NOTES

Use of Gum Tragacanth Overlay, Applied at Room Temperature, in the Plaque Assay of Fish and Other Animal Viruses

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Fish cells derived from rainbow trout gonad or Atlantic salmon are sometimes damaged by the relatively high temperature of agar overlay widely used for plaquing animal viruses. This heat-induced cell damage can be avoided by the use of gum tragacanth, which may be applied at room temperature. When the medium was buffered with tris(hydroxymethyl)aminomethane-hydrochloride and NaHCO_3 , the plaque assay could be performed without the use of a CO_2 incubator. Using this method, a number of animal viruses were plaqued on a variety of cell monolayers at different temperatures under atmospheric conditions.

Plaque assay of viruses is generally recognized as the most sensitive and accurate method of quantifying virus infectivity. The assay for fish viruses in established fish cell lines involves incubation at temperatures lower than 37 C, usually within the range of 15 to 24 C, because many fish cell types and fish viruses cannot tolerate temperatures as high as 37 C. In plaquing these viruses, some workers used media with sodium bicarbonate- CO_2 buffering in closed vessels (1, 6, 13). Others have used dish cultures of cells under partial pressure of CO_2 (4, 7, 10). Wolf and Quimby recently described various buffers and methods for plaquing eight fish viruses under normal atmosphere (12). All of these methods used hot agar or agarose in the overlay (38 to 42 C). Some fish cell lines such as fathead minnow, brown bullhead, and bluegill, with temperature tolerance of up to 35 C, can withstand the temperature of the agar overlay for the short time it takes for the agar to cool down to room temperature, and the customary agar overlay technique may be used with these cells without difficulty, provided that the cultures are subsequently incubated at the temperature that is favorable for both cell and virus growth. However, fish cell lines originating from cold-water fish (e.g., rainbow trout gonad [RTG-2] or Atlantic salmon [AS] cells) are more sensitive to heat and so readily damaged by temperatures normally encountered with agar overlays that reproducible results are often difficult to ob-

tain, even when agar just above its gelling temperature is added to chilled plates.

Nevertheless, some investigators (2, 10) studying the replication of infectious pancreatic necrosis virus (IPN) in RTG-2 cells were able to use the agarose overlay technique successfully, even though the temperature tolerance of both cell and virus was as low as 24 to 25 C. Others (5, 9) still use end-point dilution titration for assaying IPN. We have tried to replace agar with carboxymethylcellulose or gelatin (both of which may be applied cold), but found that they inhibited plaque formation. Other investigators have attempted to use starch, but found that it gave variable plaque counts with the same virus preparation (10). As another alternative we have experimented with tragacanth (3) in combination with the tris(hydroxymethyl)aminomethane (Tris)- NaHCO_3 buffer system of Wolf and Quimby (12), and found that this overlay could be applied to cells at any desirable temperature and that a number of animal viruses could be plaqued on various types of cell monolayers at different temperatures under atmospheric conditions.

The following certified cell lines were used (purchased from the American Type Culture Collection): CCL-42 (FHM); CCL-55 (RTG-2); BHK-21/C13 (baby hamster kidney); and L-929 (mouse epithelium). AS cells were the generous gift of B. Nicholson, University of Maine (8). Primary chicken embryo cells were prepared from 10- to 11-day-old embryonated eggs. The

cells were grown in plastic tissue culture plates (60-mm diameter) or in Linbro trays containing 24 flat-bottomed wells (capacity, 3 ml per well) of 22-mm diameter (ICN FB-16-24TC).

Eagle minimum essential medium (MEM) with Earle balanced salt solution was used throughout this work. The medium contained final concentrations of 100 IU of penicillin, 100 μ g of streptomycin, and 25 IU of nystatin per ml. Fetal calf serum was added to a concentration of 10% (vol/vol) for cell growth and at 5% for plaquing. For incubation of cultures in a CO₂ incubator, 3 ml of a sterile 7.5% (wt/vol) NaHCO₃ solution was added to 100 ml of medium. For incubation under atmospheric conditions, 1 ml of a sterile 7.5% NaHCO₃ solution and 1.6 ml of 1 M Tris-hydrochloride buffer (pH 7.7) was added to 100 ml of medium (12).

