American Journal of Biomedical Sciences
ISSN: 1937-9080
nwpii.com/ajbms

Standardization of Plaque Assay of Japanese Encephalitis Virus (Nakayama NIH Strain) on BHK-21 (Cl-13) Cell Line

Namrata Anand, Sandeep Kumar and Dipa Gowal

Japanese Encephalitis Vaccine Division, Quality Control Section, Central Research Institute, Kasauli (H.P.), India
***Corresponding author**
Dr. Dipa Gowal
Dy. Asst. Director
Japanese Encephalitis Vaccine Division,
Quality Control Section,
Central Research Institute,
Kasauli (H.P.), India.
Ph.: +91-9816838860
Email: gill.microbiologist@rediffmail.com

Received: 15 July 2009; | Revised: 19 September 2009; | Accepted: 28 September 2009

Abstract

For establishment of plaque assay on BHK-21(Cl-13) cell line, different parameters were standardized. A cell count of 3-4 X 10^5/ml was found suitable when grown in Eagle's minimum essential medium (EMEM) containing 5% foetal bovine serum (FBS) whereas a concentration of 2% FBS was optimum for the overlay media containing 0.2% final conc. of carboxymethyl cellulose (CMC); a pH value of 7. 8 was suitable for normal growth of BHK-21 cells as well as for the development of plaques; an inoculum size of 0.2ml per well of a 6-well TC plate at 10^{-8} dilution of the Japanese Encephalitis (JE) virus was found to be sufficient to give the optimum plaque count. A 90 minutes adsorption time was adequate for the virus to adsorb onto the BHK-21 cells. Under these conditions the development of plaques occurred in five days at 36 $\pm$ 1^0C, 5% CO_2 and >90% Relative Humidity (RH). Repeatability of the assay was tested and found to be good (Mean $\pm$ SD= 90 $\pm$ 6.6; CV=7.2%). A comparison between the JE virus plaques on BHK-21 cells and Chick Embryo Fibroblast (CEFB) cells showed comparable results. Thus, it was concluded that the continuous cell line such as BHK-21 can be used in place of CEFB cells for plaque assay of JE virus and this system can be used as an alternative approach for the potency assay of JE vaccine.

Keywords: JE virus plaques; BHK-21 cell line.

1. Introduction

The assay of antibodies against Japanese encephalitis (JE) virus is essential for laboratories engaged in the production of JE vaccine and in the sero-epidemiological studies. The major serologic techniques employed for assay of antibodies to arboviruses are neutralization (NT), complement

fixation (CF) and haemagglutination inhibition (HAI) [1]. Neutralization test has higher sensitivity compared with HI, CF or ELISA, and since the functional antibody can be measured, the neutralization test is thought to be the most appropriate [2, 3, 4]. For determination of neutralizing-Ab titre, the plaque reduction neutralization assay (PRNT), using CEFB cells, is one of the oldest methods [5, 6, 7]. In our laboratory, PRNT assay is being followed for determination of potency of the mouse brain inactivated freeze dried JE vaccine. It is the most reliable, sensitive and specific assay but on the other hand it is quite a lengthy and a laborious procedure. The substrate used here is a primary culture of CEFB cells. A large number of embryonated eggs as well as trained staff are required to carry out the assay. Because of the complicated nature of preparing CEFB cell culture, it is desirable to introduce a method using an established cell line (such as BHK-21) which could reduce labour and be simple to perform.

The WHO recommends the use of BHK-21 cells in the quality testing of JE vaccines such as the virus-inactivation test and also for potency estimation by PRNT assay [6]. Thus, an attempt has been made to propagate JEV in BHK-21 (clone-13) cell line to establish plaque assay system and further to develop a novel assay for potency testing of the JE vaccine.

2. Materials and Methods

2.1 JE virus strain

JE virus strain used for the study was the Nakayama N.I.H. supplied by Dr. Akira, Oya, Deputy Director General, National Institute of Health, Tokyo, Japan.

