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Polymerase Chain Reaction for Detection of Adenoviruses in Stool Samples

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The usefulness of the polymerase chain reaction (PCR) method for diagnosing adenovirus infections was investigated. Several primers, including primers specific for the hexon-coding region and for enteric adenovirus types 40 and 41, were evaluated. The PCR method was validated against cell culturing in routine diagnostic work and against restriction enzyme analysis of viral DNA. Sixty diagnostic specimens were selected for evaluation by the PCR method. Twenty of the 60 specimens were found positive on the basis of cytopathic effects and latex agglutination (Adenolex [Orion Diagnostica, Helsinki, Finland]), and 16 were identified and typed as adenoviruses by polyacrylamide gel electrophoresis. PCR was performed on all 60 specimens in parallel directly on diluted stool samples and on viral DNA extracted from cells inoculated with the same stool samples. When the general hexon primers were used 51 of the 60 specimens from infected cell cultures were found positive by PCR, whereas only 13 specimens were found positive when PCR was performed directly on stool samples. With the use of selective primers for enteric adenoviruses 16 of the 60 cell cultures were found to exhibit amplification products by PCR, whereas 4 were detected in stool samples. None of the 60 specimens were found positive by PCR when an adenovirus type 40-specific primer pair was used. PCR was found to be a fast, sensitive, and reliable method for the detection of adenoviruses in diarrheal disease, provided the amplifications were performed directly on diluted stool samples.

Viruses are the major etiological agents in acute diarrheal disease in children. The introduction in the 1970s of electron microscopy (8) for the examination of fecal specimens allowed the identification of rotaviruses, Norwalk agent, and enteric adenoviruses (EAds). In a prospective 1-year study in Sweden, rotaviruses and adenoviruses were detected in 45 and 13% of stool samples from children with gastroenteritis, respectively (29). Adenoviruses, particularly the enteric types 40 (Ad40) and 41 (Ad41) (7), are considered to be second only to rotaviruses as a cause of gastroenteritis in young children (6, 17, 28). Most adenovirus infections occur in children under 2 years to age and show very little seasonal variation in shedding rates (17, 28).

A549 cells are useful for the isolation of nonenteric adenoviruses. However, like most other viruses associated with diarrhea, the EAds are fastidious, with a limited capacity to grow in these cells (7). However, 293 cells, immortalized by transformation with the human Ad5 E1A and E1B region, support limited replication of EAds (23). Alternative serological methods, i.e., immune electroosmophoresis (12), enzyme-linked immunosorbent assay (13, 14), latex agglutination test (10), immunoelectron microscopy (35), and enzyme immunoassay (34), have been developed for the identification and typing of adenoviruses in diarrheal disease. Furthermore, restriction enzyme analysis of viral DNA (27, 31) and dot blot hybridization (16, 24) have been used to diagnose diahrreal disease caused by adenoviruses.

The polymerase chain reaction (PCR) is an in vitro method for primer-directed enzymatic amplification of specific target DNA sequences (20, 21). A few copies of target DNA can be amplified to a level detectable by gel electrophoresis or Southern blot hybridization.

We have studied three different PCR systems for the detection of human adenoviruses representing all six subgenera, the two EAds Ad40 and Ad41, and Ad40, respectively. The three systems have also been evaluated with clinical material for the direct detection of viruses in stools.

MATERIALS AND METHODS

Study group and specimens. The stools used in this study were selected from specimens submitted to the Regional Diagnostic Virus Laboratory at the University Hospital of Umeå from January to August 1989. The fecal specimens were collected in transport medium (phosphate-buffered saline, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.3], 1% bovine serum albumin, 5% sucrose, 50 μg of gentamicin per ml) and clarified by centrifugation for 5 min at 8,800 × *g*.

All samples originating from children who were younger than 3.5 years of age and who were suffering from diarrhea were selected, except for those positive for rotaviruses. The selected samples were inoculated onto A549 cells in roller tubes. Cell cultures showing cytopathic effects (CPE) were assayed for viral antigens by latex agglutination (Adenolex [Orion Diagnostica, Helsinki, Finland]) and were included. Of the total of 135 samples, 60 fulfilled the criteria for inclusion and 12 had to be excluded because of an insufficient amount of stool.

Twenty of the 60 samples contained adenoviruses, as determined by the Adenolex test with cultured A549 cells; only 2 of these samples were Adenolex positive when the test was performed directly on stools. The remaining 40 samples were all found negative for adenoviruses by the Adenolex test performed directly on stools; 10 of the 40 showed some CPE when cultured on A549 cells, but the Adenolex test performed on the inoculated cells gave a negative result.

