Optimal Conditions of Plaque Titration of Japanese Encephalitis Virus on BHK21 Cells

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Abstract: Optimal conditions for the infectivity titration by plaque formation (PFU) of Japanese encephalitis (JE) virus on mammalian BHK21 cells were investigated. Early virus inoculation at 6 hr after seeding low concentration of the cells (0.25×10^5/ml) produced larger and clearer plaques compared with higher concentration or longer incubation (1−2 days) of the cells. However, the virus titer increased by the incubation time until 2 days to become the highest for this inoculum cell concentration, and up to 1 day for the inoculum cell concentration of 0.5 or 1.0×10^5/ml. While the virus titer did not differ significantly by the time of cell culture for the inoculum cell concentration of 2.0×10^5/ml. Supplementation of 9% fetal bovine serum (FBS) to the cell growth medium was mandatory for clear plaque formation. Among several methyl cellulose (MC) concentrations in the overlay medium, a moderate concentration of 1.25% was optimal to produce clear and distinct plaques on 1 day's culture from 2.0×10^5 cells/ml.

Key words: Plaque formation, Infectivity titration, Japanese encephalitis virus

INTRODUCTION

Japanese encephalitis is an acute viral encephalitis characterized by high fever, headache and impaired consciousness accompanied by high mortality and grave sequelae (Shope, 1980; Monath, 1985, 1986). The causative agent, JE virus, is a member of flavivirus (Westaway *et al.*, 1985), and formerly classified as mosquito-borne group B arbovirus (Clarke and Casals, 1965). JE is present from Far East, through Southeast to South Asia and has been a great public health problem in several countries in these regions (Miles, 1960; Umenai *et al.*, 1985; Rosen *et al.*, 1986). Infectivity assay by plaque formation and its application to the neutralization (N) test by the plaque reduction have been prerequisites for accurate viral infectivity assay and N test (Dulbecco and Vogt, 1954).

In the case of JE virus, Primary cultures of hamster of pig kidney cells were used to calculate 50% tissue culture infective dose ($TCID_{50}$) by observing cytopathic effect

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(Kissling, 1957; Diercks and Hammon, 1958; Lee *et al.*, 1958). The method was improved to use stable pig kidney (PS) and baby hamster kidney (BHK21) cell lines for $TCID_{50}$ and PFU assays (Inoue and Ogura, 1962; Westaway, 1966; Karabatsos and Buckley, 1967). De Madrid and Porterfield (1969) and Hashimoto *et al.*, (1971) used PS cells with carboxymethyl cellulose (CMC) overlay on microplates for the PFU assay of group B arboviruses. While the National Institute of Health of Japan adopted the PFU assay on primary chicken embryo cell cultures (Dulbecco and Vogt, 1954; Inoue *et al.*, 1961) as the standard method for JE virus. Okuno *et. al.*, (1978, 1985) described a rapid infectivity assay by focus formation and a focus reduction N test of JE and dengue viruses by immunoperoxidase staining of intracellular viral antigens in the infected BHK21 cells. Although, their method was reported to provide rapid results, it requires several steps and reagents, and the results were not always guaranteed in our hands. We have been using Hashimoto's method modified to semimicroplates, BHK21 cells and 1.5% MC overlay medium instead of microplates, because of its simplicity and reliability even though longer time was required to obtain the results. However, the size and clearness of the plaques were not always uniform, and hazy or confluent plaques sometimes did not provide accurate data. Therefore, we examined several basic conditions of PFU assay of JE virus on BHK21 cells to present the results in this paper.

Materials and Methods

Cells: Aedes albopictus, Clone C6/36, cells (Igarashi, 1978) were used to prepare the seed of JE virus. The cells were grown at 28℃ in Roux bottles (750 cm^3) using 40 ml/ bottle of Eagle's minimal essential medium supplemented with 0.2 mM each of 7 nonessential amino acids (hereafter shown as Eagle's medium: Eagle, 1959) and 9% heat-inactivated FBS (Sanko Pure Chemicals, Japan). BHK21 cells were used for PFU assay of JE virus and were grown in 12-well semimicroplates (Linbro, Flow Laboratories, USA) using the same medium as C6/36 cells and 3 ml/ well, but at 37℃ in humidified 5% CO_2-atmosphere. Four concentrations of BHK21 cells (0.25, 0.5, 1.0, and 2.0×10^5 cells/ml) in cell growth medium were seeded to the plates and cultured for 6 hr, 1 day or 2 days before JE virus inoculation, to see the effect of cell density on plaque formation.

