

## Original Article

# Evaluation of RT-PCR as a Tool for Diagnosis of Secondary Dengue Virus Infection

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**SUMMARY:** Dengue fever and dengue hemorrhagic fever are serious illnesses in many tropical and subtropical countries. Laboratory tests are essential for the confirmation of dengue virus infection. In the present study, we examined the reliability of reverse transcriptase polymerase chain reaction (RT-PCR) in the laboratory diagnosis of dengue, especially in secondary dengue virus infections. We defined the day when fever subsided as fever day 0. In primary dengue virus infection, the dengue viral genome was detected in all of the 7 samples which were collected on fever day -1 or earlier, in 3 of 4 samples on fever day 0, and in 1 of 2 samples on fever day 1. None of the samples collected on fever day 2 or later were positive by RT-PCR. In secondary dengue virus infection, the dengue viral genome was detected in all of the 28 samples which were collected on fever day -2 or earlier, in 25 of 26 on fever day -1, in 29 of 34 on fever day 0, and in 5 of 10 on fever days 1-2. None of the samples collected on fever day 3 or later were positive. Virus isolation and direct titration were attempted using the plasma samples. When the data of secondary infection cases were analyzed based on fever day, dengue viruses were isolated from all of the 5 samples which were collected on fever day -2 or earlier, in 5 of 13 samples on fever day -1, and in 4 of 22 on fever day 0, but were not isolated from any of the 4 samples collected on fever days 1-2. Viruses were directly detected in 7 of 11 samples on fever day -2 or earlier, in 4 of 13 on fever day -1, and in 1 of 16 on fever day 0. These results indicate that RT-PCR is more sensitive than virus isolation and direct virus titration for determining secondary dengue virus infection. The results also suggest that RT-PCR is a useful diagnostic test for confirmation of dengue virus infection in secondary infection as well as in primary infection, especially when plasma samples are collected before the fever subsides.

## INTRODUCTION

Dengue viruses belong to the family *Flaviviridae*, genus *Flavivirus*. There are four types: dengue virus types 1, 2, 3, and 4. Dengue virus infection occurs mainly in tropical and subtropical areas of the world. Dengue virus infection can be asymptomatic, or cause dengue fever (DF) and dengue hemorrhagic fever (DHF) (1). DF and DHF are serious illnesses in many tropical and subtropical countries. Clinical observation is important for diagnosis of DF and DHF; however, laboratory tests are essential for confirmation. Serological and virological tests have been used for confirming dengue virus infection. Hemagglutination inhibition (HI) test has been used as a main serological test; however, it requires paired sera and has cross-reactivity with other flaviviruses (2). Enzyme-linked immunosorbent assay (ELISA) has been recently used in many laboratories (3-8). Virus isolation is a definitive test, but is time consuming (9-11). Instead of virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR) has been widely used for dengue diagnosis (12-17). It has been reported that RT-PCR is positive in serum samples collected in the febrile stage in primary dengue virus infection (17). In the present study,

we examined whether RT-PCR is a reliable diagnostic test in secondary dengue virus infection as well as in primary infection.

## MATERIALS AND METHODS

**Specimen collection and definitions:** Three hundred and ten plasma specimens were collected from 142 patients for diagnostic purposes. These patients were hospitalized in Pediatric Ward II of Sawanpracharak Hospital, Nakhon Sawan Province, Thailand from May to September 2002. Peripheral blood specimens were collected and plasma samples were separated at the Sawanpracharak Hospital. One vial of each plasma sample was kept in a liquid nitrogen tank and the other vials of the same samples were kept at -20°C. All the plasma samples were transported in dry ice to the Arbovirus Laboratory, National Institute of Health (Thai NIH), Department of Medical Sciences, Nonthaburi, Thailand. Disease day and fever day were defined as previously described (17,18). Disease day 1 is the day of the onset of symptoms. Fever day 0 is the day of defervescence; the day when the patient's temperature fell below 37°C without further significant temperature elevation. Days prior to fever day 0 are designated fever day -1, fever day -2, etc. The day after defervescence is fever day 1.

**Antibody-capture ELISA:** IgM and IgG antibody-capture ELISAs were performed as previously described by Innis et al. (4). In brief, 96-well U-bottom microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with anti-human

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IgM or anti-human IgG (Cappel, Aurora, Ohio, USA). Fifty microliters of 1:100 diluted plasma samples or control sample were added in duplicate, washed 6 times with PBS-T, and 50  $\mu$ l of dengue viral antigen was then added. Twenty-five microliters of diluted horseradish peroxidase (HRP)-human anti-flavivirus IgG conjugate (Thai NIH) were added. After washing 6 times with PBS-T, 0.5 mg/ml of OPD (O-phenylenediamine dihydrochloride, Sigma, St. Louis, Mo., USA) with 3.3  $\mu$ l/ml of 3% H<sub>2</sub>O<sub>2</sub> was added. Color development was stopped by the addition of H<sub>2</sub>SO<sub>4</sub> and the OD was read at 490 nm using a microplate autoreader (Bio-tex Instrument Model EL 311, BIO-TEX INSTRUMENTS, INC., Winooski, Vt., USA). The levels of specific antibodies were calculated from OD values as previously reported (4).

