Main Factors Influencing the Efficient Propagation of Virulent or Attenuated Strains of *Japanese encephalitis virus* in BHK-21 Cells

Man Teng · Jun Luo · Guang-Xu Xing ·
Na Cheng · Yan-Yan Yang · Rui-Guang Deng ·
Gai-Ping Zhang

Received: 2 March 2012 / Accepted: 9 July 2012 / Published online: 12 September 2012
© Indian Virological Society 2012

Abstract *Japanese encephalitis virus* (JEV) causes severe viral encephalitis in humans and some other mammalian animals. Highly efficient culture of the virus is critical for antigen preparation, vaccine production and other basic researches. We have investigated the influence of a number of variables such as the strain virulence, the state of the host cells, medium composition, infection method and others on the proliferation of distinct JEV strains in BHK-21 cells. The results showed that two distinct strategies are needed for the propagation of virulent or attenuated JEV strains. The most critical variables were the method of infection, and especially the density of the host cell. Our studies indicate the general approaches to the in vitro culture of other JEV strains using BHK-21 cell line.

Keywords JEV · In vitro culture · TCID₅₀ · BHK-21 cell line

Japanese encephalitis virus (JEV) is widely found in the Asia–Pacific regions, including the south-east Asian, the

Indian subcontinent, the west Pacific regions and the North Australia [10]. As well as causing encephalitis in humans, JEV is pathogenic in other mammals, such as pig, horse, cattle, goat, wild boar, and the bat. The domestic pig has been characterized as the critical amplifying and maintenance host involved in JEV transmission cycle [1, 3, 5]. Although underreported, the lowest estimate of human infection worldwide is about 45,000 cases per annum including an associated 10,000 deaths. Since the first case report in 1949, JEV has circulated in most regions of China for over 60 years [11]. In the last decade, China has reported an annual average of 4,606 cases and associated 172 deaths (<http://www.moh.gov.cn/publicfiles/business/htmlfiles/wsb/pyqxx/index.htm>). However, inadequacies in disease reporting especially from the wider countryside and small towns, Japanese encephalitis (JE) cases are also obviously underreported in China.

JEV is a mosquito-borne flavivirus, which is maintained in a vertebrate host-mosquito cycle between water birds and/or pigs and *Culex* mosquitoes. In the typical JEV transmission cycle, it is suggested that two cycles of amplification occurs in pigs [3, 10]. In the initial cycle, approximately 20 % of the pigs are infected and then mosquitoes become infected by feeding on these viremic pigs. After a period of incubation, they transmit the virus to other susceptible pigs, resulting in up to 100 % porcine seroconversion. After a further extrinsic incubation period in mosquitoes, human clinical cases begin to appear. In recent years, JE cases have happened frequently in pig herds causing huge losses in the pig farming industry and potentially increasing the threat to public health. The typical cyclic pattern of JEV transmission suggests three control measures—early clinical diagnosis in both pigs and humans, mosquito control, and vaccination to decrease susceptible host populations, especially of little children

M. Teng · J. Luo (✉) · G.-X. Xing · N. Cheng · Y.-Y. Yang ·
R.-G. Deng · G.-P. Zhang (✉)
Key Laboratory of Animal Immunology of the Ministry
of Agriculture, Henan Provincial Key Laboratory of Animal
Immunology, Henan Academy of Agricultural Sciences,
Zhengzhou 450002, People's Republic of China
e-mail: luojun593@yahoo.com.cn

G.-P. Zhang
e-mail: zhanggaiping2003@yahoo.com.cn

G.-X. Xing
College of Animal Husbandry and Veterinary,
Henan Agricultural University, Zhengzhou 450002,
People's Republic of China

and pigs. The elimination of mosquitoes from large areas of countryside is problematic but the remaining two countermeasures, vaccination and early diagnosis, may be more useful.

