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Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR

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Abstract

The use of the polymerase chain reaction (PCR) in molecular diagnosis is now accepted worldwide and has become an essential tool in the research laboratory. In the laboratory, a rapid detection, serotyping and quantitation, one-step real-time RT-PCR assay was developed for dengue virus using TaqMan probes. In this assay, a set of forward and reverse primers were designed targeting the serotype conserved region at the NS5 gene, at the same time flanking a variable region for all four serotypes which were used to design the serotype-specific TaqMan probes. This multiplex one-step RT-PCR assay was evaluated using 376 samples collected during the year 2003. These groups included RNA from prototype dengue virus (1–4), RNA from acute serum from which dengue virus was isolated, RNA from tissue culture supernatants of dengue virus isolated, RNA from seronegative acute samples (which were culture and IgM negative) and RNA from samples of dengue IgM positive sera. The specificity of this assay was also evaluated using a panel of sera which were positive for other common tropical disease agents including herpes simplex virus, cytomegalovirus, measles virus, varicella-zoster virus, rubella virus, mumps virus, WWF, West Nile virus, Japanese encephalitis virus, *S. typhi*, *Legionella, Leptospira, Chlamydia,* and *Mycoplasma.* The sensitivity, specificity and real-time PCR efficiency of this assay were 89.54%, 100% and 91.5%, respectively.

Keywords: Dengue virus; One-step real-time RT-PCR; TaqMan; Quantitation

1. Introduction

Dengue disease is an endemic viral disease affecting tropical and subtropical regions worldwide, predominantly in urban and semi-urban areas. Dengue fever (DF) and its more severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are now important public health problems (Gubler, 1998; Innis, 1995). Dengue disease is endemic in more than 100 countries including Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and Western Pacific, threatening more than 2.5 million people The World Health Organization estimated that there may be 50–100 million cases of dengue disease worldwide per year, which resulted in 250,000–500,000 cases of DHF and 24,000 deaths each year (Gibbons et al., 2002; Halstead, 1988; WHO, 1997).

Dengue is caused by dengue virus, a mosquito-borne flavivirus and which is the most prevalent arbovirus in tropical and subtropical regions of Asia, Africa, and Central and South America (Gubler, 1997). Dengue virus is a positive, single-stranded enveloped RNA virus. The total genome of dengue virus is approximately 11 kb in length (Halstead and O'Rourke, 1977) and the entire genome has been sequenced. The genome is composed of three structural protein genes, encoding the nucleocapsid or core protein (C), a membrane-associated protein (M), an envelope protein (E), and seven nonstructural (NS) protein genes (Deubel et al., 1988). Both ends of the open reading frame are flanked by an un-translated region (UTR). The genome organization is 5′-UTR-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′-UTR, and this order is the same for other flavivirus (Chambers et al., 1990; Leyssen et al., 2000; Rice et al., 1985). The flavivirus genome encodes a polyprotein of about 3000 amino acids, which is co-translationally and post-translationally processed by viral and host proteases (Leyssen et al., 2000; Rice et al., 1985). The

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Table 1a
Samples used for the evaluation of the real-time TaqMan RT-PCR

Category	Den 1	Den 2	Den 3	Den 4	Total
a. Acute serum from which dengue virus was isolated	25	25	25	20	95
b. Tissue culture supernatant of dengue virus	25	25	25	21	96
c. Seronegative acute samples (culture negative), but convalescents samples seroconverted	–	–	–	–	15
d. IgM positive sera	–	–	–	–	100
e. Other disease agents (negative control)	–	–	–	–	70

dengue virus complex consists of four serologically related but antigenically and genetically distinct viruses (DENV-1, -2, -3, and -4) (Nimmannitya, 1987). Infection induces a lifelong protective immunity to the homologous serotype but confers only partial and transient protection against the other three serotypes. It is generally accepted that secondary infection with various dengue virus serotypes is a major risk factor for DHF and DSS due to antibody-dependent enhancement (Burke et al., 1988; Guzman et al., 1990; Halstead et al., 1973). Other risk factors related to DHF–DSS include the virulence of the infecting virus, the age, immune status, and genetic predisposition of the patient (Bravo et al., 1978; Lei et al., 2001).

Infection with dengue virus produces a wide spectrum of clinical features ranging from asymptomatic or non-specific viral syndrome of typical dengue fever (DF), to a severe and fatal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Gubler, 1998; Innis, 1995). Studies have shown that more than 50% of infected individuals are either asymptomatic or have an influenza-like undifferentiated fever (Burke et al., 1988; Endy et al., 2002). Thus diagnosis of dengue virus infection on the basis of clinical syndromes is not reliable, and the diagnosis should be confirmed by laboratory tests. Therefore, there is a need for rapid detection and serotyping of dengue viruses.

In the laboratory, dengue virus infections are diagnosed by the isolation of the virus, direct detection of viral antigen, the detection of specific antibodies and presence of viral RNA (Gibbons et al., 2002; WHO, 1997). Currently, there are two conventional methods that are used to diagnose dengue virus infection. These are virus isolation by cell culture followed by fluorescent-antibody staining and serological diagnosis of antibodies based on capture immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assays (ELISA). Generally, virus isolation is time consuming and takes about 7–10 days, while serological diagnosis can be confusing because of the antibodies that cross-react with other flaviviruses. In contrast, identification of dengue virus by molecular methods such as reverse transcriptase polymerase chain reaction (RT-PCR) is highly indicative of an acute infection in human serum or plasma (Chang et al., 1994; Chow, 1997; Lanciotti et al., 1992). Further, this method identifies different serotypes of dengue virus by a single reaction by multiplex RT-PCR. In addition when used with real-time technology, this assay can be quantitative.

A real-time quantitative RT-PCR system based on the TaqMan technology was developed and is described below.

2. Materials and methods

2.1. Samples collection

A total of 376 samples were collected by the University Malaya Medical Centre (UMMC) in the year 2003 and used to evaluate the sensitivity and specificity of the primers. These samples included (1) acute serum from which dengue virus was isolated ($n = 95$), (2) tissue culture supernatant of dengue virus ($n = 96$), (3) seronegative acute (culture and IgM negative) samples ($n = 15$), (4) IgM positive sera ($n = 100$), and (5) a panel of negative control which consists of serum positive for other microbes ($n = 70$) (Tables 1a and 1b). The capture IgM ELISA of Lam et al. (1987) and the haemagglutination inhibition (Clarke and Cassals, 1958) for IgG antibodies were used to determine the levels of both classes of antibody in all samples.

2.2. Primer and probe design

In contrast to other PCR techniques, the TaqMan system makes use of a fluorescence-labeled probe that has to be digested by the $5' \rightarrow 3'$ exonuclease activity of the DNA polymerase to produce a fluorescence signal. Thus the digestion of an almost complete hybridized probe to the target DNA is essential. Therefore, a highly conserved region of the serotype-specific dengue virus genome must be chosen to allow optimum annealing not

Table 1b
Panel of negative control

Other disease agents	No. of sample
Herpes simplex virus	6
Cytomegalovirus (CMV)	6
Measles virus	6
Varicella-zoster virus (VZV)	6
Rubella virus	6
Mumps virus	6
WWF	6
West Nile virus (WNV)	15
Japanese encephalitis virus (JEV)	4
S. typhi	2
Legionella	1
Leptospira	2
Chlamydia	2
Mycoplasma	2
Total	70

Table shows the 14 groups of other disease agents and the number of the serum samples which were used for the evaluation of this assay.