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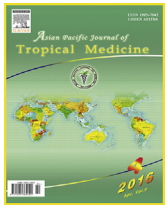

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Mechanism of Japanese encephalitis virus genotypes replacement based on human, porcine and mosquito-originated cell lines model

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ABSTRACT

Objective: To examine the multiplication efficiency Japanese encephalitis virus (JEV) genotype I (GI) and genotype III (GIII) of different cell lines which originated from human, porcine, mosquitoes in order to prove mechanism of JEV GI replacement JEV GIII since it emerging in nature recent decades.

Methods: The mixture of GI and GIII JEV isolates was inoculated on human rhabdomyosarcoma (RD), pig kidney epithelial (PS) and *Aedes albopictus* C6/36 clone (C6/36) which originated from human, porcine and mosquitoes, respectively. Plaque assays were performed to calculate virus titer and real-time RT-PCR with GI and GIII specific primer sets to quantify the number of GI and GIII RNA copies.

Results: The highest virus titer reached at the 3rd day of post infection when GI and GIII mixture was inoculated on RD and PS and that of C6/36 was at the 4th day. JEVs were amplified and maintained by C6/36 cells after 10 passages whereas that by RD and PS only limited within 8 and 6 passages, respectively. GI strain amplified and maintained more efficiently on C6/36 and PS but not RD, whereas GIII strain amplified and maintained more efficiently on RD.

Conclusions: There is a correlation between the multiplication efficiency of GI and GIII JEV strains when these two genotype strains co-infected on different cell lines with the predominance of GI strains in C6/36 and PS and the limited detection of GI strains in RD cells proving a possible mechanism of shift JEV genotypes in nature recent decades since GI emerging.

1. Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne virus of the genus *Flavivirus* in the family Flaviviridae which has circulated widely in Asian and western Pacific countries, and in northern Australia [1,2]. Approximately 67900 cases of JE typically occur annually and 10000–15000 JEV-related human deaths are reported annually [2,3]. JEV is transmitted to susceptible reservoirs by arthropods with the mainly involvement of mosquitoes, especially *Culex tritaeniorhynchus* whereas pigs, horses, birds and bats are the primary natural hosts of JEV. Humans are the dead-end host in which JEV could not be transmitted from humans to humans.

The JEV genome consists of a single-stranded positive-sense RNA in which the open reading frame encodes a large polyprotein. The N-terminal region of the polyprotein encodes the structural proteins: Capsid (C)-pre-membrane (prM)-Envelope (E) and the non-structural proteins (NS) (NS1-NS2A-NS2B-NS3-NS4-NS5) [4]. JEV is classified into five genotypes based on the nucleotide sequences of the *C/prM* and *E* protein genes [5]. However, all five genotypes have been detected in mosquitoes and reservoirs while the number of genotypes detected in humans is limited. While JEV genotypes I and III are predominant in humans, only two of JEV genotype II strains were detected so far; one in Australia and the other in Korea [5,6].

In humans, genotype I (GI) strains have displaced genotype III (GIII) strains to become the predominant genotype in many countries [7]. The emergence of JEV GI strains was first identified in mosquitoes and pigs in northern Asian countries, Korea in 1993 and Japan in 1994 [8–10] and in mosquitoes in

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China in 1979 [11]. However, GI strains have mainly been isolated from mosquitoes and swine while very few have been isolated from humans [11,12]. Therefore, the JEV GI strains are supposed more adapting to mosquitoes and pigs than to humans [14]. Among twelve haplotypes that are defined based on the four amino acid residues in the *E* protein (sites 123, 209, 227, and 408), the haplotypes and the host ranges of the GI isolates are narrower than those of the GIII isolates [15].

To identify the adaptation of JEV GI and GIII strains in different host species experimentally, we conducted the study to examine the multiplication of JEV GI and GIII strains when these two genotypes co-infected in different cell lines which originate from human, porcine and mosquitoes. This study aims to prove an evidence of the mechanism shift JEV genotypes in nature recent decades since JEV GI emerging in most of Asian countries where JEV GIII circulating before.

2. Material and methods

2.1. Cell and viruses

Pig kidney epithelial (PS); human rhabdomyosarcoma (RD); mosquito *Aedes albopictus* C6/36 clone (C6/36), baby hamster kidney-21 cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum. The cells were incubated at 37 °C with 5% CO_2 except C6/36 cell was incubated at 28 °C. The Japanese encephalitis virus (JEV) strain 93VN118 and 94VN141 were originally detected from mosquitoes in 1993 and 1994, respectively. Their genotypes were identified as genotype I-b (GI-b) (94VN141) and genotype I (GI) (93VN118) (GenBank accession number AB933310 and AB933311) [12]. Those virus strains were chosen because they are the first JEV GI and the last JEV GIII to be detected from mosquitoes in the Northern Vietnam. Virus stocks were stored at −80 °C until examined.

