

Real-time PCR for detecting circulating dengue virus in the Guangdong Province of China in 2006

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Dengue virus (DENV) causes a wide range of diseases in humans, from the acute febrile illness dengue fever (DF) to life-threatening dengue haemorrhagic fever/dengue shock syndrome. We developed four real-time quantitative PCR assays for each serotype of DENV based on computational analysis. These assays had high sensitivity and specificity without cross-reactivity for the four serotypes. To evaluate the performance of these assays in detecting and typing the virus in clinical samples, we analysed 64 serum samples from Guangdong during 2006. The results showed that 71 % of those samples were positive by the DEN-1 assay. The DENV assay results, in agreement with the serological tests and sequencing analysis, showed that the pathogen resulting in the DF explosion in Guangdong in 2006 belonged to DEN-1. Compared to the serological assays, the real-time PCR assays that we developed were much more sensitive in the 1–3 days after onset of the symptoms.

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INTRODUCTION

Dengue virus (DENV) is a member of the genus *Flavivirus* of the family *Flaviviridae* and has four serologically distinct serotypes (DEN-1, DEN-2, DEN-3 and DEN-4). DENV causes a wide range of diseases in humans, from the acute febrile illness dengue fever (DF) to life-threatening dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) (Gubler, 2002; Kalayanarooj *et al.*, 1997; Gibbons & Vaughn, 2002; Gubler, 1998). These diseases are endemic in most tropical and subtropical countries, and are transmitted by mosquitoes of the genus *Aedes*, such as *Aedes aegypti* and *Aedes albopictus* (Gubler, 2002; Aaskov *et al.*, 2006). It is estimated that the annual worldwide occurrence of DF and DHF is, respectively, 100 million and 250 000 cases, and the mortality case number is 25 000 (Gibbons & Vaughn, 2002; Halstead, 1988; Monath, 1994). Therefore, rapid detection and determination of the DENV

serotypes of past and current infections are important for patient management as well as for epidemic control.

Confirmation of diagnosis is usually established by cell culture, serological assays or PCR tests. The isolation of DENV from clinical samples through cell culture has generally been unsuccessful owing to the fastidious culture conditions, the low level of transient viraemia and the time-consuming procedure. Serological assay based on IgG or IgM has its limitations in the diagnosis of DENV. Firstly, there is extensive cross-reactivity with other flaviviruses, particularly among the DENV serotypes. Secondly, primary infections are characterized by the increase of dengue-specific IgM antibodies 4–5 days after the onset of symptoms and the increase of IgG antibodies after 7–10 days (Schwartz *et al.*, 2000; Johnson *et al.*, 2000; Martin *et al.*, 2000, 2002; Lanciotti, 2003; Vaughn *et al.*, 1997), hence the serological assay cannot provide a rapid response to the infection. Thirdly, rheumatoid factor can influence the IgM capture assay and result in false-positives for DENV (Schwartz *et al.*, 2000; Jelinek *et al.*, 2000). Most of all, virus isolation and serological assays have less impact on patient management and the control measures exercised by medical and public health personnel, such as the detection of secondary infection, which is very important to the patients. In contrast, PCR assays have the potential to detect infection at earlier time points and provide quicker results. The real-time PCR assay has many

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Abbreviations: DF, dengue fever; DHF, dengue haemorrhagic fever; DSS, dengue shock syndrome; FAM, 6-carboxyfluorescein; IFA, immunofluorescence assay; RT-PCR, reverse transcriptase-PCR; TAMRA, 6-carboxytetramethylrhodamine; TCID₅₀, 50 % tissue culture infective dose.

Figures showing the sensitivity and dynamic range of real-time PCR in detection of DENV RNA are available with the online version of this paper.

advantages over traditional PCR-based assays, including rapidity, quantitative measurement, low contamination rate, higher sensitivity, higher specificity and easy standardization.