JE virus and its PFU titration: A wild strain of JE virus, JaOArS982 (Hori *et al.*, 1986), was used throughout this study. The seed virus was inoculated to C6/36 cell cultures and infected fluid of 2% FBS in Eagle's medium was harvested 2−3 days after infection, divided into aliquots and kept at−70℃ until the experiments. The virus was serially diluted in 10-fold steps with virus diluent (5% FBS in Eagle's medium), and 0.2 ml of the diluted virus was inoculated to each well of BHK21 cell culture plates. After 2 hr adsorption at 37℃ in humidified 5% CO_2-atmosphere by spreading the inoculum over the cell sheets every 30 min, the cells were covered by the overlay medium of 2% FBS in Eagle's medium containing varying concentrations of MC (0.5, 0.75, 1.0, 1.25, 1.5, and 1.75%). The cells were again incubated at 37℃ for 5 days in humidified 5% CO_2-atmosphere. The overaly medium was removed, and the cells were gently rinsed with phosphate-buffered

saline with calcium and magnesium (Dulbecco and Vogt, 1954). The cells were fixed with cold methanol at −20℃ for 30 min, rinsed with tap water and stained with 0.1% trypan blue in 0.9% NaCl at room temperature for 1 hr. Excess dye was removed by rinsing with tap water, and the plates were dried to count the number of plaques to calculate the virus infectivity by PFU/ml.

RESULTS

Effect of BHK21 cell concentration on JE virus plaque formation

Fig. 1 shows JE virus plaques formed on BHK21 cells which were seeded at 4 different concentrations as described in the Materials and Methods, and inoculated with 3 virus dilutions; at 10^{-6} in the uper line, 10^{-5} in the middle line, and 10^{-4} in the lower line respectively. The virus was inoculated to the cells after 6 hr (Fig. 1A), 1 day (Fig. 1B), or 2 days (Fig. 1C) of the cell culture. In these panels, the wells on the left column number 1 were seeded with 0.25×10^5 cells/ml, the 2nd column with 0.5×10^5 cells/ml, the 3rd column with 1.0×10^5 cells/ml, and the right 4th column with 2.0×10^5 cells/ml respec-

