

Selection of Nonfastidious Adenovirus Species in 293 Cells Inoculated with Stool Specimens Containing Adenovirus 40

MARTHA BROWN

Department of Microbiology, University of Toronto, and Department of Virology, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

Received 12 December 1984/Accepted 30 April 1985

Of 35 stool specimens isolated and examined in 293 cells, 15 isolates contained adenovirus species 40 (Ad40), and 4 of these 15 isolates also contained a nonfastidious adenovirus species (Ad1 in two cases, Ad18, or Ad31) which was selected over Ad40 during serial passage in the 293 cells. The selection of Ad1 over Ad40 was examined in detail. Restriction analysis of intracellular DNA and the relative infectivity titers of Ad40 and Ad1 at each passage level after the inoculation of 293 cells with a particular stool specimen demonstrated that although the amount of Ad40 DNA synthesized far exceeded that of Ad1, the relative infectivity titer of Ad40 was low. The growth characteristics of Ad40 were then compared with those of Ad1, Ad18, and Ad41 in singly infected 293 cell cultures. One-step growth curves showed the same growth rate in each case, with a latent period of 12 h and a maximum titer at 24 to 36 h postinfection. Yields of infectious Ad40 virus were consistently 100- to 1,000-fold lower than those of Ad1. This difference was reflected by a reduced yield of total Ad40 virions ($\rho 1.34$) as determined by ^{35}S labeling experiments. However, the 3- to 10-fold reduction in total yield of Ad40 virions did not account for the 100- to 1,000-fold reduction in the yield of infectious virus.

Fastidious enteric adenovirus species 40 (Ad40) and 41 (Ad41) have recently been recognized as causative agents of gastroenteritis (4, 9, 10). Unlike the other adenovirus species, these species are refractory to isolation in cell lines such as HeLa, KB, HEp-2, and human embryonic kidney, but they do grow in 293 cells (8), a continuous line of human embryonic kidney cells transformed with Ad5 DNA (5).

In a previous study, we compared the use of HeLa and 293 cells for the isolation of adenovirus from clinical stool specimens (1). Of 35 specimens examined, 15 induced cytopathic effect (CPE) on primary inoculation only in 293 cells. These specimens were presumptively classified as Ad40 or Ad41. However, on serial passage of the progeny virus from 293 cells, it was found that four of these isolates acquired the ability to grow in HeLa cells as well. Further analysis demonstrated that the original stool specimen contained a small amount of a nonfastidious species (Ad1 in two cases, Ad18, or Ad31) along with an excess of Ad40. Passage in 293 cells resulted in selection of the nonfastidious species. This result was intriguing in light of the fact that 293 cells are considered permissive for the replication of Ad40 (8). For this reason, the selection of nonfastidious species over Ad40 in 293 cells was examined in detail.

MATERIALS AND METHODS

Cells and virus. The 293 cells were obtained from F. Graham, McMaster University, Hamilton, Ontario, Canada, at passage level 43 and were used in this study between passages 50 and 80. They were subcultured by using a trypsin-EDTA solution and were maintained in minimal essential medium with 10% fetal calf serum as previously described (1).

Stool specimens were suspended in a small volume of minimal essential medium and clarified by centrifugation in an Eppendorf centrifuge at $12,000 \times g$ for 5 min. The supernatant fluids were stored at -70°C and used as inocula for the infection of cell cultures.

The Ad1 and Ad18 virus stocks were passage 3 material prepared in 293 cells from clinical isolates identified by

serum neutralization tests and further characterized by restriction analysis of their DNA. The prototype strains of Ad40 (Dugan) and Ad41 (Tak) were purchased from the American Type Culture Collection, and stocks were prepared in 293 cells.

Virus titration. Infectivity titers of virus preparations were determined by endpoint dilution in 60-well (10- μl capacity) MicroTest plates. The virus preparations were incubated at 37°C in a plastic container with moistened filter paper to keep the plates from drying. CPE was scored 10 days postinfection (p.i.), and the 50% tissue culture infective dose titer was calculated by the statistical method of Reed and Muench (7). Titers were expressed as infectious units per milliliter, with 1 infectious unit equal to 0.7 50% tissue culture infective doses (3).

Virus neutralization. The adenovirus isolates were classified according to species by microneutralization tests with 293 cells. Specific antisera against Ad1 and Ad18 were obtained from Microbiological Associates and the National Institutes of Health, respectively. Antiserum against Ad40 was a generous gift from G. Wadell, Umea, Sweden.

