A Comparative Study of Agar Overlay and Standard Tissue Culture Methods for Isolation of Enteroviruses

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STANDARD FLUID MONOLAYER tissue culture methods *(1)* have, in our experience, yielded relatively few enterovirus isolates from cerebrospinal fluids and autopsy specimens. Because agar overlay tissue culture techniques had been reported to give increased numbers of enterovirus isolations from certain clinical specimens *(2–5)*, we studied 62 cerebrospinal fluids and 141 autopsy tissues by both standard tissue culture and agar overlay methods. Two kinds of tissue culture cells, primary rhesus kidney (PMK) and human epithelial (HEp-2), were used. Overlay media were prepared with and without added $MgCl_2$ (0.025M). Overlay medium with added $MgCl_2$ was included in the study because $MgCl_2$ had been shown to enhance plaque formation by a number of enteroviruses *(6)*.

Subsequent to the initial study, 975 diagnostic specimens of various types that were received for enterovirus isolation were examined by standard tissue culture methods and by an agar overlay method in which PMK cells and overlay medium with added $MgCl_2$ were used. The results of the initial study, as well as the results obtained over an 18-month period on the 975 specimens, are included in this report.

Methods

Tissue culture. The PMK and HEp-2 cells (National Institutes of Health strain) were obtained from the Tissue Culture and Media Section, Scientific Services Division, Center for Disease Control. The PMK cells were grown in tubes or in 80-ml prescription bottles (Duraglass) in a lactalbumin hydrolysate-Hanks' balanced salt solution medium (LAH) with 5 percent fetal calf serum. HEp-2 cells were grown in tubes or in 80-ml prescription bottles in Eagle's minimal essential medium (MEM) with 10 percent fetal calf serum. Maintenance media for tissue cultures were a lactalbumin hydrolysate-yeast extract-Earle's balanced salt solution (YE-LAH) without calf serum for PMK and Eagle's MEM with 2 percent fetal calf serum for HEp-2. Antibiotics used in the growth media were penicillin, streptomycin, and amphotericin B. Maintenance media also included neomycin. For the subsequent studies of the 975 diagnostic specimens by

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agar overlay, PMK cells were grown in 60-mm plastic Petri dishes in a CO_2 incubator. Growth medium was the LAH medium with 5 percent fetal calf serum.

Preparation of specimens. Extracts of autopsy specimens were prepared in YE-LAH medium with penicillin, streptomycin, neomycin, and amphotericin B added. Pieces of tissue sufficient to yield an approximate 20 percent suspension were homogenized in this medium for 5 minutes at 14,000 rpm in a Sorvall Omni Mix cup immersed in an ice bath. The resulting suspensions were centrifuged for 20 minutes at 2,000 rpm and 5°C in an International refrigerated centrifuge (model PR2). Supernatant fluids were used for inoculation of tissue cultures. Blood and cerebrospinal fluids with antibiotics added were used without further processing. Stools, throat washings, rectal swabs, and throat swabs were processed for inoculation of tissue cultures by standard methods *(1)*. The tissue extracts, blood, and cerebrospinal fluids initially studied were stored at approximately —20°C for varying lengths of time between examination by the standard tissue culture and agar overlay methods. In the subsequent studies of the 975 diagnostic specimens, extracts were stored at —20°C and examined by the standard tissue culture and agar overlay methods within a few days of one another.

Standard tissue culture procedure. Two tubes each of PMK and HEp-2 cells on maintenance media were inoculated with 0.2 ml of the autopsy and cerebrospinal fluid specimens. Inoculations were made into the tissue culture fluid in the tubes; that is, inocula were not adsorbed to the cell monolayers. Tubes were incubated in stationary racks at 36°C for 8 days and observed every other day for the presence of cytopathic effect. One blind passage was made in each cell type for those specimens showing no cytopathic effect in first passage. For the later studies of the 975 diagnostic specimens, tubes of PMK were used in the same manner.

