DETECTION OF JAPANESE ENCEPHALITIS VIRUS BY REVERSE TRANSCRIPTION/POLYMERASE CHAIN REACTION

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Summary. – A simple and rapid reverse transcription/polymerase chain reaction (RT-PCR) assay for detection of Japanese encephalitis virus (JEV) envelope (E) gene sequences in various biological samples is described. The assay successfully amplified JEV E gene sequences from infected cell cultures, *Aedes aegypti* larvae, mosquitoes and mouse blood. The sensitivity of the assay was currently 1 ng of JEV RNA and could be increased up to 1 pg on the background of 1 μg of cellular RNA by biotinylation of the PCR product, Southern blot analysis and streptavidin/alkaline phosphatase detection.

Key words: Japanese encephalitis virus; envelope gene; reverse transcription; polymerase chain reaction

Introduction

JEV, a member of the family *Flaviviridae* is the prototype of the JEV group. JEV has been implicated in periodic outbreaks of encephalitis cases reported in different countries of Asia. It is one of the most common cause of viral encephalitis (Tsai and Yu, 1994), and approximately 50,000 cases are reported from over 16 countries every year. About 25% of these cases are fatal while 50% of them results in permanent neuropsychiatric dysfunction (Burke and Leake, 1988).

Zoophilic mosquitoes of the *Cx. Vishnui* complex are the major vectors involved in transmission while wild and domestic animals are the principal hosts. A rapid and sensitive detection of JEV in clinical samples has become possible with the advent of PCR. In the present communication, we report on the development of a RT-PCR assay for JEV from a variety of biological samples.

Abbreviations: AMV = avian myeloblastosis virus; BSA = bovine serum albumin; DEPC = diethyl pyrocarbonate; DIG = digoxigenin; E = envelope; EDTA = ethylenediamine tetraacetate; GITC = guanidine thiocyanate; JEV = Japanese encephalitis virus; i.c. = intracerebral(ly); i.p. = intraperitoneal(ly); i.t. = intrathoracical(ly); MuMLV = murine Moloney leukemia virus; NP40 = Nonidet P-40; p.i. = post infection; PBS = phosphate-buffered saline; RT-PCR = reverse transcription/polymerase chain reaction; SDS = sodium dodecyl sulfate

Materials and Methods

Virus. JEV strain P 20778 (Webb *et al.*, 1964) was used in this study. A mouse brain stock virus underwent one passage in porcine kidney (PS) cell cultures. The infected tissue culture fluid was harvested after onset of cytopathic effect and titered up to 10^8 PFU/ml.

Detection of JEV in tissue cultures

RT-PCR was carried out on infected tissue culture fluids either by extraction of RNA followed by RT-PCR as described earlier (Paranjpe and Banerjee, 1996) or directly by a single tube RT-PCR assay without extraction of RNA.

RNA extraction was carried out by the methods using sodium iodide (Paranjpe and Banerjee, 1996) or guanidine thiocynate (GITC). Briefly, 100 μl of an infected tissue culture fluid was mixed with 900 μl of GITC-1 solution (120 g of GITC in 100 ml of 0.1 mol/l Tris.HCl pH 6.4, 22 ml of ethylenediamine tetraacetate (EDTA) pH 8.0 and 2.6 g of Triton X-100) and 20 μl of DNA Binder (Perkin Elmer Cetus), vortexed for 15 secs and incubated at room temperature for 10 mins. After centrifugation for 1 min, the pellet was mixed with 1 ml of GITC-2 (GITC-1 without EDTA and Triton X-100) thoroughly vortexed and centrifuged for 1 min. This step was repeated twice. The pellet was washed with 70% ethanol followed by acetone, dried at 56°C for 10 mins on open air and the bound RNA was eluted in 20 μl of water treated with diethyl pyrocarbonate (DEPC) containing 40 U of RNasin (Promega) at 56°C for 10 mins. The RNA in the supernatant obtained by centrifugation for 15 secs was saved and stored at -70°C until subjected to RT-PCR.

