Detection and Isolation of Japanese Encephalitis Virus From Blood Clots Collected During the Acute Phase of Infection

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Abstract. Clinical specimens from an encephalitis outbreak in the Lakhimpur area of Uttar Pradesh, India, were investigated for identification and characterization of the etiologic agent. IgM capture ELISA showed recent Japanese encephalitis virus (JEV) infection. JEV isolation was attempted from white blood cells (WBCs) separated from blood clots of 12 patients (9 IgM positive and 3 negative) by serial co-culturing with phytohemagglutinin P–stimulated peripheral blood mononuclear leukocytes (PBMCs) obtained from pre-screened JEV sero-negative healthy individuals. JEV was isolated from two IgM-positive blood clots. Isolate 014178 was detected in WBCs and in the first passage of PBMCs by ELISA and reverse transcriptase-polymerase chain reaction. Isolate 014173 was detectable only after a second passage in PBMC co-culture. Sequence analysis of 346 nt of the C-prM region showed homology with JEV strain GP78. This is the first report on isolation of JEV from patient blood clots. Our study shows that the co-cultures of PBMCs separated from patient blood clots provide an additional source for JEV isolation.

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. An estimated 35,000–50,000 clinical cases and 10,000 deaths caused by JE encephalitis are reported annually in Asia.4 In India, flaviviruses 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.8–9 Indian JEVs have been classified as genotype III, which is further divided into two distinct genetic clusters diverged by 6–7% from each other. 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 tissue, 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 flaviviruses including JEV multiply in monocytes and phytohemagglutinin (PHA)-stimulated peripheral blood 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.

During this work, co-culturing with PBMCs successfully isolated JEV from WBCs of patient blood clots, and the JEV was further amplified in infant mice. The virus isolate was further characterized by serologic and genetic methods.

MATERIALS AND METHODS

Clinical specimens. These studies concentrated on the Lakhimpur-Khiri and Baharaich districts of Uttar Pradesh State in India bordering Nepal (latitude, 27.34 N; longitude, 81.38 E). Patients hospitalized in the encephalitis ward of the district hospital constituted the study population for this study. High-grade fever, headache, vomiting, tonic-clonic 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 114 blood samples and 44 CSF samples collected from clinically suspected encephalitis patients (referred by the physicians of encephalitis ward) 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 and West Nile (WN) viruses at NIV, Pune. IgM
antibodies against JE and WN viruses were detected by standard ELISA. Briefly, IgM from patient CSF and sera were captured on anti-human IgM-coated wells, and JE or WN virus antigen was added overnight at 4°C. The captured antigens were probed with biotin-labeled flavivirus cross-reactive monoclonal antibody (MAb) HX-2 and avidin horseradish peroxidase. Hydrogen peroxide and O-phenylenediamine (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.

**Virus isolation.** The NIV Animal Ethical Committee approved the animal experiments, and the guidelines were strictly followed. As a routine practice, 12 CSF samples available in adequate quantity were processed for virus isolation by intracranial (i.c.) inoculation in a group (eight mice) of 2-day-old infant mice (Swiss). The mice were monitored for sickness up to 1 week. The brains of inoculated mice were harvested, and 20% suspensions in bovine albumin in phosphate-buffered saline (BAPS) were blindly passaged twice in infant mice for virus isolation. The harvested mice brains from every passage were monitored for virus amplification by reverse transcriptase-polymerase chain reaction (RT-PCR) and antigen capture ELISA.

WBCs from 13 (12 patients and 1 healthy control [014205]) blood clots were separated by lysing the red blood cells (RBCs) using ice-cold lysing solution (phosphate-buffered 0.15 M ammonium chloride and 0.1% glucose) and washed three times with cold Dulbecco modified minimal essential medium (DMEM; GIBCO BRL, US). PBMCs from healthy, unfected individuals were isolated on Ficoll-Hypaque gradient and stimulated with 1 μg/mL PHA-P (Sigma, St Louis, MO) in DMEM with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂ overnight. One million WBCs, separated from clots under study, were mixed with two million stimulated PBMCs and co-cultured for 3 days in six-well plates at 37°C. The virus was passaged three times. Antigen capture ELISA was carried out using supernatant of cultures that showed a cytopathic effect (CPE). Twenty microliters of supernatant of the cultures giving positive RT-PCR for JEV and showing an S/N ratio of ≥2 was inoculated intracerebrally in 2-day-old Swiss albino mice (eight mice), and sickness was observed for 14 days. The brains of mice showing sickness were harvested, and 20 μL of the 20% BAPS suspensions were passaged in infant mice by i.c. route for virus stock preparation. The mouse brain stocks were stored at −80°C until use.