Preparations of virions and viral DNA. Ad41 and Ad2 were inoculated onto subconfluent monolayers of 293 cells (23)

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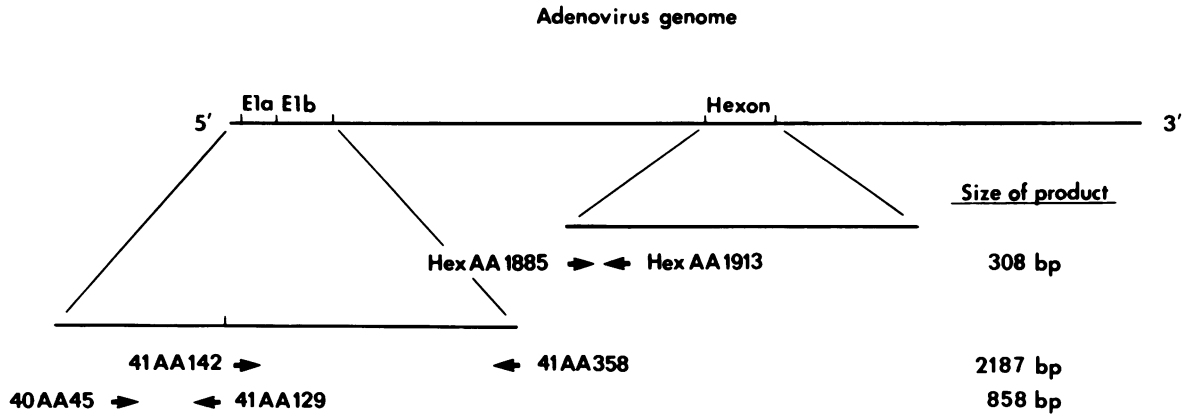

FIG. 1. Locations of the three sets of oligonucleotide primers in the 36-kb adenovirus genome.

and A549 cells, respectively, in roller tubes. Three to 5 days postinfection, virions were prepared from CsCl gradients by a modification of the method of Wadell et al. (33).

For the preparation of viral DNA, 20 to 50 μl of clarified stool suspensions or lysates of cells infected with reference strains was inoculated onto subconfluent monolayers of 293 cells in each well of a 24-well culture plate (Greiner, GmbH, Nürtingen, Federal Republic of Germany). 293 cells were used for the cultivation of all samples to ensure the isolation of fastidious adenoviruses. Each sample was inoculated into six wells, corresponding to a 12-cm^2 area of monolayered cells. After 3 or 4 days or when CPE were well developed, cells were harvested and intracellular viral DNA was extracted by the method of Shinagawa et al. (22).

Pretreatment of samples. Three different methods for disrupting the adenovirus capsid were tested. This disruption is required to initiate the PCR-mediated amplification of viral DNA in clinical specimens. The reactions were performed with clarified stool suspensions in transport medium.

For phenol-chloroform extraction, a 50-μl sample was incubated at 55°C for 1 h in the presence of 60 μg of proteinase K (Boehringer GmbH, Mannheim, Federal Republic of Germany) per ml in 10 mM Tris hydrochloride (pH 7.5). The solution was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was precipitated with ethanol in the presence of carrier tRNA (100 μg/ml). For NaOH extraction, a 50-μl sample was treated with NaOH at a final concentration of 0.5 M to denature the capsids. This treatment was followed by incubation in a 37°C water bath for 15 min and neutralization with HCl at a final concentration of 0.5 M. For heating, a 10-μl sample, undiluted or diluted 10- or 100-fold, was mixed with all the reagents necessary for PCR amplification, including the *Taq* polymerase, and heated at 95°C for 25 min directly in the programmable heat block (Hybaid Ltd., Teddington, Middlesex, United Kingdom) used for the thermalcycling procedure.

Adenovirus detection. (i) Latex agglutination test. For routine detection of adenoviruses and rotaviruses in stool specimens, the latex tests Adenolex and Rotalex (Orion Diagnostica), respectively, were used in accordance with the instructions of the manufacturer. The tests consist of particles coated with antibodies specific for adenoviruses or rotaviruses. The method is based on the agglutination of latex beads when the relevant antigens are present.

(ii) PAGE-silver staining. From each of the viral DNA preparations, 50 to 200 ng of DNA (determined by the Dipstick test [Invitrogen, San Diego, Calif.]) was used for specific adenovirus typing by polyacrylamide gel electrophoresis (PAGE). The DNA was digested with the restriction enzyme *Sma*I (Boehringer) and electrophoresed on a 5% polyacrylamide gel (0.05% bisacrylamide) with a 3% stacking gel containing 0.125% SeaKem agarose (FMC Bioproducts, Rockland, Maine) at 50 V for 16 h. DNA restriction fragments were visualized by the silver staining technique described by Beidler et al. (4).

