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Development and Evaluation of a SYBR Green—Based Real-Time Multiplex RT-PCR Assay for Simultaneous Detection and Serotyping of Dengue and Chikungunya Viruses

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Chikungunya virus (CHIKV) and dengue virus (DENV) have emerged as the two most important arbovirus diseases of global health significance. Similar clinical manifestations, transmission vectors, geographical distribution, and seasonal correlation often result in misdiagnosis of chikungunya infections as dengue cases and vice versa. In this study, we developed a rapid and accurate laboratory confirmative method to simultaneously detect, quantify, and differentiate DENV serotypes 1, 2, 3, and 4 and CHIKV. This SYBR Green I—based one-step multiplex real-time RT-PCR assay is highly sensitive and specific for CHIK and DENV. Melting temperature analysis of PCR amplicons was used to serotype DENV and to differentiate from CHIKV. The detection limit of the assay was 20, 10, 50, 5, and 10 RNA copies/reaction for DENV-1, DENV-2, DENV-3, DENV-4, and CHIKV, respectively. Our assay did not cross-react with a panel of viruses that included other flaviviruses, alphaviruses, influenza viruses, human enteroviruses, and human coronaviruses. The feasibility of using this assay for clinical diagnosis was evaluated in DENV- and CHIKV-positive patient sera. Accordingly, the assay sensitivity for DENV-1, DENV-2, DENV-3, DENV-4, and CHIKV was 89.66%, 96.67%, 96.67%, 94.12%, and 95.74%, respectively, with 100% specificity. These findings confirmed the potential of our assay to be used as a rapid test for simultaneous detection and serotyping of DENV and CHIKV in clinical samples.

Chikungunya virus (CHIKV) and dengue virus (DENV) are two of the arthropod-borne diseases that are affecting many countries worldwide, especially in the tropics and subtropics.1 Belonging to the genus Alphavirus of the Togaviridae family, CHIKV has a genomic structure of 5′cap-nsP1-nsP2-nsP3-nsP4-(junction region)-C-E3-E2-6K-E1-Poly (A)-3′.2 DENV is a flavivirus of Flaviviridae family and has a genomic structure of 5′UTR-C-prM (M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′UTR.3 Both DENV and CHIKV infections have similar geographical distribution and seasonal correlation4,5 because they share the same vectors for transmission: Aedes albopictus and Aedes aegypti mosquitoes.6 Clinical manifestations of DENV infections are often indistinguishable from CHIKV infections, which makes clinical diagnosis difficult.2,7 Although both DENV and CHIKV are single-stranded, positive-sense RNA viruses that have similar vectors, transmissibility, and clinical symptoms, DENV infections can be more severe when compared with CHIKV infections.2,7

DENV is traditionally classified into four serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. A primary
infection with one serotype of DENV is believed to confer lifelong immunity to the same serotype. On a secondary, heterologous DENV infection, antibodies predominantly directed against the initial DENV serotype are produced rapidly.8,9 Although most of the produced antibodies bind to the heterologous virus serotype, they are more likely to have nonneutralizing properties against the new serotype, and these have been found to enhance infection, a phenomenon called antibody-dependent enhancement.9,11 In addition, the dominant serotype during recent large DENV outbreaks is swapping. Therefore, accurate clinical diagnosis is important in providing health care personnel the information required for proper case management and planning preventive measures. In addition, serotyping data could contribute to warn impending epidemics. Thus, it is of great concern to develop a diagnostic kit for accurate identification of the CHIKV and DENV and at the same time to serotype DENV at the point of care.

Currently, the diagnosis of DENV and CHIKV is determined by virus isolation, detection of virus-specific antibodies by enzyme-linked immunosorbent assay, genomic RNA detection using RT-PCR, or real-time RT-PCR.2,12 Although virus isolation is the gold standard, the method is time-consuming. Enzyme-linked immunosorbent assay, especially capture of IgM, has been considered the standard for the diagnosis of arbovirus infection during the acute phase and allows diagnosis 5 to 7 days after the onset of illness but is hampered by cross-reactions between members of the Flaviviruses and Alphaviruses.13 Shu et al14 developed a SYBR Green I–based real-time RT-PCR assay for DENV serotyping, but at least four tubes of reactions are needed per sample. Chien et al15 developed a TaqMan probe–based real-time RT-PCR assay for DENV serotyping, but the method is unable to detect CHIKV simultaneously. In addition, a few TaqMan probe–based multiplex real-time RT-PCR assays for DENV and CHIKV has been developed, but these are not capable of serotyping DENV at the same time.