**Antigen capture ELISA.** JEV antigens present in PBMC culture supernatants and mouse brain suspensions were captured on ELISA wells coated with JEV-specific MAb Hs-2 by incubating overnight at 4°C. The captured antigens were detected as described above. JEV-infected tissue culture fluid (TCF) and mouse brains were used as positive controls, whereas uninfected TCF and mouse brains served as negative controls. The cultures showing an S/N ratio ≥2 were considered positive for the JEV antigen.

**Virus neutralization assay.** Porcine stable (PS) kidney cell-adapted virus pools were used for plaque titration in 24-well plates using 0.5% carboxymethylcellulose (CMC; Sigma) overlay. An in vitro neutralization test was performed by incubating two-fold serially diluted JE and WN virus-specific monoclonal or polyclonal antibodies with 100 TCID₅₀ (tissue culture infective dose) of the test virus for 1 hour at 37°C with 5% CO₂. The test virus–antibody mixture was added on a pre-formed monolayer of PS cells in 96-well plates and incubated at 37°C with 5% CO₂ for 72 hours. Controls included virus titration without antibody and with normal non-immune serum. Neutralizing titer of MAb was expressed as the reciprocal of the dilution at which 50% of virus added was neutralized.

**RNA isolation, RT-PCR, and sequencing.** The blood clot-derived WBCs suspended in DMEM, PBMC co-cultured cells, and infected mouse brains were used for RNA preparation. Viral RNA from the WBCs and PBMC co-cultured cells was isolated and purified using QIAamp Viral Mini Kit reagent (QIAGEN) according to manufacturer’s instructions. RNA from infected mouse brains was isolated using Trizol reagent (Invitrogen, Life Technologies, US) according to the manufacturer’s instructions. JEV-specific forward primer 391-GCAGAAAGCAAAAAACAAAAAGAG and reverse primer 757-ACGGATCTCCTGGTTCCGTGG were designed from the C-prM region by aligning the available sequences in GenBank. RNA was reverse transcribed using reverse primer (736) at 50°C using Superscript II reverse transcriptase (Invitrogen, Life Technologies). The cDNA was PCR amplified using the forward (391) and reverse (757) primers for detection of the JEV. PCR amplification was carried out by denaturing the DNA at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 54°C for 60 seconds, and 72°C for 60 seconds, and a final extension at 72°C for 7 minutes using Platinum Hi fidelity Taq DNA polymerase (Invitrogen, Life Technologies). Both strands of the PCR products were sequenced using Big Dye Terminator cycle sequencing Ready Reaction Kit (Applied Biosystems, US) and an automated Sequencer (ABI PRISM 310 Genetic Analyser; Applied Biosystems).

Multiple alignments of nucleotide/amino acid sequences were carried out using Clustal X 1.83. A phylogenetic tree was constructed using sequences from the C PrM region and compared with reference JEV strain sequences available in GenBank. The phylogenetic status was assessed using MEGA-3 software according to the methods described. For analysis in MEGA, Jukes-Cantor (JC) distance was used with the neighbor joining (NJ) algorithm. The reliability of different phylogenetic groupings was evaluated by using the bootstrap test (1,000 bootstrap replications) available in MEGA.

**RESULTS**

An epidemic of encephalitis was reported in August–September 2001 from 16 districts of Uttar Pradesh (India) including the Lakhimpur-Khiri district, which had the highest number of cases (i.e., 141 and 39 deaths; mortality rate, 27.6%). The UP State Government Health Service authorities reported 443 encephalitis cases and 96 deaths.

