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Dengue virus infections: comparison of methods for diagnosing the acute disease

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Abstract

Background: The control of dengue depends solely on the control of the insect vector and efficient diagnosis of human cases as no vaccines or specific treatments are currently available. Existing diagnostic methods for suspected clinical cases are complicated by the short duration of viraemia and by serological cross-reactivity with epitopes from other flaviviruses.
Objectives: To evaluate PCR-based tests (nested reverse transcription (RT)-PCR and real-time RT-PCR) for the detection and serotyping of dengue virus and compare the results with those obtained with a widely used immunological test (IgM antibody capture ELISA–MAC-ELISA).
Results and conclusions: The PCR-based methods were more effective in the first few days of infection, whereas the MAC-ELISA became more sensitive 5 or 6 days after disease onset. These results suggest that the best method for dengue diagnosis is a combination of PCR-based and immunological tests. Real-time RT-PCR was more sensitive than the nested RT-PCR approach. Furthermore, it was rapid, reproducible and highly specific, making it a potential method for the diagnosis of dengue fever.
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Keywords: Dengue virus; Diagnosis; Nested RT-PCR; Real-time RT-PCR

1. Introduction

Dengue is the most important arbovirosis in terms of numbers of humans affected. It constitutes a serious public health problem in many subtropical and tropical regions where environmental conditions allow the proliferation of insect vectors. *Aedes aegypti* is the main vector of dengue virus and is present in most countries between latitudes 35°N and 35°S. More than 2.5 billion people living in these areas are at the risk of dengue (World Health Organization, 1997). Dengue virus affects over one hundred million people per year, causing approximately 24,000 deaths (Gubler, 1998). No safe vaccines or specific treatments are currently available.

The hyperendemicity in many regions of the world has increased the occurrence of more severe forms of dengue fever (DHF, dengue hemorrhagic fever and DSS, dengue shock syndrome). In Brazil, the number of dengue virus infections is dramatically increasing every year and recent estimates suggest that more than one million cases occurred in the past 2 years in this country (Fundação Nacional de Saúde, 2003), representing more than 80% of the cases recorded in the America. Three of the four existing serotypes have been found in 23 out of 26 Brazilian States (Fundação Nacional 1de Saúde, 2003).

The control of the disease depends solely on the control of the vector and the availability of diagnostic services in transmission areas. As the symptoms of dengue are very similar to those of other fever-causing illnesses, the availability of dengue-specific laboratory diagnostic tests is of utmost importance. Serological tests, such as MAC-ELISA (IgM antibody-capture enzyme-linked immunosorbent assay), are among the most widely used. Nevertheless, they cannot consistently provide a diagnosis during the acute-phase of the

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illness and they do not identify the dengue virus serotype. Hence, they are mainly useful for the detection of dengue at later times, especially following the 7th day after onset of symptoms, when the immune response, which decreases the viral load, can be effectively detected. The main way of diagnosing dengue during the early stages of infection is to isolate the virus by cell culture or by reverse transcription (RT)-PCR (World Health Organization, 1997). Due to the long time (usually more than 7 days) required for cell culture-based tests, several RT-PCR protocols for dengue diagnosis have been developed in the past decade. These methods might eventually replace the traditional cell culture method as the gold standard for viral detection (Deubel et al., 1990; Harris et al., 1998; Henchal et al., 1991; Lanciotti et al., 1992; Morita et al., 1991; Seah et al., 1995). Very recently, real-time RT-PCR assays for the detection and quantification of dengue virus have also been described (Callahan et al., 2001; Drosten et al., 2002; Houng et al., 2000; Laue et al., 1999; Shu et al., 2003; Wang et al., 2002; Warrilow et al., 2002).

Here, we evaluate and compare a two-step nested RT-PCR protocol reported by Lanciotti et al. (1992), two TaqMan-based serotype-specific real-time RT-PCR assays for the detection of dengue virus serotypes 1 and 2, a SYBR Green I-based serotype-specific assay for the detection of dengue virus serotype 3, a previously published TaqMan-based group-specific real-time RT-PCR able to detect all four dengue serotypes (Drosten et al., 2002), and a MAC-ELISA test.

2. Materials and methods

2.1. Human serum samples

Serum samples were collected from 50 individuals with suspected dengue fever (based on epidemiological and clinical aspects) and from nine healthy individuals (negative controls). Sera were collected between 2001 and 2003 by the Public Health Services of the States of Paraná and Santa Catarina in Brazil. Of the 50 putative positive sera, 23 were collected between 0 and 3 days, 13 between 4 and 6 days and 14 at least 7 days after the onset of symptoms. The nine negative control sera were collected from healthy individuals who had never presented symptoms of dengue infection and had always lived in the city of Curitiba where no dengue epidemic has ever occurred. Three of the nine negative individuals were vaccinated against yellow fever virus around 7 days before blood collection and two were positive for hepatitis C virus (HCV). Paired serum samples (collected at least 7 days apart) from 10 patients from Rio de Janeiro, were also analyzed.

2.2. Preparation of positive controls

Two prototype strains (DEN-1 Hawaii, DEN-2 Jamaica (M20558)) and one local isolate of DEN-3 virus were propagated in *Aedes albopictus* C6/36 cells. Viral titers were determined by the focus-forming assay (Després et al., 1993). Negative human serum samples were spiked with four-fold serial dilutions (from 8.78×10^5 focus-forming units/ml (FFU/ml) to 0.21 FFU/ml) of quantified viruses prior to RNA preparation and used as positive controls and to determine the detection limits of the PCR assays.

2.3. Preparation of RNA

RNA was extracted from 140 μl serum samples and eluted in 60 μl using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions, and stored at −70 °C.

2.4. Oligonucleotide design

To design primers and probes for the serotype-specific assays, the complete genome sequences of dengue virus available in GenBank were aligned using the CLUSTAL X software (Thompson et al., 1997). The most conserved regions were selected, and primers and corresponding probes designed using the Primer Express Sequence Design Software (Applied Biosystems). Primer and probe sequences for the group-specific assay were obtained from Drosten et al. (2002). All the probes were labeled with 5-carboxyfluorescein (FAM) at the 5′ end and with 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA) at the 3′ end. The oligonucleotide sequences are listed in Table 1.

2.5. Real-time RT-PCR

One-step real-time RT-PCR assays were performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Samples were assayed in a 25 μl reaction mixture containing 5 μl of extracted RNA from the sample and the recommended concentration of MultiScribe enzyme plus RNase Inhibitor and TaqMan Universal RT-PCR Master Mix or SYBR Green Master Mix (Applied Biosystems). All reactions contained 200 nM of the specific primers; the TaqMan-based assays contained 300 nM of the corresponding probe. The PCR conditions were as follows: a 30-min RT step at 48 °C, 10-min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. For the SYBR Green I-based assay, a melting curve analysis was performed following the amplification, to ensure that the correct product had been obtained by checking its specific melting temperature (T_m). Melting curve analysis consisted of an incubation in which the temperature is increased from 60 °C to 96 °C at a rate of, approximately, 1 °C/40 s with continuous reading of fluorescence.

2.6. Nested RT-PCR

A two-step nested RT-PCR was performed as described by Lanciotti et al. (1992), except that the origi-

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