A Critique of the Plaque Assay Technique in Bottle Cultures.* (26952)

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Since its introduction Dulbecco's plaque technique (1) has been widely used for obtaining viral clones. Conditions for assuring that virus derived from a single plaque represented progeny of a single infective unit were investigated and defined by Dulbecco and Vogt (2). Modifications of the technique (4,5,6) have been proposed and adopted in many laboratories. In these instances control studies of the purity of virus isolated from plaques such as described by Dulbecco and Vogt were not mentioned.

During preliminary work with plaque purification of measles virus in bottle cultures the agent was demonstrated 11 days after inoculation in the small amount of fluid that collects in the vessel. This observation suggested that agar surfaces in bottle cultures might become widely contaminated with virus. If so, the reliability of this technique as applied to the cloning of viruses would obviously be in doubt. Accordingly, an investigation was undertaken to determine, using polio virus and HeLa cells, the distribution of virus in various regions of the system.

Methods and materials. HeLa Cells. HeLa cells from stock cultures were inoculated in milk dilution bottles measuring 40 × 40 × 105 mm, using Eagle's minimal essential medium (3) with 10% inactivated calf serum. Infection was carried out when the monolayer was confluent, usually on the fourth day.

Monkey Kidney Epithelial Cells. Monolayer cultures in screw-capped tubes were obtained from Microbiological Associates, Inc. They were maintained on medium 199 with 2% inactivated calf serum.

Polioviruses. Most experiments were carried out with a stock culture of poliovirus type I, Brunhilde strain. In some experiments poliovirus type III, Leon strain, was also used.

Plaque Technique. Stock virus of known titer was diluted to yield from 1 to 30 plaques. Bottle cultures were inoculated with 0.3 ml of the suspension and incubated at 36° C for 1 hour on a shaking machine moving at slow speed. Overlay consisted of Eagle's minimal essential medium with 5% inactivated calf serum in 1.5% agar. Cultures were inverted and incubated at 36° C. Care was exercised to maintain the cultures in the same position throughout the experiment.

Results. Presence of Virus on Agar Surface. To determine whether virus could be recovered from the agar surface of bottle cultures exhibiting a small number of plaques, points were arbitrarily marked on the glass at the time of agar overlaying. A cotton swab was touched against the agar lightly at these points on successive days. The swab was then moistened in culture medium; fluid was expressed in monkey kidney cell cultures which were incubated at 36°C for 7 days. No poliovirus was detected on the agar surface by this method prior to the appearance of plaques. When a plaque directly underlay the surface point tested, virus was recovered at the surface on the same day the plaque became visible. When a plaque was not directly under the joint tested but within 0.5 cm, virus was usually recovered one or 2 days after the plaque was first seen. At greater distances from the edge of a plaque, virus was recovered twice on the 2nd day after appearance of the plaque in 13 tests (Table I).

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TABLE I. Recovery of Poliovirus Type I from Agar Surface of Bottle Cultures.

Distance from point tested to edge of nearest plaque (cm)	No. of points tested	No. of positive cultures
.5-.9	6	1
1 or greater	7	1

The glass sides of 3 bottles were found to be free of virus. The agar surface of these was washed with 2.0 ml of sterile culture fluid. Virus recovered from the surface in this manner totalled $10^{2.1}$, $10^{2.6}$ and $10^{2.6}$ pfu from bottles with 2, 6, and 9 plaques, respectively.

Presence of Virus on Exposed Glass. To assay for the presence of poliovirus on the glass surfaces of the bottles 2.0 ml of culture fluid was run over the wall opposite the overlay, taking care to prevent the medium from coming in contact with the agar surface. Recovery of poliovirus was determined by inoculating the total volume of fluid into monkey kidney cultures or quantitated by plaque titration on HeLa cells. Poliovirus was thus detected in 14 of 24 bottles tested (58%).

The time and rate of appearance of poliovirus on the glass surface was assayed by titrating washings obtained 30 minutes after adding the agar overlay and each day thereafter through the fourth day. Results for 2 bottle cultures are shown in Table II. Absence of virus immediately following inoculation and increase of the agent following its first appearance indicate that it arose as a result of multiplication within the system.

No correlation was established between the number of plaques or their location and presence or degree of contamination of the glass walls.