Agar overlay procedure. Growth fluid was removed from the tissue culture bottles. Two bottles of each cell type were inoculated with 0.2 ml each of the specimens. The inocula were allowed to adsorb to the cells for 1 ½ hours at 36°C and were not rinsed off. Then, 7 ml of agar overlay medium was added to each bottle. Agar overlay medium consisted of 0.5 percent lactalbumin hydrolysate, 0.02 percent yeast extract, 0.22 percent $NaHCO_3$, 3 percent fetal calf serum, and 1 percent Difco purified agar in Earle's balanced salt solution with penicillin, streptomycin, neomycin, and amphotericin B. Sterile 3M $MgCl_2$ was added to the overlay medium of one bottle of each tissue culture type to give a final concentration of 0.025M. Bottles were incubated at 36°C for 10 days. For the studies of the 975 diagnostic specimens, one Petri dish of PMK was inoculated with 0.2 ml of each specimen. The inocula were adsorbed to the cells as before. Only overlay with added $MgCl_2$ was used, and the dishes were incubated for 8 days at 36°C in a CO_2 incubator.

Virus isolation and identification. After 10 days of incubation, a second agar overlay (5 ml) was added to each bottle. This overlay contained, in addition to the constituents indicated earlier, 5 percent by volume of 1:1,000 neutral red. Bottles were incubated an additional 3 days at 36°C and observed daily for the presence of plaques. Care was taken to minimize exposure to light during these observations. A plug of agar was obtained from a single, well-isolated plaque in positive bottles by means of a small metal spatula. The agar plug was homogenized in 1.5 ml of maintenance medium, and the suspension was used to inoculate tissue culture tubes. The viruses obtained were passed twice and titrated to determine the number of $TCID_{50}$ present. Each virus was identified by standard tube neutralization tests *(1)* with pools of enterovirus antisera followed by appropriate individual antisera. Procedures were the same for isolation of viruses from plaques formed by the

Table 1. Enterovirus isolations made from 62 cerebrospinal fluid specimens by agar overlay and standard tissue culture methods

Specimen No.	Agar overlay[1]				Standard tissue culture[2]	
	PMK[3]	PMK with $MgCl_2$	HEp-2[4]	HEp-2 with $MgCl_2$	PMK	HEp-2
0252	Positive	Echo 20	Positive	Negative	Echo 20	N.D.
0286	Negative	Negative	Positive	Coxsackie B1	Negative	N.D.
0643	Coxsackie B5	Positive	Positive	Coxsackie B5	Coxsackie B5	Positive
1161	Untyped	Negative	Negative	Negative	Negative	Negative
1302	Echo 4	Positive	Negative	Negative	Echo 4	N.D.
1424	Echo 30	Positive	Negative	Negative	Negative	N.D.
1439	Echo 30	Positive	Negative	Negative	Echo 30	N.D.
1444	Echo 30	Positive	Negative	Negative	Negative	N.D.
1748	Negative	Negative	Negative	Negative	Coxsackie B2	N.D.
1811	Negative	Negative	Negative	Negative	Echo 9	N.D.
1821	Negative	Echo 6	Negative	Negative	Echo 6	N.D.
1836	Negative	Untyped	Negative	Negative	Negative	Negative
Number positive ..	7	8	3	2	7	1

[1] 5 of the 12 isolations were made by agar overlay.
[2] 2 of the 12 isolations were made only by standard tissue culture.
[3] Primary rhesus kidney cells.
[4] Human epithelial cells.
NOTE: N.D. = not done.

Table 2. Enterovirus isolations made from 141 autopsy specimens by agar overlay and standard tissue culture methods