In SYBR Green I–based real-time RT-PCR assays, the melting temperature (Tm) of a PCR product depends on the concentration, length, and nucleotide composition of the amplicon so that Tm analysis has the potential to differentiate DENV serotypes. In the present study, we aimed to develop and evaluate a SYBR Green I–based multiplex real-time RT-PCR assay to detect, differentiate, and quantify DENV and CHIKV and simultaneously to serotype DENV based on Tm analysis of the PCR amplicon. Primers targeting the PrM region of the DENV genome and nsP3 region of the CHIKV genome were selected and evaluated. Detection limit of the assay was determined using serially diluted in vitro transcribed viral RNA samples. In addition, a panel of RNA viruses, including members of Togaviridae, Flaviviridae, Orthomyxoviridae, Coronaviridae and Picornaviridae, were also used to examine the cross-reactivity of our assay. Lastly, the clinical sensitivity and specificity of the assay were evaluated with a panel of serum samples collected from DENV- or CHIKV-infected and healthy individuals in Singapore.

### Materials and Methods

#### Viruses

CHIKV (EU441882.1, FJ445502), DENV-1 (S144 strain), DENV-2 (STP7 and New Guinea C strain), DENV-3 (Eden 130/05 strain), and DENV-4 (S8976 strain) were propagated in C6/36 cells, and viral titers were determined by the plaque-forming assay.16 Viral RNA of Ross River virus (RRV); Zika virus (ZIKV, MR 766 strain); Kunjin virus (KUNV, MR 61C strain); West Nile virus (WNV, Sarafend strain); human enterovirus 71 (HEV71, AF316321 strain); enteric cytopathic human orphan virus 2; enteric cytopathic human orphan virus 7; Cox sackie B2 virus (CB2) and A16 virus (CA16, World Health Organization strain); poliovirus types 1, 2, and 3 (Sabin strain); human coronavirus (CoV); and influenza A virus subtypes H1N1 and H3N2 were also used to examine the cross-reactivity of this assay. The RRV, KUNV, and WNV were provided by Prof. Mary Mah-Lee Ng (National University of Singapore, Singapore). The Human CoV and influenza A viruses were provided by Associate Professor Tan Yee Joo (National University of Singapore, Singapore).

#### Patient Sera Samples

A set of 22 serum samples from CHIKV-infected patients and 30 from uninfected individuals were collected at the National University Hospital, Singapore, with informed consent, to evaluate the clinical sensitivity and specificity of the real-time RT-PCR assay. All the serum samples were validated using a real-time RT-PCR detection assay targeting the CHIKV envelope glycoprotein 1 (E1) gene,17 and a confirmed case of CHIKV infection was defined as febrile illness associated with a positive result from the real-time RT-PCR. This part of the study was performed in accordance with a National University of Singapore Institutional Review Board approved protocol (No. 10-234). The Environmental Health Institute of the National Environmental Agency of Singapore provided a set of 25 serum samples from CHIKV-infected patients and 106 serum samples from DENV-infected patients. Besides suggestive clinical signs,

### Table 1

| Primer | Sequences |
|--------|------------|
| Dest1 F | 5′-GCGACATGGACTGCTTGGTGCCA-3′ |
| Dest1 R | 5′-CGATGTTTCCGAGCCCCCTTC-3′ |
| CHK1 F | 5′-CGAGAATACTGCCGTCGCCG-3′ |
| CHK1 R | 5′-GTCAACGCGGTCTCCCTGTTT-3′ |

F, forward; R, reverse.
In vitro RT-PCR products of DENV and CHIKV. In brief, sequence alignment software.19 A number of oligonucleotide (Table S1) and aligned using the ClustalX version 2.1 genbank retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). The full genome nucleotide sequences of 20 strains of each DENV serotype and CHIKV from recent outbreaks were obtained by standard RNA templates containing primer-binding sites synthesis from DNA templates.

**Viral RNA Extraction**

Viral RNA was extracted from 140 µL of infected cell culture supernatants or serum samples using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The RNA was eluted in a final volume of 50 µL of nuclease free water and was stored at −80°C until use.