**HI test:** Non-specific inhibitors naturally present in the plasma were extracted twice by mixing 80  $\mu$ l of plasma with 800  $\mu$ l of acetone. Plasma was dried and reconstituted with 800  $\mu$ l of borate saline buffer pH 9.0. The antigen was diluted with 0.4% bovine serum albumin in borate saline buffer to make 8 HA units/50  $\mu$ l. The treated plasma samples were serially twofold diluted from 1:10 to 1:10,240 with 0.4% BABS in a U-bottom microtiter plate. The positive and negative plasma controls were also included in the tests and they were diluted in the same way as tested plasma. Subsequently, 25  $\mu$ l of dengue viral antigen (8 HA units/50  $\mu$ l) was added in each well containing 25  $\mu$ l of diluted plasma and the plates were shaken thoroughly. The plates were incubated at 4°C overnight, and 50  $\mu$ l of 0.33% GRBC in proper VAD was added to each well. The plates were incubated at 37°C for 1 h. HI titers on the tested plates were read.

**RT-PCR:** RT-PCR was performed as previously reported with some modifications (16). Briefly, RNA was extracted from 100  $\mu$ l of plasma using a QIAamp viral RNA minikit. RT and PCR were performed in one tube using universal primer and a one-step RT kit (QIAGEN GmbH, Hilden, Germany). The reaction tube was placed in a Thermal Cycler machine (Perkin-Elmer-Cetus, Norwalk, Conn., USA). The primary PCR product was further used for nested PCR in another reaction tube. The nested PCR reaction tube was set in a thermal cycler. The secondary PCR product was subjected to agarose gel electrophoresis. Amplified DNA fragments were visualized after ethidium bromide staining.

**Virus isolation:** Dengue viruses were isolated as previously reported (19). C6/36 cells (20,21) were cultured in Leibovitz's medium (L-15, Gibco, Invitrogen Incorporation, Grand Island, New York, USA) containing 10% FBS in 24-well plates (Costar, Corning Incorporated Life Sciences, Acton, Mass., USA) at 28°C. Cells were used when full monolayers were developed. Fifty microliters of 1:10 diluted plasma were inoculated onto C6/36 cell monolayers and incubated for 90 min. The inoculums were discarded and L-15 containing 2% FBS was added. Cells were cultured at 28°C for 7 days. Culture supernatants were collected and the cells were tested for dengue virus antigens by FA test. The types of isolated dengue viruses were also determined by FA test using monoclonal antibodies (12,22). When FA tests were negative, the culture fluids were tested for the dengue viral genome by RT-PCR.

**Plaque titration assays:** Plasma samples were serially 10-fold diluted in PBS containing 10% calf plasma, and 50  $\mu$ l was inoculated onto confluent Vero cell monolayers in the 24-well plate (Costar). The assay was performed in duplicate. After adsorption for 90 min, the inoculum was discarded and

the cells were overlaid with 1 ml of 1% methylcellulose-MEM containing 2% FBS. After incubation at 32-33°C in a CO<sub>2</sub> incubator for 9-10 days, the cells were fixed with 10% formalin solution at room temperature for 1 h and rinsed gently under tap water. The cells were stained with 0.33% methylene blue solution. The plates were dried, and plaques were counted.

## RESULTS

**Demographic information of patients enrolled in the study:** A total of 118 children were hospitalized and laboratory diagnosed as having dengue virus infection. Twenty-five cases were clinically diagnosed as DF, 18 as DHF grade 1, 57 as DHF grade 2, 4 as DHF grade 3, and 8 as DHF grade 4, with 6 not clinically diagnosed as dengue virus infection. The dengue viral genome was detected by RT-PCR in plasma samples from a total of 108 cases: dengue virus type 1 in 31 cases, type 2 in 56 cases, type 3 in 12 cases, and type 4 in 9 cases. Among 118 dengue cases, 12 cases (10%) were determined to be primary infection and 96 (81%) to be secondary, on the basis of the results of HI test and ELISA. Ten cases (9%) could not be determined to be either primary or secondary infection. Twelve primary and 94 secondary infection cases were enrolled for data analysis.