**Serologic study of clinical specimens.** Sera from 114 patients collected during the outbreak showed positivity for both JE and WN viruses by IgM capture ELISA. Of these, 76 (66.6%) were positive exclusively for JEV, 10 (8.7%) were JE/WN virus cross-reactive, and 28 (24.56%) were negative for both viruses. Similarly, 26 of 44 CSF (59.09%) specimens were positive exclusively for JEV. About 60% of the speci-
mens were positive for anti-JE-IgM antibodies, confirming the involvement of JEV as an etiologic agent.

**Virus isolation.** The CSF inoculated in infant mice for virus isolation did not yield any isolate up to four passages. During every passage, the mouse brains were monitored for JEV detection by antigen capture ELISA and JEV-specific RT-PCR. However, the isolation attempts were not successful up to three blind passages, and JEV could not be detected by ELISA and RT-PCR (data not shown). Blood clots collected during the acute phase from 12 patients having JE-like encephalitis symptoms were selected for virus isolation. One blood clot (014205) collected from a healthy contact served as negative control during the experiment. Of 12 blood samples collected from the patients, 9 were JE-IgM positive and 3 were negative. The blood clot collected from the healthy control was prescreened and found to be negative for JE and WN virus antibodies. WBCs from all 12 patient blood clots were separated and processed for PBMC co-culturing and tested by ELISA and RT-PCR for virus detection after every passage. WBCs from patient 014178 and its successive passages of PBMC cultures (P-1 to P-4) showed JEV positivity by RT-PCR. WBCs from the remaining 11 blood clots and respective PBMC cultures in the first passage showed negative results by RT-PCR. However, in the second and third passages, 014173 showed JEV positivity by RT-PCR. The remaining 10 cultures were consistently negative for JEV RT-PCR up to passage 4. Virus from these cultures was passaged in mice to prepare stocks as mentioned earlier. The stocks were confirmed by RT-PCR before submission to NIV virus registry. Mouse groups inoculated with the other 10 PBMC co-cultured specimens and WBCs from control specimen 014205 did not show sickness even after three blind passages, and the virus was not detected by RT-PCR. Virus identity was further confirmed by neutralization assay, PCR, and sequencing.

**Antigen capture ELISA.** WBCs separated from the blood clots were co-cultured with PHA-stimulated PBMCs. After observing the CPE, the culture aliquots were tested by antigen capture ELISA using JE-specific Mab Hs2. JE virus could be detected in the first passage of 014178 co-cultured PBMCs, whereas it was detected in the second passage of 014173. S/N ratios were 3.6 and 2.6, respectively, for 014178 and 014173, whereas it was 1.2 in the control WBC co-culture. Mouse brain passage showed an S/N ratio of 5.7 and 4.0, respectively. JEV amplification in the PBMC co-cultures and infected mice brains was further confirmed by JEV-specific RT-PCR.

**Neutralization assay.** JEV polyclonal and monoclonal antibodies neutralized JEV isolates 014173 and 014178 and strain 733913. The polyclonal anti-WN antibodies showed no neutralization, confirming that the isolates were from the JEV (Figure 1). Although both isolates were neutralized by anti-JEV-specific MABS, there were differences in the antibody titers of each MAb. The neutralization titers of MAb Hs4 were higher with new isolates as compared with 733913. This also indicated that new isolates (this study) are antigenically distinct from JEV strain 733913.

**RT-PCR.** JEV-specific primers designed to amplify 346 nt in the C-prM region were used to detect the JEV in WBCs, PBMC co-cultures, and subsequent mouse brain passages. The JEV could be detected in WBCs separated from sample 014178 and in subsequent passages of PBMC co-culture. In case of sample 014173, JEV was not detectable in WBCs and the first passage of PBMC co-culture; however, it was detected by RT-PCR in passage 2 (Figure 2). JEV strain 733913 (human isolate from Bankura region of West Bengal, India, in 1973) was used as a positive control. Suspensions of mouse brains showing sickness also showed positive results by RT-PCR. The sequence of the RT-PCR-amplified 346-nt product confirmed it as JEV (Figure 2).

