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Prevalent Enteric Adenovirus Variant Not Detected by Commercial Monoclonal Antibody Enzyme Immunoassay

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A commercial monoclonal antibody enzyme immunoassay for the detection of enteric adenovirus types 40 and 41 (Ad40 and Ad41) in stool specimens was evaluated. Twenty-one stool specimens from children with gastroenteritis, with adenovirus particles visible by electron microscopy, and reference strains Ad40 Dugan and Ad41 Tak were tested by Ad40- and Ad41-specific and adenovirus group-reactive immunoassays. All stool specimens tested positive in the group-reactive immunoassay. However, only six specimens, containing isolates of Ad40 strain Hovi-X, an Ad40 genomic variant, and Ad41 strain Tak, reacted with the specific immunoassay, besides the reference strains. Fifteen stool specimens determined by restriction analysis to contain a genomic variant of Ad41 were negative by specific immunoassay. The positions of restriction site differences from the prototype strain Ad41 Tak were analyzed, and four mutations were mapped within the hexon gene; two others may occur in the fiber gene. The Ad41 genomic variant not detected by the enteric test is presently the most frequent cause of local adenoviral gastroenteritis. Highly specific monoclonal antibodies can fail to detect genomic variants of enteric adenoviruses, probably because of alteration of external neutralizable epitopes under immunological pressure to vary.

Adenovirus types 40 and 41 (Ad40 and Ad41) are known as enteric viruses (17) and have emerged as a major cause of pediatric gastroenteritis (3, 23). Large amounts of virus particles, in excess of 10^{11} particles per g of stool, are excreted at the peak of acute infection (5). However, these types are frequently refractory to culture in vitro, even in semipermissive cell lines (4, 7, 15), and there are still considerable difficulties in their diagnosis.

Adenoviruses are antigenically complex (16); the various capsomeres carry unique epitopes and antigens shared by members of some subgenera. Polyclonal antisera to distinguish Ad40 and Ad41 can be made only by lengthy absorption with antigens of other serogroups (12). Neutralizing antibodies to Ad40 or Ad41 are strongly cross-reactive, and hemagglutination inhibition tests cannot distinguish the two types (7). Monoclonal antibodies, however, with specificities to the unique or shared antigens of the capsomeres, have great potential as reagents in adenovirus diagnosis. A monoclonal antibody which reacts with a common antigen on the internal aspect of the hexon (16) and detects all human types is available (6). Monoclonal antibodies able to identify Ad40 (20), both Ad40 and Ad41 jointly (10), and Ad40 or Ad41 specifically (10, 24) have been developed. The antibody to Ad40 and Ad41 has been examined in an enzyme-linked immunosorbent assay system, and its practicality for sensitive diagnosis has been demonstrated (10).

Restriction analysis of Ad40 and Ad41, as a diagnostic alternative, produces patterns that are quite distinct (1) and can serve to differentiate the two types. Ad40 and Ad41 share so few comigrating bands that they were initially assigned to separate subgenera (23). Genomic variants of both Ad40 (14, 24) and Ad41 (5, 9, 13, 19, 21, 24, 28), with different banding patterns, have been found to be numerous and widespread. Further analysis may link genomic variation with the epidemiology of viral gastroenteritis in children.

This study was undertaken to evaluate a commercial enzyme immunoassay (EIA) for the diagnosis of local strains of enteric adenoviruses. This EIA, implementing the antibodies developed by Herrmann et al. (10), is the first commercial product available for diagnosis of enteric adenoviruses.

(This work was completed in partial fulfillment of a graduate degree by one of us [T.S.-T.].)