**Primer Designing**

The full genome nucleotide sequences of 20 strains of each DENV serotype and CHIKV from recent outbreaks were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank; accession numbers are provided in Supplemental Table S1) and aligned using the ClustalX version 2.1 sequence alignment software.16 A number of oligonucleotide primers for specific DENV and CHIKV were designed using National Center for Biotechnology Information primer designing tool and modified manually (Table 1). DENV serotyping primer (DEst) binding sites are conserved across all four DENV serotypes, whereas the nucleotide sequences between the forward and reverse primer binding sites are less conserved, resulting in different Tms of the PCR amplicons for DENV serotyping.

**Preparation of Viral RNA Standard**

Standard RNA templates containing primer-binding sites were obtained by in vitro synthesis from DNA templates. In vitro transcription (IVT) was performed using the MAXiScript IVT kit (catalog no. 1314, Ambion) on the RT-PCR products of DENV and CHIKV. In brief, first, a standard RT-PCR was performed using a T7 promoter sequence incorporated forward primer. PCR product containing T7 promoter was subjected to IVT at 37°C for 1 hour. The IVT products were then treated with 1 U of DNase I and incubated at 37°C for 30 minutes to remove the remaining DNA. The DNase I activity was stopped by adding 1 µL of 0.5 mol/L EDTA and heat deactivated further at 95°C for 10 minutes. The excess nucleotides and pyrophosphate were removed by NH4OAc/ethanol precipitation. The RNA pellet was suspended in diethylpyrocarbonate-treated water. The amount of IVT-generated RNA fragments was determined using the NanoDrop ND2000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and converted to molecular copies by using the following formula:

\[
Y \text{ molecules/µL} = \frac{X \text{ g/µL RNA} \times 6.02 \times 10^{23}/\text{transcript length (base)} \times 340}{1}
\]

Then 10-fold serially diluted IVT RNA samples were subjected to real-time RT-PCR to generate a standard curve for the quantification of viral RNA in terms of copy number.

**SYBR Green I—Based One-Step Real-Time RT-PCR**

All RT-PCR reactions were performed in 20-µL reactions with 2 µL of RNA template. IVT RNA of DENV-1, -2, -3, and -4 and CHIKV was included as a control in every RT-PCR run. SYBR Green I—based one-step real-time RT-PCR was performed on the Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems, Bedford, MA). Samples were assayed with 250 nmol/L of each primer in a 1× final concentration of SYBR Green ready mastermix. The RT-PCR conditions for the real-time RT-PCR consist of a 30-minute RT step at 42°C and 2 minutes of Taq polymerase activation at 95°C, followed by 40 cycles of PCR at 94°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing and extension). The fluorescence emitted was captured at the end of the extension step of each cycle. Amplification graphs were checked for the cross-point value of the PCR product. Melting-curve analysis was performed after PCR amplification to verify that the correct product was amplified by examining its specific Tm that was also used to serotype DENV.

**Preparation of Viral RNA Standard**

Standard RNA templates containing primer-binding sites were obtained by in vitro synthesis from DNA templates. In vitro transcription (IVT) was performed using the MAXiScript IVT kit (catalog no. 1314, Ambion) on the RT-PCR products of DENV and CHIKV. In brief, first, a standard RT-PCR was performed using a T7 promoter sequence incorporated forward primer. PCR product containing T7 promoter was subjected to IVT at 37°C for 1 hour. The IVT products were then treated with 1 U of DNase I and incubated at 37°C for 30 minutes to remove the remaining DNA. The DNase I activity was stopped by adding 1 µL of 0.5 mol/L EDTA and heat deactivated further at 95°C for 10 minutes. The excess nucleotides and pyrophosphate were removed by NH4OAc/ethanol precipitation. The RNA pellet was suspended in diethylpyrocarbonate-treated water. The amount of IVT-generated RNA fragments was determined using the NanoDrop ND2000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and converted to molecular copies by using the following formula:

\[
Y \text{ molecules/µL} = \frac{X \text{ g/µL RNA} \times 6.02 \times 10^{23}/\text{transcript length (base)} \times 340}{1}
\]

The primer set of the dengue virus (DENV) serotyping primer (DEst1) and the chikungunya virus (CHK1) were tested in combination for simultaneous detection and differentiation of DENV and CHIKV. The melting curve for each targeting virus is well separated, indicating that they not only are able to detect DENV and CHIKV but also have good compatibility in terms of similar annealing temperature and low tendency of forming heterodimers. The results from the combination of DEst1 and CHK1 also reveal good consistency compared with use of DEst1 alone in terms of specific melting temperatures (Tms) of amplicon from DENV-1, -2, -3, and -4.
Multiplex RT-PCR for CHIKV and DENV-1-4

Results

Designing and Validation of Primers

The consensus regions of the DENV genome were carefully selected, and 20 pairs of primers were designed for DENV serotyping. Among them, DEst1 was selected based on its capability to detect and serotype DENV and its low likelihood of forming primer dimer. The specific Tm of PCR amplicons from DENV-1, -2, -3, and -4 was 84.86°C (coefficient of variation of 0.46%), 82.61°C (coefficient of variation of 0.51%), 85.60°C (coefficient of variation of 0.54%) and 83.65°C (coefficient of variation of 0.50%), respectively (Figure 1).

