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NOTES

Rapid Plaque Assay for Encephalomyocarditis Virus

DAVID A. BAKER AND LOWELL A. GLASGOW

Departments of Microbiology and Pediatrics, University of Rochester School of Medicine, Rochester, New York 14620

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A liquid overlay plaquing technique is described which offers a rapid and simple plaque assay system for small plaque variants of encephalomyocarditis virus.

A rapid plaque assay for the small-plaque variant of encephalomyocarditis virus (EMC r^+) under a liquid overlay is reported in this communication. The inhibition of plaque formation by EMC r^+ under agar due to the presence of sulfated polysaccharides in the agar (6, 7) has led a number of investigators to develop different techniques for the plaque assay of this virus. In their original reports, Liebhaber and Takemoto demonstrated that the addition of protamine and diethylaminoethyl dextran, which bind to the sulfated polysaccharide, neutralize its inhibitory effect. Colter, et al. (4) also reported the effect of protamine, and Campbell and Colter (2) found that EMC r^+ produced large plaques under an agarose (the neutral polysaccharide of agar) or methylcellulose overlay.

It has been recognized that viruses with the capacity for cell-to-cell transmission, herpesvirus and vaccinia virus, produce circumscribed foci of cytopathic effect (CPE) under liquid medium (1, 8, 9; F. L. Black and J. L. Melnick, Fed. Proc. **13**:487, 1954). In general, an agar overlay has been necessary for the plaque assay of viruses with a rapid, lytic replication cycle. Sommerville (10), however, showed that strains of ECHO virus produced microplaques under a liquid overlay, and Holland and McLaren (5) have been able to utilize a reduced concentration of agar (0.6%) to produce poliovirus plaques in HeLa cells.

The liquid overlay technique described in this report is rapid and has several advantages over the previously reported methods for the plaque assay of EMC r^+ virus. Monolayers of L cells (L 929 obtained from the American Type Culture Collection), fetal lamb kidney (FLK) or human amnion (HA) cells prepared in 60-mm plastic petri dishes (Falcon Plastics, Inc.), were infected with serial dilutions of EMC r^+ virus. The EMC virus was originally obtained from K. K. Takemoto of the National Institutes of Health. Stock preparations of the small-plaque variant were plaque-purified and grown in L cells. Serial dilutions (0.2 ml) of EMC r^+ virus were allowed to adsorb for 1 hr and then a liquid overlay of 5 ml of Eagle's medium (MEM) with 5% fetal calf serum was added to the monolayers. After 18 to 24 hr, 0.5 ml of 20% Formalin was added to the overlay medium and allowed to remain for at least 30 min; it was then poured off and the cell monolayers were stained with methylene blue. The stain was removed; plates were washed with tap water and were immediately ready for counting. The development of circumscribed foci of CPE under liquid medium is compared with the development of plaques under agar or an agarose overlay in Figure 1. Plaques produced by the small-plaque variant of EMC virus are readily visible after 24 hr with the liquid overlay, at which time there are no plaques present with agar overlay and only tiny plaques are visible with agarose. An equal efficiency of plaquing of EMC r^+ was demonstrated with the liquid overlay technique as compared with an agarose overlay. The results of three separate assays of the stock EMC r^+ pool are presented in Table 1.

This liquid overlay technique was applicable for the small-plaque variant of EMC virus in FLK and HA cells, as well as in the standard line of L cells used routinely in our laboratory for assaying this agent. The technique was not applicable to the assay of the large-plaque variant EMC r, which produced large diffuse areas of CPE that were extremely difficult to count.

The capacity of this cytolytic virus to produce plaques under liquid medium within 24 hr is probably the result of several factors. The small-plaque variant of EMC virus has been found to bind to cells with a greater affinity than strains of the large plaque variant (3), thus EMC r^+ particles released from the cell which is undergoing lysis might be expected to be bound more readily

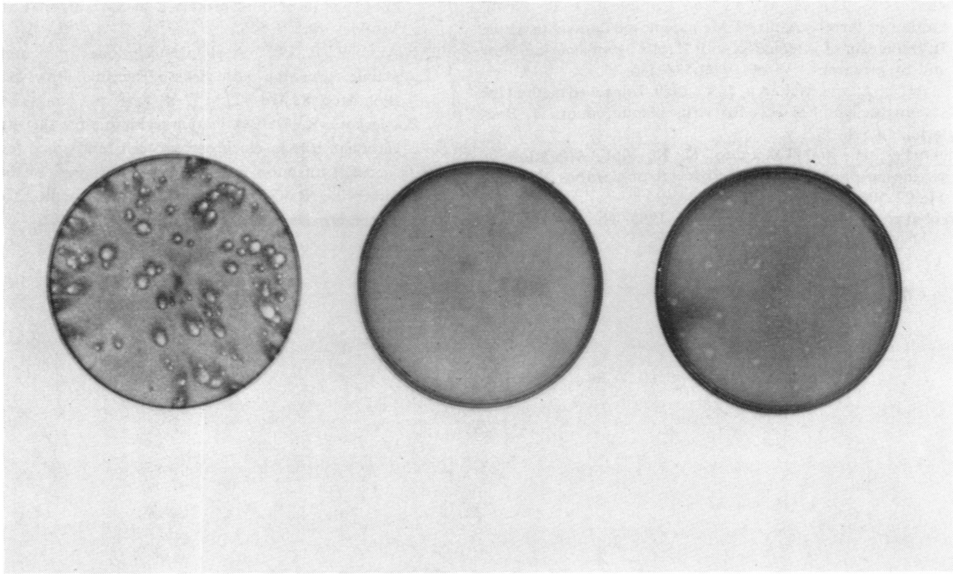

FIG. 1. *Plaques produced by small plaque encephalomyocarditis virus, after 24 hr in L cells. Left, under liquid overlay stained with methylene blue. Center, under agar stained with neutral red. Right, under agarose stained with neutral red.*

TABLE 1. *Titrations of small-plaque encephalomyocarditis virus by using the liquid overlay technique, compared with agar, and agarose overlays*[a]

Expt	Liquid overlay[b]	Agar overlay[b]	Agarose overlay[b]
1	6.65×10^8	0	3.5×10^8
2	4.0×10^8	0	4.4×10^8
3	1.02×10^9	0	1.0×10^9

[a] Plaques were counted after 24 hr of incubation at 37 C.

[b] Titer is expressed as plaque-forming units per milliliter and represents the average of three plates per dilution.

to adjacent cells. With EMC r^+ virus, there could have been a maximum of only three cycles of replication within the time required to produce plaques under the liquid overlay. The development of plaques under liquid medium suggests that with certain cytolytic viruses there is more efficient transmission of virus to adjoining cells, with development of circumscribed foci of CPE during the first several cycles of replication prior to general dissemination of virus particles throughout the culture. This concept is supported by other studies carried out in our laboratory in which EMC virus was shown to produce plaques in liquid medium in the presence of neutralizing antibody when the antibody was added after adsorption.

The technique described for assaying EMC r^+ virus has been found applicable for several other virus-host systems and appears to offer several advantages over existing techniques. (i) It is rapid and does not involve the use of agar, agarose, or polycationic substances. (ii) The fixation of the tissue permits the investigator to count plaques up to several days after addition of the Formalin by eliminating the loss of assays by the death of cell monolayers or the development of confluent plaques. Furthermore, there is almost instant visualization of plaques after staining, in contrast with the time interval required for uptake of vital dyes, such as neutral red, by cells under the standard agar overlay technique. The liquid overlay method would appear to offer a rapid and simple plaque assay for the small-plaque variant of EMC virus.

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