Journal of Virological Methods 158 (2009) 165–170

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Cloning and expression of domain III of the envelope gene of Japanese encephalitis virus: Evaluation for early clinical diagnosis by IgM ELISA

Jyoti Shukla, Rakesh Bhargava, Paban Kumar Dash, Manmohan Parida*, Nagesh Tripathi, P.V. Lakshmana Rao

Division of Virology, Defence Research and Development Establishment, Gwalior 474002, India

Article history:
Received 26 September 2008
Received in revised form 6 February 2009
Accepted 10 February 2009
Available online 27 February 2009

Keywords:
Japanese encephalitis
Recombinant IgM ELISA
Clinical diagnosis
Indirect ELISA

A B S T R A C T

Japanese encephalitis virus (JEV) is the single largest cause of childhood viral encephalitis in the world with an estimated 50,000 cases and 10,000 deaths annually. The laboratory diagnosis is based essentially on IgM ELISA owing to low transient viremia making virus isolation difficult. In addition the requirement of cerebrospinal fluid (CSF) sample for confirmatory molecular diagnosis by reverse transcription-polymerase chain reaction (RT-PCR) makes IgM ELISA the test of choice for early clinical diagnosis. The development and evaluation of a highly sensitive and specific IgM ELISA using the recombinant domain III envelope protein (rJEV-DIII) for rapid, early and accurate diagnosis of JEV is reported in the present study. The gene coding for the envelope protein of JEV was cloned and expressed in pET 30a vector followed by purification of recombinant protein by affinity chromatography. An indirect IgM microplate ELISA using purified rJEV-DIII was optimized that had no reactivity with healthy persons. The comparative evaluation accomplished with the JE-Dengue IgM Combo ELISA (PanBio, Brisbane, Australia) and JEV Chex (XCyton Diagnostic Ltd., Bangalore, India) ELISA kits, by subjecting 120 acute phase of clinical samples revealed more than 95% accordance. The rJEV-DIII ELISA and the PanBio ELISA were found to have a sensitivity and specificity of 98% and 96%, respectively. The compared positivity of the rJEV-DIII ELISA and SYBR green-I based real-time RT-PCR assay in CSF samples revealed higher positivity. The specificity of this assay was confirmed with serum samples obtained from patients with dengue and chikungunya. The recombinant domain III envelope protein based JEV specific ELISA will be useful for rapid screening of large numbers of clinical samples in endemic areas during outbreaks.

1. Introduction

Japanese encephalitis virus (JEV) is an arthropod-borne virus that belongs to the genus *Flavivirus* family *Flaviviridae* (Murphy et al., 1995). JEV is maintained in nature through a transmission cycle involving primarily *Culex* species of mosquitoes and wild or domestic animals, and human beings as incidental hosts (Rosen, 1987). During the last 50 years, the epidemiology of JEV has undergone change. While mass vaccination campaign has resulted in a decrease in the number of encephalitis cases in Japan, Taiwan, and South Korea, the geographic areas affected by the virus has disseminated to include the Indian subcontinent, China, Southeast Asia, and the Western Pacific region. Although the reasons for virus proliferation are not understood fully. They may be attributed to the increasing irrigation and animal husbandry practices that in turn favour breeding of the *Culex* mosquito vector. Approximately 2.8 billion people inhabit this vast geographical area, where JE is likely to remain an important public health problem in the 21st century (WHO, 2005).

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′ non-translated region (NTR) and 3′ NTR (Chambers et al., 1990; Monath et al., 1996; Rauscher et al., 1997). The viral envelope protein, serves as the cell receptor binding protein and the fusion protein for viral attachment and entry into the host. It is a major antigen responsible for eliciting neutralizing antibody response and protective immunity in hosts (Konishi et al., 1999; McMinn, 1997). The E protein is a homodimer containing three structurally distinct domains: a central β-barrel (domain I), an elongated dimerization region (domain II) and a C-terminal immunoglobulin (Ig)-like module (domain III) (Rey et al., 1995).

* Corresponding author. Tel.: +91 751 2233495; fax: +91 751 2351148.
E-mail address: paridamm@rediffmail.com (M. Parida).

0166-0934/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2009.02.018

In humans, JEV infection can cause severe central nervous system disorders including febrile headache, aseptic meningitis and encephalitis. Among 35,000–50,000 annual cases of JEV, about 10,000 cases are fatal, and a high proportion of survivors sustain serious neurological and psychiatric sequelae (Burke and Monath, 2001; Tsai, 1998). Humans are an incidental host, and they may be infected when living or passing in close proximity to the enzootic zone. Hence, most human infections occur in rural tropical areas, where facilities for diagnosis are limited.

The routine laboratory diagnosis of JEV infection is based on culture and serology followed by identification of the viral genome by reverse transcription-polymerase chain reaction (RT-PCR). Virus isolation which is the "gold standard" has generally been unsuccessful, owing to the low level of transient viraemia associated with the disease process, that in turn requires viable virus in samples. Serologically, JEV infection can be detected by immunoglobulin M (IgM) and IgG capture enzyme-linked immunosorbent assay (ELISA). However, confirmation and typing of virus require demonstration of fourfold or greater increase in the virus specific neutralizing antibody titre by plaque reduction neutralization (PRNT) assays as undertaken with several flaviviruses. Nevertheless both virus isolation and PRNT assays are time consuming and tedious, requiring more than a week for overall completion (Burke et al., 1982; Solomon et al., 1998).