Direct RT-PCR was carried out with 10 µl or 200 µl of a JEV-infected tissue culture fluid after virus lysis in 1 x PCR buffer (500 mmol/l KCl, 15 mmol/l $MgCl_2$ and 100 mmol/l Tris.HCl pH 8.3) at 95°C for 10 mins. After centrifugation at 14,000 rpm for 5 mins, 10 µl of the supernatant was mixed with 200 U of murine Moloney leukemia virus (MuMLV) RT (Gibco BRL), 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus), 1 mmol/l each of dNTPs (Pharmacia) and 40 U of RNasin (Promega). RT-PCR was carried out in a single tube by following the protocol described earlier (Paranjpe and Banerjee, 1996) except that cDNA was primed by the primer JEC6. The sense primer employed in this experiment was JEGAD3. The expected PCR amplified fragment size was 434 bp.

Detection of JEV in mosquito larvae

The ability, sensitivity and specificity of RT-PCR to detect JEVsequences in *Ae. aegypti* mosquito larvae infected with JEV was determined.

Mosquito larvae. Fourth instar larvae obtained from the Entomology Division of the National Institute of Virology, Pune, were employed. Serial 10-fold JEV dilutions ranging from 10^{-1} to 10^{-6} were made in phophate-buffered saline (PBS) pH 7.4. Approximately 0.2 µl of a virus dilution per larva was inoculated in the head capsule region (Mourya, 1990). The larvae were held at 28°C in water for the desired time period and then stored at -70°C. Some larvae were inoculated with West Nile virus under identical conditions. Five larvae inoculated with the 10^{-6} dilution of JEV were processed at three different time periods post infection (p.i.) for detection of JEV by RT-PCR. The larvae inoculated with West Nile virus and uninfected larvae served as negative controls.

RNA isolation and RT-PCR . The larvae were homogenised in 500 µl PBS in a microcentrifuge tube by using Kontes homogeniser (Scientific Glassware Instruments, USA). The homogenate was centrifuged at 14,000 rpm for 30 mins at 4°C. The supernatant (50 – 100 µl) was used for RNA isolation (Paranjpe and Banerjee, 1996). The RT-PCR was carried out as described earlier (Paranjpe and Banerjee, 1996). The primers RBGAD1 and JEC4 which amplified a fragment of 964 bp were utilised.

Detection of JEV in mosquitoes. The capability of RT-PCR of detecting JEV in pools of mosquitoes was determined. *Ae. aegypti* mosquitoes were infected intrathoracically (i.t.) with 10^6 LD_{50} of JEV. Mosquitoes were immobilised by keeping them on ice. Approximately 0.02 µl of the virus suspension per mosquito was inoculated by the procedure described by Rosen and Gubler (1974). After inoculation, the mosquitoes were kept in a plastic jar for 8 days. During this period, they were fed on 10% glucose solution. A single infected mosquito mixed with 99 uninfected mosquitoes represented a pool processed for RT-PCR. Briefly, the pooled mosquitoes were homogenised in 2 ml of PBS. The obtained suspension was spun at 10,000 rpm at 4°C for 30 mins, and 500 µl of the supernatant was used for isolation of total RNA by the sodium iodide method. The primers JEGAD3 and JEC1 were employed for amplification of the complete E gene. The nested PCR was carried out utilizing the primers RBGAD1 and JEC6 which amplified a fragment of 372 bp. A hundred uninfected mosquitoes were used as a negative control.

Detection of JEV in mice. Adult (21-day-old) Swiss mice were infected intraperitoneally (i.p.) with 10^6 LD_{50} of JEV per mouse in 0.02 ml. Whole blood was collected by intracardiac or intraocular route daily for 5 days p.i. and tested for the virus by RT-PCR. A hundred µl of whole blood was diluted with 200 µl of sterile DEPC-treated water and processed for RNA extraction by the GITC or sodium iodide methods. Five µl of the obtained RNA preparation was used for RT-PCR (Paranjpe and Banerjee, 1996) by employing the primers JEGAD3 and JEC1 for the first PCR and the primers JER2 and JEC4 for the nested PCR.