DENV occurred in southern China in the 1950s and disappeared for about 30 years until its recurrence in 1978. In 2006, a total of 1019 cases of DF occurred in the Guangdong Province of China. In this study, we established four sensitive and serotype-specific one-step real-time PCR assays to detect DEN-1, DEN-2, DEN-3 and DEN-4. More than 64 confirmed DENV sufferers during the 2006 epidemic in Guangdong were tested, indicating that the assays can be easily standardized across laboratories.

METHODS

Patients and serum samples. Serum samples were collected from 64 patients by the Medical Centre of Guangzhou Medical Research Institute: 44 samples were collected during the period 1–7 days after onset of symptoms and 20 were collected at 8–14 days. Those samples were kept, transported to the laboratory at -20°C and inactivated at 56°C for 1 h.

Viruses. Fourteen DENV strains including the four serotypes of the DENV were cultured in C6/36 cells and the titres were quantified by the TCID₅₀ (50% tissue culture infection dosage) method. The virus genomes were further sequenced and divided into different clades (unpublished data). We also included other arboviruses to determine the specificity of this assay, including West Nile virus, Japanese encephalitis virus, yellow fever virus, Semliki Forest virus, Chikungunya virus and Sindbis virus. The West Nile virus was provided by the Chinese Academy of Inspection and Quarantine, and the remaining viruses were provided by the Guangzhou Medical Research Institute.

Primers and probes. Due to the divergence of DENV, we used all available sequence data in GenBank for comparing genomes of DENV. Alignment of DENV sequences was performed separately with sequence data for DEN-1 (85 strains), DEN-2 (101 strains), DEN-3 (78 strains) and DEN-4 (28 strains). Within the alignments, the most conserved sequences of each serotype of DENV were selected for primer and probe designing. The primer set used for each DENV serotype is listed in Table 1, designed by Primer Express Software 3.0

(PE Applied Biosystems). The probe was labelled at the 5'-end with 6-carboxyfluorescein (FAM) reporter dye and at the 3'-end with 6-carboxytetramethylrhodamine (TAMRA) quencher dye.

Transcription of the conserved RNA sequences *in vitro*. The conserved sequences of each DENV serotype were amplified by RT-PCR and cloned into the pGEM-T Easy vector (Promega). The recombinant vectors were linearized with *Pst*I, purified with a PCR purification kit (Qiagen) and transcribed with T7 RNA polymerase using a RiboMAX Express large-scale RNA production system (Promega). The template DNA was degraded with 5 U RNase-free DNase I. The RNA transcripts were purified twice with the RNeasy kit (Qiagen), spectrophotometrically quantified, divided into aliquots and stored at -80°C . Dilutions of these transcripts (4×10^8 – 4×10^{-2} copies μl^{-1} at tenfold dilutions, 4×10^1 – 7.8×10^{-2} copies μl^{-1} at twofold dilutions) were used for the determination of detection limits and the amplification efficiency.

RNA extraction. Virus RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. Briefly, 140 μl of each sample or cell culture (10^{-3} TCID₅₀) was used for the extraction of viral genomic RNA. The RNA was eluted in 50 ml DEPC-treated water and used in the following experiments immediately or stored at -80°C .

Real-time PCR. Real-time PCR was performed using an ABI 7300 PCR thermal cycler (PE Applied Biosystems). To take the DEN-1 assay as an example, the PCR mixture included a final volume of 20 μl with 10 μl one-step RT-PCR Master kit (Qiagen), 0.5 μl (10 pmol μl^{-1}) each primer, 1.5 μl (2 pmol μl^{-1}) probe, 5.5 μl distilled water and 2 μl extracted RNA. The amplification conditions were as follows: an initial step at 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, annealing and elongation at 60°C for 60 s, with fluorescence acquisition in single mode. Negative controls included human RNA, C6/36 RNA, distilled sterile water and PCR mixture. All experiments were repeated four times. The PCR mixture for the remaining three DEN assays was the same as for DEN-1. Amplified products were detected by agarose gel electrophoresis and sequence identification. The sequencing was undertaken on an ABI PRISM 3730 DNA Sequencer.