In absence of culture isolation, RT-PCR is the method of choice for early confirmatory diagnosis with acute phase clinical samples. In addition to conventional RT-PCR, there are more rapid, sensitive and real-time PCR based assays such as the TaqMan RT-PCR and loop mediated isothermal amplification (LAMP) methods have been reported which are currently under evaluation with clinical samples (Toriniwa and Komiya, 2006; Parida et al., 2006a). However, the adores and PCR based methods exhibit several intrinsic disadvantages, requiring either a high precision instrument for amplification or else an elaborate complicated method for detection of amplified products. These intricacies jeopardize its routine use in diagnostic laboratories. Also serum is not the ideal sample for the detection of JEV genome. Therefore, the requirement of a CSF sample in indispensable for confirmatory molecular diagnosis by RT-PCR via the IgM ELISA thus making it the only test of choice for early clinical diagnosis.

Most of the JEV specific IgM ELISA test systems are based on the capture principle and rely on the use of whole virus antigens (Ravi et al., 2006). However, JEV is a slow growing and fastidious arbovirus making it difficult to produce it in bulk in culture to sufficient titre. Cross-reactivity among *Flaviviruses* may be a major shortcoming with regard to the IgM ELISA used currently for routine investigation. The only commercial kit, based on recombinant protein IgM ELISA, is the PanBio JE-Dengue IgM Combo ELISA (PanBio, Brisbane, Australia), that can be exploited for the detection of both dengue and JE virus. However, these commercial kits have undergone several modifications and consequently withdrawn time and again from the market that include high cost and reported cross-reactivity. Therefore it is essential to develop a simple, safe and cost effective recombinant protein based IgM ELISA for detection of JE infection at early stages with high degree of sensitivity and specificity.

The development and evaluation of a highly sensitive and specific recombinant protein based JEV specific IgM ELISA, by targeting the highly immunogenic domain III of envelope gene of JEV (rJEV-DIII) has been reported. The gene was expressed in *Escherichia coli* and purified in a single step, yielded 25 mg of pure protein per liter culture. An indirect microplate ELISA using this rJEV-DIII protein was standardized to detect anti-JEV IgM antibodies and evaluated with a panel of 120 suspected clinical samples. Cross-reactivity was also monitored with serum samples from patients with dengue and chikungunya. The ELISA results were compared with a PanBio ELISA kit (PanBio, Brisbane, Australia) in addition to native virus antigen based JEV Chex test kit (XCyton Diagnostic Ltd., Bangalore, India) along with real-time RT-PCR. The sensitivity, specificity and applicability of this rJEV-DIII based IgM ELISA for clinical diagnosis of JEV infection with acute phase of patient serum and CSF samples have been emphasized.

2. Methods

2.1. Materials

Japanese encephalitis virus (JaOArS982 strain) was obtained from Institute of Tropical Medicine (ITM), Nagasaki, and propagated in C6/36 cells and was used in this study. *Escherichia coli* host strain BL21 (DE3) and the plasmid vector pET 30a were obtained from Novagen (Madison, WI, USA). Luria Bertani (LB) for bacterial media preparation from Difco laboratories, Detroit, Michigan; Ni-NTA super flow resin, viral RNA mini kit, and anti-His antibody from Qiagen, Hilden, Germany. Access quick one-step RT-PCR kit (Promega, Madison, WI, USA) and TOPO-TA Vector system (Invitrogen, Carlsbad, USA). Prestained protein ladder and restriction enzymes were from MBI Fermentas, Hanover, USA. Brilliant SYBR green single step QRT-PCR Master Mix' (Stratagene, Cedar Creek, Texas). Isopropyl β-D-thiogalactoside (IPTG), diaminobenzidine (DAB), Tetra methyl benzidine (TMB), 30% Hydrogen peroxide (H_2O_2), Kanamycin and all secondary antibody-enzyme conjugates (anti-mouse, anti-rabbit and anti-human horseradish peroxidase) were from Sigma Chemical, St. Louis, MO, USA. JE-Dengue IgM Combo ELISA kit (PanBio, Brisbane, Australia) and JEV Chex (XCyton diagnostic Ltd, Bangalore, India). Amicon ultra centrifugal filter devices from Millipore (Bedford, USA). The centrifuge, model Sorvall Evolution RC was from Kendro Laboratory Products (Newtown, CT, USA). The ultrasonic homogenizer Vibracell VCX 750 model was from Sonics (Newtown, CT, USA). Automated DNA sequencer, model ABI 310 was from Applied Biosystems (Foster City, CA, USA). MX 3000P quantitative PCR system (Stratagene, Cedar Creek, Texas).

2.2. Cloning and expression in pET30a vector

Viral RNA was extracted from JE virus (JaOArS982 strain) using QIAamp viral RNA Mini Kit, in accordance with the manufacturer's instructions. The genomic region coding for domain III of envelope protein was amplified using a set of primers [JE-F 5′ CAT ATG GAC AAA CTG GCC CTG 3′ (Nde I) and JE-R 5′ CTC GAG GCT TCC AGC TTT GTG CC 3′ (Xho I)]. The purified PCR product (rJEV-DIII gene) was cloned into the pCR4-TOPO cloning vector by using TOPO-TA cloning kit as per manufacturer's protocol. This was then subcloned in pET30a vector expression vector and transformed into *E. coli* BL21 (DE3) cells. The positive clone was confirmed by nucleotide sequencing.

2.3. Expression profile and localization of recombinant protein

The logarithmic phase cultures were induced with different conc. of IPTG (0.5, 1, 1.5 and 2 mM) and checked at hourly interval for a period of 5 h. Un-induced recombinant clone and normal BL-21 cells were kept along side as controls. Following induction, cells were lysed in 1× lysis buffer and analyzed by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). In order to identify the localization of recombinant protein the cell suspension was sonicated and the resultant cell lysate later was centrifuged at 18,600 × *g* for 30 min at 4 °C. The clear supernatant and remaining pellet were collected separately and then analyzed on 12% SDS-PAGE.