2.1.1 JE Stock virus preparation

The stock virus was prepared as described in Minimum Requirement for JE Vaccine, Government of Japan, 1986 [5]; WHO 1988 [6] and I.P. 1996 [7]. Briefly, the JE virus strain was diluted to 10^{-3} in Hank's Balanced Salt solution (HBSS) (SIGMA) containing 5% Foetal Bovine Serum (FBS) (GIBCO) and 0.02ml was inoculated intracerebrally into two days old suckling mice. Brains of mice, showing typical signs of encephalitis, were harvested immediately before death and homogenized under aseptic conditions and made upto 10% w/v suspension in Hank's balanced solution containing 5% FBS. It was subjected to centrifugation at 2000 g for 30 min under refrigerated conditions. The supernatant was collected, distributed into small aliquots (0.3ml each) and stored at -70^{o}C till use and this served as the stock virus. The above prepared virus was titrated on CEFB cells (a routine system being followed in our laboratory).

2.2 Cell line

BHK-21(Cl-13) cell line was obtained from Central Drugs laboratory/ National Control Laboratory, Central Research Institute, Kasauli, H.P., India, at passage level 87 and was certified to be free from *Mycoplasma* and other adventitious agents. The cells were revived as per the standard procedure.

2.3 Parameters studied for the plaque assay

A number of parameters were studied and optimized for the establishment of plaque assay on BHK-21 cells viz. cell concentration; effect of pH on the plaque development; dilution and inoculum size of the virus; time period of adsorption of virus to the BHK-21 cells; concentration of caboxymethyl cellulose (CMC) in the overlay media and the incubation period for the development of plaques.

2.3.1 Cell concentration

BHK-21 cell monolayer grown in plastic tissue culture flask (75 cm^2, Greiner Bio-One) was detached by Trypsin- EDTA (1X, SIGMA) and the cells were suspended in cell growth medium i.e. Eagle's Minimum Essential Medium (SIGMA) supplemented with 5% FBS (GIBCO). The cell count was done using haemocytometer. Different no. of cells (2, 3, 4 and 5 X 10^5 per ml) were added to the wells of 6-well TC plates (Greiner Bio-One) and incubated at 36 $\pm$ 1^{0}C, 5% CO_2 and > 90% RH. Observations were made after overnight incubation. The cell count which gave a nearly complete monolayer formation after overnight incubation was finally selected for the study.

2.3.2 Effect of pH

To study the effect of pH on the infectivity of JE virus, different pH values between 7.0 and 9.0 (including the extreme values) were tested. The pH value which suited well for the normal growth of the BHK-21 cells as well as for the plaques of JE virus was finally used in the study.

2.3.3. Working dilution and inoculum size of the virus

Serial ten-fold dilutions of JE stock virus were prepared in chilled 1x EMEM with 5% FBS and different inocula viz. 0.2ml, 0.3ml and 0.4ml of each dilution, from 10^{-5} to 10^{-10}, were inoculated on BHK-21 cell sheet in a 6-well plastic TC plate (Greiner Bio-One). The virus dilution and the inoculum size for optimum plaque count were finally worked out.

2.3.5. Adsorption time period

To optimize the time period for the adsorption of JE virus on BHK-21 cells for proper plaque development, the JE virus (at pre- standardized dilution and inoculum) was inoculated on BHK-21 cell monolayer in three 6-well TC plates. These plates were incubated at 36 $\pm$ 1^0C, 5% CO_2 and > 90% RH for different time periods (60, 90 and 120 min.) for virus adsorption. After the completion of the adsorption time, the cell monolayer was overlaid with 1x EMEM containing CMC (Loba Chemie) and further incubated under same conditions for the development of the plaques. The adsorption time which resulted in discrete plaques formation (after overlaying and five days of incubation) with round appearance, sharp boundaries, clear margins, was selected for the study.

2.3.6 CMC concentration:

Different concentrations of CMC (0.1 % (v/v), 0.2%, 0.4% and 0.5%) were incorporated in the overlay medium. The minimum concentration of CMC, which just prevented the spread of the virus on the entire cell sheet and helped in the localization of the virus, was selected for plaque assay.

2.3.7 Incubation time period

Finally, the time period for the development and appearance of plaques was also standardized. For this three 6-well TC plates, containing BHK cell monolayer infected with JE virus, after addition of the overlay media, were incubated at 36 $\pm$ 1^0C, 5% CO_2 and > 90% RH. On 4^{th}, 5^{th} and 6^{th} day of incubation, the medium from the wells was aspirated. After washing twice with PBS (pH 7.2) and fixation, the cells were stained with 0.1% crystal violet for demonstration of plaques. On the basis of best growth and appearance of plaques in the plates, the most appropriate time period for incubation was chosen.