MATERIALS AND METHODS

Prototype viruses Ad40 strain Dugan and Ad41 strain Tak were acquired from the American Type Tissue Collection, Bethesda, Md. Stool specimens from children hospitalized with gastroenteritis, which were sent to the Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada, between 1986 and 1990, were examined by electron microscopy after concentration by direct ultracentrifugation (8) and by tissue culture. Specimens positive by electron microscopy, from children with an age range of 1 month to 7 years, were emulsified as a 10% suspension with antibiotics, clarified, and inoculated onto monolayers of HEp-2 cells, 293 cells (originally from the American Type Culture Collection), and primary rhesus monkey kidney cells (Connaught Lab, Willowdale, Ontario, Canada). Neutralization with antisera from the National Institute of Allergy and Infectious Diseases, Bethesda, Md., to Ad1 to Ad7 was performed. Isolates were further identified by restriction analysis of DNA from 3- to 5-day-old cultures from 25-cm^2 flasks which were extracted by the Hirt process (11) with proteinase K to reduce the adenovirus genomic terminal protein. Large preparations of enteric virus DNA, relatively free of cellular contamination, were made by large-scale adaptation of the method of Shinagawa et al. (18). DNA was precipitated and washed in ethanol, and 10 μl of 50-μl resuspensions of stool isolate preparations or 1 to 2 μg of quantified DNA was digested in recommended buffers for 3 h with at least 10 U of

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TABLE 1. Detection of enteric adenoviruses by specific and group-reactive EIAs

Type by restriction analysis	No. of isolates detected by:			
	Enteric EIA		Group-reactive EIA	
	Positive	Negative	Positive	Negative
Clinical isolates				
Ad41 genomic variant	0	15	15	0
Ad40 genomic variant	4	0	4	0
Ad41 strain Tak	1	0	1	0
Ad40 strain Hovi-X	1	0	1	0
Reference strains				
Ad41 strain Tak	1	0	1	0
Ad40 strain Dugan	1	0	1	0

restriction endonuclease. Electrophoresis was performed on 150-ml slabs of 0.8% agar with 0.089 M Tris borate–10 mM EDTA (pH 8.0) buffer containing 0.5 μg of ethidium bromide per ml. Gels were run on a Bio-Rad DNA subcell apparatus at 25 V/5 mA for approximately 16 h and photographed under UV light with a red filter.

Adenovirus immunoassays were carried out in accordance with the manufacturer's instructions (Cambridge Bioscience, Worcester, Mass.). A 100-μl aliquot of a 10% stool suspension or clarified tissue culture supernatant of the reference strains of Ad40 and Ad41 was added to microwells coated with adenovirus capture antibody (6). Horseradish peroxidase-conjugated monoclonal antibody (100 μl) directed against the common antigen on the internal aspect of the hexon (6) or a subgenus F (Ad40 and Ad41)-specific epitope (10) was added, mixed, and incubated at room temperature for 1 h. The wells were washed six times with deionized water before the substrate, tetramethylbenzidine, was added. The reaction was stopped after 10 min with 100 μl of normal sulfuric acid, and the plates were read at 450 nm. Positive samples were taken as those with an optical density greater than 0.150.

RESULTS

A commercial monoclonal antibody EIA for the detection of enteric Ad40 and Ad41 in stool specimens was evaluated. A high proportion of fastidious adenovirus fecal specimens, with virus evident by electron microscopy but growing poorly in conventional epithelial cell culture, failed to react with the enteric EIA, and an analysis of the untyped isolates with restriction endonucleases was initiated. Twenty-one stool specimens, including four with isolates of an Ad40 genomic variant from a previous study (9), had sufficient material and were examined by both the enteric adenovirus-specific EIA and a group-reactive EIA with a monoclonal antibody reactive with all mastadenoviruses (6). Clinical fecal isolates of Ad41 Tak and Ad40 Hovi-X, as well as supernatants of culture-grown reference strains of prototypes Ad41 Tak and Ad40 Dugan, were additionally tested. All stool specimens and supernatants tested positive in the group-reactive EIA. Only six fecal specimens, containing Ad40 Hovi-X, and Ad40 genomic variant isolate, and Ad41 strain Tak, besides the reference strains were detected by the enteric EIA. However, the enteric EIA failed to detect fastidious adenovirus in 15 of the 21 stool specimens (Table 1). These 15 specimens were found to contain a strain of Ad41, with some variation of restriction patterns from prototype Ad41 strain Tak.

