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COMPARISON OF CYTOPATHOGENICITY, IMMUNOFLUORESCENCE AND IN SITU DNA HYBRIDIZATION AS METHODS FOR THE DETECTION OF ADENOVIRUSES

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Abstract—Three different methods were compared for their efficiency at detection of adenoviruses. The samples examined for viral analysis consisted of concentrates prepared from raw sewage, chosen as providing a representation of the spectrum of viruses being intestinally shed from a large population at any given time. When using one single cell line, HEp-2, the overall numbers of adenoviruses detected using cytopathogenicity and immunofluorescence were roughly equal. In situ hybridization was approx. 40% more sensitive than either of these other methods as determined by average virus titers for the different samples, and also proved to be better by means of a nonparametric comparison. The 293 cell line was approx. 5 times more sensitive for detecting adenoviruses by cytopathogenicity as compared with the HEp-2 cell line, but proved unsuitable in our hands for quantitatively detecting indigenous adenoviruses by immunofluorescence. The relative number of indigenous adenoviruses present in the sewage concentrates we examined was, on average, 94-fold greater than that of enteroviruses. Assay of enteroviruses was performed by plaque assay in the BGM cell line.

Key words—adenovirus, cytopathogenicity, immunofluorescence, in situ hybridization, comparative assay

INTRODUCTION

Adenoviruses are causative agents of a number of human viral illnesses. Among these are pharyngitis, conjunctivitis, pharyngo-conjunctival fever (Straus, 1984) and gastroenteritis (Albert, 1986). Because of the very nature of gastroenteritis, it is reasonable that the adenoviral agents of this syndrome will be found associated with fecal material and in an infectious state. It is also possible for the adenoviruses associated with these other syndromes to likewise be present in the human intestinal tract as a result of drainage from sites of infection. As a group, the adenoviruses have been shown to be reasonably resistant to low pH conditions (Fields and Metcalf, 1975). Thus the viruses associated with these other ailments may similarly be in an infectious state at the time they are shed from the intestinal tract.

The objective of the present study was to compare different methods for detecting intestinally shed adenoviruses. In doing so we chose to evaluate two rapid assay techniques. The first of these was an indirect immunofluorescence method, and the second an in situ DNA probe hybridization approach which utilized a biotin–avidin system in conjunction with horseradish peroxidase. Both of these techniques incorporated an incubation period of 48 h between the time when cell cultures were infected, and the time that cultures were subsequently fixed for staining and examination. Both of these rapid approaches result in formation of focal areas of viral infection which can be visualized and enumerated by microscopic examination. These two methods were contrasted with the more traditional approach of isolating adenoviruses in cell culture as determined by production of characteristic cytopathogenic effects, an approach which in our hands required an incubation period of from 7 to 8 days for obtaining complete results. The samples examined in this study for presence of viruses consisted of concentrates produced from raw sewage, an approach chosen to reflect an overall sample population of those viruses being shed by members of a community (Sellwood et al., 1981).

MATERIALS AND METHODS

Cell cultures and media

The cell lines used in this study were BGM, a continuous line originating from African Green monkey kidney; HEp-2, a continuous human epidermoid carcinoma line; and 293, an adenov-5 transformed primary human embryonal kidney line. The BGM cell line was obtained from Daniel R. Dahling, U.S. EPA, Cincinnati, Ohio. Both the HEp-2 and 293 cell lines were purchased from American Type Culture Collection, Rockville, Md. Production of individual BGM cultures for performing viral plaque titration assays was done in 25 cm² tissue culture flasks. Production of tube
cultures of either HEp-2 or 293 cells for use in cytopathogenicity assays was done in screw-capped 16 x 125 mm culture tubes. The type of tube material used for cytopathogenicity assays depended upon the cell line; borosilicate glass for HEp-2 cells and polystyrene for the 293 line. Choice of the latter combination was based upon a finding that cells of the 293 line did not grow well on glass surfaces. Production of individual HEp-2 cell cultures for either immunofluorescence or in situ hybridization assays was done using 8-well chambered slides with removable gaskets (Lab-Tek, Miles Laboratories Inc., Naperville, Ill.).