**Detection of dengue viral genome by RT-PCR in patients with primary dengue virus infection:** The dengue viral genome was detected by RT-PCR in 26 plasma samples from 12 primary infection cases. When the data were analyzed based on fever days, the dengue viral genome was detected in all 7 samples which were collected on fever day -1 or earlier, in 3 of 4 samples on fever day 0, and in 1 of 2 samples on fever day 1 (Fig. 1). None of the samples collected on fever day 2 or later were positive for dengue viral genome. When analyzed based on disease days, the dengue viral genome was detected in all of the 6 samples which were collected on disease day 5 or earlier and 5 of 8 collected on disease days 6-8 (Fig. 2). None of the samples collected on disease day 9 or later were positive.

**Detection of dengue viral genome in patients with secondary dengue virus infection:** The dengue viral genome was detected by RT-PCR in 114 plasma samples from 94 secondary infection cases. When the data were analyzed based on fever days, the dengue viral genome was detected in all of the 28 samples which were collected on fever day -2 or earlier, in 25 of 26 on fever day -1 in 29 of 34 on fever day 0,

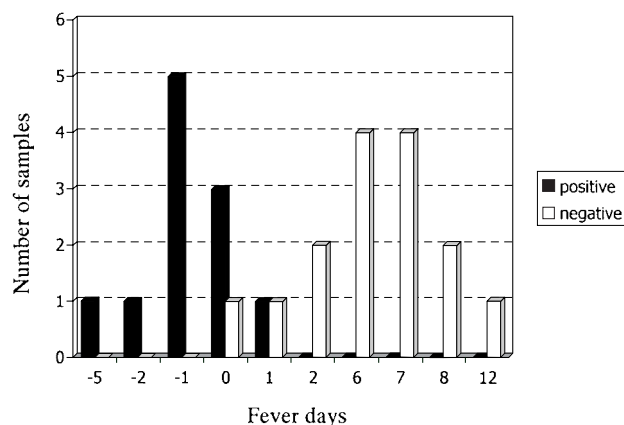


Fig. 1. Detection of dengue virus genome by RT-PCR in plasma samples from patients with primary dengue virus infection based on fever days.

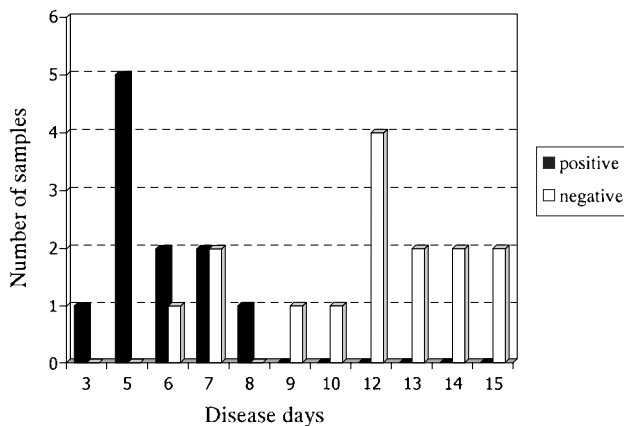


Fig. 2. Detection of dengue virus genome by RT-PCR in plasma samples from patients with primary dengue virus infection based on disease days.

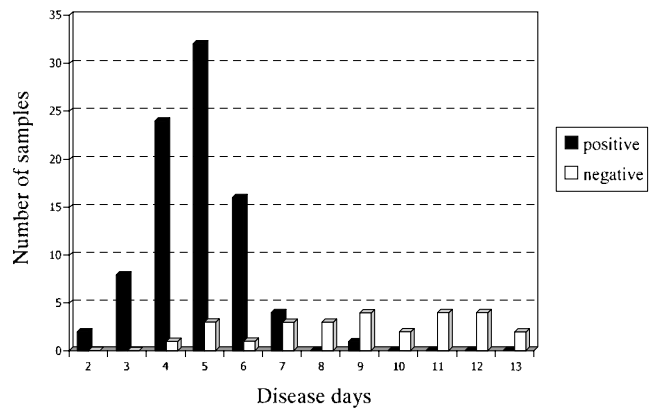


Fig. 4. Detection of dengue virus genome by RT-PCR in plasma samples from patients with secondary dengue virus infection based on disease days.

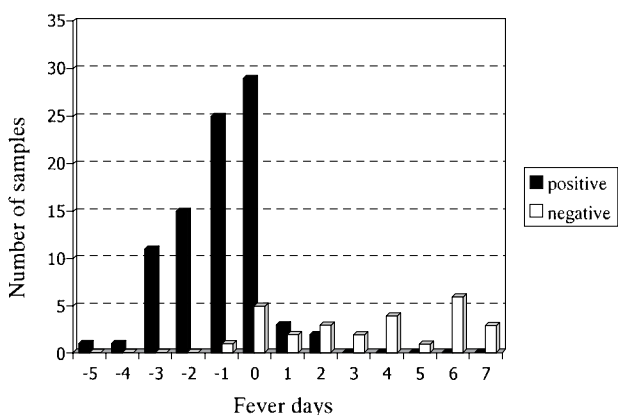


Fig. 3. Detection of dengue virus genome by RT-PCR in plasma samples from patients with secondary dengue virus infection based on fever days.