Although in recent years vaccination against JEV has contributed much to the control of disease in humans and pigs [4, 8, 12], its effectiveness is compromised by variations in vaccine quality. It is well known that the growth of virus to high titer is needed for the preparation of high quality vaccines, but it has been found difficult to obtain high titers of JEV in cell cultures. A number of cell lines, such as the mosquito *Aedes albopictus* cell line C6/36 [2], the baby hamster kidney cells BHK-21 [9], the porcine kidney cell line PK-15 [6], the African green monkey kidney epithelial cell Vero [13], and the chicken embryo fibroblast cell cultures (CEFs), all act as susceptible hosts to JEV. Of these BHK-21 cell line, because of its ease of culture on any scale, is one of the most commonly used cell lines for JEV propagation, virus titration, and vaccine preparation. From previous experiences, we found that when BHK-21 cells were used for JEV growth only very low variable titers were obtained. The rapid growth rate of BHK-21 cells, together with the differing virulence of JEV strains, is possibly the main factors influencing JEV growth but remain uninvestigated. Herein, we examined the roles of culture medium composition, primary host cell density, and method of infection on the virus propagations of both virulent and attenuated JEV strains in BHK-21 cells maintained under different conditions. The results provide us with distinct strategies for the efficient propagation of virulent or attenuated JEV strains in BHK-21 cells.

First an accurate virus titration method for measuring JEV proliferation in BHK-21 cells had to be established. Aliquots of 2.5×10^3 , 5.0×10^3 or 10.0×10^3 BHK-21 cells diluted in 100 μ l of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 8 % new-born calf serum (NCS) (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China) were seeded into the wells of 96-well plates and cultivated for 24 h at 37 °C in a 5 % CO₂ incubator. Then, growth medium of the cell monolayers, as shown Fig. 1a, were replaced with 100 μ l of a maintenance medium DMEM supplemented with 1, 2, 3, or 4 % NCS and cultured for a further 96 h, respectively. The results showed that the cells initially seeded at densities of 2.5×10^3 cells/well and maintained in DMEM plus 3 % NCS gave better growth over 72 h.

Based on these optimized conditions, a stable method was established for JEV virus titration. Briefly, batches of 2.5×10^3 BHK-21 cells were primarily seeded into 96-well plates and maintained in 100 μ l DMEM plus 8 % NCS for 24 h. The supernatants were removed and 100 μ l of aliquots of diluted culture supernatants of BHK-21 cells previously infected with any one of the three JEV strains,

two virulent strains CSF.XZ-2D and BSF.ZZ-3 isolated from diseased pigs and the other vaccine strain SA14-14-2 isolated from commercial vaccine (Wuhankeqian Animal Biological Products Co., Ltd., China), were serially 10-fold diluted in 100 μ l DMEM plus 3 % NCS before transfer to the 96-well plates. An equal volume of dilution medium was added to the last column to serve as controls. The plates were incubated for 4–5 days prior to reading the end point by cytopathic effect (CPE). Virus titers, 50 % tissues culture infective dose (TCID₅₀), were calculated according to the method of Reed and Muench [7]. Repeated titration of three stock suspensions of CSF.XZ-2D, BSF.ZZ-3 and SA14-14-2, gave values of $10^{6.1 \pm 0.1}$, $10^{5.6 \pm 0.2}$ and $10^{4.5 \pm 0.1}$ TCID₅₀/ml at 4–5 days post-infection (dpi), respectively. The level of the CPE caused by JEV infection is easily seen by microscopic examination. This provides a procedure which is easy, accurate and reproducible for the titration of JEV.