For the specific neutralization of Ad1 and Ad40 in the serial passage experiment (Fig. 1), the undiluted virus preparation was mixed with an equal volume of the appropriate antiserum and incubated at 37°C for 1 h. The remaining virus in each preparation was then titrated by endpoint dilution. Each antiserum had previously been titrated against its homologous virus and was used at a dilution which totally neutralized the virus at a concentration fourfold higher than the endpoint giving complete (4+) CPE.

Purification of adenovirus from stool. Stools were from soiled diapers of patients with adenovirus in their stools as determined by electron microscopy. The stool was suspended in approximately 500 ml of 10 mM Tris-hydrochloride (pH 8.1) with an Omnimixer and then clarified by centrifugation at $8,000 \times g$ for 10 min. The pellet was extracted with a small volume of 10 mM Tris-hydrochloride (pH 8.1) and centrifuged again at $8,000 \times g$ for 10 min. The supernatant fluids were then pooled and further clarified by

reentrifugation at $8,000 \times g$ for 10 min. Solid polyethylene glycol 6000 was added to a final concentration of 6%, and the mixture was held overnight at 4°C . The precipitate was collected by centrifugation at $3,000 \times g$ for 10 min, resuspended in a small volume of 10 mM Tris-hydrochloride (pH 8.1) layered on a preformed cesium chloride gradient (1.2 to 1.5 g/ml in 50 mM Tris-hydrochloride [pH 8.1]), and spun at $120,000 \times g$ for 1 h. The virus band with $\rho 1.34$ was diluted, layered on cesium chloride (1.4 g/ml), and centrifuged overnight at $120,000 \times g$. The virus band ($\rho 1.34$) was then harvested, dialyzed overnight against 5 mM Tris (pH 8.1)–1 mM EDTA, and stored at -20°C .

Restriction enzyme analysis of DNA. Intracellular viral DNA was extracted from infected cells by a modification of the Hirt procedure (6) as previously described (1).

Restriction enzyme *Sma*I was purchased from Boehringer-Mannheim Ltd. and was used according to the directions of the manufacturer. DNA fragments were separated by polyacrylamide gel electrophoresis and stained with silver as previously described (2).

Gradient analysis of virus production. Subconfluent monolayers of 293 cells in 60-mm petri plates were infected with Ad1, Ad18, Ad40, or Ad41 at an input multiplicity of infection (MOI) of approximately 4. [^{35}S]methionine was added to the infected cells in complete medium (30 $\mu\text{Ci/ml}$) at 12 h p.i., and incubation was continued until 48 h p.i. Cells were scraped from triplicate plates, pelleted by low-speed centrifugation ($575 \times g$ for 10 min), and resuspended in 4 ml of minimal essential medium. Virus was released by five cycles of freezing-thawing, and the lysate was clarified by centrifugation at $575 \times g$ for 10 min. Samples were taken and stored at -70°C for titration of the virus by endpoint dilution. The remainder of the lysate was layered on a preformed gradient of cesium chloride (1.2 to 1.5 g/ml) and centrifuged at $120,000 \times g$ for 1 h. Gradients were fraction-

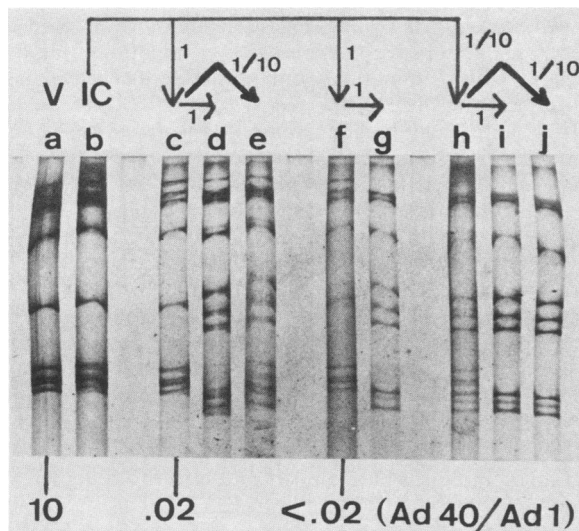


FIG. 1. *Sma*I restriction patterns of intracellular DNA during serial passage of virus from a clinical stool specimen. DNA from cells inoculated with the original stool specimen (IC) was compared with DNA from virus purified from stool (V). Progeny virus was passaged undiluted (arrows marked 1) or at a 1:10 dilution (arrows marked 1/10). Cultures represented in lanes f and g were harvested 5 days p.i. Other cultures were harvested 3 days p.i. The numbers below lanes a, c, and f represent the ratios of the Ad40 titer to the Ad1 titer in the corresponding progeny virus.

