

Single-Reaction Multiplex Reverse Transcription PCR for Detection of Zika, Chikungunya, and Dengue Viruses

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Clinical manifestations of Zika virus, chikungunya virus, and dengue virus infections can be similar. To improve virus detection, streamline molecular workflow, and decrease test costs, we developed and evaluated a multiplex real-time reverse transcription PCR for these viruses.

Zika virus is a mosquito-borne flavivirus that, in 2015, spread throughout the tropical and subtropical regions of the Western Hemisphere. In January 2016, the first autochthonous cases of Zika fever were confirmed in Nicaragua (1). The diagnosis of human Zika virus infections is confounded by a nonspecific clinical presentation, which overlaps substantially with that of dengue virus (DENV) and chikungunya virus (CHIKV) (2,3) and by cross-reaction with DENV IgM and DENV nonstructural protein 1 in assays for Zika virus (4–7).

Molecular assays can detect and differentiate these 3 pathogens during the acute phase of illness. Although a number of molecular tests have been published for detecting DENV and CHIKV, only 2 Zika virus real-time reverse transcription PCRs (rRT-PCRs) have been reported and were characterized by using human specimens (6–8). These assays are run as individual reactions, and molecular testing for all 3 viruses, using established protocols, requires multiple reactions for a single patient sample (6,9,10). We describe a Zika virus rRT-PCR that was designed to be run in multiplex with published assays for pan-DENV and CHIKV detection (11,12). We then evaluated the single-reaction multiplex rRT-PCR for Zika virus, CHIKV, and

DENV (referred to as the ZCD assay) by testing clinical samples from persons with suspected cases in Nicaragua.

The Study

The Zika virus primers and probe (online Technical Appendix Table 1 (<http://wwwnc.cdc.gov/EID/article/22/7/16-0326-Techapp1.pdf>)) were designed by using all complete or nearly complete ($\geq 10,000$ kb) Zika virus genome sequences available in GenBank ($n = 21$) accessed March 28, 2014). Target sequences were subsequently confirmed to match strains from the Americas. All rRT-PCR reactions were performed on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) by using 25- μ L reactions of the SuperScript III Platinum One-Step qRT-PCR kit (Life Technologies, Carlsbad, CA, USA) and 5 μ L of RNA template. Cycling conditions for the ZCD assay were as follows: 52°C for 15 min; 94°C for 2 min; 45 cycles at 94°C for 15 sec, 55°C for 20 sec (acquisition), and 68°C for 20 sec. Each run included a no-template control and positive controls for Zika virus, CHIKV, and DENV.

Linear range and lower limit of 95% detection (95% LLOD) for each target were determined as recommended. We determined linear range and 95% LLOD for each target as recommended (13; online Technical Appendix). The linear range of the ZCD assay extended from 10^8 to 10 copies/mL for Zika virus and DENV-3 and from 10^8 to 100 copies/mL for DENV-1, -2, -4, and CHIKV. The 95% LLOD for each target, in copies/mL of eluate (5 μ L added to each ZCD reaction), was as follows: Zika virus, 7.8; CHIKV, 13.2; DENV-1, 11.7; DENV-2, 13.5; DENV-3, 4.1; DENV-4, 10.5.

Assay exclusivity was established by testing genomic RNA from the following viruses: West Nile, Japanese encephalitis, tickborne encephalitis, yellow fever, Saint Louis encephalitis, o'nyong-nyong, Semliki Forest, Mayaro, Ross River, Getah, Barmah Forest, and Unas (12,14). No amplification was detected for any of these viruses.

De-identified serum samples, collected from Nicaraguan patients with suspected Zika virus, CHIKV, and/or DENV infections, were tested (online Technical Appendix). We tested 216 samples by using the ZCD assay and the pan-DENV-CHIKV rRT-PCR, which is a validated duplex assay containing the DENV and CHIKV primers and probes used in the ZCD assay (12). Both assays were performed on an ABI7500 (Applied Biosystems) (Table 1). A total of 173 samples were positive for DENV alone

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Table 1. Comparison of DENV and CHIKV detection in the ZCD assay and pan-DENV-CHIKV rRT-PCR*

| ZCD assay | pan-DENV-CHIKV rRT-PCR | | | | | |
|-----------|------------------------|-----|-------|-----------------|-----|-------|
| | DENV Detection | | | CHIKV Detection | | |
| | Pos | Neg | Total | Pos | Neg | Total |
| Pos | 55 | 5 | 60 | 113 | 23 | 136 |
| Neg | 3 | 153 | 156 | 12 | 68 | 80 |
| Total | 58 | 158 | 216 | 125 | 91 | 216 |

*CHIKV, chikungunya virus; DENV, dengue virus; neg, negative; pos, positive; rRT-PCR, real-time reverse transcription PCR; ZCD assay, single-reaction multiplex rRT-PCR for Zika virus, CHIKV, and DENV.