DEst1 was further tested in combination with each of 10 pairs of primers for CHIKV detection for compatibility and capability of differentiating DENV from CHIKV. The pair of DEst1 and CHK1 (Figure 2) (the specific Tm of CHIKV PCR amplicon was 88.28°C, with coefficient of variation of 0.46%) was selected because not only was it able to detect DENV and CHIKV but also it had a similar annealing temperature and low tendency of forming heterodimers. The results for the combination of DEst1 and CHK1 revealed good consistency compared with use of DEst1 alone, in terms of specific Tms of amplicons from DENV-1, -2, -3, and -4. Figure 3 lists the temperature ranges for the PCR amplicons specific to each DENV serotype and CHIKV.

Detection Limit of the Assay

The detection limit of the real-time RT-PCR assay was determined using 10-fold serially diluted standard IVT RNA samples. Figure 4 gives the standard curves of DENV-1, -2, -3, and -4 and CHIKV using the DEst1 and CHK1 primers. The detection limits were calculated to be 20 RNA copies per reaction for DENV-1, 10 RNA copies per reaction for DENV-2, 50 RNA copies per reaction for DENV-3, 5 RNA copies per reaction for DENV-4, and 10 RNA copies per reaction for CHIKV. All standard curves revealed good correlation of coefficient to the data. The PCR reaction efficiency for DENV-1, -2, -3, and -4 and CHIKV was 103.8%, 103.6%, 98.3%, 106.7%, and 97.2%, respectively.

Cross-Reactivity of the Assay

Besides the use of standard RNA samples of DENV-1, -2, -3, and -4 and CHIKV as positive controls, RNA of RRV was used as a representative member of Alphavirus; KUNV, WNV, and ZIKV were used as representative members of Flavivirus in the cross-reactivity study. In addition, a panel of other RNA viruses, including H1N1, H3N2, polio 1, polio 2, polio 3, CoV, enteric cytopathic human orphan virus 2, HEV71, CB2, and CA16, were also evaluated. All CHIKV and DENV samples produced positive results, and none of the other viral RNA samples produced false-positive results (Table 2).

Sensitivity and Specificity of the Assay

To evaluate the feasibility of the present assay for clinical diagnosis, 47 serum samples obtained from patients with confirmed CHIKV infection during the acute phase, 106 serum samples from patients with confirmed DENV infection, and 30 serum samples from uninfected individuals were tested. Our real-time RT-PCR had high sensitivity, picking up 45 of the 47 CHIKV cases (95.74% detection sensitivity; 95% CI, 84.27%–99.26%) and 106 of the 106 DENV cases (100% detection sensitivity; 95% CI, 95.64%–100%). The serotyping sensitivity for DENV-1, -2, -3, and -4 was 89.66% (95% CI, 71.50%–97.29%), 96.67% (95% CI, 80.95%–99.83%), 96.67% (95% CI, 80.95%–99.83%), and 94.12% (95% CI, 69.24%–99.69%), respectively. None of the 30 serum samples from uninfected individuals produced positive results (100% detection specificity; 95% CI, 85.87%–100%) (Table 3).
Table 2 Detection of DENV, CHIKV, and Other Viruses Using Multiplex Quantitative RT-PCR