In order to optimize growth conditions for viral growth, 25-cm² flasks were seeded with BHK-21 cells at 0.625×10^6 , 1.25×10^6 , 2.50×10^6 , 5.00×10^6 , 7.50×10^6 or 10.00×10^6 cells per flask and maintained in 5 ml DMEM plus 8 % NCS for 24 h, then the cells grow adherently and constituted the sub-confluent or confluent monolayers (Fig. 1b). The supernatants were removed and the cell layers were washed with phosphate buffered saline (PBS, pH 7.2). The cells were then incubated with 0.5 ml of viral suspension, virulent strain CSF.XZ-2D or attenuated strain SA14-14-2 at a titer of $10^{4.0}$ TCID₅₀/ml, for 1.5 h at 37 °C. After incubation, the cells were washed twice to remove unbound virus particles and then maintained in 5 ml of either mediums DMEM plus 1 % or 3 % NCS, respectively. At 60 hour post-infection (hpi), the cultures were examined and sampled for virus titration as described above. Statistical analysis was performed using statistic software SPSS 13.0 for Windows. Differences between treatments were tested using one way analysis of variance (Oneway ANOVA, LSD) and were considered significant among treatment groups at a probability level of $p < 0.05$. The results showed that at 60 hpi, cells of virulent strain-infected sub-confluent monolayers (0.625×10^6 – 2.50×10^6 cells/bottle) and maintained in DMEM plus 1 % NCS were all killed by CSF.XZ-2D while those maintained in DMEM plus 3 % NCS live cells remained. In the flasks seeded at high density, 5.00×10^6 – 10.00×10^6 cells per flask, nearly all the cells were dead, because of inadequate nutrient. Similar results are observed in SA-14-14-2-infected monolayers, however there are more surviving cells, i.e. less CPE possibly reflecting the lower virulence of the attenuated vaccine strain.

These results are reflected by the observed virus production (Table 1). Titers of CSF.XZ-2D produced in most monolayers maintained in DMEM plus 3 % NCS were

Fig. 1 Proliferation of the virulent or attenuated JEV strains on BHK-21 cells under different conditions. **a, b** BHK-21 monolayers prepared for the virus titration or the in vitro culture of JEV, respectively. Aliquots of BHK-21 cells primarily used for preparing the monolayers are shown on the left of each picture. All the experiments were triple repeated independently but for the limited space, only one representative repeat is shown here (original magnification ×200). **c, d** Virus

titers of JEV propagated in distinct initial monolayer density and maintenance medium for 60 h. **e, f** Virus titers of JEV propagated on optimal initial BHK-21 monolayers treated with various infection methods and infecting dose and maintained in optimal medium for 60 h. **g, h** Virus titer curves of JEV proliferated in BHK-21 cells under optimal conditions. Data points are the average from three independent repeat experiments

Table 1 Virus titers of the virulent or attenuated JEV strains propagated in different growth conditions for 60 h on BHK-21 monolayers

Virus titers ^c		BHK-21 cell primary seeding density per 25-cm ² and virus titers determined at 60 hpi (TCID ₅₀ /ml)					
Strains	Mediums	0.625 × 10 ⁶	1.25 × 10 ⁶	2.50 × 10 ⁶	5.00 × 10 ⁶	7.50 × 10 ⁶	10.00 × 10 ⁶
CSF.XZ-2D	MDEM plus 1 % NCS	10 ^{4.1±0.4A,a}	10 ^{4.5±0.3A,a}	10 ^{4.1±0.1A,a}	10 ^{3.9±0.1B,a}	10 ^{3.6±0.1B,a}	10 ^{3.4±0.3B,a}
	MDEM plus 3 % NCS	10 ^{5.7±0.3A,b}	10 ^{6.0±0.2A,b}	10 ^{5.7±0.3A,b}	10 ^{5.0±0.2B,b}	10 ^{4.3±0.5B,a}	10 ^{3.4±0.1B,a}
SA14-14-2	MDEM plus 1 % NCS	10 ^{3.3±0.5B,a}	10 ^{4.3±0.3A,a}	10 ^{4.9±0.6A,a}	10 ^{5.1±0.7A,b}	10 ^{4.2±0.6A,a}	10 ^{3.0±0.5B,a}
	MDEM plus 3 % NCS	10 ^{5.8±1.1A,b}	10 ^{4.6±0.8B,a}	10 ^{4.1±0.4B,a}	10 ^{3.7±0.1B,a}	10 ^{3.2±0.4B,b}	10 ^{3.2±0.7B,a}

^{A,B} Within line for each treatment of a specific JEV strain, means (±standard deviation) with different superscripts differ significantly ($p < 0.05$)

^{a,b} Within column for each treatment of a specific JEV strain, means (±standard deviation) with different superscripts differ significantly ($p < 0.05$)

^c The data shown are the average of three independent repeated experiments

Table 2 Influence of differentially added virus concentrations on the propagations of virulent or attenuated JEV strains in optimal initial monolayers and maintenance mediums