(n = 25), CHIKV alone (n = 110), or both (n = 38). The ZCD assay and pan-DENV-CHIKV rRT-PCR showed very good agreement for DENV detection ($k = 0.907$). Six of 8 discrepant samples were co-infected with DENV and CHIKV, and the 2 discrepant samples with DENV mono-infections had cycle threshold (C_t) values of 41.34 and 42.25. The 2 assays demonstrated good agreement for CHIKV detection ($k = 0.662$). C_t for the 35 CHIKV discrepant samples were reached significantly later (mean 39.8, SD ± 1.5) than the 113 concordant samples (28.7, \pm SD 9.7; $p < 0.0001$).

The first case of Zika virus infection in Nicaragua was detected with the ZCD assay during the assay comparison described above. After Zika virus identification, 133 consecutive samples were tested by using both the ZCD assay and a comparator Zika virus rRT-PCR targeting the capsid gene (6) (Table 2). When the comparator rRT-PCR was analyzed according to the published validation ($C_t \leq 38.5$ defining a positive result), these assays demonstrated only moderate agreement ($k = 0.47$), and Zika virus was detected in significantly more samples by using the ZCD assay ($p < 0.001$). Of the 31 samples positive only for Zika virus in the ZCD assay, 22 (71%) produced a $C_t (> 38.5)$ that was reached later in the comparator Zika virus rRT-PCR. These 22 samples had mean C_t of 32.06 (SD ± 2.45) in the ZCD assay and 42.09 (SD ± 1.41) in the comparator Zika virus rRT-PCR. If all samples in the comparator rRT-PCR with $C_t > 38.5$ were considered positive, the assays demonstrated very good agreement ($k = 0.81$; online Technical Appendix Table 2). Of the 56 Zika virus-positive samples in the ZCD assay, 39 were positive only for Zika virus, and 17

Table 2. Comparison of Zika virus detection in the ZCD assay and the Zika virus comparator rRT-PCR*

| ZCD assay | Zika virus rRT-PCR | | |
|-----------|--------------------|-----|-------|
| | Pos | Neg | Total |
| Pos | 25 | 31† | 56 |
| Neg | 1‡ | 76 | 77 |
| Total | 26 | 107 | 133 |

*CHIKV, chikungunya virus; C_t , cycle threshold; DENV, dengue virus; neg, negative; pos, positive; rRT-PCR, real-time reverse transcription PCR; ZCD assay, single-reaction multiplex rRT-PCR for Zika virus, CHIKV, and DENV.

†22 samples produced a late C_t in the comparator Zika virus rRT-PCR ($C_t > 38.5$).

‡Sample was also pos for a DENV-CHIKV co-infection and tested pos for Zika virus when repeated in the ZCD assay.

showed evidence of mixed infection: Zika virus-DENV (n = 3); Zika virus-CHIKV (n = 10), or Zika virus-CHIKV-DENV (n = 4).

Conclusions

The ZCD assay improved detection of Zika virus relative to the comparator rRT-PCR, and 31 samples were positive only for Zika virus by the ZCD assay when the comparator was interpreted as published (6). Notably, 22 (71%) of these 31 samples produced a late signal in the comparator Zika virus rRT-PCR ($C_t > 38.5$), indicating that these most likely are true, late-positive results. Improved sensitivity for Zika virus is needed given the low viremia detected in clinical samples and the current lack of accurate alternative diagnostics, such as serology (6,7,15). Additionally, the ZCD assay identified 17 co-infections in Zika virus-positive patients. Although preliminary, these data provide evidence for the utility of a multiplex diagnostic test for these pathogens.

The performance of the ZCD assay for DENV detection was similar to that of the pan-DENV-CHIKV rRT-PCR, and the analytical sensitivity for CHIKV was similar in both assays (12). CHIKV detection in clinical samples in the ZCD assay and pan-DENV-CHIKV rRT-PCR demonstrated good agreement, although both assays contain the same CHIKV primers and probes. Discrepant samples all had C_t of ≥ 37.36 , which correspond to 10 copies/mL of eluate and fall below the 95% LLOD. Although ZCD assay results for these CHIKV-positive samples were reproducible, the clinical significance of such low-level viremia in patients with suspected chikungunya fever is unclear and warrants further study.