| Virus (strain)          | Multiplex quantitative RT-PCR |
|-------------------------|-------------------------------|
|                         | DENV-1 | DENV-2 | DENV-3 | DENV-4 | CHIK |
| DENV-1 (S144)           | +      | –      | –      | –      | –    |
| DENV-2 (STP7)           | –      | +      | –      | –      | –    |
| DENV-2 (New Guinea C)   | –      | +      | –      | –      | –    |
| DENV-3 (Eden 130/05)    | –      | –      | +      | –      | –    |
| DENV-4 (S8976)          | –      | –      | –      | +      | –    |
| CHIKV (EU441882.1)      | –      | –      | –      | +      | –    |
| CHIKV (FJ4455002)       | –      | –      | –      | +      | –    |
| RRV                     | –      | –      | –      | –      | +    |
| ZIKV (MR 766)           | –      | –      | –      | –      | +    |
| KUNV (MRM 61C)          | –      | –      | –      | –      | +    |
| WNV (Sarafend)          | –      | –      | –      | –      | +    |
| HEV71 (AF316321)        | –      | –      | –      | –      | +    |
| ECHO2                   | –      | –      | –      | –      | +    |
| ECHO7                   | –      | –      | –      | –      | +    |
| CB2                     | –      | –      | –      | –      | +    |
| CA16 (WHO)              | –      | –      | –      | –      | +    |
| Polio virus 1 (Sabin)   | –      | –      | –      | –      | +    |
| Polio virus 2 (Sabin)   | –      | –      | –      | –      | +    |
| Polio virus 3 (Sabin)   | –      | –      | –      | –      | +    |
| CoV                     | –      | –      | –      | –      | +    |
| Influenza virus A H1N1  | –      | –      | –      | –      | +    |
| Influenza virus A H3N2  | –      | –      | –      | –      | +    |

CA16, Coxsackie A16 virus; CB2, Coxsackie B2 virus; CHIKV, chikungunya virus; CoV, coronavirus; DENV, dengue virus; ECHO, enteric cytopathic human orphan virus; HEV71, human enterovirus 71; KUNV, Kunjin virus; RRV, Ross River virus; WHO, World Health Organization; WNV, West Nile virus; ZIKV, Zika virus.

### Discussion

Since 2004, major CHIKV outbreaks causing debilitating disease have been reported in Africa, India, Europe, the Middle East, and Southeast Asia.20 The recent resurgence of chikungunya fever has drawn global attention because of its explosive onset, rapid spread, high morbidity, and myriad of clinical manifestations.21 DENV that causes dengue fever, hemorrhagic fever, and shock syndrome has been defined as “the most important mosquito-borne viral disease in the world” by the World Health Organization, with an estimated 2.5 billion people at risk for infection in tropical and subtropical regions. Global estimates vary, but approximately 50 million to 200 million dengue infections, 500,000 episodes of severe dengue infections, and >20,000 dengue-related deaths occur annually.23,24 Because of common transmission vectors and similar clinical manifestations, CHIKV and DENV can be misdiagnosed in areas where both viruses are co-circulating.25 This may lead to poor patient management at point of care.

In addition, serotyping information is important for the understanding of dengue epidemiology. For instance, serotype switch is used as an early warning of epidemics in Singapore because data have revealed that such switches are associated with epidemics. In recent studies, several DENV serotyping assays have been reported to require four tubes of tests or to be unable to detect CHIKV at the same time.26-28 Recently, a few probe-based multiplex real-time RT-PCR assays were published for simultaneous detection of CHIKV and DENV, which required individual primer and probe sets to detect each targeted virus, but none of them can be used for DENV serotyping probably because of the limitation in the number of channels required for discriminating the viruses using real-time PCR instruments. For instance, Cecilia et al.29 reported a single-step multiplex real-time RT-PCR assay for simultaneous detection and quantification of all dengue serotypes and CHIKV. The assay was developed using primers and TaqMan probes targeting DENV-1/2/3, DENV-4, CHIKV, and β-actin. The assay revealed high sensitivity for DENV (100%) and CHIKV (95.8%) with high specificity (100%) when compared with conventional RT-PCR assay. Detection limit was set within the range of 1 to 50 PFU/mL (100 PFU/mL for DENV-2 and 3, 1000 PFU/mL for DENV-1 and CHIKV, and 3250 PFU/mL for DENV-4). The assay is used primarily for detection of all four DENV serotypes and CHIKV but lacks serotyping capability. We report a rapid and accurate laboratory confirmation method to simultaneously detect, quantify, and differentiate DENV...

