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Clinical diagnosis of early dengue infection by novel one-step multiplex real-time RT-PCR targeting NS1 gene

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ABSTRACT

Background: Dengue is a mosquito-borne disease that causes a public health problem in tropical and subtropical countries. Current immunological diagnostics based on IgM and/or nonstructural protein 1 (NS1) antigen are limited for acute dengue infection due to low sensitivity and accuracy.
Objectives: This study aimed to develop a one-step multiplex real-time RT-PCR assay showing higher sensitivity and accuracy than previous approaches.
Study design: Serotype-specific primers and probes were designed through the multiple alignment of NS1 gene. The linearity and limit of detection (LOD) of the assay were determined. The assay was clinically validated with an evaluation panel that was immunologically tested by WHO and Malaysian specimens.
Results: The LOD of the assay was 3.0 $\log_{10}$ RNA copies for DENV-1, 2.0 for DENV-3, and 1.0 for DENV-2 and DENV-4. The assay showed 95.2% sensitivity (20/21) in an evaluation panel, whereas NS1 antigen- and anti-dengue IgM-based immunological assays exhibited 0% and 23.8–47.6% sensitivities, respectively. The assay showed 100% sensitivity both in NS1 antigen- or anti-dengue IgM-positive Malaysian specimens (26/26). The assay provided the information of viral loads and serotype with discrimination of heterotypic mixed infection.
Conclusions: The assay could be clinically applied to early dengue diagnosis, especially during the first 5 days of illness and approximately 14 days after infection showing an anti-dengue IgM-positive response.

1. Background

Dengue is a mosquito-borne disease that results from infection with any of 4 genetically related serotypes of the dengue virus (DENV-1–DENV-4, which have multiple genotypes within each serotype [1,2]. It is the most widespread viral disease in humans worldwide and transmitted by the mosquito *Aedes aegypti* and *Aedes albopictus* [3,4]. An estimated 390 million cases are reported annually [5–7]. Most dengue infections with one serotype (primary infection) are acute and asymptomatic or mildly symptomatic, conferring long-term homotypic immunity. The acute phase is characterized by undifferentiated fever (dengue fever) lasting 2–7 days with or without rash [8,9]. However, secondary infections with other DENV serotypes are common in endemic areas because co-circulation of multiple DENV serotypes is frequent in those areas. These heterotypic infections can cause clinical manifestations such as dengue hemorrhagic fever and dengue shock syndrome [4,7,9–11].

Early dengue diagnosis is important during the acute phase of infection, when the viral RNA or soluble secreted antigen such as nonstructural protein 1 (NS1) can be detected (0–5 days after onset of illness) [3,8,12]. Many diagnostic assays have been developed in either immunological (NS1- and IgM-based assays) or molecular platforms (reverse-transcription polymerase chain reaction [RT-PCR] assay). An IgM-based assay is useful because it is expressed earlier than dengue-specific IgG in the acute phase of infection [3,9]. However, the IgM level is detectable 3–5 days after the onset of illness and persists for approximately 2 weeks after infection, so the assay is not highly sensitive and specific for detecting acute infection [3,13,14]. An NS1-based assay is a simple method to detect NS1 antigen secreted from DENV. Many studies have explored its potential as an early diagnostic tool during the acute phase of dengue infection [12,13,15,16]. However, the sensitivities of the

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Table 1
Primer and probe sequences for the one-step DENV multiplex RT-PCR.

Serotype	Name	Primer and probe sequence	Location[a]
DENV-1	Forward	5′-GGTTGTAGGAGATGTTGCTGGG-3′	264–285
	Probe	5′-FAM-TGATTAGGCCACAACCCATGGAATACAA-BHQ1-3′	308–335
	Reverse	5′-TAGCCTTTCCCCAGCTTTTCCA-3′	364–343
DENV-2	Forward	5′-TCAAAGGAATCATGCAGGCAGG-3′	278–299
	Probe	5′-YAK-TGCGGCCTCAGCCCACTGAGCTAAAGTA-BHQ1-3′	311–338
	Reverse	5′-TCGGGGCCATCAATGAGAAA-3′	416–397
DENV-3	Forward	5′-GCCAATGAACTGAACTACATATTATGGG-3′	214–241
	Probe	5′-Cal Red-ACGGTAGTTGTGGGCGACATAATTGG-BHQ2-3′	259–284
	Reverse	5′-GGTGTTAGTGTTCTTTTCCCTTGCTCT-3′	317–291
DENV-4	Forward	5′-ATGTTCTCTGGGAAGGAGGACAT-3′	230–252
	Probe	5′-Cy5-TCACTGTAGTGGCTGGGGACGTGAA-BHQ2-3′	257–281
	Reverse	5′-TCATTCACTGGAGGTGTGAGTGCTC-3′	329–305

[a] Location is provided from the consensus sequences obtained from alignment of each serotype sequence in September 2012.