Virus titers ^c		Primary virus infection doses and virus productions determined at 60 hpi (TCID ₅₀ /ml)		
Strains	Infection methods	0.5 ml of 10 ^{1.0}	0.5 ml of 10 ^{2.0}	0.5 ml of 10 ^{3.0}
CSF.XZ-2D	Washed post-incubation	10 ^{4.7±0.3B,a}	10 ^{5.8±0.4A,b}	10 ^{4.6±0.4B,a}
	Unwashed post-incubation	10 ^{4.4±0.1A,a}	10 ^{4.7±0.1A,a}	10 ^{4.2±0.3A,a}
SA14-14-2	Washed post-incubation	10 ^{3.2±0.4B,a}	10 ^{3.7±0.3B,a}	10 ^{4.5±0.2A,a}
	Unwashed post-incubation	10 ^{3.3±0.5B,a}	10 ^{4.3±0.3B,a}	10 ^{5.7±0.5A,b}

^{A,B} Within line for each treatment of a specific JEV strain, means (±standard deviation) with different superscripts differ significantly ($p < 0.05$)

^{a,b} Within column for each treatment of a specific JEV strain, means (±standard deviation) with different superscripts differ significantly ($p < 0.05$)

^c The data shown are the average of three independent repeated experiments

universally significantly higher ($p < 0.05$), nearly 100-fold than those maintained in DMEM plus 1 % NCS. This was especially true for the sub-confluent monolayers as shown in Fig. 1c. From the data plot, the optimum seeding density for virus production is 1.25×10^6 cells per flask, although the mean titers for seeding densities of 0.625×10^6 and 2.50×10^6 are not statistically significantly different. A similar phenomenon was also observed with SA14-14-2 when maintained in DMEM plus 1 % NCS, excepting that the observed optimum seeding density was 5.00×10^6 cells per flask (Fig. 1d). When the maintenance medium contained 3 % NCS, instead of 1 %, the optimum seeding density was no different for the virulent CSF.XZ-2D strain. However for strain SA14-14-2, peak production of virus was observed at 0.625×10^6 cells per flask and declined steadily with increasing cell numbers.

Changes in virus production, as a function of the added virus concentration, were subsequently investigated. Flasks were seeded with BHK-21 cells at the optimum densities for each JEV strain as found above, i.e. 1.25×10^6 for CSF.XZ-2D and 0.625×10^6 for SA14-14-2. Following growth for 24 h in DMEM plus 8 % NCS, the monolayers were washed twice with PBS and 0.5 ml of either virus suspension was then added at $10^{1.0}$, $10^{2.0}$, or $10^{3.0}$ TCID₅₀/ml,

respectively. Each flask was prepared in duplicate. After an incubation of 1.5 h, the monolayer in one of the flasks was washed twice with PBS. After adding 5 ml of DMEM plus 3 % NCS to each flask, they were all incubated at 37 °C for 60 h. Then, the contents were taken for the determination of the virus titer. Again, differences in proliferation were found between virulent and attenuated JEV strains under identical infection protocols (Table 2). Titration assays showed that for CSF.XZ-2D, although virus production showed a similar response to infecting dose, washing the cell monolayer after the initial incubation gave a higher yield overall and particularly at an infecting dose of $10^{2.0}$ TCID₅₀/ml (Fig. 1e), with a titer of $10^{5.8±0.4}$ TCID₅₀/ml. In contrast the production of SA14-14-2 was directly related to the infecting dose and higher titers were obtained if the monolayer was not washed (Fig. 1f). The yield of SA14-14-2 was $10^{5.7±0.5}$ TCID₅₀/ml at an infecting dose of $10^{3.0}$ TCID₅₀/ml.

The last parameter to be investigated was the length of time post infection for cultures to give the maximum yield of virus. Using the optimal conditions already determined for each strain, cultures were incubated for 24, 48, 60, 72, and 96 h and the virus titers were obtained at each time point. The results for CSF.XZ-2D showed that from a titer $10^{3.8±0.7}$ TCID₅₀/ml at 24 hpi, it increased over the next

36 h to $10^{6.0 \pm 0.3}$ TCID₅₀/ml at 60 hpi, following which the virus titer declined until 96 hpi (Fig. 1g). The virus titer of SA14-14-2 started from a lower level of $10^{2.5 \pm 0.1}$ TCID₅₀/ml at 24 hpi, but steadily increased to reach a similar peak titer of $10^{5.5 \pm 0.7}$ TCID₅₀/ml until at 72 hpi (Fig. 1h).