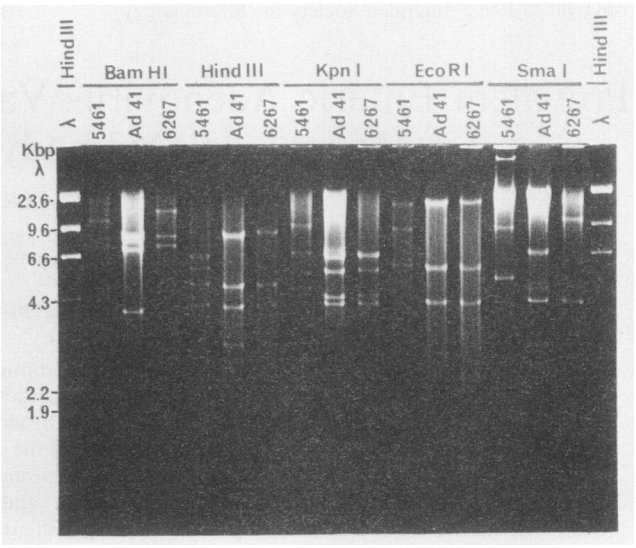

FIG. 1. DNA restriction patterns of Ad40 strain Hovi-X (isolate 5461), Ad41 strain Tak, and genomic variant Ad41 (isolate 6267) digested with *Bam*HI, *Hin*dIII, *Kpn*I, *Eco*RI, and *Sma*I in 0.8% agar electrophoresis. Lambda phage molecular size markers, from identical conditions, have been attached.

The enteric isolates demonstrated only three combinations of patterns with restriction endonucleases (Fig. 1). Fecal samples from one patient, containing clinical isolate 5461, yielded restriction patterns consistent with Ad40 strain Hovi-X (14). Another isolate, 3953 (Fig. 2), had restriction patterns characteristic of Ad41 prototype strain Tak (1). The vast majority of enteric isolates showed restriction patterns similar but not identical to those of the Ad41 prototype. These patterns were first described from virus cultured locally from stools between 1981 and 1983 (9). Figure 2

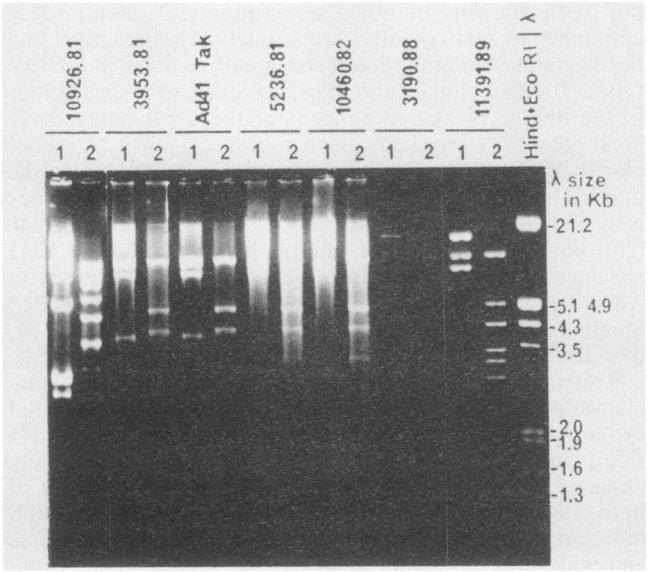

FIG. 2. *Bam*HI (1) and *Hin*dIII (2) digests of clinical isolates from 1981 to 1989. Isolates 10926 and 3953 have patterns conforming to those of strains Ad40 Hovi-X and Ad41 Tak, respectively; isolates 5236, 10460, 3190, and 11391 have variants of patterns of Ad41. The year of isolation is indicated after the clinical number.

