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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% CO₂ 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% CO₂ 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% CO₂ 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 GI detection, forward primer (5'-GGGGACAAGCA-GATTAACCA-3'), reverse primer (5'-GAAGGCCACCAC-CAAACACTT-3') were used at a final concentration of 0.2 µM and probe (FAM-TCAACAACCTTTGAAAGGGGC-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 × 10³ PFU/mL) and RD (1.6 × 10⁵ PFU/mL), and 4 d *pi.* on C6/36 (7.2 × 10⁵) 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

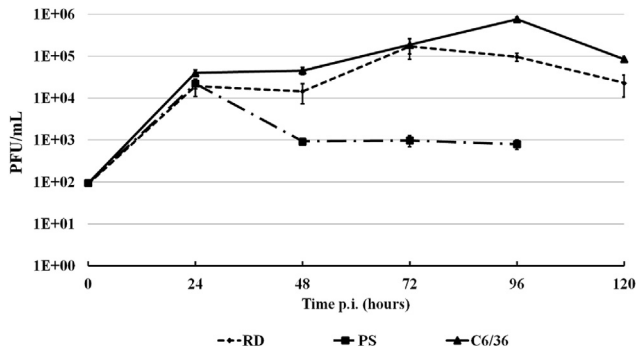


Figure 1. Multiplication kinetics by time post infection of JEV mixture of strains representative of GI and GIII genotype in C6/36, RD and PS cell lines.

highest virus titer reached after the fifth passage (1.2×10^7 PFU/mL) but not by PS or RD. The virus titer could not be determined after the 6th passage when virus mixture was inoculated on PS and after the 8th passage when the virus mixture was inoculated on RD (Figure 2).

3.3. Multiplication of GI and GIII strains in human, porcine and mosquito cells

After 93VN118 strain (GIII) and 94VN141 strain (GI) was mixed and continuously passage using PS, RD and C6/36, the numbers of RNA copy of each strain after each passage were quantified indirectly through Ct value (the higher RNA copies, the lower Ct value) by real-time RT-PCR using GI/GIII specific genotype primer sets.

In C6/36, Ct value of 94VN141 strain (GI) was lower than 93VN118 strain (GIII) meaning that the virus titer of GI strain was higher than that of GIII strain at all 10 passages (Figure 3a). Although GI strain was amplified more efficiently in C6/36 than GIII strain but both genotype strains were maintained during 10 passages because its RNA copies still could be detected by real-time RT-PCR at 10th passage.

On PS cell-line, both GI and GIII decreased gradually after each passage but the dead-end circle point of GIII strain was after 6th passage and that of GI strain was after 7th and 8th passage (Figure 3b) meaning that GI strain was maintained longer than GIII by RD cell-line.

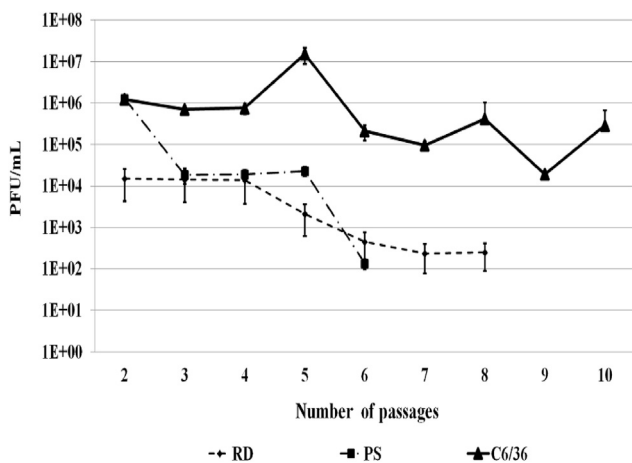


Figure 2. Multiplication kinetics within ten passages of JEV mixture of strains representative of GI and GIII genotype in C6/36, RD and PS cells.

