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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], 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

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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 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 $\log_{10}$ 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.

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

* Corresponding author at: 3375 Hillview Ave, Room 2913, Palo Alto, CA 94304, USA. Fax: +1 650 723 6918.
E-mail address: bpinsky@stanford.edu (B.A. Pinsky).
[1] Present address: Medical Research Institute, Colombo, Sri Lanka.

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Table 1
CHIKV primer and probe sequences included in the pan-DENV–CHIKV rRT-PCR.

Name	Sequence (5′ → 3′)	Concentration[a]	Location[b]
CHIKV forward	CATCTGCACYCAAGTGTACCA	200 nM	2578–2598
CHIKV reverse	GCGCATTTTGCCTTCGTAATG	200 nM	2654–2674
CHIKV probe[c]	GCGGTGTACACTGCCTGTGACYGC	100 nM	2614–2637

[a] The concentration of each oligonucleotide in the final reaction mixture is provided.
[b] Genomic locations are provided based on the reference sequence Chikungunya virus strain S27–African prototype (Genbank: AF369024.2).
[c] The 5′ fluor and 3′ quencher on the CHIKV probe were Cal Fluor 610 and BHQ-2, respectively.

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 μL using the SuperScript III Platinum One-Step qRT-PCR kit (Life Technologies) and 5 μL 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/μL 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.