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Development and evaluation of SYBR Green I-based one-step real-time RT-PCR assay for detection and quantitation of Japanese encephalitis virus

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Abstract

One-step SYBR Green I-based real-time RT-PCR assay for rapid detection as well as quantitation of Japanese encephalitis virus (JEV) in acute-phase patient CSF samples by targeting the NS3 gene was developed. The assay developed in this study was found to be more sensitive as compared to conventional RT-PCR. The specificity of the reported assay system was established through melting curve analysis as well as by cross-reactivity studies with related members of *Flavivirus*. The applicability of Real-time PCR assay for clinical diagnosis was validated with 32 suspected acute-phase CSF samples of Gorakhpur epidemic, India, 2005. The improved sensitivity of real-time RT-PCR was reflected by picking up 10 additional samples with low copy number of template in comparison to conventional RT-PCR. The quantitation of the viral load in acute-phase CSF samples was done using a standard curve obtained by plotting cycle threshold (C_t) values versus copy numbers of the RNA template. This is the first report on the application of real-time RT-PCR for detection as well as quantitation of JEV from patient CSF samples. These findings demonstrate the potential clinical application of the reported assay as a sensitive diagnostic test for rapid and real-time detection and quantitation of JEV in acute-phase clinical samples.

Keywords: SYBR Green I; Real-time PCR; Japanese encephalitis virus

1. Introduction

The Japanese encephalitis virus is the single largest cause of viral encephalitis in the world with an estimated 50,000 cases and 10,000 deaths annually. JEV a mosquito-borne virus belonging to the genus *Flavivirus* of the family *Flaviviridae*, is closely related to West Nile (WN) virus, Dengue (DEN) virus, St. Louis encephalitis (SLE) virus and Murray Valley encephalitis (MVE) virus (Monath and Hinz, 1996; Murphy et al., 1995). In nature, JEV is maintained through a transmission cycle primarily involving *Culex* species mosquitoes and wild or domestic animals, birds, and human beings (Rosen, 1987). The JEV is an enveloped virus containing a single-stranded positive-sense RNA genome of approximately 11,000 nucleotides. The genome has a single open reading frame, which encodes three structural proteins [capsid (C), pre-membrane (prM) or membrane (M), and envelop (E)] and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) between the 5′-nontranslated region (NTR) and 3′-NTR (Sumiyoshi et al., 1987).

JEV infection in humans can cause severe central nervous system disorders including high fever, headache, aseptic meningitis and encephalitis. Humans are incidental host, infected when living or passing in close proximity to this enzootic cycle. Hence, most infections of human occur in rural tropical areas, where facilities for diagnosis are limited. In the last 50 years, the epidemiology of JEV has changed. While mass vaccination campaigns have been associated with a decrease in the number of encephalitis cases in Japan, Taiwan, and South Korea, the geographical area affected by the virus has expanded to include the Indian subcontinent, China, Southeast Asia, and the Western Pacific region. The reasons for this expansion are incompletely understood, but increasing irrigation and animal husbandry favoring breeding of the *Culex* mosquito vector are thought to be important. Approximately 2.8 billion people live in

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this vast geographical area, and JE is likely to remain an important public health problem in the 21st century. The post-monsoon outbreak of JE is a common feature and outbreaks have been regularly reported from most parts of India since the mid-1950s (Banerjee, 1996). The geographical expansion of JEV ranges from the East (Calcutta, West Bengal), North (Uttar Pradesh) to South (Vellore, Tamil Nadu) of the Indian subcontinent. The virus has already established endemicity in different pockets of the country especially in Karim Nagar, Andhra Pradesh (South) and Gorakhpur, Uttar Pradesh (North) of India (Kabilan et al., 2004; Reuben and Gajanana, 1997).

The laboratory diagnosis routinely used for JEV infection is based on four basic types of assays: serology, virus isolation, immunocytochemistry, and molecular techniques. Serologically, JEV infection can be detected by immunoglobulin M (IgM) and IgG capture enzyme-linked immunosorbent assay (ELISA) (Burke et al., 1982; Endy and Nisalak, 2002; Solomon et al., 1998). However, confirmation and typing of virus are based on demonstration of fourfold or greater increase in the virus specific neutralizing antibody titer by plaque reduction neutralization (PRNT) assay with several flaviviruses. Virus isolation from clinical and surveillance samples has generally been unsuccessful, owing to the low level of transient viremia associated with the disease process, and also requires viable virus in samples.

