Multiplex real-time RT-PCR for detecting chikungunya virus and dengue virus

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1. Introduction

Chikungunya virus (CHIKV) belongs to the family Togaviridae and the genus Alphavirus[1]. It contains a single-stranded RNA genome of approximately 11.8 kb and comprises three different strains: the East, Central and South African strain, West African strain and Asian strain[2]. Dengue virus (DENV) is a member of the family Flaviviridae, genus Flavivirus with a single-stranded RNA genome of 11 kb which has been categorized into four serotypes, DENV1–4[3]. Both viruses are arthropod-borne viruses (arboviruses). Major mosquitoes vectors for the viruses are of the Aedes genus, including Aedes aegypti and Aedes albopictus. Worldwide an estimated 50 million are infected with DENV per year, culminating in about 25 000 deaths in severe cases[4]. The magnitude of problems caused by the spreading of CHIKV can be seen in the unprecedented spread of CHIKV on islands of the Indian Ocean in 2005[5] and the largest recorded outbreak with more than 1.38 million cases in 2006–2007[6,7]. Recent endemic areas are mainly urban areas in South, East and West Africa, the south-western Indian Ocean islands, India and Asia including Thailand. The report showed that the recent CHIKV outbreak started spreading in Thailand in 2008[8,9], and are of the ECSA strain confirmed by complete genome analysis[10,11].

CHIKV causes chikungunya fever (CHIKF) in both male and female individuals of any age. CHIKF is self-limiting with a very low death rate and usually presents as a rapid onset of fever, a characteristic rash, severe and incapacitating joint pain and other less common symptoms such as headache, fatigue, nausea, vomiting and conjunctivitis[12]. The clinical characteristics of dengue vary from asymptomatic to potentially fatal complications. Dengue fever (DF) may present as headache, fever, bone or joint pain while dengue hemorrhagic fever (DHF) may produce bleeding, plasma leakage, fever, and thrombocytopenia which can affect any age. A plasma leakage test is crucial as DHF usually presents plasma leakage while DF does not. DHF may lead to dengue shock syndrome and must be monitored.

Objective: To develop diagnostic test for detection chikungunya virus (CHIKV and Dengue virus (DENV) infection. Methods: We have performed a rapid, accurate laboratory confirmative method to simultaneously detect, quantify and differentiate CHIKV and DENV infection by single–step multiplex real–time RT–PCR. Results: The assay’s sensitivity was 97.65%, specificity was 92.59% and accuracy was 95.82% when compared to conventional RT–PCR. Additionally, there was no cross–reaction between CHIKV, DENV, Japanese encephalitis virus, hepatitis C, hepatitis A or hepatitis E virus. Conclusions: This rapid and reliable assay provides a means for simultaneous early diagnosis of CHIKV and DENV in a single–step reaction.
Discernment between DENV and CHIKV can be problematic because of their highly similar symptoms. For example, the Tanganyika (now Tanzania) epidemic of 1952–1953 was thought to be dengue infection while it was actually the first documented infection by CHIKV. A dengue–like epidemic on the Comoros Islands in 2005 was additionally a CHIKV outbreak[14,15]. Furthermore, the two viruses not only show similar symptoms to each other but also to other fever such as measles, influenza infection and severe acute respiratory syndrome[16]. The 2004 CHIKV epidemic in Kenya was originally thought to be malaria[17]. Additionally, there have been several reports of co-circulation and co-infection of CHIKV and DENV in Delhi, Malaysia, the Maldives and India along with the increasing number of travelers and the spread of the vectors, *Aedes aegypti* and *Aedes albopictus*, for both viruses[18–20].

Accurate and rapid diagnosis is critical, as this will facilitate treatment in case of atypical presentations and complications. Early distinction between dengue and other febrile illnesses could help identify patients with signs of complications. RT-PCR confirmed CHIKV positive samples (n=39, accession number FJ882884–FJ882922)[9] and positive DENV (n=26, accession number JF274206–JF274231), which were then confirmed by RT–PCR and NS1 antigen rapid test (SD BIOLINE Dengue Duo, Kyonggi–do, Korea) were included in this study[21–22]. A total of 290 suspected cases were recruited in this study as well, comprised of acute serum samples collected within 7 days of the onset of illness, consistent with clinical diagnosis of dengue according to WHO criteria[23] and of CHIKV samples from suspected infections in the endemic area. Moreover, 28 hepatitis C positive, 2 hepatitis A positive, 6 hepatitis E positive, 2 Japanese encephalitis virus (JEV) samples were included in this study to evaluate sensitivity and specificity of this method.

2. Material and methods

The research proposal has been approved by the ethics committee, Faculty of Medicine, Chulalongkorn University. All the study specimens were anonymized with coding numbers and permission granted by the directors of hospitals.