2.4 Plaque fixation and staining

The growth medium was aspirated from the wells of 6-well TC plates using a sterile Pasteur pipette and the cell monolayer washed twice with PBS (pH 7.2). For plaques fixation, 2ml of 10% formal saline was poured onto the cells and kept for 30 minutes at room temperature (22-25℃). After fixation, the fixative was aspirated and again the cells were washed with PBS. BHK-21 cells were stained using 0.1 % of crystal violet for 10min. The excess stain was washed off with PBS (pH 7.2); air-dried and number, size and other characteristics of plaques were measured.

3. Results and discussion

Different parameters were standardized for the plaque assay of JEV (Nakayama NIH strain) on BHK-21. The plaque assay was carried out in 6-well TC plates. The first parameter to standardize was the number of BHK-21 cells to be used per well of the 6-well TC plate. A cell concentration of 3-4 x 10^5/ml produced a complete monolayer after overnight incubation when 2ml of this suspension was used. Also at this concentration, the cells were found to remain healthy till the entire length of the assay. Cell concentration lower than 3 x10^5 failed to give a complete monolayer formation after overnight incubation. On the other hand, a higher cell concentration (>4x10^5/ml) produced a confluent monolayer. Further due to high cell density, the cells produced a thick monolayer in the next 24 hrs and a significant cell death occurred (more than 50% showed rounding and cell sheet detachment). This observation is supported by the

work of Kimura, T. et al., [8] wherein a $5x10^5$/ml cell concentration of BHK-21 cells in 76mm Petri dish was found to be optimum for plaque study under similar conditions (36 ± 1^oC, 5% CO_2).

It is known that the JE virus is quite stable at alkaline pH and the infectivity is enhanced by alkalinity upto a pH 8.5 [9]. In this study for the optimization of pH for plaque assay, different pH values ranging between 7.0 and 9.0 were tested. We observed a marked effect of the different pH values on the development of JEV plaques (Table 1**).** At pH 7.0 and 7.2, no plaque development occurred in the wells infected with the virus but a generalized cytopathic effect was noticed compared to healthy cell controls. Very small and hazy plaques were seen at pH 7.4 but the counting could not be done due to poor visibility of plaques. Plaque number increased from pH 7.6 to7.8, maximum being at pH 8.0 thereafter no plaques were seen instead rounding of the BHK-21 cells was observed in both the test and normal control wells. This might be due to the detrimental effect of the high pH of the medium. Hence, the pH range from 7.6-8.0 was considered optimum for the JEV plaque development. A pH 7.8 was used in the present study since at pH 8.0 rounding of cells occurred in the control wells. Moreover, the plaque formation at pH 8.0 was not found to be significantly different than at 7.8.

Table 1: Effect of pH on plaque development and characteristics.

pH	PLAQUE CHARACTERISTICS					
	Size (mm)	**Distribution**	**Shape**	**Centre**	**Plaque Number**	**Cell Control**
7.0[a]	-	-	-	-	-	Healthy
7.2 [a]	-	-	-	-	-	Healthy
7.4	0.5 -1.0	Uniform	Irregular	Hazy	ND[c]	Healthy
7.6	0.5-1.0	Uniform	Regular (Round)	Clear	59[d] $\pm$ 2.561	Healthy
7.8	1.5-2.0	Uniform	Regular (Round)	Clear	81[d] $\pm$ 2.857	Healthy
8.0	1.5-2.0	Uniform	Regular (Round)	Clear	92[d] $\pm$ 2.135	Partial rounding [e]
8.2	-[b]	-	-	-	-	Rounding
8.4	-[b]	-	-	-	-	Rounding
8.6	-[b]	-	-	-	-	Rounding
8.8	-[b]	-	-	-	-	Rounding
9.0	-[b]	-	-	-	-	Rounding

[a] No JEV plaques.
[b] No plaques observed due to cell rounding caused by high pH.
[c] Plaques could not be counted due to poor visibility.
[d] Mean plaque count and standard deviation of n=5 readings.
[e] Some of the cells showed rounded appearance.