Purity of Virus Derived from Plaques. When bottles were simultaneously infected with type I and type III polioviruses, both agents were subsequently recovered from the glass. Accordingly, mixed infections were carried out and the purity of virus isolated from a number of plaques was determined in the following manner.

Bottle cultures of HeLa cells were inoculated with sufficient poliovirus type I and type III to yield approximately 5 to 15 plaques of each. Five days later virus isolation from several of the resulting plaques was effected by introducing a bent capillary pipette into the plaque center and aspirating gently. The pipette was washed in 0.7 ml of Hanks' salt solution. Aliquots of 0.2 ml of the washing were incubated in each of 3 tubes. To one tube was added Type I polioantibody (monkey antiserum, N.F.), to a second Type III polioantiserum and to a third both Type I and Type III antibodies. The cultures were incubated for 7 days. In this way virus was recovered from 32 of 40 plaques, but in no instance were both types of poliovirus isolated from the same plaque. It is noteworthy that in these experiments poliovirus was recovered from the walls of 2 of the 12 bottles and that the edge of a plaque caused by one type was at times only 2 to 3 mm from that of a plaque caused by the other type of virus.

In the foregoing experiment neutral red was included initially in the overlay. With certain viruses (i.e. measles) it has been found necessary to add the dye several days after inoculation since it interferes with plaque formation. The experiment was therefore repeated in essentially the same way except that neutral red (0.5 ml of 1:2000 solution) was added on the 5th day after inoculation.

Virus isolations were attempted from 30

TABLE II. Recovery of Poliovirus Type I from Glass Walls of Bottle Cultures.

	Bottle A		Bottle B	
Elapse from inoculation	No. of plaques	pfu of virus recovered	No. of plaques	pfu of virus recovered
30 min.	0	0	0	0
1 day	0	0	0	0
2 days	1	0	8	0
3 "	3	$10^{3.1}$	11	0
4 "	3	$10^{3.3}$	11	$10^{1.5}$

plaques about 3 hours after addition of the dye. Polioviruses representing both types were recovered from each of the plaques tested.

Discussion. Lateral migration of virus through agar from the edge of plaques was demonstrated by Dulbecco and Vogt (2). It is evident from results here described that virus also diffuses vertically and can be recovered from the surface over the plaque at the time the plaque is large enough to be visible. These experiments also suggest that this surface virus remains relatively localized in undisturbed bottles, but is distributed over the entire surface of the agar if fluid drains across it.

The most likely source of virus that has been demonstrated on the glass walls of bottles would seem to be drainage between the cell layer and the agar from infected centers. That such flow can occur is suggested by the wedge-shaped extension to the edge of the cell sheet of peripheral plaques which is occasionally observed when incubation is continued several days after maximal count is obtained. It seems less likely that virus on the glass has drained from the agar surface, although this possibility cannot be excluded.

Taken as a whole our experiments suggest that if a single agar overlay is applied and maintained in the inverted position virus isolated from a plaque is very likely to be pure. If this procedure is followed the bottle technique would appear to be acceptable as a method for cloning viruses. Even so, homogeneity of viral progeny cannnot, however, be accepted on a strictly theoretical basis. A requirement for addition of a second agar overlay or fluid such as neutral red after the virus has multipled sufficiently to have diffused through the agar appears to render the procedure unacceptable unless a modification can be found that will prevent widespread dissemination of virus. It would seem that application of a second overlay of agar or of fluid would also be contraindicated when petri dishes were employed according to the procedure of Dulbecco.

Conclusion. The bottle technique cannot be confidently applied to the purification of viruses unless certain precautions are taken to prevent diffusion of virus over the agar surface. These precautions consist in a) maintenance of the system in the inverted position, b) the avoidance of fluids applied to the surface after viral multiplication has taken place.

Summary. Poliovirus was regularly recovered from the agar surface of bottle cultures exhibiting plaques. From the area immediately surrounding a plaque virus recovery was frequent. Only rarely was virus isolated at a distance therefrom. Virus was also often recovered from the glass walls. In cultures simultaneously inoculated with 2 different antigenic types of poliovirus and no material subsequently added, the agent isolated from a plaque consisted of a single antigenic type provided the bottles were maintained in the inverted position. When a solution of neutral red was added several days after inoculation of 2 antigenic types of virus, both types were recovered from a single plaque.

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