The following viruses were used: IPN, vesicular stomatitis virus, Indiana serotype, Sindbis virus, Mengo virus, encephalomyocarditis virus, reovirus type 3, and Newcastle disease virus.

Agar overlay was prepared by mixing double-strength MEM with an equal volume of sterile 1.5% aqueous ionagar solution at 43 C. To prepare overlay containing tragacanth, the gum in powdered form (Fisher Scientific Co.) was suspended in doubled glass-distilled water (1.6 g/100 ml) at 60 C with vigorous shaking. The suspension was autoclaved at 120 C for 30 min, cooled, and stored in the refrigerator. It was warmed to the desired temperature before use, and an equal volume of double-strength MEM was added. (The tragacanth gum suspension must be mixed well before use, since the colloidal bassorin which makes up part of the gum tends to settle to the bottom of the container during storage [3].)

To perform the plaque assay, the growth me-

dium was removed from the cell monolayers and virus dilutions were applied in MEM without serum. After a 1-h adsorption period at room temperature, the overlay was added (5 ml per tissue culture plate or 1.5 ml per Linbro tray well) and the cultures were incubated at the appropriate temperature for 2 to 7 days, depending on the cell-virus system used. At the end of the incubation period, the overlay was removed and the cells were fixed with a fixing solution (25% formalin, 10% ethyl alcohol, 5% acetic acid [vol/vol]) for a few minutes, stained with a 1% solution of crystal violet (in fixing solution), washed with tap water, and dried.

The comparative plaque counts obtained with various viruses under the two different overlays are summarized in Table 1. The number of plaques was the same in a given virus-cell system regardless of the kind of overlay used. In most cases, cultures overlaid with tragacanth were incubated as long as those overlaid with agar, except: (i) Newcastle disease virus plaques, assayed on chicken embryo cell monolayers, took twice as long to develop under tragacanth as under agar; and (ii) IPN plaques were visible after 2 days on fathead minnow cells using agar, but an incubation period of 4 to 5 days was needed when using tragacanth (Table 1, Fig. 1). On the other hand, the development of plaques in Sindbis virus-infected chicken embryo cell layers was more rapid under tragacanth than under agar. Plaques could be seen within 24 h (2 to 3 mm in diameter), and after 2 days of incubation the plaques were as large as 5 to 7 mm in diameter (Fig. 1).

The assay of IPN in AS or RTG-2 cells using agar overlay was not always successful due to the damage caused to the cells by the high temperature of the molten agar. This did not

TABLE 1. Comparison of plaque counts and incubation times under tragacanth and agar overlays

Virus	Cell ^a	Days of incubation		PFU/ml ^b	
		Tragacanth	Agar	Tragacanth	Agar
Encephalomyocarditis	L	2	2	5 × 10 ⁹	4.6 × 10 ⁹
Mengo	L	2	2	4.5 × 10 ⁹	4.4 × 10 ⁹
Vesicular stomatitis	L	2	2	2.0 × 10 ⁹	2.1 × 10 ⁹
Sindbis	BHK	2	2	1.5 × 10 ⁹	1.2 × 10 ⁹
Sindbis	CE	1	2	1.7 × 10 ⁹	1.2 × 10 ⁹
Newcastle disease	CE	4	2	3.2 × 10 ⁹	3.0 × 10 ⁹
Reovirus (purified)	L	7	7	1.2 × 10 ¹²	1 × 10 ¹²
Infectious pancreatic necrosis	FHM	4	2	2.6 × 10 ⁷	1.8 × 10 ⁷
Infectious pancreatic necrosis	RTG-2	2	2	1.8 × 10 ⁷	1.5 × 10 ⁷
Infectious pancreatic necrosis	AS	3	ND ^c	1.2 × 10 ⁷	ND

^a CE, Chicken embryo; FHM, fathead minnow.

^b PFU, Plaque-forming units.

^c ND, Not done.