To optimize the working dilution and inoculum size of the virus, the JE virus was diluted 10-fold serially in chilled 1x EMEM (SIGMA) + 5% FBS (GIBCO). The dilutions from

10^{-6} to 10^{-9} were tested and finally 10^{-8} dilution was selected as it gave the desired results (Table 2). For plaque assay, optimization of virus dilution and especially the inoculum is quite crucial, because the number as well as the appearance of plaques depends a lot on these two parameters.

In this study we found that at 10^{-6} dilution of virus using 0.2ml of inoculum, BHK-21 cells showed CPE on the 2nd day of infection and the entire sheet was found detached on the 4th day of infection as compared to the control wells which were healthy till the entire length of incubation. The extent of CPE was found to increase with increase in the size of the inoculum (0.3ml and 0.4ml) due to excess of the virus particles. Similar findings were observed with 10^{-7} dilution of the virus at each of the above mentioned inoculum sizes. Plaque formation was seen to occur at 10^{-8} dilution of the virus. With 0.2ml of the inoculum at this dilution, a uniform distribution of plaques was observed wherein the mean plaque count was found to be 80 (n=10; SD=3.516; CV=4.4%). With 0.3ml inoculum, though plaque formation occurred, yet plaques could not be counted due to overlapping of plaques, while at 0.4ml inoculum, plaques could not be seen due to high plaque density instead a generalized CPE was seen. At 10^{-9} dilution of the virus, very less number of plaques could be observed. Even with the highest inoculum (0.4ml) at this dilution, the mean number of plaques was just 7 (n=10; SD=0.775; CV=11.1%) which was too low to be used and hence was not suitable for the study. Hence, the inoculum size of 0.2ml/well of 6-well TC plate at 10^{-8} dilution of virus was found optimum for plaque assay of JEV on BHK-21 cells.

Table 2: Standardization of dilution and inoculum size of the virus.

Dilution of virus [£]	**Inoculum size**			**Cell control**
	0.2ml	**0.3ml**	**0.4ml**	
10^{-6}	CPE[a]	CPE[a]	CPE[a]	Healthy [c]
10^{-7}	CPE[b]	CPE[b]	CPE[b]	Healthy [c]
10^{-8}	80 ± 3.516, 4.4%[d]	-[e]	-[e]	Healthy [c]
10^{-9}	No plaques	No plaques	7 ± 0.775,11.1%[f]	Healthy [c]

[£] Dilutions were made in chilled 1x EMEM (SIGMA) containing 5% FBS (GIBCO).

a Cytopathic effect ranged between 50-60% on second day of infection and 100% on fourth day.

b Cytopathic effect ranged between 20-30% on second day of infection and 75-80% on fourth day and 100% on fifth day.

[c] Healthy cell sheet on the fifth day of incubation.

[d] Mean and standard deviation and coefficient of variation of n=10 plaque counts (74, 82, 82, 84, 80, 76, 80, 76, 84,84).

[e] Plaques could not be counted due to high plaque density/overlapping of plaques.

[f] Mean and standard deviation and coefficient of variation of n=10 plaque counts (8, 6, 8, 7, 6, 7, 7, 6, 8, 7).

The study of the effect of different adsorption times on the attachment of the JEV to the BHK-21 cells showed that 90 minutes time was appropriate. A significant difference was observed in the growth and appearance of the plaques at different adsorptions times. At time periods less than 60 min. poor plaque development occurred (data not shown). This might be due to insufficient adsorption of the virus. However, no difference in development of plaque was found at the adsorption time of 90 and 120 min. Hence an adsorption time of 90 min. at 36°C ± 1 was selected for the present study. Even in PRNT assay on CEFB cells, which is used for the potency assay of JE vaccine, an adsorption time of 90 min at 36°C ± 1 is being followed.

In order to localize the virus, different concentrations of CMC were incorporated in the overlay media (1X EMEM +2% FBS) viz.; 0.1%, 0.2%, 0.4%, 0.5%. Of the different concentrations of CMC, 0.2% was found suitable. It was observed that at higher concentrations (0.4% and 0.5%), the cells showed abnormal behaviour i.e. rounding and detachment of the cell sheet occurred even in the absence of the virus as observed in the cell control wells. The final concentration of 0.2% in overlay medium was found optimum for the plaque assay, as the BHK-21 cells did not show any abnormal behavior. Though 0.2% CMC concentration is quite low yet

it just maintained the semi-solid nature of the overlay media, which is quite necessary for the localization of the plaques. At concentration lower than this (0.1% CMC), plaques were not seen instead a generalized CPE was observed.