RT-PCR sensitivity study. The sensitivity of RT-PCR to detect JEV RNA on the background of 1 µg of cellular RNA was detertmined with JEV RNA isolated from gradient-purified JEV. The PCR product was labelled by including biotinylated-11-dUTP (Gibco BRL) in PCR as described by Lo *et al.* (1990). The amplified product was subjected to Southern blot analysis visualised by streptavidin/alkaline phosphatase. JEV RNA was isolated by the sodium iodide method. The quality and quantity of the RNA obtained was determined spectrophotometrically by recording absorbance at 260 nm and 280 nm. The concentration of RNA was adjusted to 1 µg/µl. Serial 10-fold dilutions were made in DEPC-treated water containing 40 U of RNasin (Promega). Samples containing 1 µg, 1 ng, 1 pg and 1 fg of JEV RNA with 1 µg of total cellular RNA isolated from PS cells were tested in RT-PCR. JEV RNA in the samples was converted to cDNA by using random hexamers (Paranjpe and Banerjee, 1996). The RNA:cDNA hybrids were ethanol-precipitated, pelleted in a microcentrifuge, washed with 70% ethanol, air dried, dissolved in 65 µl of bidistilled water, and subjected to PCR assay with the primers RBGAD1 and JEC5 (Lo *et al.*, 1990). The reaction mixture was overlayed with 100 µl of mineral oil and cycled in a Perkin Elmer Cetus thermocycler for 25 cycles described (Paranjpe and Banerjee, 1996).

Southern blot analysis. The amplified product was electrophoresed on a 1% agarose gel, stained with ethidium bromide (0.5 µg/µl), and blotted to a nitrocellulose membrane as described by Manak (1993). The biotinylated DNA on the blot was detected by streptavidin and alkaline phosphatase. The blot was immersed in 10 ml of a blocking buffer (2% bovine serum albumin (BSA) in PBS) and gently agitated at room temperature for 30 mins. The blot was washed three times with a washing buffer (0.1% Tween 20 in PBS) at room temperature by gentle agitation. A streptavidin/alkaline phosphatase reagent diluted in the washing buffer (1:1000) was added and allowed to react at room temperature for 30 mins under constant agitation. Then, 100 µl of NBT reagent freshly prepared in dark and 100 µl of BCIP reagent in 10 ml of a carbonate buffer (0.1 mol/l $NaHCO_3$ and 1 $MgCl_2$ pH 9.8) were added to the blot. The reaction was monitored and terminated in running tap water after the development of violet color. The blot was dried, covered with filter paper and stored at room temperature.

Results

Sensitivity of RT-PCR

The sensitivity of the assay was determined with various amounts of JEV RNA mixed with constant amount of cellular RNA. It was found that a successful RT-PCR, as evi-

denced by an amplified product of correct size in ethidium bromide-stained agarose gel, was achieved with 1 ng of JEV RNA on the background of 1 μg of cellular RNA (data not shown). The PCR labelling of the amplified cDNA with biotinylated-11 dUTP, and Southern blot analysis of the labelled DNA with streptavidin and alkaline phosphatase were capable of increasing the sensitivity 1000-fold and detecting 1 pg of JEV RNA on the background of 1 μg of cellular RNA (Fig. 1).

Tissue culture studies

We found out that for successful amplification of the full length E gene of JEV and production of fragments greater than 1 kb, random hexamers were more efficient in priming cDNA synthesis than specific PCR primers (data not shown). Fragments smaller than 1 kb (434 bp) were amplified without prior extraction of viral RNA by direct single tube RT-PCR (Fig. 2).

Mosquito larvae studies

In the mosquito larvae infected with the 10^{-6} dilution of JEV, we were able to detect JEV-specific sequences as early as 12 hrs p.i. by RT-PCR (Fig. 3) while the immunofluorescence staining was positive for JEV antigen on day 3 p.i. only (data not shown). The larvae inoculated with West Nile virus were negative by both RT-PCR and immunofluorescence with JEV-specific probes. The specificity of the detection of JEV was confirmed by cloning of the PCR product in TA vector and sequencing of the cloned products (data not shown).

Mosquito studies

The RT-PCR was capable of detecting JEV in a single infected mosquito among of 99 uninfected mosquitoes (Fig. 4). Though JEV could not be detected after the first round of PCR carried out with the primers JEGAD3 and JEC1, a JEV-specific signal was obtained after a nested PCR with the primers RBGAD1 and JEC6 which amplified a fragment of 372 bp. Our studies also revealed that although a pool of 100 mosquitoes could be used in detecting JEV, a smaller pool (50 mosquitoes) was more convinent as it made the sample preparation easier.