The medium used for routine cultivation of the BGM cell line consisted of equal parts Eagle's minimum essential medium (MEM) and Leibovitz medium L-15, supplemented with 10% v/v fetal bovine serum (FBS) and 30 ml1 of sodium bicarbonate solution. All culture media used in this study were supplemented with HEPES buffer at a level of 20 mM, penicillin G at 100 units ml-1, amikacin sulfate at 1 mg ml-1 and production of tube cultures of this same line consisted of MEM supplemented with 12.5% v/v FBS and 30 ml1 of sodium bicarbonate solution. Medium used for cultivation of the HEp-2 cell line in chambered slides was the same as that listed above for HEp-2 except for incorporation of sodium bicarbonate solution at a lower level of 5 ml1. The medium used for both routine cultivation of the 293 cell line and preparation of tube cultures of this same line consisted of MEM supplemented with 12.5% v/v FBS and 30 ml1 of sodium bicarbonate solution. All culture media used in this study were supplemented with HEPES buffer at a level of 20 mM, penicillin G at 100 units ml-1, amikacin sulfate at 30 mg ml-1, and streptomycin sulfate at 100 mg ml-1. Where indicated, guanidine hydrochloride was present in the culture media at a final concentration of 100 mg ml-1 in order to selectively suppress replication of enteroviruses while allowing unrestricted replication of adenoviruses (Hurst et al., 1988).

Preparation of sewage concentrates

Each of the sewage concentrates examined in this study was prepared from a separate 8 l vol of raw sewage. Ten sewage samples were collected during the period of mid-November through mid-December, 1986, from municipal treatment facilities serving the Cincinnati, Ohio area. Following collection, each sewage sample was adjusted to a final concentration of 0.05 M MgCl2 by addition of a 4 M MgCl2 stock solution. The samples were then stored cold until the solids could be settled to the bottom, following which approx. one-half of the supernatant volume of each sample was decanted and discarded.

The remainder of each sample was then centrifuged in airtight sealed 125 ml Erlenmeyer flasks by centrifugation for 10 min at 2800 g to pellet the solids, and the supernatant was carefully removed. The cell cultures were examined on days 2-5 post inoculation for the presence of characteristic adenoviral cytopathogenic effects. Culture tubes containing HEp-2 cells were incubated on a roller apparatus during the first 2 days post infection and on days 4-8 post infection in order to more closely be compared with those of the other viral assays used in this study, the derived TCID50 values were divided by a factor of 7 and then expressed as TCID50 ml-1.
Fixing of the infected slides was done by first removing all medium from the wells, and then removing the chambers from the slides. The slides were then immersed either in methanol for 5 min at −20°C if they were to be examined by immunofluorescence, or in Carnoy’s B fixative (by vol; 60% absolute ethanol, 30% chloroform, 10% acetic acid) for 5 min at 23°C if they were to be examined by in situ hybridization. The gaskets were removed from the slides following fixation of the cells.

**Immunofluorescence assay**

Staining of infected cells present on the slides for examination by immunofluorescence was accomplished using antiserum prepared in rabbits. This antiserum was prepared as a reference reagent by the National Institutes of Health, and was purchased from American Type Culture Collection, Rockville, Md. The antiserum was prepared against whole human adenovirus 5 virions and is therefore reactive against the group common human adenoviral antigen by immunofluorescence. The slides were washed in PBS and then incubated for 1 h at 23°C with fluorescein-conjugated staphlococcal protein A diluted in PBS. The final step in staining for immunofluorescence examination consisted of washing the slides in PBS, immersing them in 0.0025% Evan’s blue dye prepared in 0.15 M potassium chloride for 10 min at 23°C, and rinsing in PBS. The slides were then examined for enumeration of fluorescent cell foci using epi-illumination. All cells determined as positive by immunofluorescence were immediately subjected to fixation prior to immunofluorescence staining.