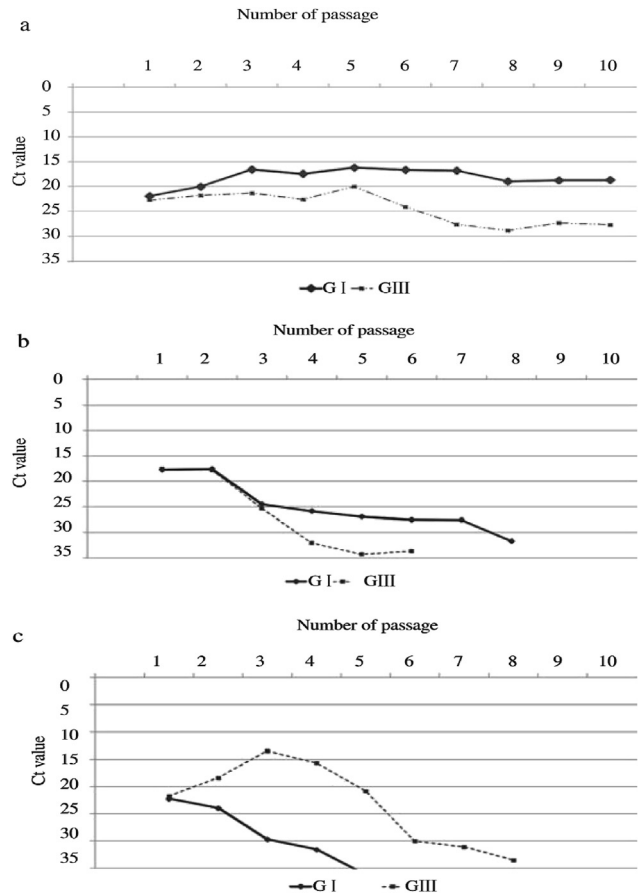


Figure 3. Multiplication kinetics of strains representative of GI and GIII genotype when co-infected in C6/36, PS and RD cells. a: C6/36 cell line; b: PS cell line; c: RD cell line.

On RD cell-line, the GI strain decreased gradually and disappeared after 5th passage gradually but GIII strain increased and got peak at 3rd passage and decreased gradually after that. However, GIII strain was maintained longer than GI strain when the dead-end point was after 8th passage (Figure 3c).

4. Discussion

The study was examined the amplification and maintenance capacity of three different cell-lines which originated from humans, porcine and mosquitoes, the three main components of JEV cycle in nature to the most two predominant JEV genotypes. The virus titer reached peak on C6/36 1 d later than that on RD and PS. It is worthy to note that multiplication of GI-b strains increased when the temperature increased [7] and the incubation temperature of C6/36 cells is 28 °C and that of RD and PS cell is 37 °C. GI-b strains were also proven to be temperate genotype whereas GIII is significantly associated with temperate climates [16].

C6/36 was the most effective tool to isolate JEV since the JEV strains could multiply and maintain high titer after 10 passages whereas the multiplication of JEV was limited within 6–8 passages when viruses were inoculated on PS and RD. When two genotype strains (GI and GIII) were co-infected *in vitro*, GI-b strain amplified more efficiently than GIII strains on C6/36 and PS cells whereas GIII strain amplified more efficiently on RD cells than GI-b strain showed that GI-b strains increased viral multiplication on C6/36 cells compared to GI-a

strains as well as other genotype strains [7]. GI-b strain could not maintain itself on RD cells, and disappeared after 5 passages. Although there were differences between *in vitro* and natural condition such as C6/36 cells lack a functional RNA interference response [7], the results provided some pieces of experimental explanation to the predominant and replacement of GI strains to GIII strains in mosquitoes and pigs [7,17] and very few GI strains have been isolated from humans [12,13].

