

Molecular Diagnostic Studies of subjects from Dhemaji district of Assam in Lieu of Determination of frequency of Japanese Encephalitis (JE)

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ABSTRACT :

In the present investigation, total of 156 samples (112 blood samples and 44 CSF samples) were collected for investigation of JE. Amongst them, 53 samples were found to be JE positive; 81 samples were found to be negative and 22 samples were categorized in equivocal category (may turn into positive with progression of time). As per IgM Elisa results, in 53 positive JE samples, 7 were JE positive Vaccinated males, 8 were positive vaccinated females, 16 were JE positive non-vaccinated males, 15 were JE positive non vaccinated females, 1 male was JE positive having unknown vaccination status and 6 females were found to be JE positive with unknown vaccination status. It was found that JE positive patients were dominant from age levels 0-40 years. The results of DNA bands of RT-PCR of positive, negative and equivocal samples were found to be in correlation with that of IgM Elisa results. It was observed that, positive samples showed that prominent DNA bands in comparison to latent bands in equivocal samples. There was no DNA band observed in negative samples.

Keywords: JEV, Dhemaji District of Assam; vaccinated and non-vaccinated, Elisa, molecular biology

INTRODUCTION:

Amongst the flaviviruses, Japanese encephalitis virus (JEV) is one of the major causes of encephalitis in India and Southeast Asia [1-3]. JEV is endemic in many parts of Asia, and a higher incidence of cases has been reported in children than in adults. Estimated 35,000–50,000 clinical cases and 10,000 deaths caused by JE encephalitis are reported annually in Asia. [4] In India, flavi-viruses including JEV are detected in many parts of the country. Thus far, JE activity has been reported from 24 states/Union Territories in India [5-7] genetically, depending on 12% divergence in the C-prM genomic region, JEVs have been classified into four genotypes. Indian JEVs have been classified as genotype III, which is further divided into two distinct genetic clusters diverged by 6–7% from each other. [8, 9]. Circulation of multiple genetic variants in the same geographic region has also been reported. Recently, introduction of newer genotypes and co-circulation of different genotypes in the same geographic areas

of Vietnam and Australia have been reported [10, 11]. These reports alarm a necessity to monitor genetic variations and introduction of newer strains even in JE-endemic areas. The majority of JEV isolates from humans have been obtained from cerebrospinal fluid (CSF) or brain tissue of fatal cases. However, in remote areas lacking the expertise necessary to drain the CSF or to obtain brain tissues, these specimens are not available for further study. Serum and CSF are the preferred specimens for serologic diagnosis of JEV infection. JEV has a peripheral multiplication cycle before it infects the brain. Thus, by the time symptoms of encephalitis ensue, immune response already sets in, making it difficult to isolate virus from serum. There are few JEV isolates from peripheral blood, leading to the belief that there is no viremia in JE patients during the encephalitis phase [12]. It is known that flavi-viruses including JEV multiply in monocytes and phytohemagglutinin (PHA)-stimulated peripheral blood

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mononuclear cells (PBMCs) [13]. Initial amplification of the virus in PBMC co-culture might be helpful for virus isolation from IgM antibody-positive JE patients. Because the volume of blood that can be collected from the pediatric age group is low, blood clots offered an easier source of white blood cells (WBCs) that could harbor the virus. This PBMC amplification method can be exploited for virus isolation. Complete characterization of the virus by various immunologic and molecular techniques can only be achieved after successful isolation. The JEV genome consists of a single-stranded positive sense RNA, approximately 11 kb in length. The open reading frame (ORF) encodes a large polyprotein that is cleaved into at least 10 proteins. The N-terminal region of the polyprotein encodes the structural proteins (C-prM-E), followed by the nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4-NS5) [14]. JEV is transmitted to susceptible reservoirs by arthropods. The natural cycle of JEV is maintained with the involvement of mosquitoes, especially *Culex tritaeniorhynchus*. Pigs, horses, birds, and bats are the primary natural hosts of JEV, and humans are the dead-end host. Based on the nucleotide sequences of the C/prM and envelope (E) protein genes, JEV is classified into five genotypes and the cut-off value for the nucleotide differences between each genotype is 12% [15]. Monitoring for the presence of JEV in mosquitoes can be used to estimate levels of potential JEV exposure, intensity of viral activity, and genetic variation of JEV throughout surveyed areas. The present study was thus performed in order to determine the comparative differences between the positive and equivocal patients (expected) of Japanese encephalitis (JE) on the basis of their genomic DNA.

MATERIALS AND METHODS

In total population of 7, 13, 178 people in Dhemaji district of Assam, India, the vaccination programme to control Japanese Encephalitis (JE) was conducted in which, 3, 03, 038 adults and 1, 76, 28 children were vaccinated. The range of age distribution was from 0 to above 51 years. The study was conducted from July, 2013 to April, 2015.