FIG. 1. Comparative plaque characteristics of seven viruses grown under tragacanth overlay. (1) IPN on fathead minnow cells (agar overlay) incubated for 2 days at 22 C; (2) IPN on fathead minnow cells incubated for 5 days at 22 C; (3) Sindbis virus on chicken embryo cells incubated 2 days at 37 C; (4) Newcastle disease virus on chicken embryo cells incubated 4 days at 37 C; (5) Mengo virus on L cells incubated 2 days at 37 C; (6) reovirus on L cells incubated 7 days at 37 C; (7) encephalomyocarditis virus on L cells incubated 2 days at 37 C; (8) vesicular stomatitis virus on L cells incubated 2 days at 37 C.

occur with the tragacanth overlay, since it could be applied at ambient temperatures. A number of other animal viruses were also titrated on different cells at 37 C, using a humidified incubator with normal atmosphere. In most (but not all) cases, the time of incubation necessary for plaque development was the same for the two overlay systems (Fig. 1, Table 1). The viruses listed in Table 1, with the exception of IPN, were also titrated by using tragacanth in a humidified CO₂ incubator at 37 C, utilizing the standard bicarbonate-CO₂ buffer. The results were similar to those given in Table 1.

Tragacanth is easy to prepare, and the cells used for the present study tolerated long exposure to the material, e.g., L cells infected with reovirus for 7 days. It is easier to remove from the cell layers than agar. The stained, dried plates or trays constitute a permanent record of titration. Gum tragacanth is inexpensive and is especially suitable for use in teaching institutions where large classes are conducted in experimental virology.

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LITERATURE CITED

1. Campbell, J. B., and K. Wolf. 1969. Plaque assay and some characteristics of Egtved virus (virus of viral hemorrhagic septicemia of rainbow trout). *Can. J. Microbiol.* 15:635-637.
2. Cohen, J., A. Poinard, and R. Scherrer. 1973. Physicochemical and morphological features of infectious pancreatic necrosis virus. *J. Gen. Virol.* 21:485-498.
3. Hossein, M., and F. Rapp. 1968. A new overlay for plaquing animal viruses. *Proc. Soc. Exp. Biol. Med.* 129:13-17.
4. Jorgensen, P., E. Vestergard, and N. P. Kehlet. 1971. Infectious pancreatic necrosis (IPN) virus in Danish rainbow trout. *Nord. Veterinaarmed.* 23:568-575.
5. Kelly, R. K., and P. C. Loh. 1975. Replication of IPN virus: a cytochemical and biochemical study in SWT cells. *Proc. Soc. Exp. Biol. Med.* 148:688-693.
6. McCain, B. B., J. L. Fryer, and K. S. Pilcher. 1971. Antigenic relationships in a group of three viruses of salmonid fish by cross neutralization. *Proc. Soc. Exp. Biol. Med.* 137:1042-1046.
7. Moss, L. H., and M. Gravel. 1969. Ultrastructure and sequential development of infectious pancreatic necrosis virus. *J. Virol.* 3:52-58.
8. Nicholson, B. L., and C. Byrne. 1973. An established cell line from the Atlantic Salmon (*Salmo salar*). *J. Fish. Res. Board Can.* 30:913-916.
9. Nicholson, B. L., and J. Dunn. 1974. Homologous viral interference in trout and Atlantic salmon cell cultures infected with pancreatic necrosis virus. *J. Virol.* 14:180-182.
10. Wolf, K., and R. W. Darlington. 1971. Channel catfish virus: a new herpes virus of ictalurid fish. *J. Virol.* 8:525-533.
11. Wolf, K., and M. C. Quimby. 1970. *In Progress in sport fishery research*, p. 86-95. 1969. Resource Publ. no. 88. U.S. Dept. of the Interior, Washington, D.C.
12. Wolf, K., and M. C. Quimby. 1973. Fish viruses: buffers and methods of plaquing eight agents under normal atmosphere. *Appl. Microbiol.* 25:659-664.
13. Wolf, K., and P. E. Vestergard Jorgensen. 1970. Salmonid viruses: double infections of RTG-2 cells with Egtved and infectious pancreatic necrosis viruses. *Arch. Gesamte Virusforsch.* 29:337-342.