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Detection of dengue virus by simple RT-PCR using universal degenerate primers
Observations from a preliminary study

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Ανίχνευση του ιού του δάγγειου με απλή RT-PCR με τη χρήση γενικών εκφυλιστικών ανιχνευτών: Πρόδρομη μελέτη

Περίληψη στο τέλος του άρθρου

OBJECTIVE **Dengue hemorrhagic fever (DHF) is caused by a mosquito-borne flavivirus with four distinct serotypes (dengue types 1–4: DEN1–4). All four serotypes are endemic in most of the countries in the tropical and subtropical regions. Because of the high sensitivity and specificity of PCR diagnostic techniques, several PCR-based strategies for the detection of dengue have been developed recently. In this study aimed to develop a one-step reverse transcription (RT)-PCR assay using universal primer for rapid detection of viral RNA of all dengue serotypes.** METHOD **A reverse transcriptase polymerase chain reaction (RT-PCR) was developed by using a degenerate primer pair specific to the non-structural (NS1), which bears conserve region in the dengue genome of all serotypes. The primer was designed based on the genome sequence of dengue strains collected in Thailand. The RT-PCR assay using the newly designed primers was optimized and evaluated by the detection of dengue RNA in mosquito-cell culture and patient blood.** RESULTS **The NS1 region of dengue (1–4) was successfully amplified giving an RT-PCR product of the expected size (637 bp). Since dengue virus is thought to replicate in mononuclear cells *in vivo*, the RNA extracted from peripheral blood mononuclear cells of patients suspected of being infected with DHF was used as template for RT-PCR. The results indicate that detection of viral RNA in mononuclear cells could be an effective alternative technique to the determination of immunoglobulin changes in plasma.** CONCLUSIONS **According to this study, this technique is rapid, convenient and provides accurate diagnosis in suspected dengue.**

Key words

Dengue
Mononuclear cells
Reverse-transcription PCR

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Dengue fever (DF) and dengue hemorrhagic fever (DHF) have been proclaimed as global public health problems since first emergence in the 18th century.[1] Nowadays, the areas endemic for dengue include Southeast Asia, the Western Pacific, Africa, the Americas and the Eastern Mediterranean.[2] DF and DHF are caused by a single-strand RNA virus of the genus Flavivirus, transmitted to humans by *Aedes* mosquitoes (*Aedes aegyptii* and *A. albopictus*). In Thailand, DF, DHF and dengue shock syndrome (DSS) are diseases included in the national surveillance system. All of the four serotypes (DEN1–4) have been found in the country. The National Institute of Health (NIH) reported a DHF incidence of 82/100,000 with a case fatality rate of 0.06% in 2008.[3] Early diagnosis of dengue infection can reduce the numbers of cases of DHF and DSS. The primary diagnosis of DF and DHF is usually based on clinical symptoms and hematological testing, proceeding to definitive diagnosis by serological testing or virus isolation if needed.[4]

Several PCR-based strategies, which are rapid and sensitive, have recently been developed for the detection of dengue virus in clinical specimens.[5–7] In this study, it was aimed to develop one-step reverse transcription (RT)-PCR assay using a universal primer for the rapid detection of the viral RNA of all dengue serotypes. Primers were designed based on the genome of dengue virus collected in Thailand. In addition, it was investigated whether peripheral blood mononuclear cells (PBMC) provide higher sensitivity than plasma for viral RNA detection.

MATERIAL AND METHOD

Sample collection

Five finger prick blood samples were collected from patients

with a provisional diagnosis of dengue infection. After all the samples had been routinely tested for hematological values, they were used for this study, and also tested for antibodies against dengue using IgG/IgM immunochromatography (Dengue Duo Cassette, Panbio, Australia).

Isolation of peripheral blood mononuclear cells (PBMC)

Mononuclear cells were isolated by the density gradient centrifugation method using Lymphoprep™ (Nycomed, Oslo, Norway). Briefly, 0.5 mL whole blood was centrifuged at 900 g for 10 min. Plasma was then separated and kept for further study. The cell pellet was resuspended in 1 mL phosphate-buffered saline (PBS). The cell suspension was gradually layered on top of 0.5 mL Lymphoprep™ and centrifuged at 600 g for 20 min. The layer containing PBMC was transferred into a new micro-centrifuge tube and washed with PBS. The washed PBMC was stored at -20 °C until used.

RNA extraction

Viral RNA was extracted from either mononuclear cells or plasma as total RNA using the Nucleospin® RNAII extraction kit (MACHEREY-NAGEL Inc, USA) according to the manufacturer's recommendations. RNase inhibitor (QIAGEN, CA) was added to the RNA solution immediately after elution. RNA from a mosquito cell culture of dengue (kindly given by Assistant Professor Wanla Kulwitchit at the Faculty of Medicine, Chulalongkorn University) was also extracted using the same protocol.

