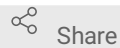




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Phylogenetic analyses of the JEV gene sequence

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ABSTRACT

The E gene was amplified by semi-nested PCR.

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MATERIALS TEXT

mosquito grinding supernatant, CSF sample that shown positive by detection of JEV by quantitative real-time reverse-transcription (q-RT)-PCR; Tianlong Nucleic Acid Automatic Extractor, Tianlong Nucleic Acid Extraction Kit, Ready-To-Go kit, random primers (pdN6), GoTaq Green Master Mix Kit (Promega, Madison, WI, USA). The primers used for the first round of E gene PCR were JEV-E-1F (TTCATAGAAGGAGCCAGTGGA) and JEV-E-1R (TCGTTTAAACTCGCGACTGA), the primers used for the second round were JEV-E-1F and JEV-E-2R (TTTCCCGAAAAGTCCACATC). The primers used for the first round of C+PrM gene PCR were JE-C+PrM-1F (CGTTCTTCAAGTTTACAGCATTAGC) and JE-C+PrM-1R (CCYRTGTTYCTGCCAAGCATCCAMCC), and the primers used for the second round were JE-C+PrM-1F and JE-C+PrM-2R (CGYTTGGAATGYCTRGTC CG).

Nucleic acid extraction

- 1 Use Tianlong nucleic acid extraction instrument (automatic nucleic acid extraction instrument, model: np968. C, manufacturer: Suzhou Tianlong Biotechnology Co., Ltd.), and supporting nucleic acid extraction kit: Tianlong nucleic acid extraction kit (magnetic bead method EX-RNA/DNA virus), manufacturer: Suzhou Tianlong Biotechnology Co., Ltd., for nucleic acid extraction. Perform the following operations in a biosafety cabinet. Add 200 µl of patient cerebrospinal fluid and serum specimens and mosquito grinding supernatant to each well in columns 1 and 7 of the 96-well plate provided with the nucleic acid extraction kit

cDNA library preparation

- 2 33 µl of RNA extract was placed in a 65 ° C water bath for 10 min.
- 3 Ice bath for 2 min
- 4 32 µl of RNA samples were pipetted into the first chain reaction tube in a Ready-To-Go kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and allowed to stand at room temperature for 1 min.
- 5 1 µl of random primer (pdN6) (TaKaRa, Japan) was added to every sample.
- 6 37 ° C water bath for 1 h.

The E gene amplification

The E gene sequence of JE virus was amplified by semi-nested PCR. The first round of

- 7 amplification , using primers: JEV 3F: TTCATAGAAGGAGCCAGTGGGA and JEV 3R: TCGTTTAAACTCGCGACTGA. The gene amplification system was 25 μ l, including: 2 μ l of cDNA template, 12.5 μ l of GoTaq $\text{\textcircled{R}}$ Green Master Mix-2 \times (Promega, Madison, WI), 1 μ l of upstream and downstream primers at 10 μ mol/L, and 8.5 μ l of RNase Free Water. The reaction program was: 94 $^{\circ}$ C, 8 min for 1 cycle; 94 $^{\circ}$ C, 1 min, 55 $^{\circ}$ C, 1 min, 72 $^{\circ}$ C, 1 min for 35 cycles; 72 $^{\circ}$ C, 10 min, keep in 4 $^{\circ}$ C. The second round of amplification sequence was 300 bp, using the first round of PCR product as a template with the forward primer JEV 3F and the reverse primer JEV 2R: TTTCCCGAAAAGTCCACATC.
- 8 The second round of amplification sequence was 300 bp, using the first round of PCR product as a template with the forward primer JEV 3F and the reverse primer JEV 2R: TTTCCCGAAAAGTCCACATC, the amplification system and reaction program was same as the first round.

C+PrM gene amplification

- 9 The C+PrM gene sequence was also amplified by semi-nested PCR according to the same amplification system and procedure as E gene. The primers used for the first round of PCR were JE-C+PrM-1F (CGTTCTTCAAGTTTACAGCATTAGC) and JE-C+PrM-1R (CCYRTGTTYCTGCCAAGCATCCAMCC)
- 10 primers used for the second round were JE-C+PrM-1F and JE-C+PrM-2R (CGYTTGGAATGYCTRGTCGG). The product of the first round of PCR was used as the template of the second round. The second round also use the same amplification system and procedure as E gene.

agarose gel electrophoresis

- 11 5 μ L of the amplification product was detected by 1% agarose gel electrophoresis .

Nucleotide sequence determination

- 12 Nucleotide sequence determination was done by Sangon Biotech Co. Ltd. (Shanghai, China, Beijing Sequencing Department).

Phylogenetic analyses

- 13 The viral gene nucleotide sequences were spliced and corrected using SeqMan II software (DNA Star, Madison, WI, USA).
- 14 The JEV gene sequences used for phylogenetic analysis were downloaded from GenBank(Table S1) .

- 15 The ClustalW multiple sequence alignment was performed using BioEdit (Version 7.0, Hall 1999).
- 16 Neighbor-joining phylogenetic trees were drawn using MEGA6.0 with 1000 bootstrap replicates.