Specimen No., type of tissue	Agar overlay[1]				Standard tissue culture[2]	
	PMK[3]	PMK with $MgCl_2$	HEp-2[4]	HEp-2 with $MgCl_2$	PMK	HEp-2
0423, brain	Untyped	Positive	Negative	Negative	Negative	Negative
0453, brain	Negative	Echo 9	Positive	Positive	Negative	Negative
0456, epiglottis	Coxsackie B4	Negative	Negative	Negative	Negative	Negative
0482, meninges	Coxsackie B5	Negative	Negative	Negative	Negative	Negative
0482, cerebellum	Positive	Coxsackie B5	Positive	Negative	Negative	Negative
0482, cerebrum	Negative	Echo 9	Negative	Negative	Negative	Negative
0547, heart blood	Negative	Coxsackie B5	Negative	Negative	Negative	Negative
0547, intestine	Negative	Negative	Positive	Coxsackie B5	Negative	Negative
0586, brain	Negative	Coxsackie B5	Negative	Coxsackie B5	Negative	Negative
0754, blood	Coxsackie B5	Positive	Positive	Coxsackie B5	Coxsackie B5	Positive
0756, intestine	Negative	Coxsackie B5	Negative	Negative	Negative	Negative
1088, liver	Negative	Echo 31	Negative	Negative	Negative	Negative
Number positive	5	9	4	4	1	1

[1] 11 of the 12 isolations were made only by agar overlay.
[2] None of the 12 isolations were made only by standard tissue culture.
[3] Primary rhesus kidney cells.
[4] Human epithelial cells.

diagnostic specimens in the Petri dishes, except that only a single agar overlay containing neutral red was used. Viruses isolated by standard tissue culture techniques were identified by the same methods applied to plaque isolates.

Results

Cerebrospinal fluid specimens. The enterovirus isolations made from 62 cerebrospinal fluid specimens by the agar overlay and standard tissue culture methods are shown in table 1. Of the 62 specimens studied, 12 were positive—5 only by agar overlay, 2 only by the standard method, and 5 by both overlay and standard procedures. The remaining 50 specimens were negative by both methods. Three of the five cerebrospinal fluids negative by the standard technique were not tested in HEp-2 cells. One of these three spinal fluids (No. 0286) was positive by agar overlay only with HEp-2 cells. Accordingly, the results may be biased in favor of the overlay method by omission of HEp-2 fluid tissue cultures with this particular spinal fluid and, possibly, with the other two spinal fluids similarly not tested.

The number of plaques observed with the overlay procedure was usually small (one to eight plaques per 0.2 ml inoculum). One specimen (No. 0643), however, showed many plaques with both types of cells and with both overlay media. Since the plaques observed with a given specimen appeared relatively uniform in the various overlay systems, virus usually was isolated and typed from only one of the systems found positive. In all instances where both overlay and standard tissue culture procedures were positive, the same virus type was found in both. Most of the viruses isolated were typed as known enteroviruses (three coxsackie B and seven echoviruses). However, two isolates (Nos. 1161 and 1836) could not be typed with antisera against polioviruses 1–3, coxsackieviruses B1–6 and A9, and echoviruses 1–33. Both these isolates produced a cytopathic effect typical of the enteroviruses in PMK cells but were not characterized further. Of the agar overlay modifications studied, PMK cells with 0.025M $MgCl_2$ in the overlay yielded the most viral isolations, followed closely by PMK cells without $MgCl_2$. HEp-2 cells, with or without $MgCl_2$ in the overlay, yielded fewer viral isolates than PMK cells under overlay. However, as indicated previously, in one case (No. 0286) a virus was isolated only with the HEp-2 cells under overlay.

Autopsy specimens. The enteroviruses isolated from 141 autopsy specimens by the agar overlay and standard tissue culture methods are shown in table 2. Of these 141 specimens, 11 were positive only by agar overlay, none were positive only by the standard procedure, 1 was positive by both overlay and standard procedures, and 129 were negative by both methods. Each specimen was tested in all agar overlay and standard tissue culture systems. The number of plaques formed was very small. Numbers ranged from 1 to 7 plaques per 0.2 ml inoculum, but in many instances (15 of 22 cases where plaques were formed), only 1 plaque was observed. The viruses isolated were typed as known enteroviruses (8 coxsackie B and 3 echoviruses) with one exception, No. 0423, which could not be typed with antisera against polioviruses 1–3, coxsackieviruses B1–6 and A9, and echoviruses 1–33. This virus produced a cytopathic effect characteristic of the enteroviruses in PMK cells but was not characterized further. In one case (No. 0482), isolation of two types of enteroviruses from different tissues of the central nervous system suggested that the patient might have had a dual infection. PMK cells with 0.025M $MgCl_2$ in the overlay again yielded the most viral isolations. There was little difference in the number of viruses isolated between PMK without $MgCl_2$ in the overlay and HEp-2 with or