Primer design and RT-PCR

Twelve sequences of dengue serotype 1–4 used for primer design were retrieved from GENBANK. All are of Thai strains collected between 1987 and 2001. The primers were designed to have a sequence complementary to the NS1 region of the dengue genome of all serotypes as shown in figure 1.

The newly designed primers were evaluated by RT amplification of the dengue RNA extracted from the mosquito cell culture. The concentration of the primer and assay condition were varied until the best RT-PCR result was achieved. Viral RNA extracted from the PBMC and plasma of patients suspected of being infected with dengue was then amplified using the optimum conditions as follows. The mixture of 2 µL viral RNA, 100 picomole of forward and reverse primers (DDU1 and DDU2) and RNase/DNase-free water to the final volume of 12 µL was heated at 75 °C for 2 min and immediately chilled on ice, then 8 µL of ONE-STEP RT-PCR PreMix (iNtRON Biotechnology, Inc, Korea) was added to the reaction tube. The RT-PCR reaction was performed by a RT step at 45 °C for 30 min and a denaturation step at 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 sec, 48 °C for 30 sec, 72 °C for 30 sec, and a final period of 72 °C for 8 min.

Primer DDU1 & DDU2	5'GTBCACACHTGGACAGA3' 2492 2508	...	3'ACCTCGTTAGGWCAHGA3' 3119 3135
DEN1-TH1991	GTTCACACTTGGACAGA	...	TGGAGCAATGGAGTTCT
DEN1-TH1994	--C----------	...	----------
DEN1-TH 2001	----------	...	----------
DEN2-TH1988	--G-----A--------	...	----------G--
DEN2-TH1998	--G-----A--------	...	----------G--
DEN2-TH2001	--G-----A--------	...	----------G--
DEN3-TH1987	--C-----C--------	...	----------T--A--
DEN3-TH1994	--C-----C--------	...	----------T--A--
DEN3-TH1998	--C-----C--------	...	----------T--A--
DEN4-TH1991	--G----------	...	----------G--
DEN4-TH2000	--G----------	...	----------G--
DEN4-TH2001	--G----------	...	----------G--

Figure 1. Forward (5'→3') and reverse primers (3'→5') containing sequences complementary to part of the NS1 region in the genome of DEN-1, -2, -3 and -4 (IUPAC-IUB symbols: B=G/C/T, H=A/C/T, W=A/T).

RESULTS

RT and cDNA amplification of dengue viruses were performed using universal degenerate primers (DDU1 and DDU2) specific to the NS1 region of dengue genome (serotype 1–4). The evaluation of newly synthesized primers and investigation for appropriate assay conditions were conducted by RT amplification of dengue RNA extracted from a mosquito cell culture. The results demonstrated that RT-PCR provides a DNA band of the expected size (637 bp) as shown in figure 2.

Detection of dengue RNA in clinical specimens was performed using the conditions established above. The method detected viral RNA in 2 of 5 PBMC samples, one of which was a serologically negative case. Three RT-PCR negative samples were samples with IgM and or IgG positive. However, there was no amplification product detected in any RNA extracted from plasma (fig. 2, tab. 1).

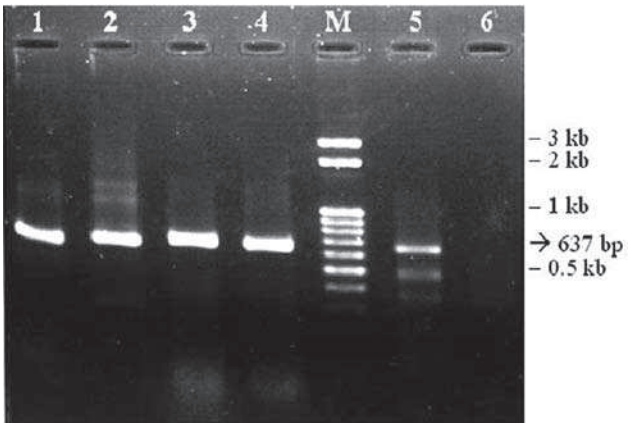

Figure 2. Reverse transcription (RT)-PCR of RNA from 4 serotypes of dengue viruses from cell culture (lane 1: DEN-1, lane 2: DEN-2, lane 3: DEN-3, and lane 4: DEN-4), a peripheral blood mononuclear cells (PBMC) blood specimen (lane 5) and a plasma sample (lane 6).

Table 1. Detection of dengue infection from blood samples using reverse transcription (RT)-PCR in peripheral blood mononuclear cells (PBMC) and plasma, and serological assay.