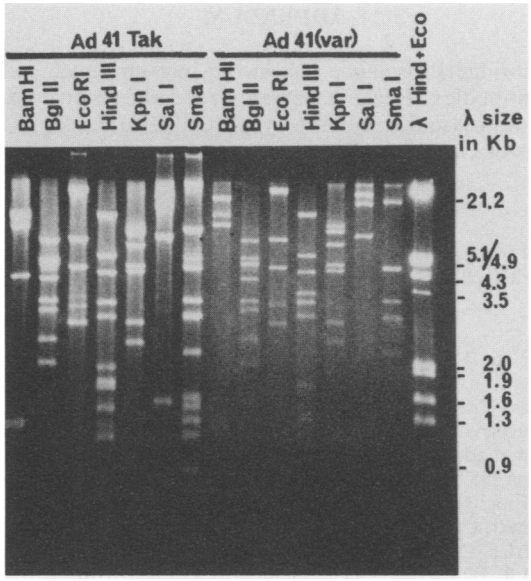

FIG. 3. Ad41 strain Tak and a variant Ad41 isolate cleaved with a variety of restriction endonucleases for comparison of banding patterns.

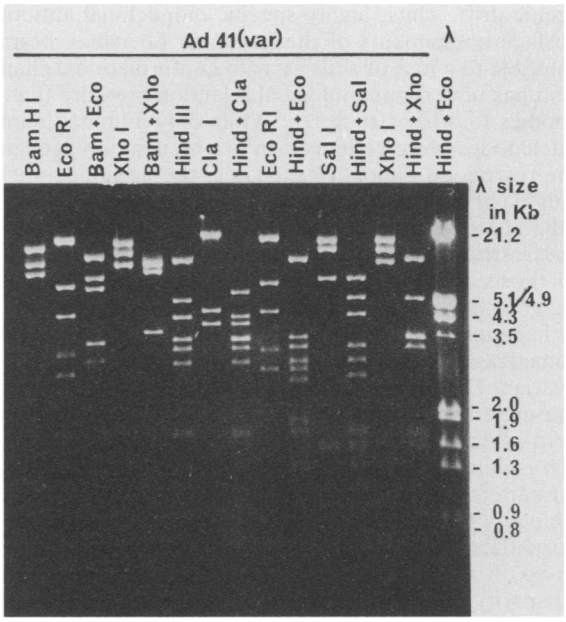

FIG. 4. Double digestion (with *Bam*HI, *Hin*dIII, and other enzymes together) of the Ad41 variant DNA, extracted by the method of Shinagawa et al. (18) to map mutated restriction sites.

shows the *Bam*HI and *Hin*dIII restriction patterns of various clinical specimens from 1980, 1981, 1982, and more-recent years. The restriction patterns for these enzymes of the local Ad41 variant have been consistent over an 8-year period.

Comparison of restriction patterns generated by electrophoresis of genomic DNA of prototype Ad41 Tak and the variant Ad41 strain with frequently used enzymes (Fig. 3) shows patterns for *Bgl*II, *Kpn*I, and *Sal*I to be the same for both strains, while *Bam*HI, *Hin*dIII, and *Sma*I produced variations in two fragments or more. A few isolates of the variant strain, for example, 6267 in Fig. 1, show an alteration to the *Eco*RI D fragment. This *Eco*RI aberration was the only variation noted in patterns of different isolates of the Ad41 variant. The *Bam*HI A fragment of the genomic variant was elevated relative to that of strain Tak, and the D band was missing from the genomic variant digest in Fig. 3. With enzymes *Hin*dIII and *Sma*I, the genomic variant patterns likewise demonstrate both elevated and absent bands. The position of altered fragments in the Ad41 variant strain genome was investigated by double digestion of variant DNA and electrophoresis (Fig. 4). The uppermost *Bam*HI band of the variant was digested by *Eco*RI and *Xho*I, both of which subdivided the *Bam*HI D fragment of strain Tak. This finding shows that the A and D fragments are contiguous and combined in the variant genome, implying a mutation disguising the *Bam*HI restriction site positioned at approximately map unit 60 (2) on the prototype genome. Fragments F and I of the *Hin*dIII digest of Tak strain DNA are similarly combined in the pattern of the variant strain, as is evident from the reduction of this fragment by the central restriction sites of enzymes *Sal*I, *Eco*RI, and *Cla*I between map units 51 and 56 (25). The enzyme *Cla*I, used for double digestion because of its simple four-fragment pattern with Ad41 strain Tak (25), also shows (Fig. 4) a missing restriction site at map unit 55 between fragments A and B. Thus, three restriction site diversities between Ad41 strain Tak and the local variant fall between map units 51 to 60 along the genome. Two further anomalies, a novel variant restriction site cleaving the prototype *Hin*dIII G band and the mutation causing a combination of the *Sma*I A and B bands (Fig. 3) in the variant pattern, occur between map units 85 to 90.