Mouse studies

JEV sequences were detected in the blood of mice infected with 10^6 LD_{50} of JEV as early as 24 hrs p.i. The intensity of the band on ethidium bromide stained gels increased on day 3 p.i. (Fig. 5). As the mice were bled for 5 days p.i.only, an end point assay was not performed. The virus load was

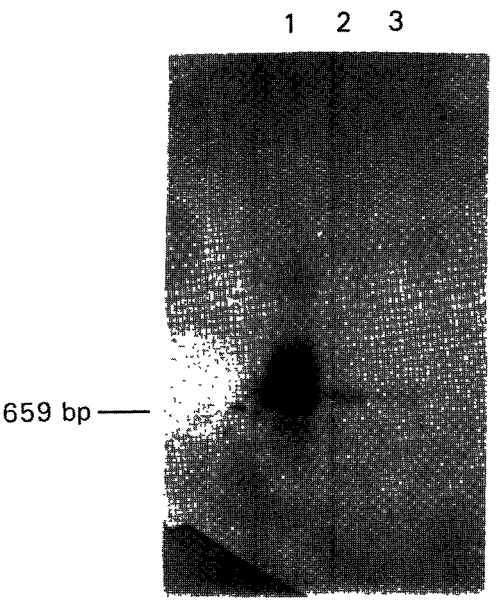

Fig. 1
RT-PCR of JEV RNA using biotinylation of DNA and streptavidin/alkaline phosphatase detection
One μg (lane 1), 1 ng (lane 2) and 1 pg (lane 3) of JEV RNA. Primer pair RBGAD1/JEC5 was used. The expected size of the RT-PCR product was 673 bp.

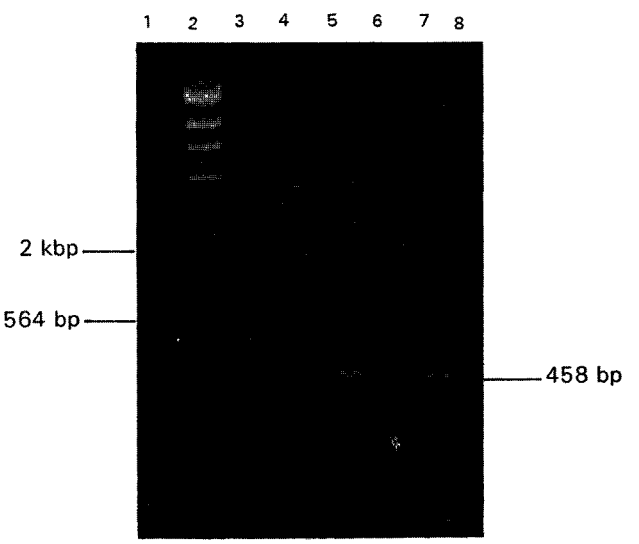

Fig. 2
Detection of JEV in JEV-infected tissue culture fluids by direct RT-PCR
Ten μl (lane 1) and 200 μl (lanes 4–7) of infected tissue culture fluid. Uninfected tissue culture fluid (lane 3), reagent only (lane 8) and *Hind*III/lambda DNA size marker (lane 2).