There is a correlation between the high multiplication efficiency of GI JEV strains when two genotype strains, GI and GIII, were co-infected on porcine and mosquito cells with the predominant of GI strains in mosquitoes and porcine and the limited detection of GI strains in humans. The high multiplication efficiency of GIII strains on humans also corrected well with the predominant of GIII strain in humans.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

- [1] Erlanger TE, Weiss S, Keiser J, Utzinger J, Wiedenmayer K. Past, present, and future of Japanese encephalitis. *Emerg Infect Dis* 2009; **15**(1): 1-7.
- [2] Solomon T. Control of Japanese encephalitis-within our grasp? *N Engl J Med* 2006; **355**(9): 869-871.
- [3] Campbell GL, Hills SL, Fischer M, Jacobson JA, Hoke CH, Hombach JM, et al. Estimated global incidence of Japanese encephalitis: a systematic review. *Bull World Health Organ* 2011; **89**(10): 766-774.
- [4] Lindenbach BD, Murray CL, Thiel HJ, Rice CM. Flaviviridae: the viruses and their replication. In: Knipe DM, Howley PM, editors. *Fields virology*. 6th ed. New York: Lippincott Williams & Wilkins; 2013.
- [5] Williams DT, Wang LF, Daniels PW, Mackenzie JS. Molecular characterization of the first Australian isolate of Japanese encephalitis virus, the FU strain. *J Gen Virol* 2000; **81**(10): 2471-2480.
- [6] Schuh AJ, Li L, Tesh RB, Innis BL, Barrett ADT. Genetic characterization of early isolates of Japanese encephalitis virus: genotype II has been circulating since at least 1951. *J Gen Virol* 2010; **91**(Pt 1): 95-102.
- [7] Schuh AJ, Ward MJ, Brown AJL, Barrett ADT. Dynamics of the emergence and establishment of a newly dominant genotype of Japanese encephalitis virus throughout Asia. *J Virol* 2014; **88**(8): 4522-4532.
- [8] Yun SM, Cho JE, Ju YR, Kim SY, Ryou J, Han MG, et al. Molecular epidemiology of Japanese encephalitis virus circulating in South Korea, 1983–2005. *Virol J* 2010; **7**: 127.
- [9] Tang WF, Ogawa M, Eshitac Y, Aonoa H, Makino Y. Molecular evolution of Japanese encephalitis virus isolates from swine in Oita, Japan during 1980–2009. *Infect Genet Evol* 2010; **10**(2): 329-336.
- [10] Ma SP, Yoshida Y, Makinoet Y, Tadano M, Ono T, Ogawa M. Short report: a major genotype of Japanese encephalitis virus currently circulating in Japan. *Am J Trop Med Hyg* 2003; **69**(2): 151-154.
- [11] Wang HY, Takasaki T, Fu SH, Sun XH, Zhang HL, Wang ZX, et al. Molecular epidemiological analysis of Japanese encephalitis virus in China. *J Gen Virol* 2007; **88**(Pt 3): 885-894.
- [12] Do LP, Bui TM, Hasebe F, Morita K, Phan NT. Molecular epidemiology of Japanese encephalitis in northern Vietnam, 1964–2011: genotype replacement. *Virol J* 2015; **12**: 51.
- [13] Wang L, Fu S, Fu SH, Zhang HL, Ye XF, Yu DS, et al. Identification and isolation of genotype-I Japanese encephalitis virus from encephalitis patients. *Virol J* 2010; **7**: 345.
- [14] Nga PT, del Carmen Parquet M, Cuong VD, Ma SP, Hasebe F, Inoue S, et al. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. *J Gen Virol* 2004; **85**(Pt 6): 1625-1631.
- [15] Han N, Adams J, Chen P, Guo ZY, Zhong XF, Fang W, et al. Comparison of genotypes I and III in Japanese encephalitis virus reveals distinct differences in their genetic and host diversity. *J Virol* 2014; **88**(19): 11469-11479.
- [16] Schuh AJ, Ward MJ, Brown AJ, Barrett AD. Phylogeography of Japanese encephalitis virus: genotype is associated with climate. *PLoS Negl Trop Dis* 2013; **7**(8): e2411.
- [17] Pan XL, Liu H, Wang HY, Fu SH, Liu HZ, Zhang HL, et al. Emergence of genotype I of Japanese encephalitis virus as the dominant genotype in Asia. *J Virol* 2011; **85**(19): 9847-9853.