DISCUSSION

The first commercial test available for diagnosis of enteric adenoviruses is unable to detect the most common strain of Ad41 in Manitoba. Genetic variation within one type of adenovirus can evidently disrupt the diagnostic capability of highly specific monoclonal antibodies. Variation in the restriction sites of enzymes *Sma*I and *Hin*dIII between the Ad41 variant and strain Tak occur toward the right terminus within the confines of the fiber gene on the Ad2 genome (2). The variation in the restriction sites of enzymes *Bam*HI, *Cla*I, and *Hin*dIII was determined to lie between map units 51 and 60, which places these mutations within the Ad41 hexon gene (22). The mutation abrogating the restriction site between *Sma*I fragments E/F and H was also found in the hexon, at variance with the current *Sma*I restriction map (2). Discrepancies in the *Hin*dIII map of Ad41 Tak, involving the placement of fragment I adjacent to fragment F, were also discovered, and these have been corrected elsewhere (submitted for publication). The Ad40- and Ad41-specific monoclonal antibody used for enteric adenovirus detection was shown by radioimmunoprecipitation to react with a hexon epitope (10). The inability of the monoclonal antibody to detect the Ad41 variant can thus be ascribed to hexon gene mutation underlying the variation in restriction endonuclease patterns. Similarly, the neutralizing activity of other monoclonal antibodies was dependent on the presence of restriction sites at map units 52 and 56 (24). The internal aspect of the hexon is highly conserved (22), and the monoclonal antibody used in the group-reactive test cross-reacts with all *Mastadenovirus* types of various animal hosts (6) and picks up genomic variants. On the other hand, the outer surface of the hexon and the fiber forming most of the external capsid are both thought to be neutralizable determinants (16) and must be most prone to alteration by

antigenic drift. Thus, highly specific monoclonal antibodies to surface components of the hexon or fiber may be more susceptible to a loss of activity with conformational changes in epitopes under immunological selection pressure than are antibodies to other structures. While only a limited number of nucleotides were examined in the recognition sequences of the restriction endonuclease sites, the preponderance of mutations in the hexon gene would indicate that the hexon is not the ideal target for reagent antibodies.

The restriction patterns of the local Ad41 variant differ from those of prototype strain Tak for enzymes *Bam*HI, *Cla*I, *Hin*dIII, and *Sma*I. The differences are limited and have been interpreted as being largely due to a combination of some fragments due to a lack of some restriction sites in the variant DNA. The *Bam*HI pattern observed is common to the majority of Ad41 isolates (13, 24), while the *Hin*dIII pattern of the variant was seen in a large number of the Dutch isolates and a single Belgian strain (24). The variant *Sma*I pattern, with band A combined with B and band E/F combined with H relative to strain Tak, corresponds to the *Sma*I pattern of all 15 Ad41 isolates called Ad41a gathered from South Africa, eastern Canada, and Europe (13). While the disparity of *Sma*I patterns was influential in the original definition of two enteric types (23), only the *Sma*I restriction pattern of many isolates of Ad40 and Ad41 showed complete uniformity (13, 14), a useful diagnostic and epidemiological feature (5, 28). The initial consistency of Ad41 *Sma*I patterns has not held true, however, with six patterns found altogether among the known strains collected from a wide geographic area (24). The combination of Ad41a *Sma*I and other restriction patterns exhibited by the local variant conforms most probably with the strain designated Ad41 D12 (24). Whereas this variant was found established as the locally prevalent isolate when first investigated as far back as 1981 (9), Ad41 D12 has only recently risen to dominate Dutch and Belgian isolates from initial emergence in the Netherlands in 1982, implying some movement in the spread of this strain.

