

ENTEROVIRUS PLAQUE TECHNIQUE: UTILIZATION OF MAINTENANCE MEDIUM ON AGAR OVERLAY WITHOUT NEUTRAL RED¹

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ABSTRACT

A plaque technique for quantitating enteric viruses using liquid growth medium on the agar overlay was developed. Deletion of neutral red and staining of plaques using crystal violet, following plaque development, facilitated the technique. This method utilized a minimal number of monkey kidney epithelial cells for testing the greatest number of samples.

There is a need for a plaque technique which does not incorporate neutral red in the agar overlay for use in quantitating enteric viruses. The addition of neutral red dye in agar overlay using HeLa cell cultures and polio virus, type I, reduced plaque formation fifty per cent (Darnell et. al., 1958). Monkey kidney monolayers exposed to white light in the presence of neutral red even at a dilution of 1:40,000 undergo degeneration within 24 hr after exposure (Klein & Goodgal, 1959). Mouse embryo, various species of monkey kidney epithelium and rabbit kidney cell cultures were all damaged by photodynamic action in the presence of neutral red (Holtz, 1962). Viable cell sheets under agar overlay must be maintained for 14 days to insure time for the development of slow plaque-forming viruses. In conventional agar overlay systems degeneration of cell sheets occurs due to the utilization of available nutrients, the accumulation of toxic waste materials and the effects of neutral red dye. This cellular degeneration is enhanced when sewage samples are tested for the presence of enteric viruses due to the toxic materials contained in a given volume of the inoculum being tested. It is desirable to utilize a minimal number of monkey kidney epithelial cells when testing a large number of samples for the presence of enteric viruses. This is a report of a modification of the standard plaque technique which overcomes the disadvantages mentioned. This modified technique does not incorporate neutral red in the agar overlay and by the addition of fluid maintenance medium over the agar it is possible to maintain cell viability for 14 days. Substituting 1 oz French square bottles makes it possible to utilize to the fullest extent the monkey kidney epithelial cells.

METHODS AND MATERIALS

Sewage samples for use in isolating enteric viruses were obtained by placing gauze pads in flowing sewage and after collection the pads were held at -20°C prior to use (Moore, 1948). The liquid content of the pad was extracted with pressure. The sample was cleared of gross particles by centrifugation and the supernatant fluid was treated with 120 mg streptomycin and 30,000 units of penicillin. The specimen remained at room temperature for 4 hr before sterility tests were made. Only samples free of bacteria were used.

Cortices from *Macacus cynomolgus* monkey kidneys were used to prepare monolayer tissue cultures. The tissue was prepared by the method of Melnick et. al., (1955). Approximately 900,000 cells in 3 ml were seeded into each 1 oz square bottle and incubated in a stationary position at 37°C until a cell sheet developed.

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The growth medium was removed from the French square bottles containing confluent sheets of epithelial cells. The cultures were inoculated with varying volumes of the processed sewage samples containing the unknown viruses to insure the formation of plaques that were separate and discrete. Immediately following inoculation the bottles were placed at 37°C for 1 hr to allow the viruses to attach to the cells. The bottles were rotated every 15 min to assure distribution of the inoculum over the surface of the monolayer. Excess inoculum was removed from the bottles and the cell sheets were covered with 2.5 ml of 1.5 per cent Noble agar (Difco) made up in lactalbumin hydrolysate medium with 2 per cent inactivated calf serum (Melnick and Riordan 1952). After the agar overlay solidified, 3 ml of maintenance medium were added to each culture bottle. The inoculated cultures were incubated at 37°C and observed daily for 14 days for plaque development. The liquid maintenance medium was changed periodically during the incubation period to prevent cell monolayer degeneration. A Bausch and Lomb series B, stereomicroscope with a variable power pod of 7X to 30X magnification was used to observe cell sheets for the presence of developing plaques.

The cultures were fixed with 70 per cent ethanol for five minutes to prevent confluent lysis of the cell sheet 96 hours after the first visible appearance of a plaque. After the alcohol was removed, the cells were stained with 0.1 per cent crystal violet stain for five minutes and then washed three times with tap water. This staining procedure greatly facilitated plaque counting and preserved the plaques until counts could be made on the large number of samples involved.

RESULTS

The first signs of plaque development occurred between the first and ninth

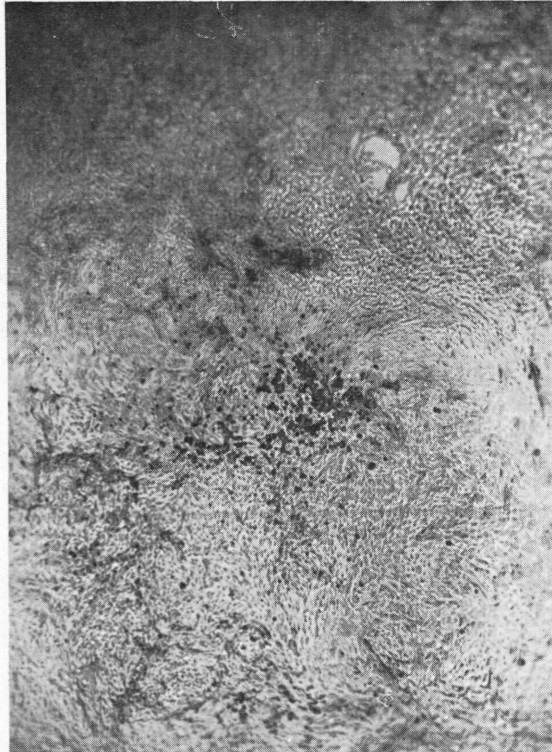


FIGURE 1. Individual clustering of cells throughout the cell monolayer prior to full plaque development fixed and stained with crystal violet (11X).