**In situ hybridization assay**

Staining of infected cells present on the slides for examination by the in situ hybridization technique was accomplished by first incubating the cell monolayers with a "probe" solution containing whole human adenovirus 5 genomic DNA labelled with biotin-linked deoxyuridine. Following incubation with the probe solution, the slides were incubated with an avidin-horseradish peroxidase conjugate. This was followed by incubation with diaminobenzidine hydrochloride and hydrogen peroxide to yield a brown precipitate over the position of the infected cells. The slides were then counterstained by mounting coverslips on them using a 0.5% w/v solution of trypan blue in 0.15 M NaCl. Examination of the slides for the presence of focal areas of infected cells was accomplished using transmitted bright light microscopy. The results of this assay are expressed in terms of flu of virus per ml of inoculum.

**RESULTS**

The first part of this study consisted of comparing the sensitivities of cytopathogenicity, immunofluorescence and in situ hybridization for adenovirus detection. In making this evaluation we considered it important that the same cell line be used for all three detection methods so as to avoid possible cell line-dependent differences in viral sensitivities. Cells of the HEp-2 line have been chosen for this comparison because of difficulties which we have experienced when attempting use of the 293 line for adenovirus immunofluorescent foci assays. These difficulties with the 293 line have been 2-fold. Firstly, a tendency of the cells to round-up severely during the course of adenoviral infection such that the positive status of fluorescing cells cannot be confirmed through examination of the cell's internal morphology. Secondly, a poor adherence of cultures of this cell line to either glass or plastic microscope slides, such that the produced monolayers readily detach during efforts at fixation prior to immunofluorescence staining.

The results of comparing these three different detection methods for quantitating adenoviruses present in the processed sewage concentrates are presented in Table I. It can be seen that viral titers yielded by the cytopathogenicity and immunofluorescence methods were, overall, approximately...
Table 2. Nonparametric comparison of techniques for assaying indigenous adeno-
viruses present in sewage concentrates*

| Sample designation | Cytopathogenicity | Immunofluorescence | In situ hybridization |
|--------------------|-------------------|--------------------|-----------------------|
| 1                  | 1                 | 2                  | 3                     |
| 2                  | 1                 | 2                  | 3                     |
| 3                  | 1                 | 3                  | 2                     |
| 4                  | 2                 | 1                  | 3                     |
| 5                  | 1                 | 2                  | 3                     |
| 6                  | 2                 | 1                  | 3                     |
| 7                  | 1                 | 2                  | 3                     |
| 8                  | 2                 | 1                  | 3                     |
| 9                  | 2                 | 1                  | 3                     |
| 10                 | 3                 | 2                  | 2                     |

Total = 16 Total = 17 Total = 28

*Comparison made from "mean" values for each sample/assay technique combina-
tion as presented in Table 1. For each particular sample, the method yielding the
highest assay titer was assigned a numerical value of 3, second highest assigned
a numerical value of 2 and lowest assigned a numerical value of 1.

Table 3. Assay titers for indigenous viruses in concentrates from raw
sewage

| Sample designation | Enterovirus* plaque assay (BGM cell line) | Adenovirus+ cytopathogenicity (293 cell line) |
|--------------------|------------------------------------------|---------------------------------------------|
|                    | Mean | SD   | Range   | Mean | SD   | Range   |
| 1                  | 424  | 319  | 220-875 | 155  | 143  | 14-350  |
| 2                  | 348  | 198  | 170-625 | 293  | 180  | 170-350 |
| 3                  | 330  | 75   | 250-430 | 393  | 254  | 180-750 |
| 4                  | 245  | 158  | 25-390  | 198  | 198  | 170-625 |
| 5                  | 188  | 122  | 25-320  | 245  | 158  | 25-390  |
| 6                  | 10   | 5    | 25-180  | 188  | 122  | 25-320  |
| 7                  | 3    | 7    | 25-180  | 10   | 5    | 25-180  |
| 8                  | <1   |     |         | <1   |     |         |
| 9                  | <1   |     |         | <1   |     |         |
| 10                 | <1   |     |         | <1   |     |         |