Table 3. Repeatability of plaque assay of JE virus (Nakayama NIH strain) on BHK-21 cells.

	PLAQUE COUNTING[a]			
S.No.	Operator-I	Operator-II	Operator-III	Operator-IV
1	74[b]	93	86	90
2	82	98	90	95
3	82	99	92	86
4	84	92	98	92
5	80	99	90	87
6	76	87	94	93
7	80	96	93	87
8	76	94	90	95
9	84	97	94	89
10	84	93	91	91
MEAN±SD	80 ± 3.516	95 ± 3.572	92 ± 3.059	91 ± 3.106
CV (%)	4.4	3.8	3.3	3.4
Overall Mean ±SD= 89 ± 6.42, CV(%) = 7.2				

[a] Plaque assay in 6-well TC plate.
[b] Plaque count per well.

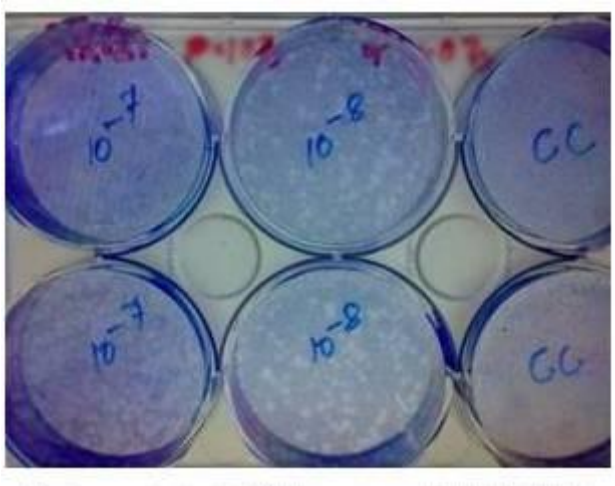

Fig 1: JEV plaques on BHK-21 cells

The standardization of incubation time period for the development of plaques concluded that the plaque development occurred best between 4- 5 days of incubation at 36 ± 1°C, 5% CO_2 and > 90% RH. This observation is supported by the work of Kimura et al. [8] wherein JEV plaques appeared on BHK-21 cells on 5th day of virus infection. In another study, plaque formation was seen to occur in just 52hrs using Actinomycin D in the overlay medium [10].

The plaques were seen as clear, distinct, uniformly distributed and with sharp boundaries, measuring app. 1.5-2.0 mm in diameter (Fig-1). The microscopic appearance of normal BHK-21 cells and the JE virus (Nakayama NIH) plaques (marked arrow) respectively have been shown in Fig -2 and -3.

A comparison was made between the characteristics of plaques on BHK-21 cells and CEFB cells in 70mm glass Petri dishes (Fig- 4). The JE virus plaques were found comparable in terms of number (mean count 95 and 89 in CEFB [data not shown] and BHK-21 [Table 3] cells respectively) also in various characters viz. shape, distribution, margins etc except for size 2-3mm on CEFB cells compared to 1.5-2.0mm on BHK-21 cells. This finding is in accordance with the study of Kimura et al. [8] wherein the ratio of mean plaque size on BHK-21 cells to that on the CFEB

cells was observed to be less than one with Nakayama NIH virus strain.