The diversity of adenovirus restriction patterns (1), even within one type (13, 19, 24), is a well-established characteristic. Restriction analysis has potential in elucidating sources of infections, geographical spread of strains, and other epidemiological features of adenovirus infection. An Ad41 genomic variant has been found to predominate as the single most important cause of gastroenteritis in young children in Manitoba. No sign of the Ad40 genomic variant, observed at a much higher frequency than Ad40 Hovi-X between 1981 and 1983 (9), was seen after 1986. This result may follow the epidemiological trends in other studies; the genomic type represented by strain Hovi-X is highly prevalent, and Ad40 is relatively homogeneous by current restriction analysis (14, 21, 24). Also, the proportion of Ad40 among enteric isolates has diminished in recent years in the Newcastle area (28) and the Netherlands (24) and could be a general trend. The prototype Ad41 strain Tak, on the other hand, is a relatively rare strain with distinctive *Sma*I and *Hin*dIII patterns. First isolated in Holland in 1973 (7), Tak-like patterns were evinced by only a small proportion of recent isolates from the Netherlands (5, 24). This is the first report of isolation of Ad41 strain Tak from another area and shows the distribution to be incompletely mapped as yet. The proportion of locally prevalent enteric strains has been shown to change with time. It is very likely that Ad40 and Ad41 undergo strain succession like other species of adenovirus (26, 27). This augurs poorly for the continued usefulness of species-specific monoclonal antibodies in enteric adenovirus diagnosis.

ADDENDUM

Cambridge Bioscience has in the interim developed further antibodies. Reagent samples recently received at the Cadham Provincial Laboratory were able to detect 15 of 16 isolates of enteric Ad41.

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LITERATURE CITED

1. **Adrian, T. H., G. Wadell, J. C. Hierholzer, and R. Wigand.** 1986. DNA restriction analysis of adenovirus prototypes 1 to 41. Arch. Virol. **91**:277–290.
2. **Akusjarvi, G., and G. Wadell.** 1987. Genetic maps of human and animal adenoviruses, p. 78–82. *In* S. J. O'Brien (ed.), Genetic maps 1987, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Brandt, C. D., H. W. Kim, W. J. Rodriguez, J. D. Arrobio, B. C. Jeffries, E. P. Stallings, C. Lewis, A. J. Miles, M. K. Gardner, and R. H. Parrott.** 1985. Adenovirus and pediatric gastroenteritis. J. Infect. Dis. **151**:437–443.
4. **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.
5. **Buitenwerf, J., J. J. Louwerens, and J. C. de Jong.** 1985. A simple and rapid method for typing adenoviruses 40 and 41 without cultivation. J. Virol. Methods **10**:38–44.
6. **Cepko, C. L., C. A. Whetstone, and P. A. Sharp.** 1983. Adenovirus hexon monoclonal antibody that is group specific and potentially useful as a diagnostic reagent. J. Clin. Microbiol. **17**:360–364.
7. **de Jong, J. C., R. Wigand, A. H. Kidd, G. Wadell, J. G. Kapsenberg, C. J. Muzerie, A. G. Wermenbol, and R. G. Firtzlaff.** 1983. Candidate adenoviruses 40 and 41: fastidious adenoviruses from human infant stool. J. Med. Virol. **11**:215–231.
8. **Hammond, G. W., P. R. Hazelton, I. Chuang, and B. Klisko.** 1981. Improved detection of virus by electron microscopy after direct ultracentrifuge preparation of specimens. J. Clin. Microbiol. **14**:210–221.
9. **Hammond, G. W., G. Mauthe, J. Joshua, and C. Hannan.** 1985. Examination of uncommon clinical isolates of human adenoviruses by restriction endonuclease analysis. J. Clin. Microbiol. **21**:611–616.
10. **Herrmann, J. E., D. M. Perron-Henry, D. Stobbs-Walro, and N. W. Blacklow.** 1987. Preparation and characterization of monoclonal antibodies to enteric adenovirus types 40 and 41. Arch. Virol. **94**:259–265.
11. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell culture. J. Mol. Biol. **26**:365–369.
12. **Johannson, M. E., I. Uhnoo, L. Svensson, C. A. Pettersson, and G. Wadell.** 1985. Enzyme linked immunosorbent assay for detection of enteric adenovirus 41. J. Med. Virol. **17**:19–27.
13. **Kidd, A. H.** 1984. Genomic variants of adenovirus 41 (subgroup G) from children with diarrhoea in South Africa. J. Med. Virol. **14**:49–59.
14. **Kidd, A. H., F. E. Berkowitz, P. J. Blaskovic, and B. D. Schoub.** 1984. Genome variants of human adenovirus 40 (subgroup F). J. Med. Virol. **14**:235–246.
15. **Kidd, A. H., and C. R. Madeley.** 1981. In vitro growth of some fastidious adenoviruses from stool specimens. J. Clin. Pathol. **34**:213–216.
16. **Norrby, E.** 1969. The structural and functional diversity of adenovirus capsid components. J. Gen. Virol. **5**:221–236.
17. **Petric, M., S. Krajden, N. Dowbnia, and P. J. Middleton.** 1982. Enteric adenoviruses. Lancet **i**:1074–1075.
18. **Shinagawa, M., A. Matsuda, T. Ishiyama, H. Goto, and G. Sato.** 1983. A rapid and simple method for preparation of adenovirus DNA from infected cells. Microbiol. Immunol. **27**:817–822.