NS1 antigen-based assays are variable, ranging from 24% to 94% in published reports [13,15,17,18].

RT-PCR assays have been developed for detecting and serotyping DENV, which target various regions of DENV genomes [19–36]. However, numerous RT-PCR assays have not been validated based on an evaluation of accuracy and quality. Only approximately 10% of published methods showed optimal performance and more than 80% of methods lacked sensitivity and/or specificity, demanding improvement [37]. Recent evaluation of commercial assays showed sensitivities ranging from 83.3% to 100% and 100% specificity [38,39].

2. Objectives

To create a more sensitive and accurate molecular diagnostic method than previous approaches, we developed a simple one-step multiplex real-time RT-PCR assay that allowed to simultaneously detect, quantify, and identify serotypes of DENV in a single reaction, especially from the early phase of dengue infection.

3. Study design

3.1. Primers and probes design

The NS1 sequences of 4 dengue serotypes were collectively obtained from a dengue virus database (http://www.ncbi.nlm.nih.gov/genomes/VirusVariation/) in September 2012 (923 DENV-1, 317 DENV-2, 166 DENV-3, and 7 DENV-4) and aligned to designate the serotype-specific primers and probes. The region of interest was further analyzed to define a highly conserved region in each serotype. Primers were designed to have few substitutions among sequences ranging from 0 to 5; 3 or fewer were preferred within 10 bases from the 3' end of primers. Melting temperatures was to be 56–59 °C and GC content, 40–55% (Vector NTI 10.3.0, Invitrogen, USA). Probes were designed to have at least 5 °C higher melting temperatures than primers. To avoid misidentification of non-dengue viruses, the sequences of the Flaviviridae family such as West Nile virus, Japanese encephalitis virus, and Chikungunya virus were cross-checked. The primers and probes are listed in Table 1.

3.2. Preparation of reference DNA and RNA template

The consensus sequence of the selected region for each serotype (DENV-1, DENV-3, and DENV-4) was synthesized as a positive template (Cosmo Genetech Co., Ltd., Republic of Korea) to determine the limit of detection (LOD) and linearity of the assay (Table 2). A plasmid containing DENV-2 NS1 gene was kindly provided by Dr. Seong Kug Eo (Chonbuk National University, Republic of Korea). The NS1 genes were subcloned into pGEM-T Easy Vector (Promega, USA) and *in vitro* transcribed using RiboMAX™ Large Scale RNA Production System-T7 (Promega, USA).

3.3. One-step multiplex real-time RT-PCR assay

The assay was optimized using the iScript™ one-step RT-PCR kit for Probes (Bio-Rad Laboratories Inc., USA). A 20-μL reaction contained serotype-specific primers (300 nM) and probes (200 nM for DENV-1, 300 nM for DENV-2 and DENV-4, and 150 nM for DENV-3). Finally, 10% (v/v) of RNA was added to the reaction. RT-PCR was performed using a CFX96 thermocycler (Bio-Rad Laboratories Inc., USA) as follows: reverse transcription at 50 °C for 10 min, pre-denaturation at 95 °C for 5 min, and 40 cycles at 95 °C for 15 s and 56 °C for 14 s. A non-template control (NTC) was included to determine positivity or negativity. In case the cycle threshold (Ct) value of NTC was not assigned, a sample showing a Ct value before 40 was considered positive. Every amplification was conducted in duplicate and repeated at least twice. The serotype was determined by the pattern of signals obtained from 4 particular probes (green, yellow, orange, and red).

3.4. Linearity and determination of the limit of detection (LOD)

The assay was evaluated according to a previous report with modification [40]. Linearity was analyzed with 10-fold serially diluted *in vitro* transcribed RNAs of each DENV serotype (10^0–10^{10} copies). The copy number of RNA was calculated using the following formula: [X g/μL RNA/(transcript length in nucleotides × 340)] × 6.022 × 10^{23} = Y molecules/μL. The linear range was determined using a standard curve generated with diluted reference RNAs, and the best-fit line to the raw data was established by linear regression analysis with 95% confidence intervals (GraphPad Prism, CA, USA). The LOD was determined as the lowest concentration of reference RNA detectable during a linearity experiment.

3.5. Conventional RT-PCR

Conventional RT-PCR was carried out according to previous protocol described by Lanciotti et al. [41] and Harris et al. [28]. Viral RNAs was reverse-transcribed by ReverTraAce qPCR RT kit (Toyobo, Japan) or HelixCript™ 1st-Strand cDNA synthesis kit (NanoHelix Co., Ltd., Republic of Korea). cDNA was subsequently amplified using Platinum Blue PCR SuperMix (Invitrogen, USA) and resultant PCR product was electrophoresed to determine positivity.

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