Sample no	Viral RNA detection in		Serological test	
	PBMC	Plasma	IgM	IgG
1	Positive	Negative	Positive	Negative
2	Positive	Negative	Negative	Negative
3	Negative	Negative	Negative	Positive
4	Negative	Negative	Positive	Positive
5	Negative	Negative	Positive	Positive

DISCUSSION

This study demonstrated the development of a rapid and simple method for dengue detection by RT amplification of viral RNA. The use of degenerate primers enabled the detection of 4 serotypes of dengue in a single step. This technique is different from earlier methods because it can be used with a small amount of blood, as in the case of small children. Since it has been reported that dengue viruses usually infect peripheral blood leukocytes,[8,9] it was attempted to detect viral RNA in PBMC. Previous reports have also suggested that higher sensitivity was obtained when using PBMC samples instead of plasma for viral RNA detection.[10,11] According to the serological pattern in this study, the negative results of RT-PCR in samples with positive IgG might be caused by the low level of viruses in later days of the disease.[12,13] Another possibility could be that the viremia drops rapidly in the case of secondary infection.[13] For these reasons the conditions of RT-PCR testing need to be modified to obtain higher potency. This study was also limited by the lack of negative control specimens carrying other related viral infections to evaluate the specificity of the method. In spite of these limitations, on the basis of this study to the researchers propose this technique as a simple alternative in the diagnosis of dengue infection by using finger prick blood samples at the time of provisional diagnosis. Further studies with increased numbers of clinical samples would be necessary to confirm such a particular advantage.

Additionally, there are some valuable observations arising from this study. It can be seen that there is a discrepancy in the diagnosis of dengue infection. Some serologically positive cases have a negative PCR result while some PCR positive cases have a negative serological result. This can be explained by the fact that in the latter situation the usefulness of the PCR testing for early diagnosis at the time of presentation to the physician is confirmed, while the former case demonstrates the persistence of antibody after the viremia stage. This indicates the pitfalls of PCR and serology testing if used singly. Indeed, patients in endemic areas usually give no clear-cut history of illness, which makes it hard to estimate the exact timing of the disease. A combination of finger prick blood PCR and serology testing can be particularly helpful for diagnosis in such cases.

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ΠΕΡΙΛΗΨΗ

Ανίχνευση του ιού του δάγγειου με απλή RT-PCR με τη χρήση γενικών εκφυλιστικών ανιχνευτών: Πρόδρομη μελέτη

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ΣΚΟΠΟΣ Ο δάγγειος αιμορραγικός πυρετός (ΔΑΠ) οφείλεται σε τέσσερις διαφορετικούς οροτύπους φλαβοϊού (DEN1–4). Και οι τέσσερις ορότυποι είναι ενδημικοί στις περισσότερες χώρες των τροπικών και των υποτροπικών περιοχών. Λόγω της υψηλής ευαισθησίας και της ειδικότητας της διαγνωστικής τεχνικής με την PCR, πρόσφατα έχουν αναπτυχθεί διαφορετικές μέθοδοι PCR για την ανίχνευσή τους. Σκοπός της μελέτης ήταν η ανάπτυξη τεχνικής ενός βήματος RT-PCR με τη χρήση γενικών ανιχνευτών για ταχεία ανίχνευση του ιικού RNA όλων των οροτύπων του δάγγειου. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Η RT-PCR εφαρμόστηκε με τη χρήση ενός ζεύγους ανιχνευτών, ειδικών ως προς το μη δομικό (NS1) που

επιτρέπει τη διατήρηση της περιοχής στο γονιδίωμα του δάγγειου όλων των οροτύπων. Ο ανιχνευτής σχεδιάστηκε με βάση τις αλληλουχίες στο γονιδίωμα των στελεχών δάγγειου που συλλέχθηκαν στην Ταϊλάνδη. Αυτή η μέθοδος RT-PCR βελτιώθηκε και αξιολογήθηκε με ανίχνευση του δάγγειου μέσω RNA σε καλλιέργεια κυττάρων κουνουπιού και αίματος ασθενούς. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Καταδείχθηκε ότι η περιοχή NS1 του δάγγειου (1–4) εφαρμόστηκε επιτυχώς, παρέχοντας ένα προϊόν RT-PCR αναμενόμενου μεγέθους (637 bp). Αφού ο ιός του δάγγειου θεωρείται ότι αναπτύσσεται *in vivo* στα μονοπύρηνα κύτταρα, χρησιμοποιήθηκε RNA από μονοπύρηνα κύτταρα περιφερικού αίματος ασθενών με υποψία δάγγειου ως πρότυπο για την RT-PCR. Τα αποτελέσματα δείχνουν ότι η ανίχνευση του ιικού RNA σε μονοπύρηνα κύτταρα θα μπορούσε να είναι μια αποτελεσματική εναλλακτική λύση για την τεχνική προσδιορισμού των μεταβολών ανοσοσφαιρίνης στο πλάσμα. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Σύμφωνα με την παρούσα μελέτη, φαίνεται ότι η εν λόγω τεχνική είναι ταχεία, εύκολη και παρέχει τη δυνατότητα ακριβούς διάγνωσης σε υποψία δάγγειου.

Λέξεις ευρετηρίου: Δάγγειος, Μονοπύρηνα, RT-PCR

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