A limitation to our study is the use of a single comparator Zika virus rRT-PCR. This assay was 1 of 2 rRT-PCRs developed for the 2007 Yap Island Zika virus strain (6). The second rRT-PCR, targeting the membrane gene, was evaluated for the current study but proved consistently less analytically sensitive. Therefore, performance of this second Zika virus assay most likely would not have affected result interpretation.

In conclusion, the single-reaction multiplex ZCD assay detected and differentiated Zika virus, CHIKV, and DENV. This assay should streamline molecular workflow and decrease test costs while improving detection of these 3 human arboviruses.

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Ministry of Health, and the Sustainable Sciences Institute in Managua, Nicaragua.

Patent applications or provisional patent applications that cover the ZCD multiplex assay and the primers and probes described in this report have been filed (J.J.W. and B.A.P.).

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.



Single-Reaction Multiplex Reverse Transcription PCR for Detection of Zika, Chikungunya, and Dengue Viruses

Technical Appendix

Assay Development and Analytical Evaluation

The single-reaction, multiplex real-time reverse transcription PCR (rRT-PCR) for Zika virus, chikungunya virus (CHIKV), and dengue virus (DENV) (referred to as the ZCD assay) was interpreted on the ABI7500 instrument (Applied Biosystems, Foster City, CA, USA) on the linear scale. Crossing thresholds were set manually for each target during analytical evaluation. Thresholds were set to cross amplification curves in the linear range of the assay at the start of the logarithmic phase of amplification. An exponential curve crossing the threshold was considered positive.

Linearity and lower limit of 95% detection (95% LLOD) studies were performed using quantitated, synthesized single-stranded DNA (ssDNA) that, individually, contained target sequences for each DENV serotype, CHIKV, and Zika virus. Linearity was evaluated by testing serial 10-fold dilutions from $8.0 \log_{10}$ copies/ μL to 1 copy/ μL of eluate. Four replicates of each concentration 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 for this line was ≥ 0.99 . To establish the 95% LLOD, 10 replicates of five 2-fold dilutions extending from, and including, the lower limit of the linear range were tested on a single run. The 95% LLOD was then calculated using probit analysis.

All ZCD assay testing was performed in the molecular diagnostics laboratory at the Centro Nacional de Diagnóstico y Referencia (Managua, Nicaragua). To limit the possibility for contamination, this laboratory has dedicated areas for master mix preparation, nucleic acid extraction and assay set-up, and RT-PCR performance. Additionally, each run of the ZCD assay,

pan-DENV-CHIKV rRT-PCR, and Zika virus comparator rRT-PCR included a negative control (water). The negative control was negative on all runs performed.

Statistical Analysis

The 95% LLOD was calculated by probit analysis using SPSS software (IBM, Armonk, NY, USA). Basic statistics were performed using Excel (Microsoft Corp, Redmond, WA, USA). Mean cycle threshold (C_t) values were compared by using Welch's t test. Fisher exact tests, κ statistics, and Welch's t tests were performed using GraphPad software (GraphPad Software, Inc., LaJolla, CA).

Clinical Evaluation

De-identified serum samples were obtained from: 1) a cohort study of DENV, CHIKV and Zika virus transmission in children based in District II of Managua; 2) a hospital-based study of dengue and chikungunya severity based in the Infectious Diseases Ward of the Hospital Infantil Manuel de Jesús Rivera, also in Managua; and 3) the national surveillance system of reportable illnesses for the country of Nicaragua (3–5). Samples were extracted with the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) by using 140 μ L of serum and a 60- μ L elution volume. Research protocols were approved by the Institutional Review Boards at the Nicaraguan Ministry of Health; the University of California, Berkeley; and Stanford University.

The comparator Zika virus rRT-PCR, which was used to evaluate the clinical sensitivity of the ZCD assay for Zika virus, was a published rRT-PCR targeting the capsid gene (including the primers Zika virus 1086 and Zika virus 1162c and the probe Zika virus 1107-FAM) (6). Samples with a $C_t \leq 38.5$ in the Zika virus rRT-PCR were considered positive; samples with a $C_t > 38.5$ were considered negative, as described in the initial publication (6).

The DENV and CHIKV components of the ZCD assay were compared with the pan-DENV-CHIKV rRT-PCR performed on the ABI7500 rather than the CFX96, as previously described, because the ABI instrument demonstrated improved sensitivity (1). C_t values on the CFX96 instrument were, on average, 3.91 cycles later than on the ABI7500, which resulted in a clinical sensitivity for the pan-DENV-CHIKV assay of only 45.2% on the CFX96 compared with

the ABI7500 (data not shown). These findings highlight the need to verify assay performance characteristics whenever a molecular test is implemented on a new real-time instrument.