Average value = 2.5 Average of means = 243.4 Average CV value = 0.69

*Values are expressed in terms of pfu ml^-1 and represent results of a
single trial. As such, calculation of standard deviations, ranges or average CV would not be meaningful.

+Values are expressed in terms of TCID ml^-1 and indicate the mean
of 3 independently conducted trials with 1 SD and the range of
the 3 trial values.

| CV = coefficient of variation; average value represents the mean of
separate CV values as calculated for the 10 different samples. |
tures of HEp-2 cells grown in chambered slides. The procedure used for this inoculation, as well as subsequent fixation of the infected HEp-2 cells in methanol and staining of them for examination by indirect immunofluorescence, was the same as that described in Materials and Methods for directly assaying sewage concentrates in the HEp-2 cell line. All of the 56 adenovirus positive culture tubes of 293 cells which we examined as a part of this confirmation were subsequently found to be positive by immunofluorescence in the HEp-2 cells. This indicated that the much higher viral titers obtained when performing the cytopathogenicity assay with cells of the 293 line did indeed reflect numbers of viable adenoviruses which were not capable of initially replicating in HEp-2 cells. All of these viruses were, however, capable of subsequent replication in the HEp-2 cell line following their first passage in 293 cells. It is suggested by the efforts of Takiff and coworkers (1981) that this difference of adenoviral titers yielded by cytopathogenicity in 293 cells vs HEp-2 cells reflects that portion of the total adenovirus population which is enteric in nature, i.e. is of serotypes 40 and 41, which are causative agents of human gastroenteritis. The variability associated with detecting adenoviruses by cytopathogenicity using the 293 cell line was greater in our hands (Table 3) than it was for detecting adenoviruses by either of the three techniques when using HEp-2 cells.

**DISCUSSION**

Findings obtained from comparing the techniques of cytopathogenicity, immunofluorescence and in situ hybridization for detecting adenoviruses were of interest in that each is based upon identifying a different viral effect. By our procedure, producing a positive result for cytopathogenicity should necessitate production of progeny virions which are fully infectious for the same cell culture line as was inoculated. The reason for assuming this necessity of full virus replication for our cytopathogenicity assay is the relatively low number of viral units which would have been present in the end-dilutions of the sewage concentrates being examined, likely far fewer than would be needed to initially infect 5% of cells in the culture. In addition, nearly all of the positive tubes reached a point where 25% or more of the cells demonstrated cytopathic effects. The immunofluorescence assay required that viruses be capable of proceeding through their replicative cycle to the stage of producing progeny capsid proteins. The similarity of our results from these two assay techniques would suggest that, on average, those virions which are capable of producing capsid proteins within 48 h will subsequently be able to progress in their replicative cycle through the stage of assembling fully infectious progeny if given an additional 6 days of incubation. Our discovery that the number of viruses detected by in situ hybridization was greater than that found using immunofluorescence may suggest that some members of the adenovirus populations contained in our samples were capable of progressing to the stage of generating progeny DNA within 48 h, however were unable to progress significantly further given the constraints of the cell line and the allowed amount of incubation time.