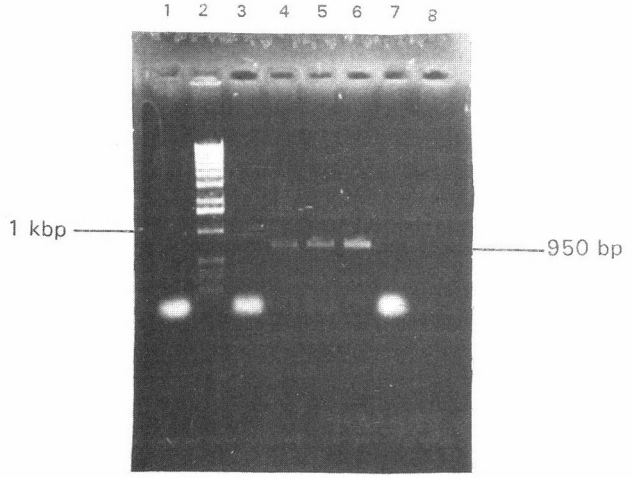

Fig. 3
Detection of JEV in JEV-infected mosquito larvae by RT-PCR
Isolated RNA was subjected to RT-PCR with primer pair RBGAD1/JEC4. The expected size of the RT-PCR product was 964 bp. Larvae 12 hrs (lane 4), 24 hrs (lane 5) and 36 hrs (lane 6) p.i. West Nile virus-infected larvae (lane 1), uninfected larvae (lane 3), reagent only (lane 7) and 1 kb DNA ladder (lane 2).

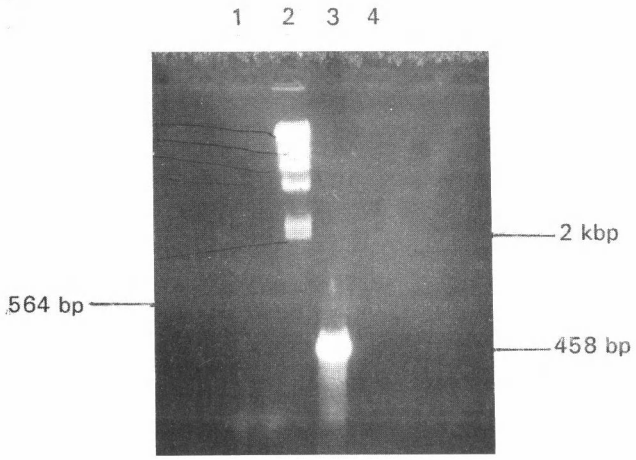

Fig. 4
Detection of JEV in JEV-infected mosquitoes by RT-PCR
Isolated RNA was subjected to RT-PCR. Primer pair JEGAD3/JEC1 was used in the first PCR, and primer pair RBGAD1/JEC6 in the second, nested PCR. The expected size of the RT-PCR product was 372 bp. JEV-infected (lane 3) and uninfected (lane 1) mosquitoes. Reagent only (lane 4) and *Hind*III/lambda DNA size marker (lane 2).

very low as determined by infant mouse brain titration (data not shown). Also, JEV sequences were detected only after a nested PCR was carried out indicating low level of viremia. The presence of JEV sequences in the brain of sick mice was confirmed by PCR (data not shown) which indicated a rapid replication of JEV in the mouse brain.

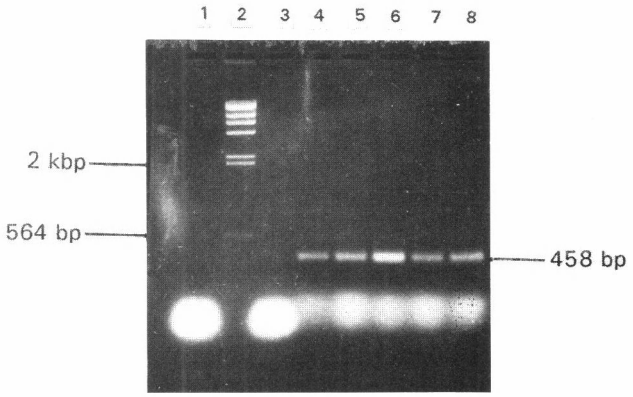

Fig. 5
Detection of JEV in the blood of JEV-infected mice by RT-PCR
Isolated RNA was subjected to RT-PCR. Primer pair JEGAD3/JEC1 was used in the firs PCR, and primer pair JER2/JEC4 in the second, nested PCR. The expected size of the RT-PCR product was 488 bp. Mice 1 day (lane 4), 2 days (lane 5), 3 days (lane 6), 4 days (lane 7) and 5 days (lane 8) p.i. Uninfected mouse (lane 1), reagent only (lane 3) and *Hind*III/lambda DNA size marker (lane 2).