19. **Shinozaki, T., K. Araki, M. Kobayashi, Y. Fujita, T. Abe, and H. Ushijima.** 1988. Genome variants of human adenovirus type 40 and 41 (subgroup F) in Japan. J. Clin. Microbiol. **26**:2567–2571.
20. **Singh-Naz, N., and R. K. Naz.** 1986. Development and application of monoclonal antibodies for specific detection of human enteric adenoviruses. J. Clin. Microbiol. **23**:840–842.
21. **Takiff, H. E., S. E. Straus, and C. F. Garon.** 1981. Propagation and in vitro studies of previously non-cultivable adenoviruses in 293 cells. Lancet **ii**:832–834.
22. **Toogood, C. I. A., and R. T. Hay.** 1988. DNA sequence of the adenovirus type 41 hexon gene and predicted structure of the protein. J. Gen. Virol. **69**:2291–2301.
23. **Uhnoo, I., G. Wadell, L. Svensson, and M. Johansson.** 1983. Two new serotypes and enteric adenovirus causing infantile diarrhoea. Dev. Biol. Stand. **53**:311–313.
24. **van der Avoort, H. G. A. M., A. G. Wermenbol, T. P. L. Zomerdijk, J. A. F. W. Kleijne, J. A. A. M. van Asten, P. Jensma, A. D. M. E. Osterhaus, A. H. Kidd, and J. C. de Jong.** 1989. Characterization of fastidious adenovirus types 40 and 41 by DNA restriction enzyme analysis and by neutralizing monoclonal antibodies. Virus Res. **12**:139–158.
25. **van Loon, A. E., T. H. Rozija, J. C. de Jong, and J. S. Sussenback.** 1985. Physiochemical properties of the DNAs of the fastidious adenovirus specimens 40 and 41. Virology **140**: 197–200.
26. **Wadell, G., M. K. Cooney, A. D. C. Linhares, L. de Silva, M. L. Kennett, R. Kono, R. Gui-Fang, K. Lindman, J. P. Nascimento, B. D. Schoub, and C. D. Smith.** 1985. Molecular epidemiology of adenovirus: global distribution of adenovirus 7 genome types. J. Clin. Microbiol. **21**:403–408.
27. **Wadell, G., J. C. de Jong, and S. Wolontis.** 1981. Molecular epidemiology of adenovirus: alternative appearance of two different genome types of adenovirus 7 during epidemic outbreaks in Europe from 1958 to 1980. Infect. Immunol. **34**:368–392.
28. **Willcocks, M. M., M. J. Carter, F. R. Laidler, and C. R. Madeley.** 1988. Restriction enzyme analysis of faecal adenoviruses in Newcastle upon Tyne. Epidemiol. Infect. **101**:445–458.