SPECIMEN	DNA PATTERN	TITRE (Ad40/Ad1)
Original Stool	Ad 40	10/1
1/5 → P 1	Ad 40	7/1
P 2 ← 1	Ad 40	0.02/1
P 2 ← 1/10	Ad 40/1	< 0.02/1

1/100 → P 1	Not Done	0.7/1
P 2 ← 1/10	Ad 1	No Ad 40

FIG. 2. Selection of Ad1 over Ad40 by serial passage of virus from a stool specimen. Virus from a clinical stool specimen was passaged in 293 cells with different dilutions of the inoculum, as indicated. The data above the dotted line correspond to the data shown in Fig. 1; the data below the dotted line represent a separate experiment.

ated from the bottom of the tube, and the amount of ^{35}S in each fraction was determined by liquid scintillation counting.

RESULTS

Serial passage of an Ad40-Ad1 specimen. It was initially observed that with four stool specimens shown to contain Ad40 on primary inoculation of 293 cells, a nonfastidious species (Ad1 in two cases, Ad18, or Ad31) was the only species detectable by passage 3 (1).

To examine this process of species selection in detail, the serial passage of one of the Ad40-Ad1 specimens was repeated, with the original stool specimen used as the initial virus inoculum. When the specimen was initially received by the laboratory and shown by electron microscopy to be positive for adenovirus, soiled diapers were obtained from the patient, and adenovirus was purified from the stool by cesium chloride centrifugation as described above. Thus, the intracellular viral DNA at each passage level could be compared by restriction analysis with the DNA from virus purified directly from the stool (Fig. 1, lanes a and b). Cells infected with undiluted progeny virus also had a DNA restriction pattern characteristic of Ad40 (Fig. 1, lane c), whereas the restriction pattern of DNA from cells infected with diluted progeny (lane h) contained bands characteristic of Ad1 as well as Ad40. Infection of cells with passage 2 material gave rise to an Ad1 restriction pattern in which Ad40 bands were minor (Fig. 1, lanes d and e) or not detected (lanes g, i, and j).

These results suggested that even though infected cells contained a large amount of Ad40 DNA relative to Ad1 DNA, the progeny virus was enriched in infectious Ad1 virions relative to infectious Ad40 virions. This possibility was confirmed by titration of the infectious virus in the progeny corresponding to lanes a, c, and f in Fig. 1. Samples of each virus preparation were treated with one of the following: (i) antiserum specific for Ad1, (ii) antiserum specific for Ad40, (iii) mixed antisera specific for Ad1 and Ad40, and (iv) minimal essential medium, for the titration of (i) Ad40, (ii) Ad1, and (iv) total virus, respectively. After incubation for 1 h at 37°C , the mixtures were titrated by endpoint dilution. The total virus was neutralized in each preparation.

TABLE 1. Comparative yields of Ad1, Ad18, Ad40, and Ad41 in 293 cells

Virus	Yield ^a in expt:		
	1	2	3
Ad1	1.1×10^9	6.6×10^8	3.8×10^8
Ad18		3.2×10^6	2.5×10^5
Ad40	2.1×10^6	2.2×10^6	2.5×10^6
Ad41	2.1×10^7	4.4×10^6	4.4×10^6

^a Infectious units per milliliter. Cells were harvested when CPE was complete (48 to 72 h p.i.).

A comparison of lanes c, f, and h in Fig. 1, which represent different dilutions of the same inoculum and thus different input MOI, indicated that the selection of Ad1 was favored when the inoculum was diluted. This observation is further documented in Fig. 2, which summarizes the relative amounts of Ad1 and Ad40 intracellular DNA and infectious virus at each passage level in two separate serial passage experiments.

Comparative yield experiments. The results described above were consistent with a higher yield of infectious Ad1 than Ad40 progeny in singly infected cells. The relative yields of Ad1 and Ad40 were then compared in cultures singly infected with Ad40 or Ad1. In this and further experiments, another fastidious enteric adenovirus species, Ad41, was also included, as was Ad18, a member of subgroup A considered to grow poorly in culture. Cells were infected at an input MOI of approximately 4 and harvested when CPE was complete (48 to 72 h p.i.). Virus was released by repeated freezing and thawing (five times) and titrated by endpoint dilution. The results (Table 1) demonstrated that the relative yields of Ad40, Ad41, and Ad18 were comparable, but that the yield of Ad1 was 150- to 500-fold higher than the yield of Ad40.