TABLE 1. Oligonucleotide primers for PCR amplification of adenoviruses

Virus type (region)	Position	Primer[a]	Sequence	Amplimer length (bp)
Ad40 (hexon)	18858–18883[b]	hexAA1885	5′-GCCGCAGTGGTCTTACATGCACATC-3′[c]	308
Ad41	19136–19158[b]	hexAA1913	5′-CAGCACGCCGCGGATGTCAAAGT-3′[c]	
Ad41 (E1B)	1421–1446[d]	41AA142	5′-TCTGATGGAGTTTTGGAGTGGCTA-3′	2,187
Ad41 (E1B)	3585–3608[d]	41AA358	5′-AGAAGCATTAGCGGGAGGGTTAAG-3′	
Ad40 (E1A)	453–477[e]	40AA45	5′-ATTGCTGTTGGCGCTTTTGACATAG-3′	858
Ad41 (E1A)	1297–1320[f]	41AA129	5′-TCAAGAGGACTTGGGGCGCTTTAA-3′	

[a] Primers were named in such a way as to describe the adenovirus type or adenovirus gene, the initials of the individual who discovered the primer, and the sequence position with the last figure deleted.
[b] The sequence positions of the hexon primers are referred to the Ad2 hexon region (1).
[c] These primer sequences are shared among Ad2 (1), Ad40 (26), and Ad41 (25).
[d] The sequence position is from Allard et al. (unpublished data).
[e] The sequence position is from vanLoon et al. (30).
[f] The sequence position is from Allard and Wadell (2).

TABLE 2. Adenovirus prototype and reference strains

Subgenus	Type[a]	Strain or description
A	12	F-3072-86, prototypelike pattern (Huie)
	31	1315, prototype
B	3p	GB, prototype
	3a	7919-86, pattern like that of representative strain B-0164
	7	Gomen, prototype
	11p[2]	Pattern like that of representative strain 5647/80
C	2	960-87, prototypelike pattern
	5a[4]	33399 (9)
D	9	Prototypelike pattern
	17	Prototypelike pattern
	19a	875-87, pattern like that of representative strain 73-20435 (32)
	20	931, prototype
	24	3153, prototype
	30	BP-7, prototype
	38	LJ, prototype
	39	D335, prototype
E	4	RI-67, prototype
F	40	HoviX, prototype
	41	D389, pattern like that of representative strain N597

[a] Some genome types are also shown.

(iii) PCR. PCR was performed with 10 μl of pretreated stool suspensions diluted 10- or 100-fold in distilled water or with 5 to 20 ng of extracted viral DNA. Amplification of adenoviral target sequences was carried out in a 50-μl reaction mixture containing 16.6 mM $(NH)_4SO_4$, 67 mM Tris hydrochloride (pH 8.8) at 25°C, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 200 μM each deoxynucleoside triphosphate (i.e., dATP, dGTP, dCTP, and dTTP), 100 μg of BSA per ml, 0.08 μM each primer (Symbicom AB, Umeå, Sweden) needed for the specific reaction, and 2 U of thermostable *Taq* DNA polymerase (Perkin-Elmer, Cetus). The samples were overlaid with 75 μl of mineral oil to prevent evaporation. Thermal cycling of the amplification mixture was performed in a programmable heat block (Hybaid) for a total of 30 to 35 cycles. A cycle represents denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and primer extension for 30 s at 70°C; in the first cycle, the denaturation step continued for 4 min at 94°C.

The enhance the binding of primers to possible mismatched sequences, we lowered the annealing temperature to 45°C and extended the annealing time from 30 to 45 s. As positive controls in all experiments, 10 pg of Ad2 or Ad41 DNA was used; PCR mixtures without DNA were used as negative controls.

After the last cycle, 10 μl of the reaction mixture was analyzed for amplification products by gel electrophoresis on a 1% SeaKem agarose gel after ethidium bromide staining (18). All gels were blotted onto nylon filters (Hybond-N; Amersham), which were used for hybridization. The entire genomes of Ad31 (subgenus A), Ad3 (subgenus B), Ad2 (subgenus C), Ad19a (subgenus D), Ad4 (subgenus E), and Ad40 and Ad41 (subgenus F) were labeled with [α-^{32}P]dCTP (Amersham) by the multiprime DNA labeling technique (Amersham) to an activity of 10^8 to 10^9 dpm/μg and used as probes under the stringent hybridization and washing conditions described before (3). Filters were exposed to Cronex 4 film (Dupont) for 16 to 40 h at −70°C.