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Technical Appendix Table 1. Primers and probes included in the ZCD assay*

| Primers and probe | Sequence (5' → 3') | Concentration† | Location‡ |
|--------------------|--------------------------|----------------|-------------------|
| Zika virus | | | |
| Forward | CAGCTGGCATCATGAAGAAYC | 300nM | 7332–7352 |
| Reverse 1 | CACTTGTCCCATCTTCTTCTCC | 300nM | 7411–7432 |
| Reverse 2 | CACCTGTCCCATCTTTTTCTCC | 300nM | 7411–7432 |
| Probe§ | CYGTTGTGGATGGAATAGTGG | 100nM | 7355–7373 |
| CHIKV | | | |
| Forward | CATCTGCACYCAAGTGTACCA | 300nM | 2578–2598 |
| Reverse | GCGCATTTTGCCTTCGTAATG | 300nM | 2654–2674 |
| Probe§ | GCGGTGTACACTGCCTGTGACYGC | 100nM | 2614–2637 |
| DENV | | | |
| -1, -2, -3 Forward | CAGATCTCTGATGAACAACCAACG | 350nM | 86–109 |
| -2 Forward C→T | CAGATCTCTGATGAATAACCAACG | 350nM | 87–110 |
| -3 Forward C→T | CAGATTTCTGATGAACAACCAACG | 300nM | 85–108 |
| -4 Forward | GATCTCTGGAAAAATGAAC | 450nM | 81–99 |
| -1, 3 Reverse | TTTGAGAATCTCTTCGCCAAC | 300nM | 179–199, 178–197† |
| -2 Reverse | AGTTGACACGCGGTTTCTCT | 350nM | 152–171 |
| -2 Reverse A→G | AGTCGACACGCGGTTTCTCT | 350nM | 152–171 |
| -4 Reverse | AGAATCTCTTCACCAACC | 450nM | 173–190 |
| Probe A | CTCGCGGTTTCAGCATAT | 100nM | 136–154 |
| Probe B | CTCTCGCGTTTCAGCATAT | 100nM | 137–155 |
| Probe C | CTCTCAGGTTTCAGCATATTG | 100nM | 135–155 |
| Probe D | CTCACGCGTTTCAGCATAT | 100nM | 135–153 |

*CHIKV, chikungunya virus; DENV, dengue virus; ZCD assay, single-reaction, multiplex real-time reverse transcription PCR for Zika virus, CHIKV, and DENV

†Concentration of each oligonucleotide in the final reaction mixture is provided. DENV and CHIKV primers and probes have been published previously (1,2).

‡Genomic locations are based on the following reference sequences: Zika virus strain MR766-NIID (GenBank accession no. LC002520.1), CHIKV strain S27–African prototype (GenBank accession no. AF369024.2), DENV-1 U.S./Hawaii/1944 (GenBank: EU848545.1), DENV-2 New Guinea C strain (GenBank accession no. AF038403.1), DENV-3 strain H87 (GenBank accession no. M93130.1), and DENV-4 strain H241 (GenBank accession no. AY947539.1). For the DENV 1, 3 reverse primer, positions are listed in the order DENV-1, DENV-3.

§5' fluor (excitation and emission wavelengths) and 3' quencher pairs were the following: ZIKV, Cal Fluor Orange 560 (538 nm and 559 nm) and BHQ-1; CHIKV, TAMRA (557 nm and 583 nm) and BHQ-2; DENV, FAM (495 nm and 520 nm) and BHQ-1. DENV probes are BHQplus probes (Biosearch Technologies, Petaluma, CA, USA).

Technical Appendix Table 2. Comparison of Zika virus detection in the ZCD assay and comparator Zika virus rRT-PCR if samples cycle threshold values >38.5 in the comparator rRT-PCR were considered positive

| ZCD Assay | Zika virus rRT-PCR | | Total |
|-----------|--------------------|----------|-------|
| | Positive | Negative | |
| Positive | 47 | 9 | 56 |
| Negative | 3* | 74 | 77 |
| Total | 50 | 83 | 133 |

*For 2 of 3 patients, another pathogen was detected in the ZCD assay (DENV–CHIKV co-infection and CHIKV mono-infection). CHIKV, chikungunya virus; DENV, dengue virus; rRT-PCR, real-time reverse transcription PCR; ZCD assay, multiplex rRT-PCR for Zika virus, CHIKV, and DENV.