The RT-PCR with JEV-infected mouse blood was initially attempted without an isolation of RNA. An aliquot (2 µl) of blood was diluted 100-fold in the PCR buffer IX, boiled for 5 mins and subjected to RT-PCR. However, a successful amplification was not achieved. The isolation of RNA using

GITC or sodium iodide was essential for removal of inhibitors of RT-PCR.

Discussion

RT-PCR conditions were optimised for detection of JEV in infected tissue culture fluids.The influence of parameters like the reverse transcriptase and RNA isolation procedure on successful amplification of JEV E gene sequence was tested. Of the reverse transcriptases tested the MuMLV enzyme was more efficient in priming longer cDNAs than was the avian myeloblastosis virus (AMV) enzyme. A possible reason for this difference might be a higher RNase H activity of AMV.

It appears that for amplification of smaller fragments (below 1 kb), the PCR primers used were efficient in priming the cDNA synthesis.

The isolation of RNA with sodium iodide or GITC followed by glass milk binding and elution was found to be a comparatively rapid procedure. This, however, may not be an ideal procedure for a high number of samples, because it requires frequent opening of tubes and may increase chances of tube to tube contamination during sample processing.

Deubel *et al.* (1993) and Laille *et al.* (1991) used Nonidet P-40 (NP40) for cell lysis and phenol with 1% sodium dodecyl sulfate (SDS) for RNA extraction. A treatment with GITC followed by purification of RNA by cesium chloride density gradient centrifugation was used by Eldadah *et al.* (1991) for purification of RNA from infected mouse brain tissues. Morita *et al.* (1991) compared four different protocols for their speed and suitability for isolation of dengue virus RNA from infected tissue culture fluids. The NP40 treatment for only 1 minute at room temperature without further purification of RNA yielded as good a template for RT-PCR as did purified RNA. The procedure could be completed within 2 – 3 hrs. However, this method has never worked in our hands when used for detection of JEV in infected cell culture fluids. We have also developed a rapid procedure for amplification of JEV sequences from infected cell culture fluids. Our method did not require a detergent treatment. A template suitable for RT-PCR was prepared by boiling as little as 10 µl of tissue culture fluid for 10 mins in 1 x PCR buffer. Furthermore, this was a single tube assay, i.e. both RT and PCR were carried out in a single tube and required minimal handling.

Although the reported rapid procedures for RT-PCR seem to work well on infected tissue culture fluids, they need be extensively tested for their suitability for clinical samples in which the viral load is possibly an important factor. In infected tissue culture fluids, virus titres are often high. This may not the case of clinical samples. Also, clinical samples are likely to contain inhibitors of Taq DNA polymerase, e.g. porphyrins (Gadkari, 1995). In such cases, simple procedures like boiling may not work.

The detection of flaviviruses by inoculation in mosquito larvae is more sensitive and more rapid than by intracerebral (i.c.) inoculation of mosquitoes (Lam *et al.*, 1986). The possibility to detect the virus by immunofluorescence depends on the proportion of the brain tissue of inoculated larvae in head – squeeze preparations (Kuberski and Rosen, 1977).

We were able to detect 4 PFU of JEV in infected *Ae. aegypti* larvae as early as 12 hrs p.i. The PCR assay developed by Chan *et al.* (1994) was capable of detecting dengue virus in infected larvae inoculated with 4 PFU of the virus on day 2 p.i. The earlier detection of JEV in larvae by RT-PCR in our study was possibly due to the fact that JEV is a neurotropic virus, and hence its rate of multiplication in the larval neural cells is likely to be faster as compared to dengue virus. A rapid and specific detection of JEV is thus possible by combining the larvae inoculation with RT-PCR. Experiments to test the feasibility of the RT-PCR assay developed to detect JEV in field samples are in progress in our laboratory.

The RT-PCR assay for detection of JEV in mosquitoes developed by us is simple and rapid as the purification of RNA is not essential and the assay may be extremely useful for detection of JEV in pools of field-caught mosquitoes as it is fairly rapid, sensitive and does not require amplification of the virus in cell cultures or mice.

A detection of dengue viruses by PCR in infected mosquitoes has been reported (Tardieux and Poupel, 1990; Lee *et al.*, 1992). Dengue virus was detected by PCR in mosquitoes fed on infectious blood and a virus-specific signal was obtained in as few as 1000 midgut cells of a single mosquito (Tardieux and Poupel, 1990). However, this method required a prior purification of RNA by proteinase K digestion followed by ethanol precipatation.