**Partial genetic analysis.** Phylogenetic analysis of the 346-nt sequence of 014173 and 014178 classified them within genotype III of the JEV. Further comparison of percent nucleotide identity (PNI) of the 346-nt sequences of 014178 and 014173 isolates showed 99.16 ± 0.48% PNI with each other. The 014178 and 014173 sequences showed 94.43 ± 1.21% and 94.71 ± 1.18% PNI with Indian prototype strain P20778. Similarly, 014178 and 014173 sequences showed 97.49 ± 0.82% and 97.21 ± 0.87% PNI with JEV strain GP78 (human isolate from Gorakhpur, UP, in 1978). The PNI between P20778 and GP78 was 94.61 ± 1.17%. Compared with P20778 (human isolate from Vellore, TN, in 1958), within the 346-nt sequence, the 014178 and 014173 isolates showed 21 nucleotide changes. However, compared with the complete amino acid frame of P20778, these isolates recorded five amino acid changes at M185-A, K218-R, V232-E, M249-T, and I267-V. Similarly, compared with GP78, within the 346-nt sequence, the 014178 and 014173 isolates showed eight nucleotide changes at T404-C, G467-T, T551-C, T602-G, C668-T, A695-G, I267-V, and C728-T. Compared with the GP78 complete amino acid coding frame, only one amino acid change (V232-E) was recorded in both isolates. Among the 014178 and 014173 isolates, three nucleotide changes at T417-C, A421-T, and T609-C were recorded. However, compared with the 014178 isolate, 014173 showed only one amino acid substitution at T211-I.

**DISCUSSION**

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.

In this study, the clinical specimens from hospitalized patients in district hospitals of UP were transported in the cold to NIV, Pune, and processed for virus isolation and characterization. IgM capture ELISA of sera from 114 patients showed 66.6% positivity exclusively for JEV, whereas 8.7% were cross-reactive to JE-WN viruses, and 24.56% were nega-
tive for flaviviruses, indicating that the JEV was the main cause of the encephalitis outbreak. Of 76 JE IgM-positive sera, WBCs from 9 randomly selected IgM-positive clots were processed for virus isolation by PBMC co-culture. WBCs from four JE IgM-negative clots (three patients and one healthy individual) were processed as negative controls. Co-culturing of WBCs from patient blood clots with PHA-P-stimulated PBMCs from normal individuals resulted in amplification of the virus. Virus amplification was detected after every passage, and eventually the virus was established by inoculation in the infant mice. Of nine JE IgM-positive blood clots, two isolates could be established. Three JE IgM-negative clots from patients and one from a healthy individual, which were processed for virus isolation, did not show virus amplification even after three passages of PBMC co-culture and its inoculation in mice up to four passages. Similarly, JEV-specific RT-PCR did not detect JEV up to three passages of the PBMC co-cultures and mice, respectively. Kedarnath et al\textsuperscript{13} reported the replication of JEV in PHA-P and Pokeweed mitogen-stimulated human PBMC cultures. The PBMC co-culture technique has been successfully exploited for JE and WN virus isolations from peripheral blood of en-
cephalitis patients from the 1981–1982 JE encephalitis epidemic in Kolar district, India.\textsuperscript{12} However, the serum-PBMC co-cultures were processed in the field laboratory immediately after collecting the patient’s samples, and thus, blood clots were not used for the virus isolations. In this study, successful isolation of JEV from IgM antibody-positive samples indicated that, even in the presence of anti-JE antibody, the patient harbors JEV in peripheral blood.