However, all the above techniques are time consuming, labor intensive and are often cumbersome to adopt for routine clinical use. Although isolation of the virus from samples is essential to make a definitive diagnosis, recently RT-PCR has been used to detect *flavivirus* genome in a rapid and specific test (Eldadah et al., 1991; Igarashi et al., 1994; Kuno, 1998; Meiyu et al., 1997; Paranjpe and Banerjee, 1998; Parida et al., 2006a; Puri et al., 1994; Tanaka, 1993). Two-step RT-PCR assay requires agarose gel analysis for the detection of amplicons after PCR cycling. So, the assay is labor-intensive and has a very high risk of contamination. Recently, in addition to conventional RT-PCR, more rapid and sensitive real-time PCR-based assays, such as TaqMan RT-PCR, nucleic acid sequence-based amplification (NASBA), reverse transcription loop mediated isothermal amplification (RT-LAMP) and branched DNA methods, have been reported and are currently under extensive evaluation with human and field mosquito samples (Chan and Fox, 1999; Dong-Kun et al., 2004; Huang et al., 2004; Igarashi, 1978; Parida et al., 2006b; Pyke et al., 2004; Shirato et al., 2005).

The real-time PCR assay has many advantages over conventional RT-PCR methods, including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity, and easy standardization. Thus, nucleic acid-based assays or real-time quantitative assay might eventually replace virus isolation and conventional RT-PCR as the new gold standard for the rapid diagnosis of virus infection in the acute-phase serum samples. In the present study, we report the development of a SYBR Green I-based real-time RT-PCR assay for rapid and real-time detection of JE virus. The data on the sensitivity, specificity of the method is reported, and applicability of the assay for clinical diagnosis of JE patients is validated with acute-phase CSF samples collected during Gorakhpur epidemic, 2005.

2. Methods

2.1. Designing of primers

The oligonucleotide primers used for real-time amplification of JEV were designed from the nonstructural (NS3) gene. Nucleotide sequence of the NS3 gene of the strain JaOArS982 was retrieved from GenBank (accession no. M18370) and was aligned with the available NS3 gene sequences of other strains of JEV (AB269326, AY508813, AF486638, AB1969242, AF416457) including the circulating strains in India responsible for recent epidemics to identify the conserved regions using Clustal W programme available in the Laser gene 5 package (DNAStar, USA). Potential target regions were selected, and primers were synthesized and evaluated for use in quantitative real-time RT-PCR. Nucleotide sequence of the JEV specific primer pair and the characteristics of the amplicon are shown in Table 1.

2.2. Viruses

Japanese encephalitis virus (strain JaOArS982) was used for preparation of antigen to be used as a positive standard in the assay system. For this purpose, the infected culture supernatant of $C_{6/36}$ cell culture adapted virus was used (Gould and Clegg, 1985; Igarashi, 1978). In addition, the four dengue virus serotypes (DEN-1, Hawaii; DEN-2, ThNH7/93; DEN-3, PhMH-J1-97; DEN-4, SLMC 318), West Nile (WN) virus strains (Eg 101) and St. Louis encephalitis (SLE) virus (Parton strain) were also used in the present study for checking the cross-reactivity.

2.3. Clinical samples

The CSF samples used in this study were collected from patients with suspected JE virus infections during an outbreak in Gorakhpur, UP, India 2005. The acute-phase CSF samples collected between days 1 and 7 after the onset of symptoms were

Table 1
Primer sequences and the characteristics of JEV amplicon generated by SYBR Green I-based real-time RT-PCR assay

Primer	Sequences (5′–3′)	Genomic region[a]	Amplicon size (bp)	Avg T_m (observed range) (°C)
JEF	5′AGA GCG GGG AAA AAG GTC AT3′	5739–5758 (NS3)	162	80.7 (80.3–81.1)
JER	5′TTT CAC GCT CTT TCT ACA GT 3′	5900–5881 (NS3)		

[a] Prototype strain JaOArS982 (GenBank accession no. M18370).