The method reported by Lee *et al.* (1992) was also capable of detecting dengue virus in experimentally infected mosquitoes stored for several years at -60°C. However, an RNA extraction by phenol/cresol followed by two rounds of precipitation with lithium chloride was necessary for detection of the virus in an 1/300th of an infected mosquito. Yields of cDNA from the mosquito extracts were comparatively lower than those from infected tissue culture fluids.

Present methods for detection of JEV in field-caught mosquitoes may require at least 10 days for an additional passage in either mosquitoes or mice and positive identification. The adaptation of flaviviruses to cultured cells or mouse brain may result in their significant changes at the genetic and antigenic level (Lee *et al.*, 1992, 1997). Different vector species and hosts may also influence flavivirus evolution (Paranjpe *et al.*, 1997) and virulence (Monath, 1982). The understanding of selection pressures exerted by the vectors requires characterisation of uncloned viruses. The detection of flaviviruses in infected mosquitoes by RT-PCR followed by direct sequencing of the reaction products may reduce the problems associated with characterisation of uncloned viruses.

Our RT-PCR assay developed for detection of JEV in blood of infected mice may be useful for detection of viremia in experimental infection studies and clinical samples, and for amplification of JEV sequences from pig blood. The viremia in JEV infection is often transient and virus isolation from blood is difficult because the virus titre is low and difficult to quantify by infant mouse brain titration.

A comparison of our RT-PCR assay with a conventional plaque assay on PS cells was reported earlier (Gadkari *et al.*, 1991). The RT-PCR was found to be over 1000-fold more sensitive than the plaque assay. A similar sensitivity of RT-PCR for detection of Saint Louis encephalitis virus (SLEV) as compared to classical infectivity titration by i.c. inoculation of suckling mice was reported by Eldadah *et al.* (1991). This RT-PCR assay was capable of detecting SLEV RNA on a background of 500 pg of total host RNA. The assay developed by Pierre *et al.* (1994) was capable of detecting

5 PFU of dengue virus after hybridisation of the PCR product labelled with dUTP-11 digoxigenin (DIG) with 100 ng of a recombinant plasmid.

A further increase in sensitivity of the assay for flaviviruses was achieved by labelling of the PCR primer with biotin and incorporation of multiple DIG residues in each amplicon (Chang *et al.*, 1994). A signal amplification was achieved by including an ELISA for DIG-DNA. This assay was found to be 64-fold more sensitive than that consisting of agarose gel electrophoresis followed by ethidium bromide staining.

During the study of the PCR sensitivity it became evident that a tube to tube contamination was possible. RNA isolation methods like those using GITC or sodium iodide require frequent washing and opening of tubes is unavoidable. This contamination may represent a problem in end point dilution experiments. A simple test was developed by us to determine whether the tube to tube contamination had occurred. A few samples were labelled by addition of a known DNA molecule, e.g. lambda DNA, and processed for RNA isolation and RT-PCR. Then a second PCR employing primers specific for lambda DNA was carried out. If the tube to tube contamination had not occurred, all samples except those labelled with lambda DNA would be negative for lambda DNA. This test proved that the tube to tube contamination took place in some cases.

The analysis and understanding of the extent of strain variation in flaviviruses is essential for molecular evolutionary studies of these viruses (Paranjpe and Banerjee, 1996; Lewis *et al.*, 1992). A careful choice of primers for PCR detection is necessary. JEV strains isolated from different hosts in diverse geographical locations at various time periods have been successfully detected (Paranjpe and Banerjee, 1996; Paranjpe, 1997). Suitable primers can therfore be used to detect and characterise different JEV strains at the genetic level and are useful for molecular epidemiological studies. An improper selection of primers may result in failure of the PCR assay (Puri *et al.*, 1994). It is also possible that mutations in the primer binding region as a result of adaptation of the virus may lead to unsuccessful amplification (Kameoka *et al.*, 1995). The use of degenerate PCR primers may help to solve the problems associated with detection of virus variants (Henchal *et al.*, 1991).

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