Table 3. Results of a comparative study of the agar overlay and standard tissue culture methods for isolation of enteroviruses from 975 diagnostic specimens

Type of specimen	Number of specimens tested	Number positive by agar overlay only	Number positive by standard procedure only	Number positive by both overlay and standard procedures	Total positive
Cerebrospinal fluid	210	6	5	10	21
Throat swab	179	2	0	9	11
Rectal swab or stool	499	6	14	47	67
Autopsy	87	3	1	9	13
Total	975	17	20	75	112

without $MgCl_2$ in the overlay. However, in one instance (No. 0547, intestine) a virus was isolated only with the HEp-2 cells under overlay.

Diagnostic specimens. In subsequent studies, 975 diagnostic specimens were examined by standard methods using PMK cells and with PMK cells under overlay containing 0.025M $MgCl_2$. The results of these studies are shown in table 3. Seventeen specimens were positive only by agar overlay, 20 were positive only by the standard procedure, 75 were positive by both overlay and standard procedures, and 863 were negative by both methods. The viruses isolated only by the agar overlay method were four coxsackie B viruses (types 1,3,5), three echoviruses (types 4,9), eight polioviruses (types 1,2), and two viruses which could not be typed with the available enterovirus antisera. Viruses isolated only by the standard procedure were four coxsackie B viruses (types 1,5) and 16 echoviruses (types 2,4,6,9,11,14,17,20,25,31). With the overlay procedure, the number of plaques observed ranged from 1 to 11 per 0.2 ml of inoculum.

Discussion

In each part of this study, enteroviruses were isolated by the agar overlay method but not by the standard tissue culture technique. Conversely, some specimens that were positive by the standard tissue culture method were negative by the agar overlay procedure. This was particularly true in the later studies of the 975 diagnostic specimens received. The differences in numbers of isolations between the overlay and the standard procedures were not significant except for the initial studies on the 141 autopsy specimens shown in table 2. Since each procedure isolated some viruses that the other did not, however, use of both methods would be desirable to isolate as many enteroviruses as possible from clinical specimens.

If large numbers of specimens were being processed, the amount of work involved might make it difficult to use the overlay method in all cases. But, the procedure appears to be worthwhile for specimens for which viral isolation might be particularly significant or of special interest (for example, with cerebrospinal fluids, autopsy specimens, material from possible vaccine-associated cases of poliomyelitis, and samples from epidemic cases of illness found negative by standard procedures). In addition to isolating further enteroviruses, the agar overlay procedure might provide a preliminary clue as to the kind of enterovirus present from the size and type of plaques produced and might indicate the presence of more than one kind of virus if different types of plaques were observed. Possibly, further increases in sensitivity of the overlay method for isolating enteroviruses could be achieved by using other cell cultures such as human embryonic lung fibroblasts *(7)*, by inoculating more Petri dishes or bottles of tissue culture with each specimen, by using larger inocula, or by using other solidifying agents such as agarose *(8)* or starch *(9)*.

In all instances where the agar overlay procedure succeeded in isolating a virus from specimens found negative by standard tissue culture, the number of plaques formed was small (usually less than 10 plaques per 0.2 ml). Similar observations were made by others with overlay isolation procedures *(3-5)*. Isolation of enteroviruses from such specimens by the overlay procedure might be due to localization of the viral progeny of initially infected cells by the overlay with subsequent infection of adjacent cells eventually leading to plaque formation. In the standard tissue culture method, on the other hand, the virus particles produced by initial infection of a few cells might diffuse away and might not infect a sufficient total number of cells to produce a detectable cytopathic effect. Since inocula were adsorbed to the cell monolayers in the overlay procedure but not in the standard tissue culture method, this also could have influenced the number of enterovirus isolations. Adsorption of the inocula might facilitate infection of the cells and thus favor isolation of any virus present. Variation in sampling of the specimens could likewise have been a factor in the additional isolations by the overlay procedure.