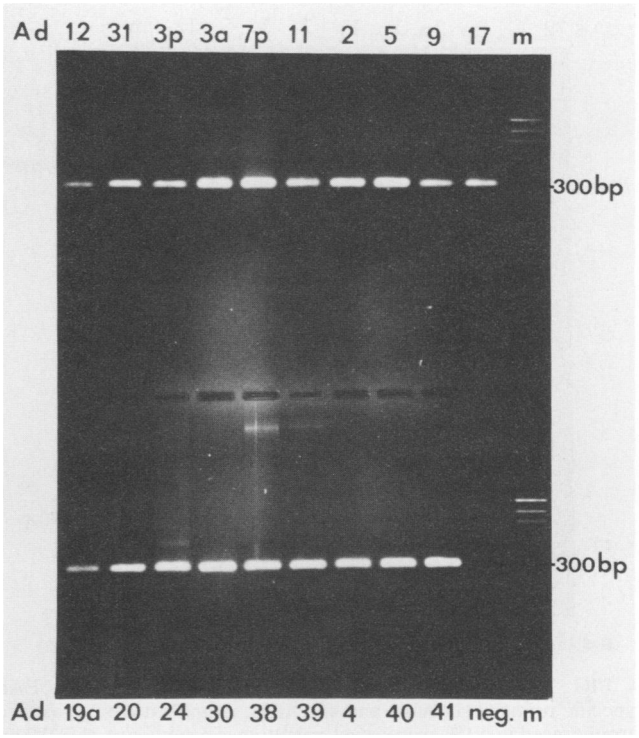

FIG. 2. PCR amplification of DNA from 18 different adenovirus types (see Table 2) representing all six subgenera with the general hexon region primers hexAA1885 and hexAA1913. Agarose gel electrophoresis of amplified products shows the characteristic 300-bp band. Two genome types of Ad3 were used. Lane m, Molecular weight standards (φX174 *Hae*III digest).

Primers. The DNA sequences of the open reading frames of the hexon genes of Ad2 (1), Ad40 (26), and Ad41 (25) were examined with the computer program developed by Harr et al. (11) to locate suitable target sequences for the detection of adenoviruses. For the specific detection of EAds Ad40 and Ad41, primers were chosen mainly from the genes encoding early regions E1A and E1B (3, 30) (Fig. 1 and Table 1).

RESULTS

Specificities of primers. The specificities of the general hexon region primers hexAA1885 and hexAA1913 were tested on 18 different adenovirus types, representing all six subgenera (Table 2). Positive results were obtained with all types when analyzed on a 1% agarose gel (Fig. 2). Southern blot hybridization of the gel was in complete concordance with ethidium bromide staining when a mixture of six adenovirus probes representing all subgenera was used (data not shown).

The specific primers for the two EAds, 41AA142 and 41AA358, were tested against the same collection of adenovirus types as the general hexon region primers were. Amplification was only detected when the two EAds Ad40 and Ad41 were used as templates. All PCRs for adenovirus types belonging to subgenera A to E were negative when assayed in ethidium bromide-stained agarose gels or in Southern blots (Fig. 3).

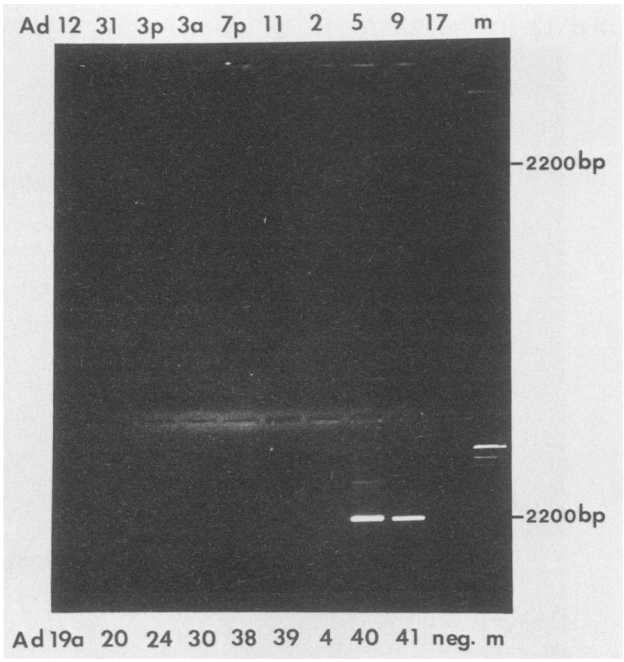

FIG. 3. Adenovirus DNA amplified by PCR with the EAd-specific primers 41AA142 and 41AA358. Amplified DNA was electrophoresed in a 1% agarose gel, resulting in a sequence of 2,187 bp. Lane m, Molecular weight standards (λ *Hin*dIII digest).

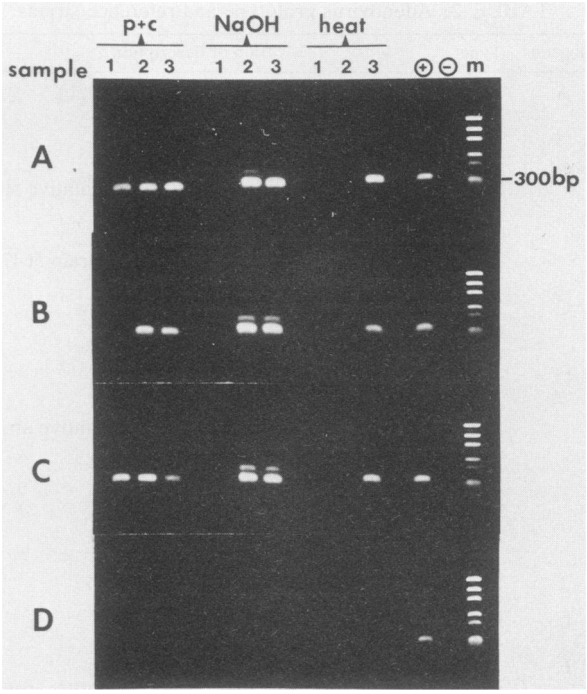

FIG. 4. Comparison of the effectiveness of three techniques for the extraction of adenovirus DNA from stools for detection by PCR. The phenol-chloroform (P+C), NaOH, and heating (heat) extraction methods were compared with undiluted (lanes 1), 10-fold-diluted (lanes 2), and 100-fold-diluted (lanes 3) stool specimens. Samples A, B, and C were typed as Ad41 by PAGE. Sample D was negative for adenoviruses and was used as a control. The hexon region primers hexAA1885 and hexAA1913 were used in all reactions. Lane m, Molecular weight standards (ϕX174 *Hae*III digest).

