3.0	34.15		1.15	3.36	1.46	4.26	1.73	5.07
DENV-4	7.0	17.08		0.12	0.69	0.41	2.42	0.42	2.49
	5.0	24.10		0.97	4.01	0.65	2.69	1.02	4.24
	3.0	33.71		2.44	7.25	1.90	5.63	2.75	8.17
CHIKV	7.0	17.08		0.13	0.75	0.28	1.64	0.30	1.75
	5.0	24.24		0.98	4.06	0.42	1.73	0.91	3.74
	3.0	31.89		0.35	1.09	0.76	2.38	0.81	2.54

^aValues in log₁₀ copies/μL of eluate SD, standard deviation; %CV, percent coefficient variation.

Table 3

Comparison of DENV detection in the pan-DENV-CHIKV rRT-PCR with detection in the hemi-nested DENV RT-PCR.

		Hemi-nested DENV RT-PCR		
		Positive	Negative	Total
pan-DENV-CHIKV	Positive	74	7 ^a	81
rRT-PCR	Negative	1	100	101
	Total	75	107	182

^aFive of seven patients also tested positive for DENV by IgM MAC-ELISA or Inhibition ELISA.

Table 4

Comparison of CHIKV detection in the pan-DENV-CHIKV rRT-PCR with detection in the reference CHIKV rRT-PCR.

		<u>Comparator CHIKV rRT-PCR</u>		
		Positive	Negative	Total
pan-DENV-CHIKV	Positive	57	0	57
rRT-PCR	Negative	0	24	24
	Total	57	24	81