Growth curves. The growth kinetics of Ad1, Ad18, Ad40, and Ad41 were also compared in one-step growth curves.

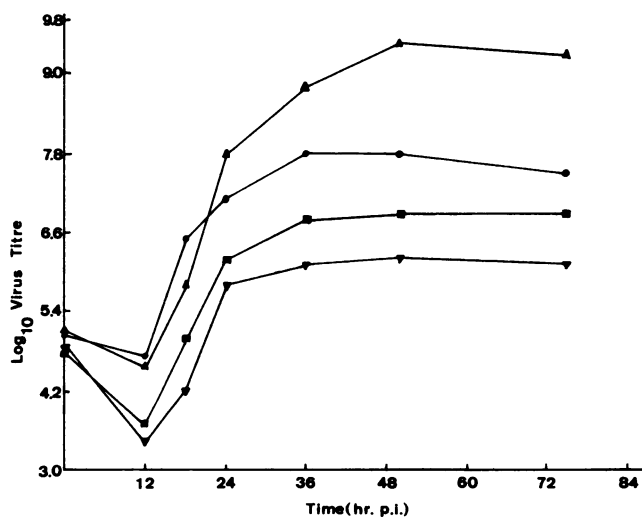


FIG. 3. One-step growth curves of Ad1, Ad18, Ad40, and Ad41 in 293 cells. Subconfluent cells in 60-mm petri plates were infected at an input MOI of approximately 4. Duplicate plates were harvested at the times indicated, and infectious virus was titrated by endpoint dilution. Symbols: ▲, Ad1; ●, Ad18; ■, Ad41; ▼, Ad40.

The four growth curves were comparable, with a latent period of 12 h (Fig. 3). However, Ad1 grew to a higher titer.

Total virus yield in infected cells. It was apparent from the results in Table 1 and Fig. 3 that the infectious yield of Ad1 was greater than that of Ad40. However, it was not clear whether the overall yield of virus particles was lower in Ad40-infected cells or whether there was a proportionately higher yield of noninfectious particles in Ad40-infected cells. Accordingly, infected cells were labeled with [³⁵S]methionine from 12 to 48 h p.i. The cells were disrupted by freezing-thawing, and the intracellular viral particles were purified by cesium chloride density gradient centrifugation. The experiment was repeated several times, and representative results are shown in Fig. 4. The yields of complete virions ($\rho 1.34$) from Ad18-, Ad40-, and Ad41-infected cells were all lower than the yield from Ad1-infected cells. The peak at $\rho 1.30$, representing incomplete particles, was not correspondingly reduced in Ad18-, Ad40-, or Ad41-infected cells, nor was the peak increased relative to the Ad1 peak at $\rho 1.30$.

The relative yields of complete virus particles from each culture were determined by adding the counts per minute of the ³⁵S in the fractions forming the peak at $\rho 1.34$. The data from three separate experiments and the infectious titer of each corresponding cell lysate are presented in Table 2. It is apparent from Table 2 that the yield of virus particles ($\rho 1.34$) was reduced in Ad18-, Ad40-, and Ad41-infected cells relative to the yield in Ad1-infected cells. However, the 100- to 1,000-fold reduction in yield of infectious Ad40 relative to Ad1 was not accounted for by the 3- to 10-fold reduction in yield of virus particles.