To distinguish between the two enteric types, Ad40 and Ad41, we tested an Ad40-specific pair of primers, 40AA45 and 41AA129, against the 18 adenovirus types. Ad40 was amplified whereas the 17 other adenoviruses were negative in ethidium bromide-stained agarose gels or in Southern blots (data not shown). The reverse primer 41AA129 binds to both Ad41 and Ad40 E1A regions, but two mismatches to the Ad40 sequence can be found within the primer. The Ad41 sequence is used as a reference sequence and, to match the Ad40 genome to the Ad41 genome, position 4 within the primer has to be changed from an A to a G and position 8 has to be changed from a G to a C. However, the specificity is provided by the lack of homology of the forward primer, 40AA45, to Ad41, due to a deletion in the Ad41 genome at that primer position (30).

Evaluation of optimal methods for pretreatment of the stool specimens. Virions have to be disrupted to make the viral DNA available for PCR amplification. To evaluate the three different methods described in Materials and Methods, we used four stool samples from an outbreak of diarrhea in Karlskrona, Sweden, in 1988. Three of these samples contained high concentrations of viral particles which had been typed as Ad41 by PAGE-silver staining of *Sma*I-digested viral DNA extracted from infected 293 cells. The fourth sample was negative for adenoviruses and was used as a control. Undiluted and 10- and 100-fold-diluted samples were analyzed by PCR after the different pretreatment protocols (Fig. 4). The general hexon region primers hexAA1885 and hexAA1913 were used in all reactions.

The phenol-chloroform method was the best for providing positive results in PCR for samples A and C, independent of concentration. The phenol treatment eliminates biological contaminants in the stool suspensions that may inhibit the activity of the *Taq* polymerase. The alkali method was almost as effective as the phenol-chloroform method when samples were diluted 10- or 100-fold before the PCR amplifications.

Treatment with NaOH followed by neutralization with HCl resulted in a relatively high concentration of salt ions which, together with inhibitor factors in the original stool suspension, may have further impaired the function of the *Taq* polymerase. This is probably why the NaOH method requires dilution to work. Positive PCR results were also obtained when dilutions of stools were heated for 25 min at 95°C to disrupt the viral capsid. Only the 100-fold dilution gave results comparable to those given by the other two treatments.

Southern blots of the gels shown in Fig. 4 were hybridized to the entire genome of Ad41, but no additional specimens became positive.

After this evaluation, we chose the alkali method. It was more simple than and as effective with diluted stool specimens as phenol extraction and more sensitive than the heating method. However, the heating method was simpler, since it could be performed directly on the PCR mixture. The first denaturation step was extended from 4 to 25 min at 95°C. This extended heating of the *Taq* polymerase did not affect the activity of the enzyme (data not shown).

Sensitivity. Ad2 and Ad41 virions were isolated by CsCl centrifugation and dialyzed twice against 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA, and the amounts were determined spectrophotometrically. After NaOH treatment, the samples were diluted in eight steps to obtain a theoretical range of virus particles from 10^8 to 1 per reaction mixture, based on a ratio of 10^{12} particles per ml at an optical density