IgG and IgM indirect immunofluorescence assay (IFA). For detecting DENV-specific IgG and IgM, serum samples were tested by 'in-house' indirect IFAs as described by Wulff & Lange (1975) with some modifications. Taking DEN-1 as an example, C6/36 cells were infected with DEN-1, and cultured for 72 h. Infected cells were

Table 1. Primers and probes used in group-specific and serotype-specific real-time assays for DENV

| Virus serotype detected | Primer/probe | Nucleotide sequence | Fluorophore |
|-------------------------|--------------|---------------------------------------|-------------|
| DEN-1 | DEN-1F | 5'-GAACAGTTTCGACTCGGAAGCTT-3' | |
| | DEN-1R | 5'-CTCTCGCGTTTCAGCATATTGA-3' | |
| | DEN-1Probe | 5'-AACAAACCAACGGAAAAAGACGGGAAAAACC-3' | FAM/TAMRA |
| DEN-2 | DEN-2F | 5'-TTATCAGTTCAAATCCAATGTTGGT-3' | |
| | DEN-2R | 5'-AGGAGGAAGCTGGGTTGACA-3' | |
| | DEN-2Probe | 5'-CATCGTCGTACACAGCTTCCATGTTC-3' | FAM/TAMRA |
| DEN-3 | DEN-3F | 5'-AAAGCTATGAACGCCATAACCAA-3' | |
| | DEN-3R | 5'-GGCGAAGAGATTCTCAAGAGGAT-3' | |
| | DEN-3Probe | 5'-TTCATTGGTCCTTGCCGTTTCAGC-3' | FAM/TAMRA |
| DEN-4 | DEN-4F | 5'-GAGAACTCTTACCAACCCTTGA-3' | |
| | DEN-4R | 5'-GAAAAATGAACCAACGAAAAAAGGT-3' | |
| | DEN-4Probe | 5'-CTGGGTCAGCACGTTTGTTCATT-3' | FAM/TAMRA |

harvested, mixed with 0.5-fold non-infected cells, washed in PBS, spotted on slides, and fixed with ice-cold acetone. Fixed slides were stored at -70°C until use. All serum samples were screened at a dilution of 1:40 in PBS. Fifty microlitres of each serum sample was spotted per well and incubated for 30 min at 37°C . After washing for 10 min in PBS, 20 μl fluorescein isothiocyanate-conjugated anti-human IgG diluted 1:40 in buffer containing Evans blue was added to each well and incubated for 30 min, embedded in glycerine, and examined by immunofluorescence microscopy. Positive samples were further tested in twofold serial dilutions to determine the end point titre. A titre of 1:20 was considered positive. The specificity of the IFA was previously demonstrated.

RESULTS AND DISCUSSION

Sensitivity and specificity of the real-time PCR assay

Standards for RT-PCR were generated by *in vitro* transcription of RNA from linearized plasmid template with T7 polymerase. A linear range of *in vitro*-transcribed virus RNA dilutions was tested. The range was wide: $40-4 \times 10^7$ copies per reaction ($R^2=0.991822$) for DEN-1 (Fig. 1), $20-2 \times 10^7$ copies per reaction ($R^2=0.988355$) for DEN-2 (Supplementary Fig. S1 in JMM Online), $80-8 \times 10^7$ copies per reaction ($R^2=0.998509$) for DEN-3 (Supplementary Fig. S2 in JMM Online) and $15-1.5 \times 10^7$ copies per reaction ($R^2=0.9991$) for DEN-4 (Supplementary Fig. S3 in JMM Online).