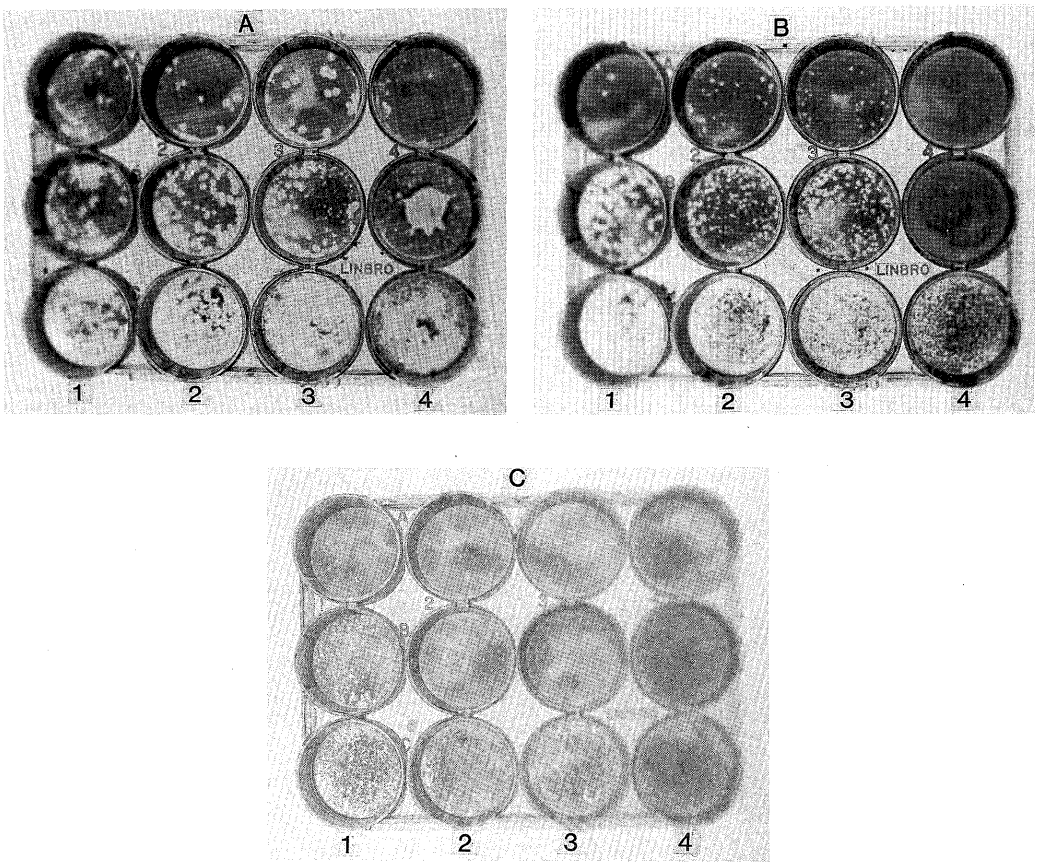

Fig. 1. Plaques of JE virus on BHK21 cells prepared from different concentrations of the cell inoculum. The details were described in the text.

Table 1. Infectivity titer (PFU/ml $\times 10^{-6}$) of JE virus on BHK21 cells prepared by different concentration of the cell inoculum

Number of cells seeded ($\times 10^5$/ml)	Days of BHK21 cell culture before virus inoculation		
	0	1	2
0.25	7.5	31	70
0.50	12	50	55
1.00	26	58	38
2.00	31	37	27

tively. After virus adsorption, the cells were covered by the overlay medium containing 1.5% MC for PFU assay. The result showed the largest plaque size, when the lowest cell concentration was used and the virus was inoculated after 6 hr of cell cultures. The plaque size decreased as the inoculum cell concentration and incubation time were increased to form semiconfluent or confluent sheets at the time of virus inoculation.

The virus titer obtained in this experiment increased up to 2 days of the cell culture for the cell inoculum of 0.25×10^5/ml, and up to 1 day for the inoculum of 0.5 and 1.0×10^5 /ml, but did not significantly differ for the inoculum of 2.0×10^5/ml. Based on this result, we used this concentration of the cell inoculum and 1 day's culture before virus inoculation in the following experiments.

Effect of FBS concentration in the cell growth medium on JE virus plaque formation

Fig. 2 shows the plaques formed on BHK21 cell cultures prepared with 4 different concentrations of FBS; 0% (result was not shown because of complete cell damage), 2% in Fig. 2A, 5% in Fig. 2B, and 9% in Fig. 2C respectively, using tetraplicate wells for each virus dilution. In this experiment also, overlay medium containing 1.5% MC was used, and the virus titer and cell condition after 1 day's culture were shown in Table 2. The cells

Table 2. Effect of FBS concentration in BHK21 cell growth medium on JE virus infectivity and cell growth

Percent of FBS in cell growth medium	Virus titer (PFU/ml $\times 10^{-6}$)	Cell condition after 1 day's culture
0	uncountable	Cell growth was not observed
2	8.5	Small number of the cells showed cell division
5	49	Cells grew out to form almost monolayers
9	88	Cells grew out to form complete monolayers