### 2.1. Case selection and Clinical samples

RT–PCR confirmed CHIKV positive samples (n=39, accession number FJ882884–FJ882922)[9] and positive DENV (n=26, accession number JF274206–JF274231), which were then confirmed by RT–PCR and NS1 antigen rapid test (SD BIOLINE Dengue Duo, Kyonggi–do, Korea) were included in this study[21–22]. A total of 290 suspected cases were recruited in this study as well, comprised of acute serum samples collected within 7 days of the onset of illness, consistent with clinical diagnosis of dengue according to WHO criteria[23] and of CHIKV samples from suspected infections in the endemic area. Moreover, 28 hepatitis C positive, 2 hepatitis A positive, 6 hepatitis E positive, 2 Japanese encephalitis virus (JEV) samples were included in this study to evaluate sensitivity and specificity of this method.

#### 2.2. Nucleic acid extraction and reverse transcription

Viral RNA was extracted using a viral nucleic acid extraction kit (BBC Biocience, Taipei, Taiwan) following the manufacturer’s specifications. The extracted RNA was stored at −80°C until further use. RNA was transcribed into cDNA by using ImProm–II™ reverse transcriptase (Promega, Madison, WI). Briefly, the template was mixed with random primers diluted in RNase–free water. The mixture was then incubated at 70°C for 5 minutes and quickly chilled at 4°C for 5 minutes and then held on ice. Reverse Transcription Mix was added and annealing was performed at 25°C for 5 minutes, followed by extension at 42°C for 60 minutes. Reverse transcriptase was inactivated by holding the mixture at 70°C for 15 minutes.

#### 2.3. Primer and probe design

Fifteen CHIKV and 11 DENV nucleotide sequences were retrieved from GenBank and aligned using CLUSTAL W (http://www.ddbj.nig.ac.jp/searches–e.html). The sequence accession numbers were FJ807891, EU703762, AF192898, GU301779, GU301780, GU301781, FJ807893, FJ445511, AM258994, FJ513655, EU244823, FJ445492, FJ807896, NC004162, and AF192907 for CHIKV and HQ166035, GQ398255, GQ398265, GQ398266, FJ744718, FJ744719, GQ398257, FJ744740, FJ744734, AY618993, AY618992 for DENV. Primer and probe sequences are shown in Table 1.

### 2.4. Plasmid preparation

The target regions between positions 10237 and 10544

<table>
<thead>
<tr>
<th>Virus</th>
<th>Probe/primer</th>
<th>Sequence (5’–3’)</th>
<th>Region, position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHIKV</td>
<td>ChikF10378–10398</td>
<td>GCATCAGCTAGCTCCGGGTC</td>
<td>E1, 10378–10398</td>
</tr>
<tr>
<td></td>
<td>ChikR10487–10508</td>
<td>CAATGTTCATCGTGAGGACC</td>
<td>E1, 10487–10508</td>
</tr>
<tr>
<td></td>
<td>ChikP10443–10456</td>
<td>Cy5–ATTCAAAACGGGCACTATCCGTCA—BBQ</td>
<td>E1, 10433–10456</td>
</tr>
<tr>
<td>DENV</td>
<td>DenF10520–10541</td>
<td>GACTAGYGGTTAGAGGACC</td>
<td>3’UTR, 10520–10541</td>
</tr>
<tr>
<td></td>
<td>DenR10674–10694</td>
<td>CHRAGAAGACGAGGATCTCTG</td>
<td>3’UTR, 10674–10694</td>
</tr>
<tr>
<td></td>
<td>DenP10610–10634</td>
<td>JOE–AAGGACTACMGTTAGWGGAGACC—BBQ</td>
<td>3’UTR, 10610–10634</td>
</tr>
</tbody>
</table>

*positions were approximated based on complete genome sequences.*
of the CHIKV E1 region and between positions 10019 and 10695 of the DENV 3’ UTR (positions were approximated from complete genome) were amplified, sequenced, cloned into pGEM®-T Easy vector (Promega, Medison, WI) and transformed into Competent JM109 *Escherichia coli*, which were subsequently spread onto LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C. The white colonies (*Escherichia coli* with inserted region) were selected for further growth in LB broth. The plasmid was extracted using the HiYield™ Plasmid Mini Kit (RBC Bioscience, Taipei, Taiwan). The presence of the inserted region and its direction were confirmed by PCR and direct sequencing and submitted to GenBank (Accession number JF297648–JF297652).

2.5. Thermal profile and optimization conditions

Separate reaction mixtures were carried out for each real-time RT-PCR with parameters adjusted for each virus target. The amplification reaction was performed in a Corbett Research Rotor–Gene 3000 in a 10 μL volume. The mixture contained 5 μL of 2× reaction mix, 0.2 μL of SuperScript™ III RT/Platinum® Taq Mix, 0.5 μM of ChikF 10378–10398 and ChikR 10487–10508, 0.2 μM of ChikP 10443–10456, 0.25 μM of DenF 10520–10541 DenR 10674–10694 and 0.125 μM of DenP 10610–10634, 1.25 mM of MgSO₄, 1.4 μL of distilled water and 1 μL of template. Initial melt was at 95°C for 2 minutes followed by 40 cycles of 95°C (15 seconds), 55°C (10 seconds), 60°C (10 seconds) and 72°C (20 seconds). Fluorescence signals were detected at the end of each 72°C step. Subsequently, both real-time RT-PCR were combined to perform in a multiplex format.

