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# Sensitivity of *Aedes albopictus* C6/36 cells line for the detection and infectivity titration of dengue virus

Zamree, I., Drakes, N.*, Rohani, A. and Lee, H.L.
Medical Entomology Unit, Infectious Disease Research Centre, Institute for Medical Research, Kuala Lumpur.
*Queen Elizabeth Hospital, Martindale Road, St. Michael, Barbados.

**Abstract.** Plaque assay procedure was carried out to determine the infectivity titration of dengue virus using *Aedes albopictus* C6/36 mosquito cell line. Cells were seeded in 6 wells plates and incubated until monolayer C6/36 cell lines were formed. Each well was exposed to a different dilution of dengue infected culture fluid, followed by addition of first and second overlay medium. Cells were observed under an inverted microscope and plaque staining was carried out. The results showed that countable plaques were not achieved and plaques formed were restricted to the central section due to the significant partial lysis of the C6/36 cells in the plates. However, the plaque assay technique using C6/36 cell line reported here appears to be promising and merits further detailed studies.

## INTRODUCTION

One of the important procedures in research, which involves viral infection, is the measurement of the concentration of the virus in a sample. Inoculating serial dilutions of virus into host cell cultures and monitoring for evidence of virus replication determine this parameter. There are a number of assays that can be used to determine the number of infectious viral particles in a preparation. Plaque Assay is one of the well-known methods to determine the infectivity titration of certain viruses. In viral plaque assays, visible structures are formed in a cell culture contained within nutrient medium. By propagating within the cell cultures, the viruses generate zones of the cell destruction known as plaques. Plaques are clear, lyses areas, which developed as the viral particles are released from the infected cells during incubation. One of the primary advantages of the plaque assay in quantitation is that it gives a number rather than a reciprocal dilution for a titer, as do other infectious assays (Manning & Collins, 1979). By detecting and evaluating these plaques, a researcher can gauge virus activity and effectiveness, as well as enumerate effective viruses.

Mammalian cell lines such as LLC-MK2 and BHK cells are ideal for the propagation of virus for plaque assay technique (Rosen & Gubler, 1974; Rudd *et al.*, 1980). However, the ability to maintain mosquito cell culture such as C6/36 at room temperature in a quiescent state for up to 14 days without supplying $CO_2$, provides access for viral isolation and titration. This obviates the need of daily trypsinization of cell monolayer, which reduces the workload and assures rapidity of the virus tissue culture infection tests. However, there are no successful reports on the use of C6/36 cells for plaque assay technique. In this study, efforts were made to use C6/36 cells line for plaque assay to determine the infectivity titer of dengue virus.

## MATERIAL AND METHODS

*Aedes albopictus* C6/36 cell line was maintained at the Medical Entomology

Unit, Institute for Medical Research, Kuala Lumpur. Minimal Essential Medium (MEM) with 10% bovine serum was used as cell maintenance medium and 2% bovine serum was used as the cell growth medium. Six well plates were seeded with $1x10^6$ cells/ml of C6/36 per well and maintained for 1-2 days at room temperature (25°C – 28°C). After the cells had monolayered, they were exposed to a culture fluid containing Dengue II viruses. The uninfected culture fluid was used as a negative control. The ICF was diluted and serial dilutions of the dengue virus were then introduced into the C6/36 cultures. After two hours of incubation period, the inoculum was aspirated out from each well. The culture was next overlaid with 2.5ml first overlay medium consisted of 0.35g Difco Bacto-agar melted and cooled below 42°C. The overlay medium was allowed to solidify at room temperature for 10-15 minutes. A second overlay medium containing 0.33% neutral red was added to the cultures after 4 days. Cells were then observed under an inverted microscope. The study was repeated accordingly by changing the incubation time, inoculum volume and temperature.

## RESULTS AND DISCUSSION

The major problem encountered which affected the formation of countable plaques was that of significant partial lysis of the C6/36 cells in the six well plate as seen during certain intervals of the study research. Variables appeared to have had an impact on the lysis of the C6/36 cells include: incubation period, inoculum volume, and temperature.