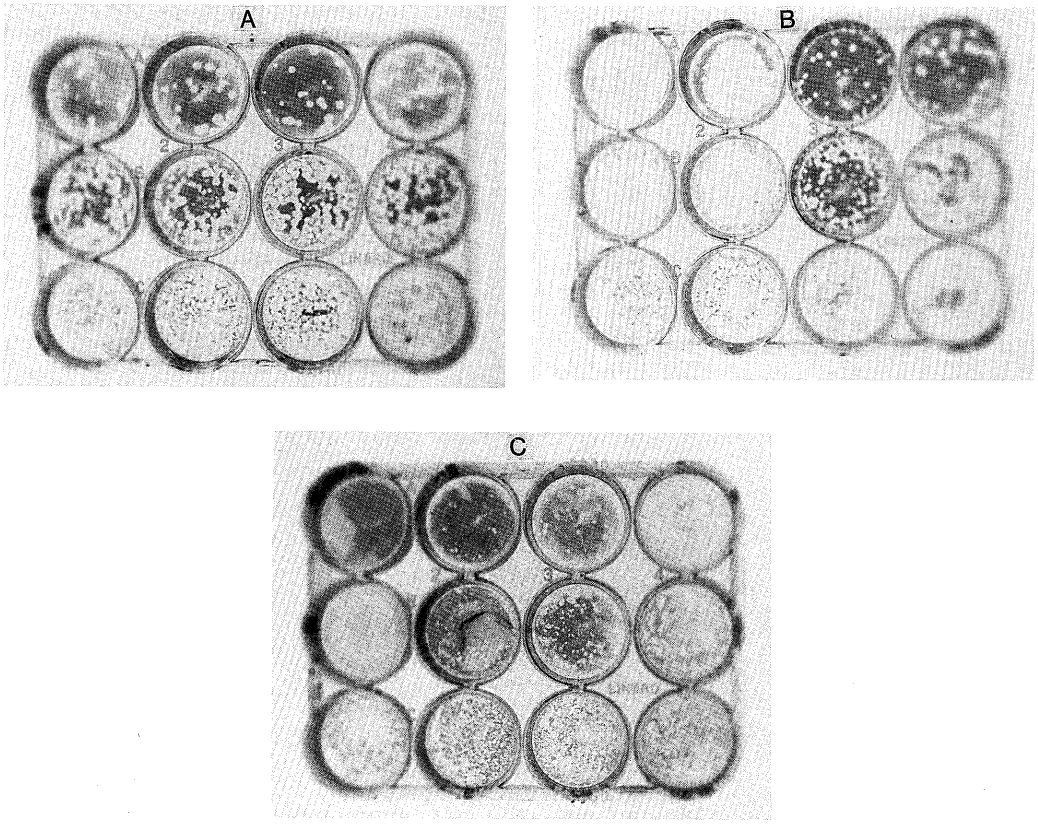

Fig. 2. Effect of FBS concentration in the cell growth medium on the plaque formation of JE virus on BHK21 cells. The details were described in the text.

did not grow and no plaques were observed on the cell cultures without FBS (Table 2 line 1), probably because of poor cell condition. At low concentration of 2% FBS, large−sized plaques were observed (Fig. 2A), but the virus titer was relatively low (Table 2 line 2). When the FBS concentration was increased to 5% and 9%, the plaque size became smaller, but the virus titer increased instead (Fig. 2B, Table 2 line 3; and Fig. 2C, Table 2 line 4, respectively).

Effect of MC concentration in the overlay medium on the plaque formation of JE virus

Fig. 3 shows the plaques formed on BHK21 cells under overlay medium containing 6 different concentrations of MC; 0.5% in Fig. 3A (columns 1 & 2), 0.75% in Fig. 3A (columns 3 & 4), 1.0% in Fig. 3B (columns 1 & 2), 1.25% in Fig. 3B (columns 3 & 4), 1.5% in Fig. 3C (columns 3 & 4), and 1.75% in Fig. 3C (columns 1 & 2), respectively. The virus titer did not change significantly by the MC concentrations as shown in Table 3. At lower concentration of 0.5 and 0.75% MC, plaques were hazy and opaque and difficult to count individually (Fig. 3A). While at moderate to higher concentration of 1 and 1.25% (Fig. 3B) or 1.5% MC (Fig. 3C columns 3 & 4), plaques were clear, distinct and easy to count.