To determine the specificity of these four real-time PCR assays, 14 dengue strains covering four serotypes, the six other arboviruses, C6/36 cells and human RNA were tested. The four assays reacted negatively to the other arboviruses tested, C6/36 cells and human RNA. The serotypes of all isolates were confirmed using the four real-time PCR assays developed by us. The typing results agreed perfectly with the serological tests and the genome sequencing of these 14 strains (Figs 2 and 3). DENV is a positive-strand RNA virus and thus is apt to mutate, resulting in nucleotide differences between genotypes and also even within one genotype.

With the pan-distribution of DENV in the tropics and subtropics, many variant strains exist within each DENV serotype. Therefore, it is essential that all genotypes and strains within a DENV serotype be detected by an assay. The four DENV assays that we developed are specific to the corresponding DENV serotype, and have no cross-reactivity with the other DENV serotypes. Real-time PCR assays are rapid and are among the most sensitive methods for determination of whether a given pathogen is present. Therefore, the specificity is very important for real-time PCR to detect the potential pathogen. The computational search for conserved sequences that we have described above minimizes the expensive and time-consuming *in vitro* work needed to identify specificity and provided greater assurance than sequences chosen on a simply

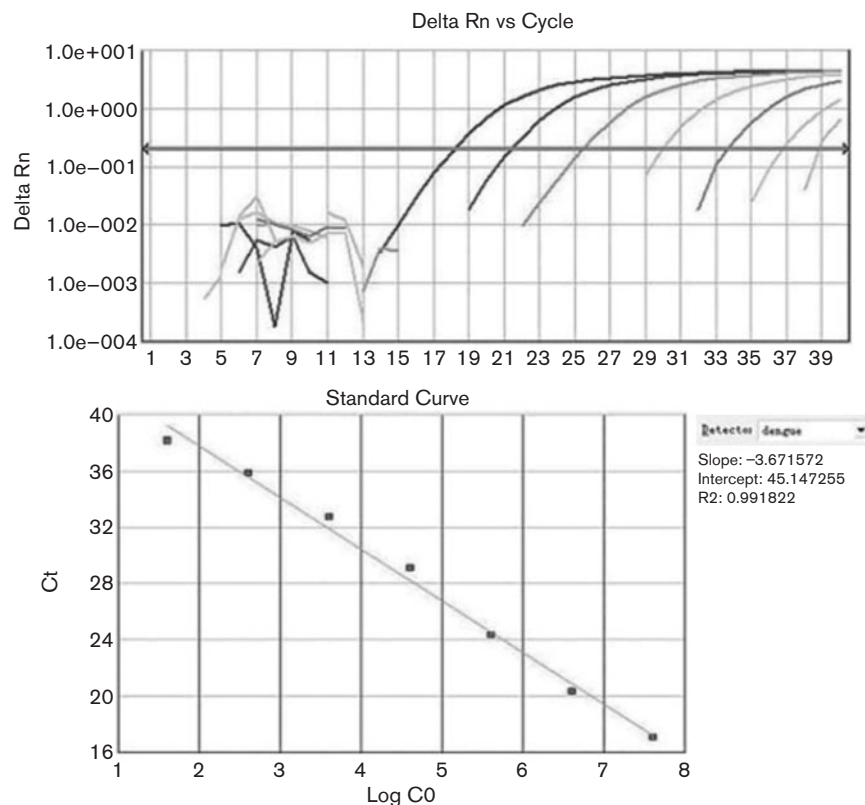


Fig. 1. Sensitivity and dynamic range of real-time PCR in detection of DEN-1 RNA. Serial dilutions of *in vitro*-transcribed DEN-1 conserved RNA sequence were tested. A wide linear range ($40-4 \times 10^7$ copies of the control RNA per reaction) was determined in this assay.