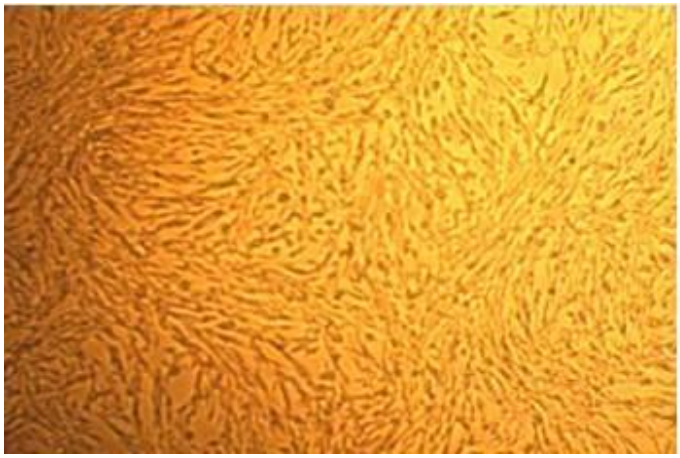

Fig 2: Normal BHK-21 cells

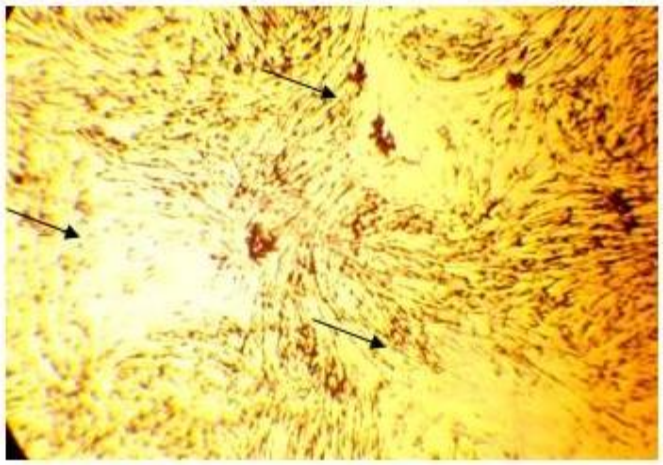

Fig 3: JEV plaques on BHK-21 cells on 5th day

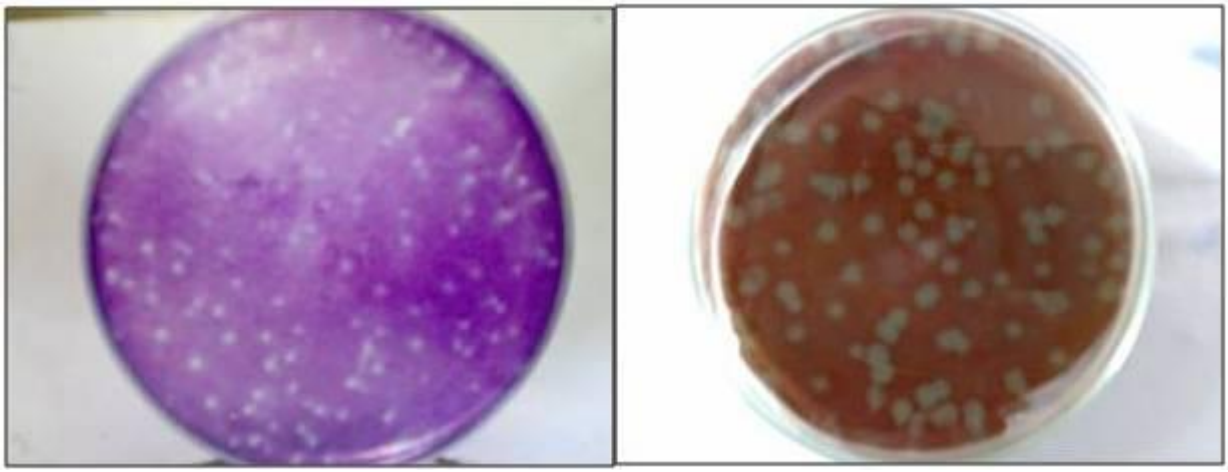

Fig 4: JEV plaques on BHK-21 cells (left) and CEFB cells (right) in 70mm glass petridish.

The efficiency and repeatability (inter-assay precision) of JE virus plaquing on BHK-21 cell monolayer was also tested and was found to be good. Repeatability was tested by repeating the plaque assay on BHK-21 cells on different occasions by different operators (Table 3). Repeatability of the assay was established by calculating the coefficient of variation (ranged between 3.3 -4.4) which was found to be quite less, indicating thereby a good precision (inter-assay).

Therefore, on the basis of above results it can be concluded that the plaque formation on BHK-21 cells is a stable and characteristic property of JEV (Nakayama NIH) virus strain. Hence, BHK-21 can be used in place of CEFB cells for the demonstration of plaque assay of JE virus. The procedure is less time consuming and easy to perform. Study on the establishment of plaque reduction neutralization (PRNT) assay using this cell line is in the progressing phase in our laboratory.

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