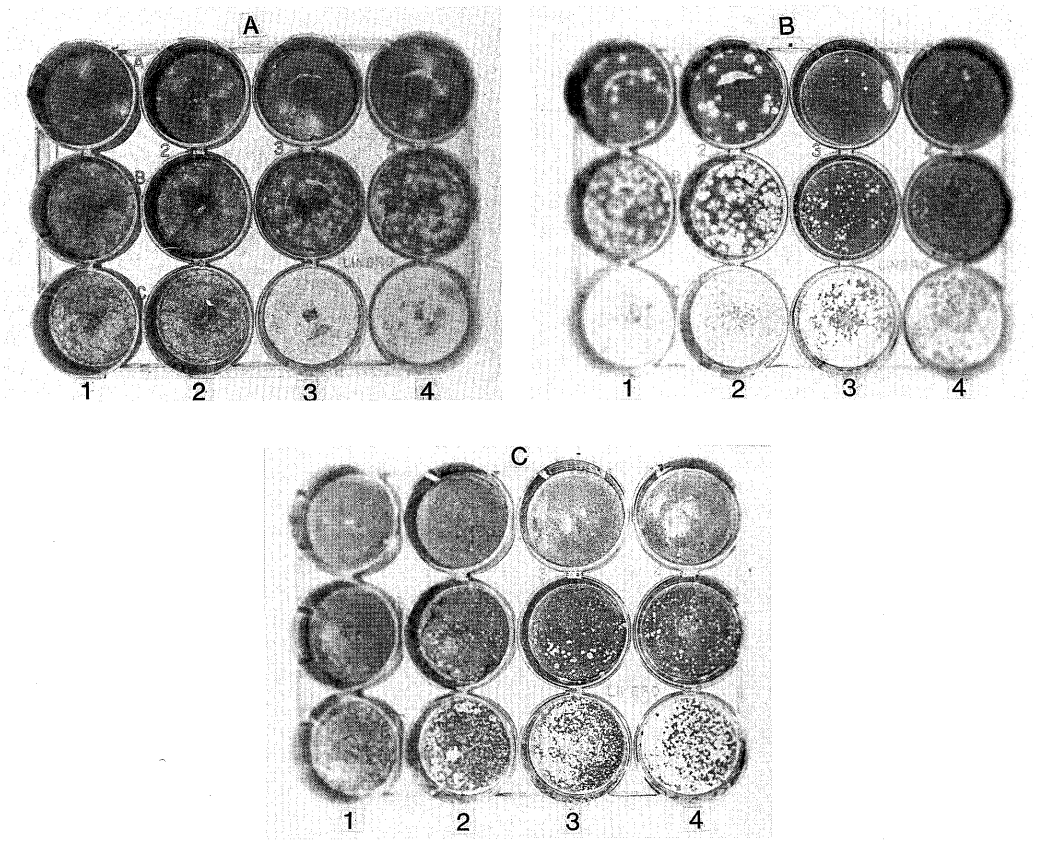

Fig. 3. Effect of MC concentration in the overlay medium on the plaque formation of JE virus on BHK21 cells. The details were described in the text.

Table 3. Effect of MC concentration in the overlay medium on the plaque formation of JE virus on BHK21 cells

MC concentration (%)	JE virus infectivity PFU/ml × 10^{-7}	Plaque morphology
0.5	5.0	Opaque and hazy
0.75	7.4	Unclear and clustered
1.0	9.7	Large and relatively clear
1.25	7.0	Small but clear
1.5	6.8	Small but clear
1.75	6.0	Small and not so sharp

Discussion

The data shows that confluent or semiconfluent cell sheets at the time of virus inoculation gave higher virus titer than the sparse cell sheets. For the cell culture of 2.0×10^5/ml, 1 day's culture was sufficient, but longer incubation time was required for the cultures with lower concentration of cell inoculum. However, the plaque size became smaller when the virus was inoculated to higher density of the cells. Roughly, reverse relation was observed between the virus titer and plaque size. Regarding FBS concentration in the cell growth medium, higher concentration gave higher virus titer, and again reverse relation was observed between the virus titer and plaque size. Our data showed that 9% of FBS in the cell growth medium should be used for the maximal PFU assay of JE virus on the cell culture of 2 days after seeding 0.25×10^5 cells/ml. The cultures of 1−2 days of $0.5-1.0\times10^5$ cells/ml gave almost similar virus titers, while the highest cell concentration of 2.0×10^5 cells/ml gave slightly less titer even when the cultures were inoculated with the virus after 6 hr or 1 day's incubation.

Regarding the MC concentration in the overlay medium, 1.25% or higher concentration gave almost the same virus titer and plaque size. From operational point of view, the overlay medium of lower MC concentration is easier to handle because of less viscosity, and 1.25% MC could be used for easy handling without losing the virus titer.

Liprandi (1981) documented PFU assay of yellow fever virus 17D vaccine strain by 1% sodium CMC overlay medium and crystal violet staining of infected Vero cells. Similar method was also reported by Buckley and Gould (1985) for the same virus and cells but 1.5% CMC overlay medium and naphthalene black staining. The first disadvantage of these PFU assays by staining infected cells is longer incubation time of 5 days for JE virus compared with the Okuno's method of immunoperoxidase staining which could provide the results within 1−2 days, but requires relatively tedious steps and counting the foci under a microscope. The second disadvantage is the inability to recover progeny viruses from the plaques formed on the host cells, which will require agar or agarose overlay medium instead of MC or CMC.

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