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Isolation and Identification of Adenoviruses in Microplates[1]

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A procedure for isolating and identifying adenoviruses in microplates is described. Comparison tests with standard tube methods show an agreement of 92%. Virus isolations are greatly facilitated by the microplate method. This method is sensitive, economical, and especially applicable to large-scale epidemiological surveys.

The conventional method of isolating viruses in vitro is an expensive, cumbersome, and time-consuming operation. The propagation and maintenance of host cultures for this purpose requires large quantities of cells, media, and utensils (tubes, caps, racks, etc.) as well as adequate space for manipulation and incubation of cultures. The isolation process is a lengthy one. A period of 4 to 6 weeks may elapse after inoculation of the tissue culture monolayer with the specimens before virus isolation work can be completed. During this time, cells are observed, media are changed, and passages are made, all requiring additional materials and handling of the culture tubes.

This report describes the use of the microplate tissue culture system for isolation of viruses. This technique has been employed for viral serology, especially where large numbers of tests are required (1). Virus isolation involves the simultaneous inoculation of patient specimen and seed tissue cells in replicate wells of a microplate. Three 7-day passes are carried out without change of media. Isolates are typed in plates when extensive cytopathology (CPE) occurs. Microscopic observation is facilitated, since several specimens are contained in a single plate. This technique is particularly applicable for large-scale epidemiological surveys enabling one technician to handle large numbers of specimens rapidly and economically.

MATERIALS AND METHODS

Microplate equipment. (i) Disposable polyvinyl "U" plates (Cooke Engineering Co.) were treated as previously described (1) and exposed to ultraviolet light for 1 hr for sterilization. (ii) Lightweight plastic covers (Linbro Chemical Co.) were also irradiated. (iii) Calibrated transfer loops and droppers were standard Microtiter equipment.

Cell cultures and media. HeLa cells obtained from V. V. Hamparian, Children's Hospital, Columbus, Ohio, were used routinely for adenovirus isolation. WI-38, HEp-2, secondary rhesus monkey kidney, and human embryonic kidney cells have also been used for isolation of other viruses. Cells for microplate cultures were trypsinized by the residual trypsinization technique and diluted to 2×10^5 cells/ml. Growth medium consisted of Eagle's minimum essential medium (MEM) in Earle's balanced salt solution (EBSS) supplemented with 10% fetal calf serum. Antibiotics were added in the following concentrations; penicillin, 200 units/ml; streptomycin, 200 μg/ml; and amphotericin B, 5 μg/ml. HeLa cells for tube cultures were prepared in the same manner. Each tube was seeded with 1 ml of cells at a concentration of 10^5. After 48 hr of incubation, cells were changed to a maintenance medium consisting of MEM in EBSS supplemented with 5% fetal calf serum. Diluent used for microplates consisted of 0.5% lactalbumin hydrolysate in EBSS.

Specimens. Specimens obtained from Naval recruits included nasal washings and throat and anal swabs collected in veal infusion broth supplemented with 0.5% bovine albumin. Diluent for swabs contained antibiotics in the concentration stated in the growth medium. The nasal washes did not contain antibiotics.

Typing sera. Typing sera were prepared in rabbits by using prototype adenovirus strains as immunizing antigens. Antibody titers and dosage for virus-typing tests were determined by the end point dilution technique.

Microplate isolation procedures. Isolation plates were set up as follows: each specimen was inoculated into one row of eight wells, each well in the row receiving one drop (0.025 ml) of diluent, two drops of specimen, and one drop of cells in growth medium. A row of control cells was seeded in wells between specimen rows. Thus a plate could accommodate six specimens and six control rows (Fig. 1). The amount of specimen in the eight wells was equivalent to that normally inoculated into duplicate tubes in the stan-

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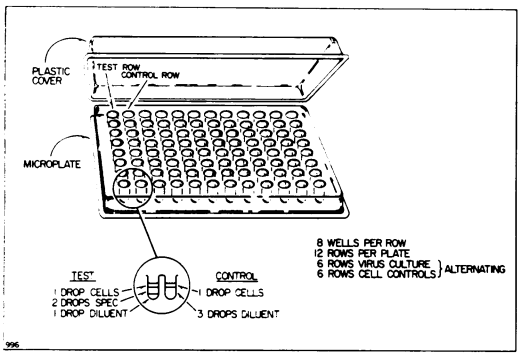