Clinical specimens

These studies were concentrated in Dhemaji district of Assam, India. Patients were hospitalized in the encephalitis ward of the district hospital constituted the study population for this study. High-grade fever, headache, vomiting, chronic convulsions, altered sensorium, and a progressive comatose condition characterized the clinical presentations of hospitalized patients in the acute phase of illness. Additionally, a few patients featured hemiparesis and neck rigidity. Cerebral malaria was ruled out by differential clinical and microscopic analysis by the local investigators.

Operative case definition of acute fever along with altered sensorium was considered as suspected encephalitis during this outbreak study. Informed consent from patients or their parents or guardians was obtained, and human experimentation guidelines of the Indian Council of Medical Research, New Delhi, were strictly followed during the study. Blood samples by venipuncture were collected from patients with suspected encephalitis presentation. The blood was collected in sterile gel-lined vacutainers (Becton Dickinson, US). Serum was separated from the clot by the gel and stored for serologic studies. The 112 blood samples and 44 CSF samples were collected from clinically suspected encephalitis patients (referred by the physicians of encephalitis ward) and were transported on ice to the laboratory for further study.

IgM capture ELISA

The CSF and sera collected from suspected encephalitis patients were tested by IgM capture ELISA for JE at IgM antibodies against JE viruses were detected by standard ELISA. The studies were conducted at District Public Health Laboratory, North Lakhimpur Civil Hospital, Saboti, Lakhimpur, Assam, India. Briefly, IgM from patient CSF and sera were captured on anti-human IgM-coated wells, and JE virus antigen was added overnight at 4°C. The captured antigens were probed with biotin-labeled flavi-viruses cross-reactive monoclonal antibody (MAb) HX-2 and avidin horseradish peroxidase. Hydrogen peroxide and O-phenylene diamine (OPD) were used as substrate and chromogen, respectively. Reaction was terminated using 4 N H₂SO₄ and read at 492 nm. The test included known strong- and weak positive samples as positive controls, and JEV IgM-negative samples were used as negative controls. Specimens giving a signal to noise (S/N) ratio of > 2 were treated as positives.

RNA extraction, real-time RT-PCR and conventional RT-PCR

The genomic RNAs were extracted from the infected cell culture fluids and CSF specimens with the QIAamp Viral RNA Mini Kit (Qiagen Sciences, Germantown, MD, USA), according to the manufacturer's instructions. To detect and differentiate GI and GIII directly in the CSF specimens, the total RNA was amplified with the Superscript® III Platinum® One-Step qRT-PCR Kit (Life Technologies, Carlsbad, CA, USA) and genotype-specific primer sets for GI (JE-E1-2140 F 5'- GGGGACAAGCAGATTAACCA-3'; JE-E1-2325R 5'-GAAGGCACCAC CAAACACTT-3'; and JE-E1-2200Probe 6-FAM-TCAACAACCTT-GAAAGGGGC-TAMRA) and GIII (JE-E3- 1978 F 5'- CCTTGCAAATTCGGATTGT -3', JEE3-2222R 5' TGAGCTCCCTTCAAAGTCGT - 3', and JE- E3-2038Probe: 6-FAM CTGGTGACAGTGAACCCC

TT-TAMRA) [15]. To amplify the E gene of JEV for sequencing and the phylogenetic analysis of nine JEV strains, conventional RT-PCR was performed with the Qiagen One Step RT-PCR Kit (Qiagen GmbH, Hilden, Germany), with a primer set specific for the E gene [15].

RESULTS AND DISCUSSION

In the present investigation, total of 156 samples (112 blood samples and 44 CSF samples) were collected for investigation of JE. Amongst them, 53 samples were found to be JE positive; 81 samples were found to be negative and 22 samples were categorized in equivocal category (may turn into positive with progression of time).

As per IgM Elisa results, in 53 positive JE samples, 7 were JE positive Vaccinated males, 8 were positive

vaccinated females, 16 were JE positive non-vaccinated males, 15 were JE positive non vaccinated females, 1 male was JE positive having unknown vaccination status and 6 females were found to be JE positive with unknown vaccination status. It was found that JE positive patients were dominant from age levels 0-40 years. The results are shown in **Table 1** and **Figure 1**. The results of DNA bands of RT-PCR of positive, negative and equivocal samples were found to be in correlation with that of IgM Elisa results. It was observed that, positive samples showed that prominent DNA bands in comparison to latent bands in equivocal samples. There was no DNA band observed in negative samples. The results are shown in **Figure 2**.