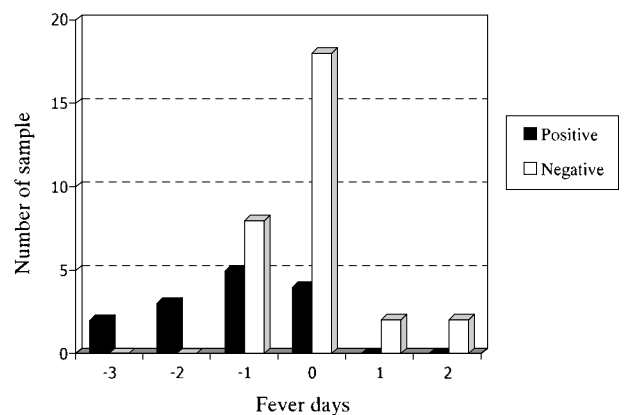


Fig. 5. Isolation of dengue virus from plasma samples from patients with secondary dengue virus infection based on fever days.

and in 5 of 10 on fever days 1-2 (Fig. 3). None of the samples collected on fever day 3 or later were positive. When analyzed based on disease days, the dengue viral genome was detected in all of the 10 samples which were collected on disease day 3 or earlier, in 24 of 25 on disease day 4, in 32 of 35 on disease day 5, in 16 of 17 on disease day 6, and in 4 of 7 on disease day 7 (Fig. 4). Only one of 20 samples collected on disease day 8 or later was positive.

**Virus isolation:** Fifty-one acute samples were used for virus isolation, using C6/36 cell culture. Dengue viruses were isolated from the samples: type 1 from 3 samples, type 2 from 9, type 3 from 1, and type 4 from 2. Of the 15 samples, 14 were from secondary infection cases and one was from a primary case. When data of the secondary dengue virus infection cases were analyzed based on fever days, dengue viruses were isolated from all of the 5 samples which were collected on fever day -2 or earlier, from 5 of 13 samples on fever day -1, and from 4 of 22 on fever day 0 (Fig. 5). Viruses were not isolated from any of the 4 samples collected on fever days 1-2.

**Direct plaque titration:** Direct plaque titration was performed using 47 plasma samples. Viruses were directly detected at titers from  $1.6 \times 10^3$  to  $5.6 \times 10^5$  pfu/ml in 12 samples. All of them were from secondary dengue virus infection cases. When the data were analyzed based on fever day, viruses were directly detected in 7 of 11 samples on fever day -2 or earlier, in 4 of 13 on fever day -1, and in 1 of 16 on fever day 0 (data not shown).

## DISCUSSION

We attempted to detect the dengue viral genome by RT-PCR in plasma samples collected from dengue patients at various days of illness. We also attempted to isolate the virus and to assess dengue viral titers in the plasma samples. The dengue viral genome was detected in most plasma samples collected on fever 0 or earlier in both primary and secondary dengue virus infections, but not from those collected on fever days 2 and 3 or later. We could isolate the virus from plasma samples collected on fever day 0 or earlier.

Although the results of dengue virus isolation were generally consistent with RT-PCR results, the positive rate of dengue virus isolation was not as high as that of RT-PCR. We assume that this may have been due to freeze-thaw process the plasma samples. The results of direct virus titration were consistent with virus isolation. Both methods detected dengue viruses in samples collected on fever day 0 or earlier. Moreover, our results demonstrated that dengue virus was detected by RT-PCR for a longer period compared to virus isolation, suggesting the higher sensitivity of RT-PCR as a laboratory diagnostic test (23,24).

Detection of IgM by IgM-capture ELISA is widely used for laboratory diagnosis and considered to be a reliable serologic test (4,8,17,18,25). In the present study, IgM was detected in more than 90% of the plasma samples collected on fever day 5 or later in primary dengue virus infection. In secondary dengue virus infection, IgM was detected in

approximately 60% of the plasma samples (data not shown). This result is consistent with the previous finding that IgM responses are low or sometimes undetectable in secondary dengue virus infection.

It has been previously reported that RT-PCR is a reliable diagnostic test when plasma or serum specimens were collected in the febrile stage in cases of primary dengue virus infection (17). The results in the present study demonstrated that RT-PCR is a reliable diagnostic test in secondary dengue virus infections as well as in primary infections when plasma samples are collected in a febrile stage. We conclude that the combination of RT-PCR, and IgM and IgG ELISA increases the accuracy and the sensitivity of the laboratory diagnosis of dengue virus infection, especially in secondary dengue virus infection.

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