Results of the plaque assay by the standard protocol method showed a 50-55% lyses of the C6/36 cells, with the periphery being totally devoid of cells. Countable plaques were not achieved and any plaques formed were restricted to the center of the culture plate (Plate 1). Plaque visualization by the staining method was poor and cells stained were not well demonstrated.

The inoculum volume, which was used in the study, appeared to have contributed to the primary effect of the lysis. It is believed that, the lysis observed was due to the retention of the suspension around the periphery of each well as a result of the small volume used. Therefore, there was an apparent need for an increase in the inoculum volume, so as to adequately cover the surface area of each of the six wells, without retention around the circumference. Hence the initial inoculum volume of 100 µl was increased to 500 µl. Using this method, 75-80% of the cells in the cell culture plate were detached after a subsequent peeling-off of the overlay agar medium. However, countable plaques were again not achieved, and plaques,

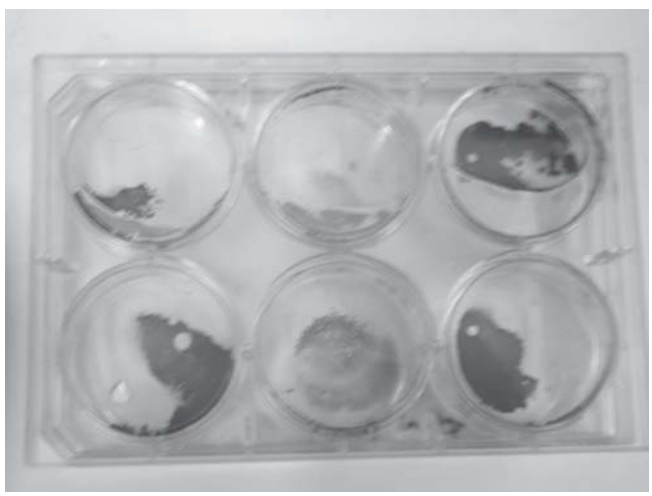

Plate 1. Demonstration of plaque by the standard method.

which had developed, were demonstrated within the remaining intact areas of the culture.

Consequently, the incubation period was changed from the initial two hours to one hour. Similar results were generated using this method as compared to the use of the standard plaque assay technique, but a more favorable percentage of structurally, viable and intact cells were demonstrated with 60-65% of the cells appeared to be "free of lysis". Plaques formed were now restricted to the centre of the culture well but still no countable plaques were achieved.

The test results showed that, by using C6/36 cell lines, 42°C might not be a suitable temperature to apply the Plaque Assay's overlay medium. Thereafter, the recommended two hours incubation period for the inoculation of the virus on the C6/36 cell monolayers was maintained according to the protocol, but the temperature for the application of the overlay medium was changed. Subsequently, different, varying temperatures ranging from 32°C to 39°C were attempted, with each resulting in the lysis of the cells after a time interval of between five minutes to 1-2 days. The result showed that, 35°C may be the best temperature to apply the overlay medium during the performance of the assay. At this temperature, 50% of the cells, which remained, have shown a formation of plaques.

Once a considerable amount of the infected cells had remained intact and unlysed, the application of the overlay medium also appeared to have contributed to lysis as well as detachment of cells from the six well culture plate. Other measure used to counteract the problem of cell lysis was to release the overlay medium very gently and carefully. This technique showed an appreciable difference in the structure of the cells after immediate solidification of agar, with less lysis but by day 3 of the incubation period, all cells had lysed.

In this study, the problems were due mainly to the specific characteristic properties of the C6/36 cell line, since the nature, type, and origin of the C6/36 cell-line is known to have some influence on its bionomics. Unlike mammalian cell-lines, insect cell lines appear to be more sensitive and less rigorous to certain conditions performed during the analysis of the plaque assay technique (Paul *et al.*, 1969). However, the plaque assay technique using C6/36 cell line reported here appears to be promising and merits further detailed studies. The formation of plaques in the insect cell line will open the possibility of studying arbovirus *in vitro*, in as close to the natural environment as facilitated in the mosquito host structure. This phenomenon has great implication in the development of theories and principles surrounding the concept of diseases, as a consequence of arbovirus infection.

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