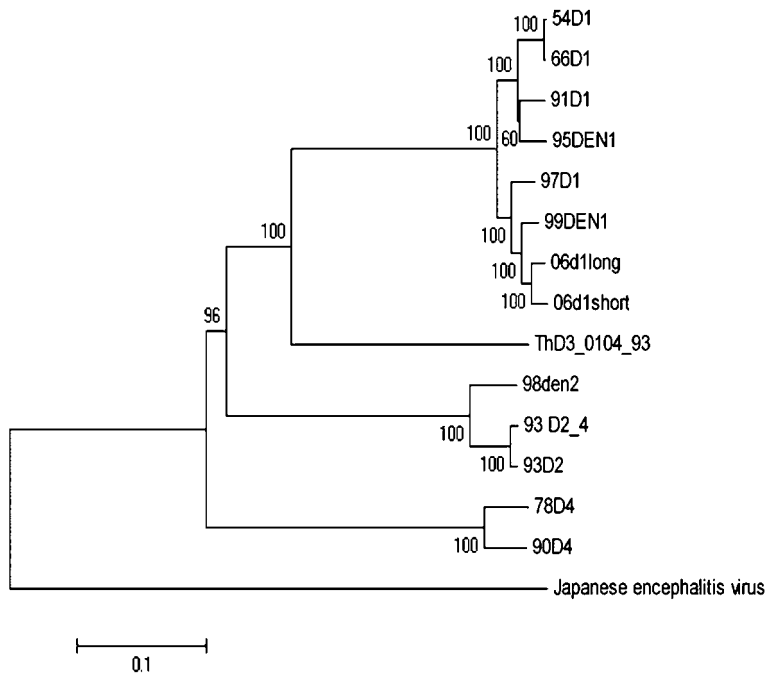


Fig. 2. Phylogenetic tree of complete nucleotide sequences of DENV isolated in Guangdong Province. The tree was constructed using the neighbour-joining method generated using MEGA software with Japanese encephalitis virus (NC001437.1) as the out-group. Significant bootstrap values are shown as percentages derived from 1000 resamplings. The reference DEN-3 sequence (ThD3_0104_93; AY676375) used for the construction of the phylogenetic tree was obtained from GenBank.

empirical basis. Because we searched for sequences conserved among multiple existing strains, it is highly possible that these sequences are conserved in newly evolving strains as well. Furthermore, real-time PCR assays allow sensitive detection of DENV in clinical samples.

A major advantage of the real-time PCR over conventional (frequently nested) RT-PCR is that the amplification and analysis of real-time PCRs are completed in closed systems. Thus the risk of contamination confounding conventional (frequently nested) RT-PCR protocols can be markedly reduced (Sudiro *et al.*, 1997; Seah *et al.*, 1995; Lanciotti *et al.*, 1992).

Comparison of real-time PCR with IFA

To evaluate the performance of these assays in detecting and typing clinical samples, we analysed 64 serum samples. A total of 44 serum samples tested positive in the DEN-1 assay; these samples reacted negatively in the remaining assays. The positive samples were also amplified by common PCR and the products were sequenced. The results of real-time PCR and sequencing were in agreement with those of the serological test. The positive rate for the DEN-1 assay was 71 %, whereas the positive rate for IgM and IgG was 54.85 % and 34 %, respectively (Table 2, Fig. 3). During the first 3 days after the onset of symptoms, the detection rate for DEN-1 was 86.36 %, while the detection rates of the serological assay based on IgM and IgG were 18 % and 9.09 %, respectively. In the following 3 days, the detection rate for DEN-1 declined to 67 %. By comparison, the IgM assay reached 77 % and the IgG assay reached 36 %. From the 7th day after the onset of symptoms, the detection rate for IgG increased to 75 %, but that for IgM decreased a little. This was in agreement with the results reported by Vaughn *et al.* (1997). Viral load ranged from 30 to 3.75×10^7 copies ml^{-1} , with a decreasing viraemia trend for samples drawn after the 3rd day of onset of disease symptoms. The median viral loads were 8.29×10^5 copies ml^{-1} on the first 3 days, 4.33×10^3 copies ml^{-1} on the following 3 days, and 9×10^2 copies ml^{-1} from day 6. A total of 21 of 24 samples obtained during the first 3 days which did not have any anti-DENV antibody were detected by the DEN-1 assay. This demonstrated that in the first few days after onset of symptoms the real-time PCR might be better than the serological tests at detecting DENV.