Isolation of enteroviruses by the standard tissue culture method but not by the overlay procedure might again have been due to variation in sampling of the specimens. Futhermore, most of the specimens that yielded an enterovirus by the standard procedure but not by the agar overlay procedure were examined in the later studies of the 975 diagnostic samples. Neutral red was incorporated in the overlay throughout the incubation period in this part of the study rather than being added in a second overlay, as was done during the tests on the initial cerebrospinal fluid and autopsy

specimens. Incorporation of neutral red has been found in some instances to reduce plaquing efficiency of enteroviruses *(10)*. Although care was taken to minimize exposure of the plates to light after addition of the overlay, the presence of neutral red might have contributed to failure of the overlay method to isolate enteroviruses from the specimens.

In the comparative experiments on cerebrospinal fluids and autopsy specimens, the largest number of viral isolations was made with PMK cells under agar overlay with added $MgCl_2$. Fewer isolations were made with HEp-2 cells under overlay with or without added $MgCl_2$. However, since the total number of isolations and the differences in isolation rates were small, conclusions about the superiority of PMK cells over HEp-2 cells or of overlay with added $MgCl_2$ compared to overlay without added $MgCl_2$ must be made with caution. With such small numbers of plaque-forming units of virus present in the specimens, sampling variation in inoculating the various systems could be an important factor influencing the results. It is interesting to note, however, that six of the viruses isolated with PMK cells under overlay containing $MgCl_2$ but not with PMK cells under overlay without $MgCl_2$ were of the types shown by Wallis and associates *(6)* to grow better under agar overlay with added $MgCl_2$. This finding suggests that addition of $MgCl_2$ to the overlay had a beneficial effect in isolation of these six viruses.

The possibility should be considered that standard tissue culture methods could be made more sensitive for isolation of enteroviruses by addition of $MgCl_2$ to the medium. In this connection, Wallis and Melnick *(11)* reported that an increased number of poliovirus isolations was made from stool specimens with standard PMK tissue cultures containing 0.025M $MgCl_2$ in the medium. It is also possible that the sensitivity of standard tissue culture methods could be increased by adsorption of the inocula to the cell monolayers. Further studies are necessary to assess the influence of both these factors on the isolation of enteroviruses by standard tissue culture methods.

The viruses isolated only by the overlay procedure were mostly recognized enterovirus types. However, five viruses were isolated only in PMK cells which could not be typed with available antisera. These viruses produced a cytopathic effect like that of the enteroviruses, but since they were not characterized further we cannot be certain of their exact classification. The possibility of isolating simian agents cannot be excluded in these cases. All the viruses isolated only by the standard tissue culture procedure were recognized enterovirus types.

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SYNOPSIS

HATCH, MILFORD H. (Center for Disease Control), and MARCHETTI, GEORGE E: ***A comparative study of agar overlay and standard tissue culture methods for isolation of enteroviruses. Public Health Reports, Vol. 90, January–February 1975, pp. 29–33.***

A study was made of the isolation of enteroviruses from 62 cerebrospinal fluids and 141 autopsy specimens with standard fluid monolayer tissue culture methods and tissue cultures under agar overlay with and without added $MgCl_2$ (0.025M). Sixteen virus isolations were made only by the overlay method, two only by the standard technique, and six by both procedures. Additional studies were carried out on 975 diagnostic specimens of various types with standard tissue culture methods and with primary rhesus kidney cells under agar overlay containing 0.025M $MgCl_2$. Seventeen virus isolations were made only by the overlay procedure, 20 only by the standard tissue culture method, and 75 by both techniques. Since there were instances in which either the agar overlay or the standard method alone succeeded in isolating a virus, use of both types of procedures would be desirable to isolate as many enteroviruses as possible from clinical specimens.