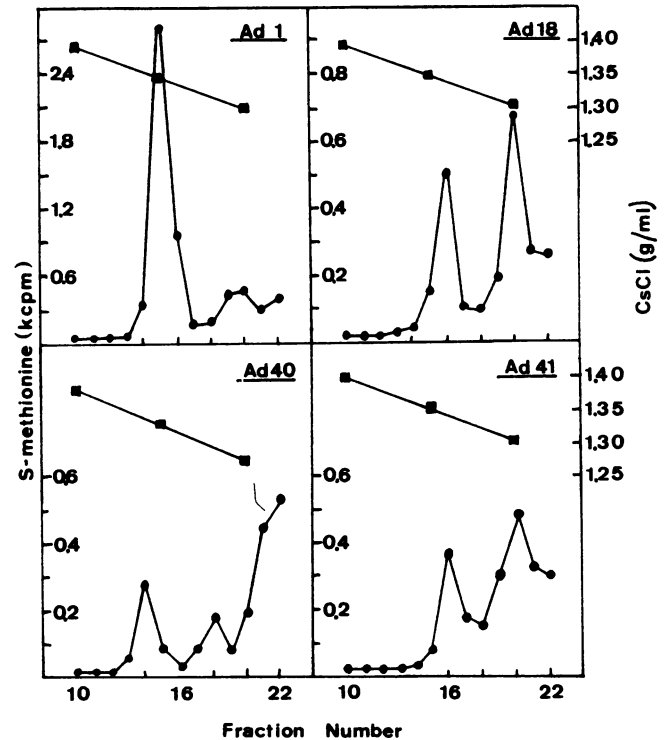


FIG. 4. Total virus yield of Ad1, Ad18, Ad40, and Ad41 in 293 cells. Subconfluent cells were infected at an input MOI of approximately 4 and labeled with [³⁵S]methionine in complete medium (30 μ Ci/ml) at 12 to 48 h p.i. Triplicate plates were harvested, and the virus was purified on cesium chloride gradients (1.2 to 1.5 g/ml) centrifuged at $120,000 \times g$ for 1 h.

TABLE 2. Comparison of the total and infectious virus yields of Ad1, Ad18, Ad40, and Ad41 in 293 cells

Virus	Virus yield in expt:					
	1		2		3	
	³⁵ S cpm ^a	IU/ml ^b	³⁵ S cpm	IU/ml	³⁵ S cpm	IU/ml
Ad1	6,058	6.1 × 10 ⁸	6,311, 3,573	4.1 × 10 ⁸	5,300, 4,287	4.8 × 10 ⁷
Ad18	860	1.1 × 10 ⁷	847, 407	1.4 × 10 ⁶	1,292, 754	1.1 × 10 ⁵
Ad40	1,009	7.9 × 10 ⁵	1,725, 1,304	4.4 × 10 ⁶	677, 413	4.4 × 10 ⁴
Ad41	ND ^c	ND	1,950, 604	3.7 × 10 ⁵	938, 615	5.1 × 10 ⁴

^a Sum of the ³⁵S counts per minute in fractions which make up the virus peak at ρ1.34 in a cesium chloride density gradient. Cell lysates were divided in half and processed separately; hence, there are two values each for experiments 2 and 3.

^b Infectious units per milliliter.

^c ND, Not done.

DISCUSSION

The isolation and serial passage of adenovirus from four stool specimens resulted in the selection of a nonfastidious adenovirus species even though the original stool specimen contained predominantly Ad40. The selection of Ad1, in particular, was investigated in detail and could be explained by a higher yield of infectious Ad1 virions in singly infected cells. As illustrated by the data in Fig. 2, the selection of Ad1 over Ad40 was favored by dilution of the inoculum, that is, a lower input MOI. Under such conditions, a greater proportion of the cells would be singly rather than multiply infected. Given that the yield of infectious Ad1 virus was shown to be higher than that of Ad40 (Tables 1 and 2, Fig. 3), the proportion of Ad1 in the progeny would be expected to increase at each passage level, as demonstrated (Fig. 1 and 2). Given the infectious titers and dilutions of the virus preparations used as inoculum, only a small proportion of the cells would have been multiply infected. Thus, we can say that Ad1 was selected over Ad40 in coinfecting cultures but not in coinfecting cells. In cultures infected with undiluted inocula (i.e., with higher input MOI), cells infected with Ad1 would likely be coinfecting with Ad40. Under these conditions, the selection of Ad1 was not favored, suggesting that Ad40 may interfere with the production of infectious Ad1 virus. It would have been interesting to examine the interaction between Ad1 and Ad40 in coinfecting cells, but the titer of Ad40 virus preparations was not sufficiently high, and attempts to produce stocks with higher titer were unsuccessful.

As was pointed out in a previous report, Ad18 was also selected after serial passage of two stool specimens containing predominantly Ad40 (1). This result was interesting, given that the yield of Ad18 in singly infected cells was not far in excess of the Ad40 yield (Tables 1 and 2). Moreover, when the serial passage of one of these specimens was repeated, Ad18 was not selected (data not shown). A review of the conditions under which Ad18 was initially isolated from this specimen indicated that cells in a 24-well culture plate had been inoculated with the original stool specimen at various dilutions and that the progeny virus for serial passage was obtained from a well inoculated with a high dilution of the specimen. It appears, therefore, that in this case, Ad18 was inadvertently selected from the original specimen by terminal dilution.