FIG. 1. *Diagram of microplate virus isolation system showing constituents of wells in test and control rows.*

dard method (0.4 ml). Plates were covered with plastic covers and incubated in a humidified incubator (at least 90% relative humidity) in 2% CO_2 atmosphere at 34 C. Twenty-four hours after inoculation, an additional drop of HeLa cells was added to each well. Cells were observed for 7 days without change of medium and then were subcultured. Three 7-day passes were routinely made before cultures were terminated.

Passage was made as follows. The cell sheets were disrupted with the dropper tip, and the entire contents of the eight wells were drawn up into the dropper. Two drops were then passed to each of eight wells in a new plate. Control wells were also passed in the same manner. Excess passage material may be frozen at this time.

Typing of isolates was done when CPE was complete. Positive specimens were diluted 1:2 by transferring one drop (0.025 ml) with a diluting loop to each of several wells containing 0.025 ml of diluent. One drop of typing sera, containing 10 to 20 neutralizing doses of adenovirus antibody, was added to each well. Virus and serum controls were also prepared in the same plate. Preincubation for 1 hr was carried out as described above. One drop of cell suspension was added to all test wells, and the plates were reincubated. Test results were evaluated at 2 and 5 days.

Tube isolations. Duplicate tubes in maintenance medium were inoculated with 0.2 ml of specimen and rolled at 34 C. Tubes were read and media were changed three times weekly. Passage was made at the end of 2 weeks by one cycle of freezing and thawing and transferring 0.2 ml to new cell cultures. Typing of isolates was carried out in microplates as previously described.

RESULTS AND DISCUSSION

Examination by microscope of plates 24 hr after inoculation showed various degrees of toxicity in most specimens. As would be expected, anal specimen toxicity was most pronounced. To overcome this problem, an additional drop of cells was added to the wells at this time. These cells overgrew the original clumped toxic cells in

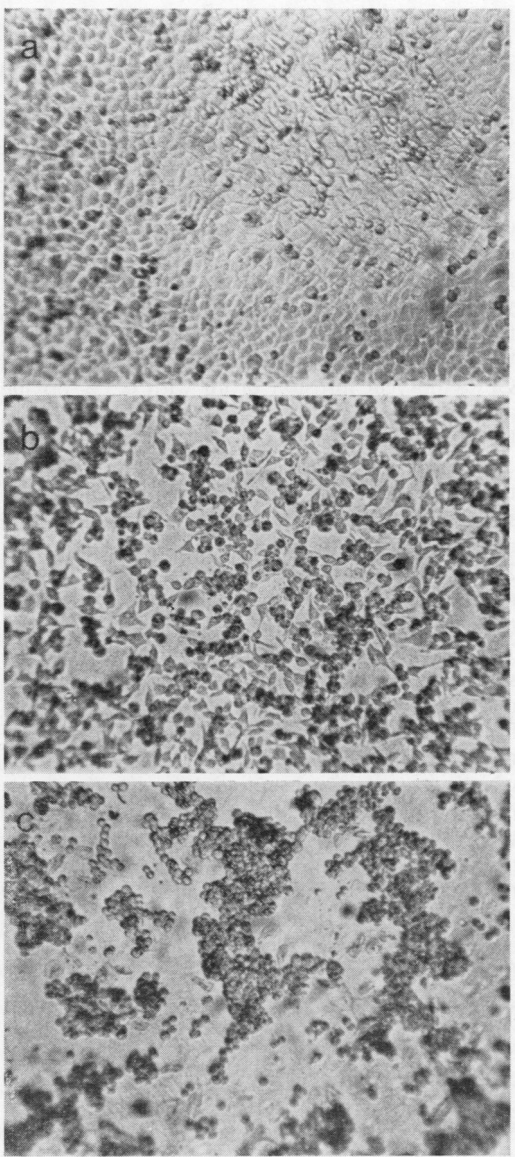

FIG. 2. *Microscopic appearance of HeLa cells in microplate wells (× 300). (a) Uninfected control cells, (b) cells showing toxic effect of specimen, (c) infected cells showing virus cytopathology.*

48 hr, and although the cell sheet was not as complete as in subsequent passes, it was considered adequate; the remaining toxicity could be distinguished from viral CPE (Fig. 2). In extremely toxic specimens, subcultures were initiated earlier than 7 days.