2.2. Viral infection

The virus titers of two isolates were determined by plaque assay, and then diluted to be at the same titer (100 PFU/mL) in MEM with 2% fetal calf serum (FCS).

The mixture of two strains whose titer was 100 PFU/mL was inoculated on the monolayer of RD, PS and C3/36 cell lines for 60 min at 37 °C and 28 °C, respectively. The un-absorbed viruses were removed from cells. The infected RD, PS cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere and C6/36 in 28 °C. The supernatants were harvested continuously for 5 d after inoculation in order to determine time-point when virus titers reached peak by using plaque assay. The virus mixture was then inoculated continuously 10 passages on RD, PS and C3/36 cell lines. The supernatants were harvested at the determined peak day in every passage and stored at −80 °C. Plaque assays were performed to determine which cell line could maintain and amplify JEV efficiently and TaqMan RT-PCR were performed to determine which genotype amplified efficiently by each cell line.

2.3. Plaque assay

Serial ten-fold dilutions of JEV GI and GIII mixture at each determined time-point were prepared in chilled MEM with 2% FCS from 10^{-1} to 10^{-6}. Diluted virus samples were inoculated on three cell lines and then MEM with 2% FCS plus with 1.25 methylcellulose was overlaid after viral absorption for 90 min. Each dilution was performed in triplicate. The baby hamster kidney-21 cells were incubated at 37 °C with 5% CO_2 for 7 d. Cells then were stained with 0.1% crystal violet after saline formal fixation for demonstration of plaques. The excess stain was washed off with tap water; air-dried. The number of plaques was measured in each well and calculated the virus titer. Plaque assays were repeated three times at the same conditions.

2.4. RNase treatment and nucleic acid extraction from supernatant

In order to measure only RNAs of JEV on the cell, before RNA extraction, the infected supernatants were treated with RNase. One unit of Rnase A (Qiagen Science, Germantown, MD, USA) diluted 1:10 or 1:100 with nuclease free water was added to 200 μL of each sample and incubated at 37 °C for 30 min. Rnase A activity, thereafter was inhibited by 18U of Rnase inhibitor (Qiagen) and incubated at room temperature for 30 min. The RNAs were then extracted using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

2.5. TaqMan real-time RT-PCR assay

JEV GI and GIII strains quantification was performed separately after each passage with a specific primer set with SuperScript® III Platinum® One-Step qRT-PCR Kit w/o ROX (Invitrogen, Carlsbad, CA, USA) as described previously.

For GIdetection, forward primer (5′-GGGGACAAGCAGATTAACCA-3′), reverse primer (5′-GAAGGCACCACCAAACACTT-3′) were used at a final concentration of 0.2 μM and probe (FAM-TCAACAACTTTGAAAGGGGC-36TAMSp) at a final concentration of 0.1 μM. The same primer concentration was used for GIII, primer set with forward primer (5′-CCTTGCAAAATTCCGATTGT-3′), reverse primer (5′-TGAGCTCCCTTCAAAGTCGT-3′) and probe (FAM-CTGGTGACAGTGAACCCCTT-36TAMSp). An Eppendorf Mastercycler ep Realplex (Eppendorf AG, Hamburg, Germany), was used for analysis. The thermal profile included incubation at 50 °C for 15 min, at 95 °C for 2 min for reverse transcription. DNA was amplified with 40 cycles at 95 °C for 15 s, 60 °C for 30 s.

3. Results

3.1. Determination of time-point of virus titer peak on different cell-lines

To determine the time point when virus titers in the supernatant were the highest, we inoculated the virus mixture on three cell-lines: PS, RD and C6/36. With the same virus titer inoculation (100 PFU/mL) and the same virus mixture volume (100 μL), the highest mixture virus titers reached after 3 d post infection (*p.i.*) on PS (1.3×10^3 PFU/mL) and RD (1.6×10^5 PFU/mL), and 4 d *pi.* on C6/36 (7.2×10^5) when measured by plaques assay (Figure 1).

3.2. Most efficient cell-line for JEV multiplication

When continuously inoculated 10 passages on cell-lines, the virus was maintained by C6/36 during 10 passages and the