The selection of Ad1 over Ad40 represents a different situation and can be explained by the 100- to 1,000-fold-higher yield of infectious Ad1 virus in singly infected cells. Thus, Ad40 grows poorly even in 293 cells, which are considered permissive for the growth of Ad40 (1, 8). Experi-

ments were designed to compare the growth characteristics of Ad40 with those of Ad1 as well as Ad41, another fastidious adenovirus species associated with gastroenteritis, and Ad18, a group A adenovirus known to grow poorly in culture. Both total and infectious yields were comparable for Ad18, Ad40, and Ad41 and were considerably less than the corresponding yields of Ad1 (Fig. 3 and 4, Tables 1 and 2). Therefore, the lower yield of Ad40 in 293 cells is not a unique property of the fastidious enteric adenovirus species (Ad40 and Ad41) since Ad18, which does not exhibit the limited in vitro host range characteristic of Ad40 and Ad41, also grows poorly.

The reason for these low yields for Ad18, Ad40, and Ad41 in 293 cells is not understood. However, it is clear that the low virus yields are not compensated for by an increase in the yields of incomplete particles (ρ1.30). In Ad40-infected cells, the yields of both complete (ρ1.34) and incomplete (ρ1.30) particles were reduced relative to Ad1-infected cells, whereas in cells infected with Ad18 or Ad41, only the yield of complete particles (ρ1.34) was reduced, while the yield of incomplete particles (ρ1.30) was comparable to that in Ad1-infected cells (Fig. 4). These results are consistent with observations made by electron microscopic examination of thin sections of infected cells (data not shown).

Even though the total yield of Ad40 was lower than that of Ad1, the 3- to 10-fold reduction in total yield does not account for the 100- to 1,000-fold reduction in infectivity. It would appear therefore that Ad40 progeny contains a greater proportion of noninfectious particles than does Ad1 progeny, possibly reflecting a greater sensitivity or fragility of Ad40 virions to procedures such as freezing-thawing. If Ad40 virions are indeed more fragile, this would suggest a structural or functional difference in one or more of the capsid components. Such differences should become apparent through detailed analysis of the protein composition of Ad40 virions. These studies are currently in progress as part of an ongoing study to characterize Ad40 and Ad41.

ACKNOWLEDGMENTS

This work was supported by grant MA8656 from the Medical Research Council of Canada and by funds obtained from The Hospital for Sick Children Research Institute. M.B. is a Scholar of the Medical Research Council of Canada.

The capable technical assistance of Antonietta Cifelli is greatly appreciated. The contribution of Martin Petric in helpful discussion is also appreciated.

LITERATURE CITED

1. Brown, M., M. Petric, and P. J. Middleton. 1984. Diagnosis of fastidious enteric adenoviruses 40 and 41 in stool specimens. J.

- Clin. Microbiol. 20:334-338.
2. Brown, M., M. Petric, and P. J. Middleton. 1984. Silver staining of DNA restriction fragments for the rapid identification of adenovirus isolates: application during nosocomial outbreaks. *J. Virol. Methods* 9:87-98.
 3. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, Jr. 1973. *Microbiology*, 2nd ed., p. 1037-1045. Harper and Row Publishers, Inc., Hagerstown, Md.
 4. de Jong, J. C., R. Wigand, A. H. Kidd, G. Wadell, J. G. Kapsenberg, C. J. Muzerie, A. G. Wermolenbol, and R. G. Firtzlaff. 1983. Candidate adenoviruses 40 and 41: fastidious adenoviruses from human infant stool. *J. Med. Virol.* 11:215-231.
 5. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59-72.
 6. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
 7. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent end-points. *Am. J. Hyg.* 27:493-497.
 8. Takiff, H. E., S. E. Straus, and C. F. Garon. 1981. Propagation and in vitro studies of previously non-cultivable enteric adenoviruses in 293 cells. *Lancet* ii:832-834.
 9. Uhnöo, I., G. Wadell, L. Svensson, and M. E. Johansson. 1983. Two new serotypes of enteric adenovirus causing infantile diarrhoea. *Dev. Biol. Stand.* 53:311-318.
 10. Uhnöo, I., G. Wadell, L. Svensson, and M. E. Johansson. 1984. Importance of enteric adenoviruses 40 and 41 in acute gastroenteritis in infants and young children. *J. Clin. Microbiol.* 20:365-372.