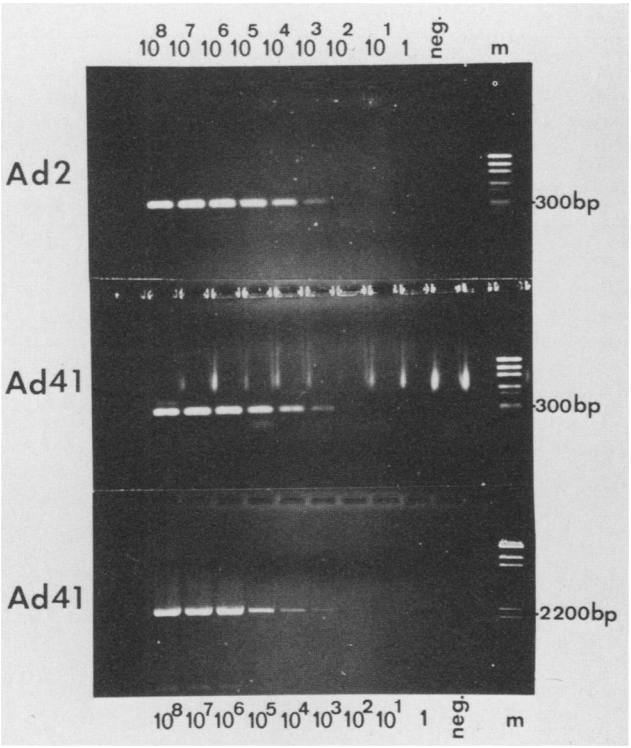

FIG. 5. Sensitivity of PCR for the detection of adenovirus DNA from virions prepared from CsCl gradients and extracted by the NaOH method. The numbers above and beneath the figure represent the numbers of viral particles per genome copy contained in 10 μl (one-fifth) of the reaction mixture prior to amplification. (Top panel) DNA from Ad2 virions was amplified with the general hexon primers hexAA1885 and hexAA1913. (Middle panel) DNA from Ad41 virions was amplified with the same primers as those used in the top panel. (Bottom panel) DNA from Ad41 virions was amplified with the EAd-specific primers 41AA142 and 41AA358, creating a long amplimer of 2,187 bp. Lane m, Molecular weight standards (φX174 *Hae*III digest [top and middle panels], and λ *Hin*dIII digest [bottom panel]).

at 280 to 330 nm of 1.0. After PCR amplification with both hexon primers (hexAA1885 and hexAA1913) and EAd-specific primers (41AA142 and 41AA358), the results were analyzed on a 1% agarose gel, which was transferred to a nylon filter by the Southern method and hybridized to the Ad2 and Ad41 probes. Dilutions containing 100 virus particles of Ad2 or Ad41 were visualized after 30 cycles of PCR with the hexon primers by both agarose gel and hybridization analyses. A dilution representing 10^3 virus particles yielded the 2,000-bp EAd-specific amplimer after 30 cycles of PCR (Fig. 5).

Adenovirus detection in clinical specimens. (i) Silver staining after PAGE. All 60 specimens were first inoculated on A549 cells as a routine procedure in diagnostic work. Thirty of the specimens displayed CPE suggestive of adenovirus infection in A549 cells. Twenty of these 30 were also Adenolex positive. All 60 specimens were also cultivated in 293 cells. Only 16 of the specimens induced CPE in these cells. The CPE in the two different cell lines were not concordant.

DNA extracted from 293 cells infected with all 60 specimens was digested with restriction enzyme *Sma*I and separated by PAGE. Among the 20 Adenolex-positive samples, no DNA restriction fragments were detected in 4 samples,

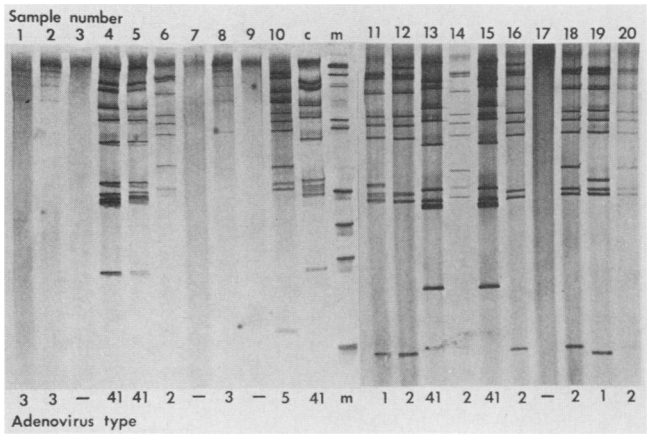

FIG. 6. Adenovirus DNA extracted from 293 cells infected with stool samples 1 to 20. The DNA was typed by *Sma*I digestion and silver staining after PAGE. No restriction fragments were detected in samples 3, 7, 9, and 17. Lane c, Control DNA, i.e., DNA extracted from 293 cells inoculated with Ad41 and analyzed simultaneously with and by the same procedure as the stool samples. Lane m, Molecular weight standards (λ *Hin*dIII digest and φX174 *Hae*III digest).

but the other 16 samples showed typical adenovirus DNA restriction fragment profiles (Fig. 6). All 16 were typed by comparison with DNA from reference adenovirus types separated by PAGE. Six Ad2, four Ad41, three Ad3, two Ad1, and one Ad5 type were identified. No DNA restriction fragments were detected after PAGE of the remaining 40 samples (Table 3).

(ii) PCR performed on DNA extracted from virus-infected cell cultures. The PCR method was tested on DNA extracted from cultures of 293 cells inoculated with the 60 stool samples. With the hexon primers hexAA1885 and hexAA1913, all 20 Adenolex-positive samples exhibited the expected 300-bp amplimer when analyzed on an agarose gel (Fig. 7). An autoradiogram of the Southern blot of the gel showed that all amplimers hybridized to the mixture of labeled adenovirus probes.