Hjelle et al.\textsuperscript{21} reported the use of patient PBMCs, blood clots, and plasma for direct detection of the Muerto Canyon virus by RT-PCR. However, progressive and significant RNA loss has been reported at the time of clot formation in cases of hepatitis C virus.\textsuperscript{22} Similarly, detection of virus-specific antigens by ELISA in peripheral blood leukocytes and clot and tissue specimens of immuno-tolerant cattle, persistently infected with pestivirus, has been reported.\textsuperscript{23} Use of anticoagulants such as heparin is preferred for blood collection from patients; however, it inhibits virus attachment and penetration by blocking receptors.\textsuperscript{24,25} In this study, the blood clots were collected and transported in sterile gel-line vacutainers from hospitals to NIV, Pune, without the use of any anticoagulant.
The use of blood clots from patients of pediatric age for JEV isolation and diagnosis is advantageous over CSF in two ways. First, the expertise necessary to drain the CSF may not be available in the field, and second, the process is painful. However, CSF examination is essential to exclude other possible treatable causes such as bacterial disease and CSF IgM ELISA for JEV, which is the acceptable standard for diagnosing JEV encephalitis. We used the WBCs from blood clots for virus isolation and characterization, whereas patient sera and CSF were used for preliminary serodiagnosis of the suspected etiologic agent responsible for the outbreak. We used JEV-specific antigen capture ELISA and highly sensitive RT-PCR assays for detection of the virus in blood clots and subsequent passages in culture. Detection of JEV by RT-PCR in WBCs of specimen 014178 (Figure 2) indicates its usefulness in JEV isolation. However, low genomic copy number in the specimen can be a limiting factor. The amount of viral load in the clinical specimen depends on the duration of disease onset and time of sample collection. PBMC co-culture offers a natural host system for propagation and amplification of the virus. RT-PCR could not amplify the JEV RNA in the case of specimen 014173, which was only detected passage 2 onward in PBMC co-culture, indicating the necessity of primary amplification of the virus in a supporting system. Introduction of host cell–dependent genetic alterations has been mainly reported in structural proteins of flaviviruses. Use of human PBMC co-culture for preliminary amplification of the virus from blood clots will avoid such host-dependent genetic alterations. Comparison of the 346-nt sequences of RT-PCR–amplified products directly from 014178 WBCs, PBMC co-culture passage 3, and mouse brain stocks did not show such alterations in the C-prM region. Presence of the JEV in mouse brain stocks of both 014178 and 01473 was confirmed by JEV-specific RT-PCR, sequencing, and antigen capture ELISA using JEV-specific MAb Hs2. The isolates were further confirmed by a neutralization test using JE-specific MAbs and polyclonal antibodies raised against JEV strain 733913. JEV-specific antibodies neutralized the two isolates and did not neutralize the WN virus–specific polyclonal antibodies.

JEV has been classified into four genotypes using a 240-nt fragment within the C-prM region. We used a larger fragment (346 nt), which covers the 240-nt region, to amplify and sequence our JEV isolates. Indian JEV strains have been classified as members of genotype III, which are further divided into two distinct genetic clusters. This 346-nt sequence was used to determine the genotypic relationship between newly isolated JEVs (014178 and 01473) and strains (P20778 and GP78) isolated from different geographic regions of India. The phylogenetic analysis indicated that, compared with the south Indian isolate P20778, both of the 2001 isolates from northern India are closely related to GP78 (Figure 3). Comparison of the 346-nt sequence of 014178 and 01473 isolates with other Indian isolates P20778 and GP78 showed major difference with P20778 (21 nucleotide changes) compared with GP78 (8 nucleotide changes). Similarly, the amino acid sequence also revealed a closer relationship with GP78 (one amino acid change) than P20778 (five amino acid changes). Only three nucleotide changes leading to only one amino acid change among 014178 and 01473 suggest that these are the variants of the same strain circulating in northern India. The comparative nucleotide identity analysis of the 346-nt sequence of Indian prototypes strains P20778 and GP78 showed 5.39% divergence. Our new isolates 014178 and 01473 showed 5.3% and 5.5% divergence with P20778 and 2.5% and 2.8% divergence with GP78, respectively, indicating their closer relationship with GP78. These findings clearly indicate that a single strain (representing GP78 isolated in 1978 from UP) is circulating in UP and is undergoing microevolutionary changes at genomic level. Genetic alterations imposed by either circulation through different hosts in nature or time span of 23 years must have led to a 2.5% to 2.8% divergence within the strain circulating in UP.

It is possible to use blood clots as an additional source for virus isolation where CSF and/or brain tissues are not available. The virus isolates can be characterized by using various serologic and genetic tools. As seen from the results of this study, the 014178 isolate could be detected in the WBCs and first PBMC passage itself. The observations indicate the possibility of use of WBCs from patient blood clots for JEV identification and isolation in human PBMC co-culture.

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