day after inoculation. Some samples produced plaques after 24 hr incubation. In other samples the initial signs of plaque formation occurred after ten days incubation. These are referred to as slow plaque-forming viruses. Regardless of the incubation period for the many samples tested, small areas of degeneration of the cells were the first sign of plaque development. This was followed by the formation of small discrete clusters of cells in the cell sheets as observed in figure 1. The number of developing plaques was dependent upon the volume of the inoculum and the concentration of virus in the specimen. The plaques (fig. 2) could be

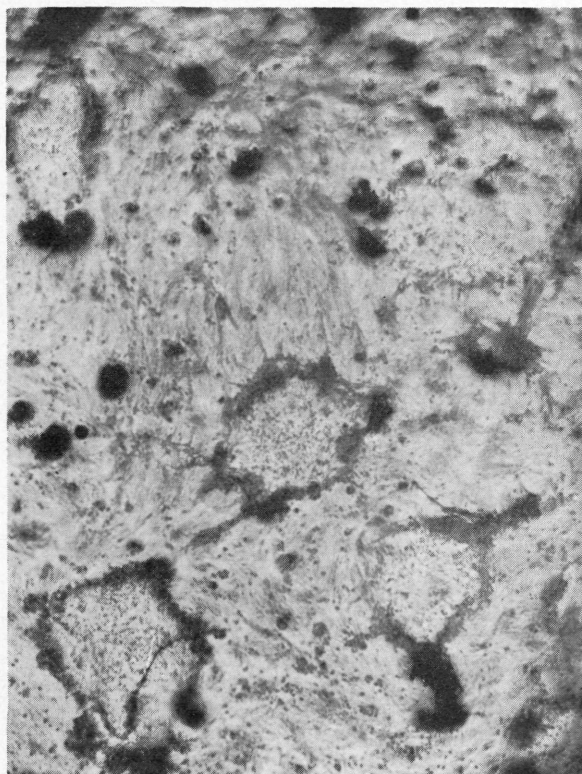


FIGURE 2. Plaque development 48 to 72 hr after the appearance of small areas of degeneration, fixed and stained with crystal violet (11X).

observed within 48 to 72 hr after the appearance of small areas of degeneration. These areas of degeneration and plaque development were compared with normal control cell monolayers (fig. 3). At no time did secondary plaques develop, following the onset of initial plaque development. This would discount the possibility of secondary plaque development resulting from possible virus spread at the fluid-agar cell interface.

DISCUSSION

It has been observed that each plaque develops from a single viable virus particle (Dulbecco and Vogt, 1954). At the onset of this investigation using conventional agar overlay techniques, cellular degeneration occurred routinely within seven to ten days following inoculation of the monolayers with the sewage samples. In many instances degeneration occurred prior to plaque development. To overcome this problem neutral red was not incorporated in the agar overlay. By adding an additional 3 ml of fluid maintenance medium over the agar, cell

viability could be maintained for 14 days. The liquid maintenance medium was changed periodically during the incubation period to insure cell sheet viability. Most conventional plaque methods routinely incorporate neutral red in the overlays 12 to 24 hr prior to plaque formation. By using sewage samples which contained unknown types of enteric viruses it was impossible to add neutral red 12 to 24 hr prior to plaque development as plaque development occurred anywhere between the second and tenth day following inoculation.

With the small amount of reagents used in the one ounce square bottles it was possible to determine the virus content of many more dilutions than would have been possible if larger amounts of reagents were required. The method described

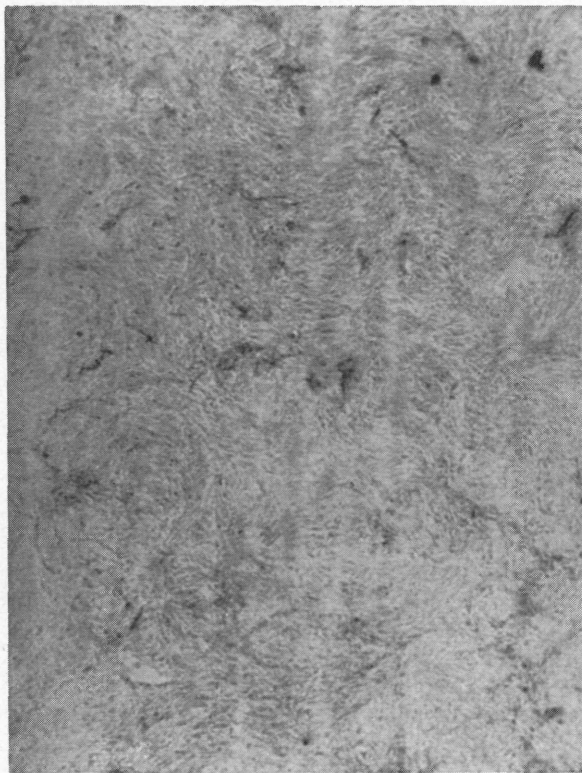


FIGURE 3. Normal control cell monolayer fixed and stained with crystal violet (11X).

is most helpful when large numbers of samples must be tested, as after fixation of the cell sheet and the addition of the dye the cultures can be set aside until time permits counting of the plaques.

This plaque technique was used in obtaining quantitative data for use in studying the removal of enterovirus from sewage by the activated sludge treatment. The results of this study are published in the *Water Pollution Control Federation Journal* (Mack et. al., 1962).

SUMMARY

A modified plaque technique for quantitating enteric viruses using liquid maintenance medium on the agar overlay was developed. Deletion of neutral red and staining of plaques using crystal violet following plaque development facilitated the technique. This method utilized a minimal number of monkey kidney epithelial cells for testing the greatest number of samples.

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