DNA extracted from the 20 positive samples was also tested with the EAd-specific primers 41AA142 and 41AA358. Nine of the 20 samples gave rise to more or less intense bands of 2,000 (Fig. 8). Only four of them were identified as Ad41 by PAGE. Samples 3 and 9 could possibly have contained virus particles of Ad41, but no DNA was visible on the polyacrylamide gels. Samples 2, 8, and 20 were found positive for EAds by PCR. However, the presence of Ad3, Ad3, and Ad2, respectively, was revealed by the *Sma*I restriction pattern on the polyacrylamide gels. These results can only be explained by double infections or persistent adenovirus infections. When a sample is cultivated on cells, the less fastidious types overgrow the fastidious EAds and then dominate in the DNA extraction yield. This fact may also explain the discordance between the results PAGE and EAd-specific PCR; the latter method detects both DNA types, even the Ad41 DNA representing the minor portion. Southern blots of the amplified samples were hybridized to an Ad41-specific probe, resulting in the same signals as those visualized on the agarose gels. However, the samples giving Ad41 restriction profiles in PAGE, 4, 5, 13, and 15, gave much stronger signals than did the other positive amplified samples.

The same procedure was repeated with the Ad40-specific primers 40AA45 and 41AA129. None of the 20 extracted

TABLE 3. Detection of adenoviruses in stools of 60 children

Sample	Patient age	Symptom(s)	Result for:						
			Adenolex testing of stools	Adenolex testing of A549 cell-cultured stools[a]	PAGE of DNA from 293 cell-cultured stools	PCR (hexon primers) of stools[b]	PCR (hexon primers) of DNA from 293 cell-cultured stools[b]	PCR (EAd primers) of stools[b]	PCR (EAd primers) of DNA from 293 cell-cultured stools[b]
1	10 mo	Fever, rhinopharyngitis, conjunctivitis	−	+	Ad3	−	++	−	−
2	3 yr	Fever, diarrhea	−	+	Ad3	−	++	−	++
3	2.3 yr	Fever, diarrhea	−	+	ND[c]	−	++	−	++
4	15 mo	Fever, diarrhea	−	+	Ad41	++	++	++	++
5	21 mo	Diarrhea	+	+	Ad41	++	++	++	++
6	2 yr	Fever, diarrhea	−	+	Ad2	++	++	−	−
7	3.5 yr	Fever, neurological symptoms	−	+	ND	−	++	−	−
8	21 mo	Fever, pneumonia	−	+	Ad3	++	++	−	++
9	18 mo	Diarrhea	−	+	ND	−	++	−	++
10	18 mo	Fever, encephalitis, fits, exanthema	−	+	Ad5	++	++	−	−
11	13 mo	Fits	−	+	Ad1	−	++	−	−
12	15 mo	Diarrhea	−	+	Ad2	++	++	−	−
13	12 mo	Diarrhea	+	+	Ad41	++	++	++	++
14	2 mo	Diarrhea	−	+	Ad2	++	++	−	−
15	9 mo	Diarrhea	−	+	Ad41	++	++	++	++
16	10 mo	Fever, diarrhea, headache	−	+	Ad2	++	++	−	−
17	4 mo	Fever, diarrhea	−	+	ND	−	++	−	−
18	8 mo	Fever, diarrhea	−	+	Ad2	++	++	−	−
19	3.5 yr	Fever	−	+	Ad1	++	++	−	−
20	2 yr	Long period of fever, diarrhea	−	+	Ad2	++	++	−	++
21	19 mo	Previous diarrhea	−	−	ND	−	−	−	−
22	16 mo	Diarrhea	−	−	ND	−	−	−	−
23	17 mo	Diarrhea	−	−	ND	−	−	−	−
24	2.5 yr	Fever, pain in joints	−	−	ND	−	++	−	++
25	12 mo	Protracted diarrhea	−	−	ND	−	+	−	−
26	7 mo	Diarrhea	−	−	ND	−	+	−	−
27	13 mo	Diarrhea	−	−	ND	−	++	−	++
28	1 yr	Diarrhea, rhinopharyngitis	−	−	ND	−	+	−	++
29	9 mo	Diarrhea with blood	−	−	ND	−	+	−	−
30	3 yr	Diarrhea	−	−	ND	−	++	−	−
31	2 wk	Fever, diarrhea	−	−	ND	−	+	−	−
32	5 mo	Diarrhea	−	−	ND	−	+	−	−
33	2 yr	Diarrhea	−	−	ND	−	+	−	−
34	2.5 yr	Diarrhea	−	−	ND	−	−	−	−
35	16 mo	Diarrhea	−	−	ND	−	−	−	−
36	22 mo	Diarrhea	−	−	ND	−	+	−	−
37	2 yr	Protracted diarrhea	−	−	ND	−	+	−	−
38	1 yr	Diarrhea	−	−	ND	−	+	−	−
39	15 mo	Diarrhea	−	−	ND	−	−	−	−
40	6 mo	Fever, diarrhea	−	−	ND	−	−	−	−
41	15 mo	Fever, diarrhea	−	−	ND	−	+	−	−
42	1 mo	Diarrhea	−	−	ND	−	++	−	++
43	1 yr	Diarrhea	−	−	ND	−	+	−	−
44	2 mo	Diarrhea	−	−	ND	−	+	−	−
45	2 yr	Diarrhea	−	−	ND	−	+	−	−
46	16 mo	Protracted diarrhea	−	−	ND	−	+	−	−
47	1 wk	Diarrhea	−	−	ND	−	+	−	−
48	7 mo	Fever, diarrhea	−	−	ND	−	−	−	−
49	16 mo	Diarrhea	−	−	ND	−	+	−	−
50	10 mo	Diarrhea, vomiting	−	−	ND	−	+	−	−
51	14 mo	Diarrhea, fever	−	−	ND	−	++	−	++
52	18 mo	Protracted diarrhea	−	−	ND	−	+	−	−
53	8 mo	Diarrhea	−	−	ND	−	+	−	−
54	18 mo	Protracted diarrhea	−	−	ND	−	+	−	−
55	9 mo	Fever, diarrhea	−	−	ND	−	+	−	−
56	4 mo	Diarrhea	−	−	ND	−	+	−	−
57	1 mo	Fever, diarrhea	−	−	ND	−	++	−	++
58	2 mo	Diarrhea	−	−	ND	−	++	−	++
59	3 mo	Diarrhea	−	−	ND	−	+	−	−
60	17 mo	Diarrhea	−	−	ND	−	−	−	−