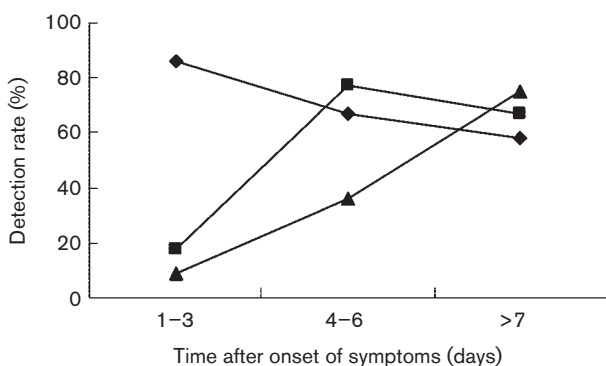


Fig. 3. Real-time PCR and IgM and IgG analysis of samples. ◆, Real-time PCR detection rate; ■, IgM detection rate; ▲, IgG detection rate.

Table 2. Summary of clinical samples

| Specimen | Total samples | 1–3 days | | 4–6 days | | ≥7 days | |
|---------------|---------------|----------|----------|----------|----------|----------|----------|
| | | Positive | Negative | Positive | Negative | Positive | Negative |
| Real-time PCR | 64 | 19 | 3 | 20 | 10 | 7 | 5 |
| IgM | 64 | 4 | 18 | 23 | 7 | 8 | 4 |
| IgG | 64 | 2 | 20 | 11 | 19 | 9 | 3 |

Whether different positive rates reported for various DENV assays reflect true differences in assay performance, or merely differences in specimen type or differences in sample preparation, will only become apparent after comparative quality control tests using identical samples in the various assays and laboratories. RNA integrity is a critical determinant of sensitivity in real-time PCR DENV assays. Protocols adopted by the various hospitals for sample collection, handling and storage were not uniform. Nonetheless, DEN-1 assay analysis produced consistent results for all 46 cases of matching serum samples. These assays that we described can be performed wherever a real-time quantitative PCR machine is available. The reagents and the machine are standardized, this method gives rapid results (sequencing is not necessary) and decreases the likelihood of error.

Early diagnosis of DENV is pivotal to any licensed antiviral therapy and epidemic control. The diagnosis is achieved serologically by detecting dengue-specific IgM or IgG antibodies, which generally appear 5–10 days after the onset of symptoms (Schwartz *et al.*, 2000; Johnson *et al.*, 2000; Martin *et al.*, 2000, 2002; Lanciotti, 2003). Both have less value for early diagnosis. The detection of dengue serotype is very important, especially in secondary infection, because a heterologous serotype often leads to fatal DHF and DSS (Halstead, 1988). Therefore, early typing is a prerequisite for proper patient management. Our results show that DENV can be readily detected in early serum samples before IgM antibodies are present by real-time PCR. Even in the presence of IgG and IgM, viral RNA can be detected at least at an early point during IgG production. The DEN-1 assay has greater sensitivity than cell culture and serological assay in the early stages, with a cut-off detection of 1–5 copies per sample, as measured comparatively to plasmid DNA quantification.

In conclusion, we applied bioinformatic methods to real-time PCR. The sequences that we chose were species-specific for the identification of DENV. These methods minimized the cost and time for evaluating the assays. Our results showed that the assays that we developed in this study are rapid, sensitive and specific and give an early response in the typing of DENV. Also, we demonstrated that the real-time PCR was a suitable tool for collecting data on the viral load in the serum of DF patients 1–6 days after onset of the symptoms. The viral load and clinical data combined may provide important information on the

pathogenesis of the different dengue-associated syndromes such as DHF and DSS.

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