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SHORT COMMUNICATION

STUDIES ON CHIKUNGUNYA VIRUS

I. PLAQUE TITRATION ON AN ESTABLISHED CELL LINE[1]

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As the first step in a series of studies on Chikungunya virus, one of the causative agents of Thai hemorrhagic fever, a plaque technique for measuring infectivity was developed. This method uses an established cell line of African green monkey kidney cell (VERO) and is preferable to other reported methods using primary cells (HENDERSON and TAYLOR, 1960; PORTERFIELD, 1960).

Chikungunya virus, prototype African strain at the 174th passage level in suckling mouse brain (SMB), and BaH 306 strain, isolated in Bangkok, at the 7th SMB passage level, were kindly supplied by Dr. Sompop Ahandrik of this Institute in Bangkok. These strains were used after 2 more passages in SMB. Infected SMB were homogenized with 9 volumes of YLE (0.1% yeast extract and 0.5% lactalbumin hydrolyzate in Earle's balanced salt solution with 0.22% $NaHCO_3$, 0.55% glucose, 100 units/ml of penicillin and 100 μg of streptomycin, pH7.4). The homogenate was centrifuged at 3,000$\times g$ for 15 min and the supernatant was kept at -70°C and used as the virus material in these experiments.

VERO cells were kindly supplied from Dr. A. Oya's laboratory in the National Institute of Health, Tokyo, Japan, at the 184th passage level, and was used up to the 250th passage level in these experiments. The cell line was transfered every 3 days at a density of 10^5 cells/ml in YLE supplemented with 4% bovine serum. For plaque titration, cell monolayers were prepared by growing 5×10^5 cells in 5 ml of medium in 2 ounce rubber-stoppered prescription bottles at 37°C for 3 days.

Before plaque assay, virus samples were diluted serially with 0.2% gelatin in YLE, and 0.2 ml of each dilution was inoculated on each cell sheet after removing the medium. Virus was allowed to become adsorbed at 37°C for 2 hours spreading it over the cell sheets every 30 min. The first agar overlay medium was prepared by mixing 2% melted agar (Difco Special Noble) with an equal volume of double strength YLE (in this case containing 0.2%

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glucose). Five ml of the overlay medium at a temperature of around 40°C was introduced onto each inoculated cell sheet. After the agar had solidified, the bottles were inverted and incubated at 37°C for 2 days. Then they were covered with 1 ml of the second overlay medium, which was the same as the first overlay medium but contained 0.02% neutral red. Incubation at 37°C was continued before plaques were counted. About 90% of the plaques were visible 24 hours after the second overlay was made and had a diameter of 1 mm. After 48 hours no increase in plaque number was observed but there was an increase in plaque size.

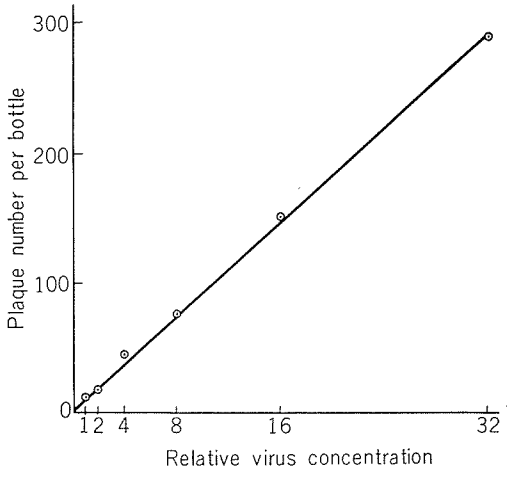

FIGURE 1 Relationship between Relative Virus Concentration and Number of Plaques.

There is a linear relationship between the relative concentration of virus inoculated and the plaque number formed with less than 300 plaques per bottle (Fig. 1). The reproducibility of the method was tested on different days with a single virus sample, as shown in Table 1. The plaque forming units (PFU) of the sample were of almost the same degree of magnitude as the $TCID_{50}$ measured by observation of CPE in roller tubes (Table 1), and were also of the same order as the LD_{50} measured by SMB titration. Titers of the BaH strain were somewhat lower than those of the African strain, and the plaque size of the former was a little smaller than that of the latter.

TABLE 1 *Reproducibility of the Plaque Method and Comparison with Tube $TCID_{50}$*

Experiment	log PFU/ml	log $TCID_{50}$/ml
1	8.26	8.50
2	8.20	8.25
3	8.41	8.63
4	8.12	8.00

Virus sample BaH 306 strain, was diluted serially 10 fold. For $TCID_{50}$ titration, 0.1 ml of each dilution was inoculated into roller tube cultures of VERO cell after removal of culture medium, 5 tubes for each dilution. Virus was allowed to become adsorbed at 37°C for 2 hours. Then 1 ml of YLE was added to each tube and the tubes were incubated at 37°C for 7 days. The $TCID_{50}$ value was calculated by the method of Reed and Muench.

The observed variation in the plaque number of different bottles inoculated with a single virus sample is not considered significant. When 16 bottles were inoculated with an average of 110 plaques of virus per bottle, the χ^2-value was calculated to be 13.38 and the probability was between 0.5 and 0.75.

Infective agents could be recovered from plaques and they were neutralized by immune serum against Chikungunya virus. No plaques or plaque-like foci were formed by inoculating a homogenate of normal mouse brain.

TABLE 2 *Comparison of the Plaque Method with the LD_{50} value for Suckling Mice*

Virus strain and Passage level	log PFU/ml	log LD_{50}/ml
African	9.09	9.20
176	9.46	9.37
BaH	8.17	8.37
9	8.13	8.00

Virus samples were diluted serially 10 fold. For LD_{50} titration, 0.02 ml of each dilution was inoculated intracerebrally into 1-3 day old suckling mice, using 8 mice for each dilution. The LD_{50} value was calculated according to Reed and Muench's method after 2 weeks observation.

Later it was found that the presence of protamine sulfate (NBC) at a concentration of 400 μg/ml in the first overlay medium makes plaques sharper and larger, and this protamine effect can be reversed by an equal amount of sodium dextran sulfate. Thus it is concluded that some sulfates of polysaccharides in the agar have inhibitory effects on plaque formation of Chikungunya virus as in the case of dengue virus (SCHULZE and SCHLESINGER, 1963a, b). Using protamine sulfate, the second overlay can be performed on the day following virus inoculation and plaques can be counted on the second and third days after inoculation. This made it possible to obtain rapid results but the final PFU titer was not very different from values obtained without protamine.

These results show that this plaque titration system can be used as a simple rapid and reliable assay method for measuring Chikungunya virus.

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