In our study, adenoviral cytopathogenic effects generally appeared by day 7 post inoculation. Our experiments could not be carried out longer than 8 days because of progressive changes in health of the cell monolayers. These changes were apparently associated with presence of guanidine hydrochloride in the culture media. In the case of cytopathogenicity assays, guanidine also seemed to make the cells somewhat more sensitive to cytotoxic effects of compounds present in sewage which concentrated along with the viruses (Hurst and Goyke, 1983). While this increased sensitivity to cytotoxicity did result in some suppression of viral titers for undiluted sewage concentrate, it was generally not a problem for our study because the high numbers of adenoviruses present in the processed sewage concentrates necessitated their dilution by a factor of at least 10-fold prior to inoculation for that type of assay. Changes in cell appearance caused by guanidine did not appear to be a problem with the immunofluorescence or in situ hybridization assays.

The large difference in numbers of adenoviruses detected by cytopathogenicity using the 293 line versus the HEp-2 line was to some extent surprising, but in retrospect need not have been. The 293 line is considered by some to be more sensitive for recovery of the enteric adenoviruses, serotypes 40 and 41, as compared with the HEp-2 line (Takiff et al., 1981). Based upon the distinctions of Takiff and coworkers, the difference in cytopathogenicity titers which we have observed between these two cell lines may represent those viruses which were of serotypes 40 and 41. While use of such distinction might seem to constitute a harsh dicotomy, it would suggest that of the total population of infectious adenoviruses which are intestinally shed by members of the community, and therefore present in raw sewage samples, approx. 80% are of enteric nature.

That adenoviruses are naturally present in sewage in greater numbers than enteroviruses, has been shown true by the studies of both Irving and Smith (1981) and Krikels et al. (1985), and is confirmed by our results. Our finding that the average number of adenoviruses present in sewage concentrates, as measured by cytopathogenicity using the 293 cell line, was 94-fold greater than for the enteroviruses as determined by plaque assay, contrasts with the findings of the above investigators who have reported differences of approx. 3-fold or less. This finding could be associated with three possible factors. Firstly, that adenoviruses might have been recovered more efficiently by the elution and concentration procedures which we used in our study than were the
enteroviruses, a possibility that we have begun to explore further in our laboratory. Secondly, that our collection of samples for use in this study may have coincided with a peak period for the presence of adenoviruses, and simultaneously a minimum period for the presence of enteroviruses. This could well be, as our samples were collected during the time period of November–December. Krikelis and coworkers noted in their study that enterovirus titers peaked in August and September as should be expected for the northern hemisphere, but that the titer of adenoviruses peaked in the months of April and June. Studies by other groups on the prevalence of adenoviral gastroenteritis in the northern hemisphere would indicate the illness to have two annual peaks, one occurring in November and the other from April to May (Cevenini et al., 1985; Cusi et al., 1986; Isaacs et al., 1986; Payne et al., 1986).

Thirdly, we believe that the large difference in the number of adenoviruses vs enteroviruses detected in the present study can also be attributed in part to our use of a cell line, 293, which is more sensitive for adenovirus detection than were those used in the studied by Irving and Smith (1981) and Krikelis et al. (1985). Clearly, the utilization of guanidine as a media additive to suppress replication of indigenous enteroviruses was also a benefit in our being able to detect adenoviruses. Without the use of either guanidine or some similar compound capable of selectively suppressing replication of enteroviruses, the assay titers obtained for adenoviruses in any study similar to ours might be artificially low due to rapid overgrowth of the cell cultures by enteroviruses. This overgrowth effect has previously been even more apparent to us when performing the rapid assay techniques, for which samples are inoculated at lower dilutions due to both the time constraints involved in reading the assays and the reagent costs.

As a routine matter, use of either the immunofluorescence or in situ hybridization assays for adenovirus detection would result in an obvious savings of time when compared to cytopathogenicity. Efforts to reduce the incubation period for either of these two assays from 48 h down to a period of 24 h were unsuccessful in our hands. The cause for this lack of success was due to reduced formation, within the shorter time period, of viral capsid proteins and nucleic acid within the infected cells. The optimum choice for detection of adenoviruses may, however, still rest with use of a cytopathogenicity assay when performed with a highly sensitive cell line such as 293.

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