Examining these experiments, it appears clear that maximal viral production will depend on providing a sufficient number of growing host cells and the virulence of the strain being grown. For the two strains of JEV used in this study, the virulent CSF.XZ-2D and the attenuated strain SA-14-14-2, initial host cell density, infecting dose, and the incubation time are critical and different. So for the attenuated strain SA-14-14-2, lower cell density, more viruses, and a greater opportunity for infection plus a longer time for growth are needed to produce a high viral yield comparable to that of the virulent strain. These findings are meaningful and they underline the care required to establish strain specific conditions for the maximal production of virus in tissue culture. As well known that highly efficient propagation of the virus is critical for antigen preparation, vaccine production and basic research, our research provides the general approaches to the in vitro culture of other JEV strains using BHK-21 cell line. Additionally, it is also meaningful for the new adventurers who explore in the research field of JEV.

Acknowledgments This work was supported by the Special Fund for Agro-scientific Research in the Public Interest (Grant No. 201203082-5). The authors also gratefully acknowledge the critical review by Prof. Norman A. Gregson (ION, UCL, London, UK).

References

- Gajanana A, Rajendran R, Samuel PP. Japanese encephalitis in South Arcot district, Tamil Nadu, India: a three-year longitudinal study of vector abundance and infection frequency. *J Med Entomol.* 1997;34:651–9.
- Igarashi A. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. *J Gen Virol.* 1978;40:531–44.
- Konno J, Endo K, Agatsuma H, Ishida N. Cyclic outbreaks of Japanese encephalitis among pigs and humans. *Am J Epidemiol.* 1966;84:292–300.
- Ohrh H, Tandan JB, Sohn YM, Shin SH, Pradhan DP, Halstead SB. Effect of single dose of SA 14-14-2 vaccine 1 year after immunisation in Nepalese children with Japanese encephalitis: a case-control study. *Lancet.* 2005;366:1375–8.
- Peiris JS, Amerasinghe FP, Amerasinghe PH, Ratnayake CB, Karunaratne SH, Tsai TF. Japanese encephalitis in Sri Lanka: the study of an epidemic: vector incrimination, porcine infection and human disease. *Trans R Soc Trop Med Hyg.* 1992;86:307–13.
- Pirtle EC. Variation in the modal chromosome number of two PK-15 porcine kidney cell lines. *Am J Vet Res.* 1966;27:747–9.
- Reed LJ, Muench H. A simple method for estimating fifty percent endpoints. *Am J Hyg.* 1938;27:493–7.
- Sohn YM, Park MS, Rho HO, Chandler LJ, Shope RE, Tsai TF. Primary and booster immune responses to SA14-14-2 Japanese encephalitis vaccine in Korean infants. *Vaccine.* 1999;17:2259–64.
- Stoker M, Macpherson I. Syrian Hamster fibroblast cell line BHK-21 and its derivatives. *Nature.* 1964;203:1355–7.
- Van den Hurk AF, Ritchie SA, Mackenzie JS. Ecology and geographical expansion of Japanese encephalitis virus. *Annu Rev Entomol.* 2009;54:17–35.
- Wang HY, Takasaki Y, Fu SH, Sun XH, Zhang HL, Wang ZX, Hao ZY, Zhang JK, Tang Q, Kotaki A, Tajima S, Liang XF, Yang WZ, Kurane I, Liang GD. Molecular epidemiological analysis of Japanese encephalitis virus in China. *J Gen Virol.* 2007;88:885–94.
- Xin YY, Ming ZG, Peng GY, Jian A, Min LH. Safety of a live-attenuated Japanese encephalitis virus vaccine (SA14-14-2) for children. *Am J Trop Med Hyg.* 1988;39:214–7.
- Yasumura Y, Kawakita M. The research for the SV40 by means of tissue culture technique. *Nippon Rinsho.* 1963;21:1201–19.