2.6. Sensitivity and specificity compared with RT–PCR

The clinical specimens were detected by conventional semi-nested RT–PCR using the primers previously defined by S. Chutinimitkul for DENV detection[24] and Naresh Kumar C.V.M. for CHIKV detection[25] and then compared to the real time RT–PCR for an evaluation of the sensitivity and specificity. Conventional RT–PCR serving as the reliable method because after RT–PCR, the result was confirmed by sequencing the PCR products and BLAST with GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Result

3.1. Design of primers and probes

The specificity of primers and probes were tested against CHIKV and the four serotypes of DENV as shown in Figure 1A and Figure 1B, respectively. The amplification plot of the Cy5 signal showed that only CHIKV could be amplified in the assay as the signal of DENV in the Cy5 channel remained low at the base line. Likewise, the JOE signal appeared for DENV1–4 at 10–13 cycles and there was no cross–reactive signal of CHIKV. The control reaction performed without template did not produce amplification as evidenced by the low level signal (Figure 1A and Figure 1B) suggesting that we were able to detect CHIKV and four serotypes of DENV successfully.

3.2. Sensitivity and specificity

The result showed that our assay could detect all CHIKV and DENV confirmed positive samples and there was no signal for any of the other viruses tested.

3.3. Evaluation of assay using clinical specimens

In a total of 290 clinical samples, conventional RT–PCR was used to detect CHIKV and DENV. The results of conventional RT–PCR showed that 125 samples were positive for either CHIKV or DENV. The sensitivity, specificity and accuracy of real time RT–PCR were 97.65%, 92.59% and 95.82%, respectively when compare to conventional RT–PCR. Among 125 positive samples, 60 samples were positive for CHIKV and 65 for DENV. All positive samples tested by conventional RT–PCR were also positive for real–time RT–PCR. Out of 162 negative samples diagnosed by conventional RT–PCR, 12 tested positive by real–time RT–PCR. As a result, real–time RT–PCR was found to be more sensitive than conventional RT–PCR.

![Figure 1.](#) 

A: in Cy5 channel labeled for CHIKV probe; B: in JOE channel labeled for DENV probe.
4. Discussion

CHIKV and DENV diagnosis may be approached via several methods such as conventional RT-PCR, commercial rapid test, ELISA, HI test, virus culture and antibody screening (IgM, IgG). Screening for antibody of virus is commonly used but IgM of CHIKV may cross-react with other alphaviruses such as O’nyong nyong virus\cite{26-28} and the antibody of DENV may cross react with other members of the Flaviviridae family such as JEV\cite{29}, resulting in false positives. Virus culture has shown reliable results but is laborious and time consuming to perform. To our knowledge, there is no method available to detect both viruses simultaneously and thus we have developed a method to detect CHIKV and DENV in a single tube.

The challenge of evaluating the assay is the difficulty in defining a gold standard for detecting the viral genome. Conventional RT–PCR has been traditionally used as the gold standard, but from our results real–time RT–PCR yields higher sensitivity than this method. Applying this assay, we were able to detect viral DNA at titers too low for amplification by normal RT–PCR (\(n=12\)). Additionally, real–time RT–PCR also has an advantage over conventional RT–PCR in terms of expediency. There are no post–amplification processes such as gel electrophoresis, gel visualization and sometimes confirmation by direct sequencing. Compared to semi–nested RT–PCR for DENV amplification, a two–step procedure, real–time RT–PCR requires less time to arrive at a diagnosis. By using multicolor probes, we were able to design oligonucleotide sequences of specific probes labeled with different colors\cite{30}. Primers and probes were designed so that they could be used under similar amplification conditions but provide immediate viral identity feedback. Additionally, regional mutations were taken into account during the design process. During the largest outbreak of CHIKV on islands in the Indian Ocean, a signature mutation, E1-A226V, was found in the E1 region. Hence, we designed a set of primers and probe that do not target on this spot so that we are able to detect CHIKV regardless of E1-226 wild–type or mutant. For DENV detection, our method is able to detect all four serotypes of DENV. Although detecting serotypes of DENV may be advantageous, the clinical treatments for all serotypes are identical and it may not be effective to use different probes to detect different serotypes. Evaluation of detection at minimum concentration revealed that this method can detect concentrations as low as 10 copies/\(\mu\)L. When testing the specificity of our assay, we found no cross reactivity between CHIKV, DENV, hepatitis C virus, hepatitis A virus, hepatitis E virus and JEV. Dual infection of CHIKV and DENV confirmed by conventional RT–PCR produced identical results with multiplex real–time RT–PCR, suggesting that this method remains useful for detecting two viruses in a single step reaction.

In conclusion, our set of primers and probes allows for the rapid and early detection of CHIKV and DENV infection in a single step reaction. This method presents a useful tool for studying seroprevalence and quantitative determination of viral infection and may shed light on the correlation between clinical severity and immune response. Detection of CHIKV and DENV presents a challenge, as these viruses may only be traceable during the acute phase, 3–7 days post infection, making other techniques crucial in confirming infections. Awareness of virus dissemination will help the people to take preventive measures and the governments to develop vector control policies.

Conflict of interest statement

We declare that we have no conflict of interest.

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References