Table 3 Performance of Real-Time RT-PCR Assay on Serum Specimens

| Type of serum samples | No. of samples | No. of samples correctly serotyped | Serotyping sensitivity, % (95% CI)* | Detection sensitivity, % (95% CI)1 | Detection specificity, % (95% CI)1 |
|-----------------------|----------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| DENV-1                | 29             | 26                                | 89.66 (71.50–97.29)               | 100 (93.58–100)                   | 100 (85.87–100)                   |
| DENV-2                | 30             | 29                                | 96.67 (80.95–99.83)               | 100 (93.58–100)                   | 100 (85.87–100)                   |
| DENV-3                | 30             | 29                                | 96.67 (80.95–99.83)               | 100 (93.58–100)                   | 100 (85.87–100)                   |
| DENV-4                | 17             | 16                                | 94.12 (69.24–99.69)               | 100 (93.58–100)                   | 100 (85.87–100)                   |
| CHIKV                 | 47             | 45                                | 95.74 (84.27–99.26)               | 100 (93.58–100)                   | 100 (85.87–100)                   |

Healthy samples        | 30             | 0                                 |                                    |                                   |                                   |

*Number of specimens with correct serotype detected/(number of specimens with correct serotype detected + number of specimens with wrong serotype detected) × 100%.

1Number of positive specimens/(number of positive specimens + number of false-negative specimens) × 100%.

1Number of negative specimens/(number of negative specimens + number of false-positive specimens) × 100%.
and CHIKV infection with DENV serotyping capability by a SYBR Green I–based one-step multiplex real-time RT-PCR assay.

SYBR Green I–based assays are more cost-effective than probe-based ones, and only two detection channels (ROX and SYBR Green) are needed, increasing adaptability of the assay to the whole range of real-time PCR instruments. However, the primary disadvantage of the SYBR Green I dye chemistry is that it binds to any double-stranded DNA, including nonspecific PCR products and primer dimers, which may lead to false-positive results. To counter the problem, primer sets used in this study were carefully designed and examined based on heterodimer formation likelihood and thermal compatibility. In brief, DENV serotyping primers were designed based on genome alignment of multiple strains of all four serotypes of DENV. The primer-binding sites selected were well conserved across all four DENV serotypes, whereas the nucleotide sequences in primer-binding sites selected were well conserved across all serotyping primers were designed based on genome alignment. DEst1 (the targeting PrM region of the DENV genome) was less conserved to yield different Tm of the PCR amplicons. between, four DENV serotypes, whereas the nucleotide sequences in primer-binding sites selected were well conserved across all serotyping primers were designed based on genome alignment. For CHIKV, primers were designed to preferably target nonstructural protein-coding regions because they were usually well conserved. CHK1 (the targeting nsP3 region of the CHIKV genome) was selected based on its exceptionally good compatibility with DEst1, reproducibility, sensitivity, and specificity in primer verification. In addition, a temperature range, instead of a fixed value, for PCR amplicons is given for each virus so that fluctuations of the Tm from the respective PCR amplicons (ie, sample variance, strain variations) could be tolerated.

We have modified the primer sequences and optimized the PCR thermal profile to achieve balanced sensitivity to all of the targeted viruses without sacrificing assay specificity. Furthermore, SYBR Green I dye chemistry limits the use of internal controls because the fluorescent signal from internal controls would be nondifferentiable from targeting viruses due to same fluorescent channels (SYBR Green I) being used. To overcome this issue, external positive controls (ie, standard viral RNA) were included in every RT-PCR run throughout this study.

The detection limit of the assay was determined as 20 RNA copies/reaction for DENV-1, 10 RNA copies/reaction for DENV-2, 50 RNA copies/reaction for DENV-3, 5 RNA copies/reaction for DENV-4, and 10 RNA copies/reaction for CHIKV. In general, DENV infection in human results in high viral load (approximately 10^9 copies/mL), with even higher viral load in DHF cases. However, the viral titer starts to decline rapidly after the acute phase, and more severe clinical symptoms start to appear. An assay that is able to detect low RNA copy number with wide dynamic range for viral detection could benefit both the operators and patients at the point of care.

In conclusion, this multiplex real-time RT-PCR assay for DENV serotyping has superior analytical and clinical performance with high sensitivity and specificity. This enabled our assay to be a useful tool for epidemiologic surveillance and for the molecular diagnosis of large-scale analyses, such as during CHIKV and DENV outbreaks. To our knowledge, this is the first SYBR Green I–based real-time RT-PCR assay reported that is able to detect, quantify, differentiate, and serotype DENV and CHIKV simultaneously.

Acknowledgments

The RRV, KUNV, and WNV were provided by Prof. Mary Mah-Lee Ng, and the human CoV and influenza A viruses were provided by Prof. Tan Yee Joo (National University of Singapore, Singapore).

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.jmoldx.2015.06.008.

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