0-10 Years		11-20 Years		21-30 Years		31-40 Years		41-50 Years		Above 51 Years	
Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
19	22	3	6	1	0	1	1	0	0	0	0

Table 1: JE positive subjects age Distribution by IgM ELISA

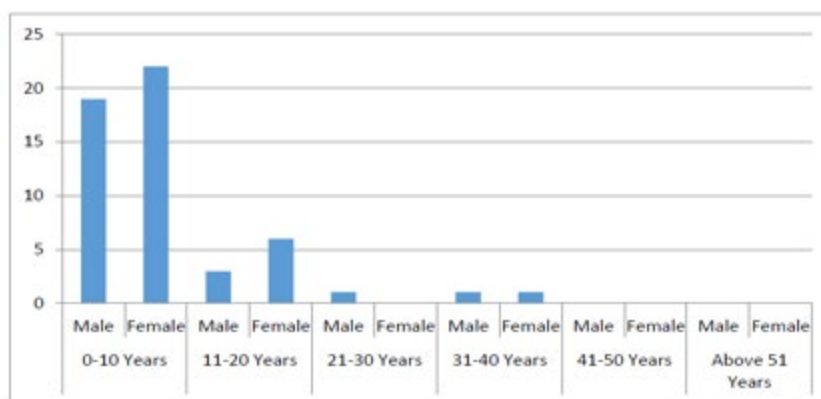


Figure 1: JE positive subjects age Distribution by IgM ELISA

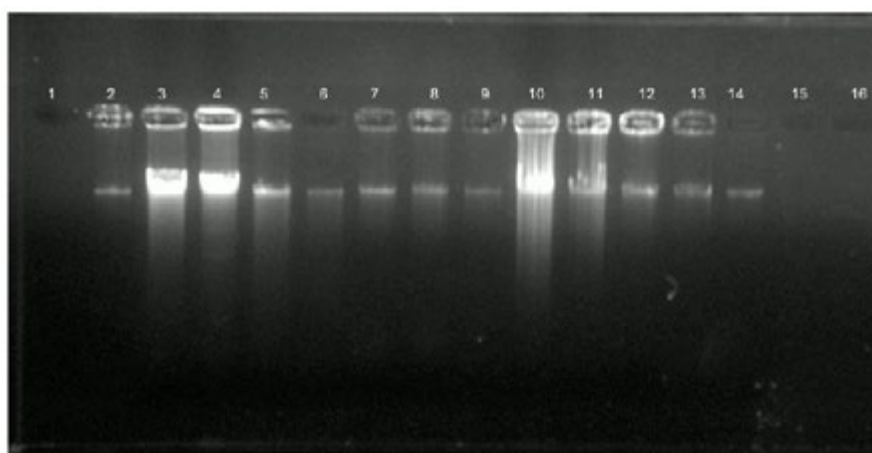


Figure 2: Bands of DNA observed after RT- PCR

(*Sample No. 3,4,10 and 11 were found to be JE-positive; sample no. 2,5,6,7,8,9, 12,13 and 14 were JE-equivocal; sample no. 1,15 and 16 were JE-negative)

A method of JEV isolation directly from WBCs from patient blood clots was successfully attempted, and the isolates were partially characterized to establish their antigenic and genetic relatedness with currently circulating JEV strains in India. Isolation of the virus from clinical specimens such as CSF and brain tissues is ideal for confirmation of the etiologic agent in encephalitis. If successful, the virus isolate can be used further to monitor genetic and antigenic variation introduced in the newer strain. In the case of JE encephalitis, virus isolation from serum or CSF specimens has always been difficult because of the presence of neutralizing antibodies elicited against the virus. The majority of the JEV isolations are reported from mosquitoes or human brain tissues. As per our knowledge, few virus isolation attempts from serum have been successful, and thus only CSF has been recommended for isolation against serum. In addition, it is not always possible to obtain CSF and tissue specimens at all or in sufficient quantities to carry out the diagnostic assays and isolation. Lack of expertise necessary to obtain these specimens in remote and rural areas makes isolation attempts more difficult.

CONCLUSION

In the present study, Japanese Encephalitis Virus (JEV) positive subjects were screened and determined as per ELISA and molecular biology results. The results of IgM Elisa were found to be in accordance with that of molecular biology results. On agarose gel, after performing RT-PCR, the prominent band of DNA was observed on agarose gel in the samples of positive subjects, while latent band of DNA was observed from equivocal subjects. The presence of latent band confirmed the confirmation of infection of Japanese Encephalitis (JE) at later stages of life. No band of DNA appeared in JE-negative subjects. It is hereby concluded that, the infection of JEV in the people can be determined at molecular level in the form of DNA and thus later in the form of protein. Further studies are however needed in order to determine the effect of JEV on the genes within DNA.

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