Besides specimen toxicity, it was discovered that the veal infusion broth in the swab diluent also contributed to this problem. In testing other media that would be suitable for sampling fluids,

it was found that EBSS supplemented with 0.5% bovine albumin was the least toxic. Although this medium was not toxic, it was found that the development of virus CPE was delayed. In one experiment, 38 specimens were collected in veal infusion broth with 0.5% bovine albumin or EBSS with 0.5% bovine albumin, respectively. Eight viruses (adenovirus 4) were recovered eventually from the same specimens collected in either medium. However, six were obtained in first pass (7 days) and two in second pass with veal infusion broth compared to one in first pass, five in second pass, and two in third pass from the sampling with EBSS. Because of this delayed CPE, the veal infusion broth with 0.5% bovine albumin was chosen to be the collecting medium for further tests.

Comparison of the tube and microplate systems for adenovirus isolation was made. Of 263 specimens cultured by both the standard tube method and the microplate procedure described, 241 or 92% showed agreement either by the recovery of a virus or by negative results in both tests. Fifty-eight isolations (51 type 4, seven type 7) and 183 negatives were obtained from the same specimens by both methods. The 22 specimens showing disagreement were distributed as follows: nine which were positive in tubes were negative in microplates. On the other hand, 13 which were positive in microplates were negative in tubes. In these disagreements, adenovirus types 4 and 7 were randomly distributed in the two tests. None of the above differences in tests were statistically significant.

During these studies it was found that viral isolates were recovered earlier in microplates than in tubes. In a comparison test of 50 specimens observed daily for appearance of CPE, 29 isolates were recognized in microplates within 13 days of incubation as compared to 19 in tubes. The difference within this period is statistically significant ($P = .045$) however, the total number of positives eventually recovered at the end of 21 days was not (31 in microplates, 26 in tubes). The median time required for virus isolation was 7 days for microplates and 9 days for tubes.

In connection with these experiments, certain variations in the microtechnique were appraised. There was no apparent advantage to inoculating specimens on preformed or established monolayers, nor was it advantageous to freeze and thaw the plates between passages. It was also found that 7 days of incubation per passage was the optimal time for recovery of viruses. Most of the isolations were made within the first two passes (14 days).

In the course of these experiments, many other advantages, in addition to the obvious ones of economy of time and materials, became apparent. Since an established cell monolayer was not necessary, specimens could be inoculated upon receipt, thus reducing the risk of loss of virus due to frozen storage and subsequent thawing. Also the use of microplates facilitates the detection of CPE. An entire well can be quickly scanned by microscope and any cellular change can be noted. In scanning tubes, often many fields encompassing the entire cell monolayer must be examined before CPE is detected.

Another desirable feature was the ease of harvesting. Plates could be frozen intact for further passing, thus eliminating transfer of material to small vials as is necessary to conserve space in tube isolation procedures. Also, harvest time was not as critical, as an additional drop of cells could be added to wells, thus delaying time of harvest.

Although sufficient numbers have not been tested, viruses of other groups have been isolated successfully using micromethods. Herpesvirus, influenza, rubella, poliovirus, echo, and rhinoviruses have all been isolated and identified. It is obvious, however, that this method as presently carried out, is not optimal for isolation of groups of viruses requiring special cultural requirements such as incubation of tubes in a roller drum apparatus for rhinovirus isolation. Also it should be emphasized that care must be exercised in performing this technique to avoid cross-contamination of cultures.

Although more adenoviruses were recovered by the microplate method in these studies, we do not wish to imply that the tube method is less sensitive. Other comparison tests have indicated reverse results. Neither of these situations is believed to be statistically significant with agreement of tests ranging from 85 to 95%. The microplate method does, however, have decided advantages when a large number of isolations need to be performed.

LITERATURE CITED

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