[a] The samples displayed CPE suggestive of adenovirus infection. This impression was supported by Adenolex positivity.
[b] ++, Positive after 30 cycles of PCR; +, positive after 35 cycles of PCR; −, negative.
[c] ND, No DNA detected.

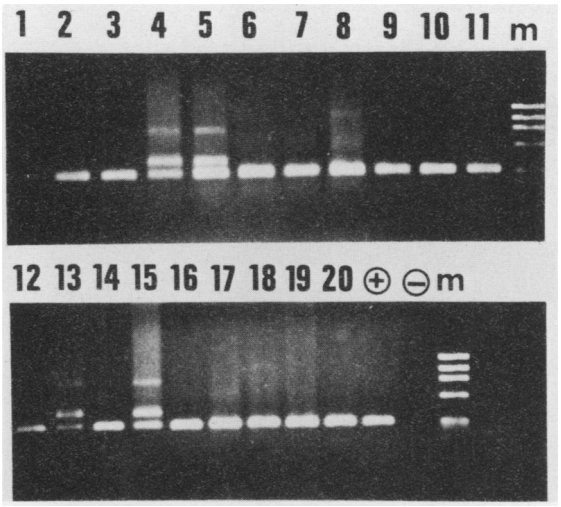

FIG. 7. Detection of PCR-amplified DNA extracted from 293 cells inoculated with fecal samples 1 to 20 by use of the general hexon primers. Lanes m, *Hae*III-digested ϕX174 DNA size markers (1,353, 1,078, 872, 603, 310, 281, 234, 194, 118, and 72 bp).

DNAs were amplified with this primer combination. When PCR was performed with the hexon primers hexAA1885 and hexAA1913 on DNA extracted from samples 21 to 60, seven samples were weakly positive. When the number of cycles was increased from 30 to 35, the bands of the 7 samples were intensified, and 22 additional samples were amplified (Fig. 9). In a separate PCR experiment done with an annealing temperature of 45°C, no additional samples became PCR positive. Southern blots of the agarose gels did not reveal additional amplified products (Fig. 9).

Samples 21 through 60 were also tested with the EAd-specific primers 41AA142 and 41AA358. Seven samples, 24,

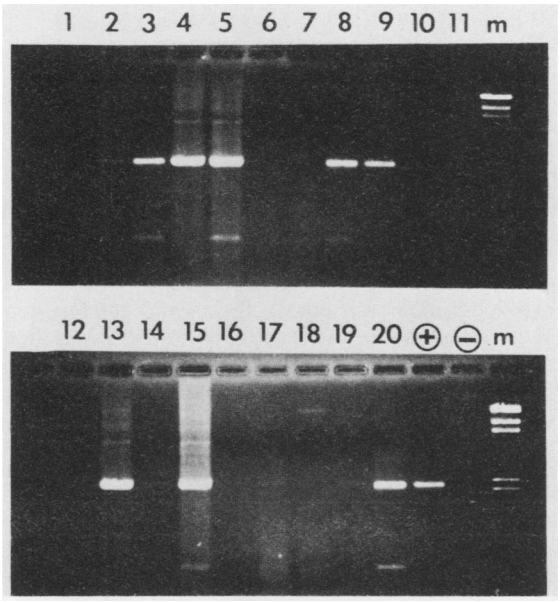

FIG. 8. Detection of EAd-specific PCR amplification of DNA extracted from 293 cells inoculated with Adenolex-positive samples 1 to 20 by use of primers 41AA142 and 41AA358. Samples 2, 3, 4, 5, 8, 9, 13, 15, and 20 were suggested to be positive. Lanes m, *Hin*dIII-digested λ DNA size markers (23,130, 9,419, 6,557, 4,371, 2,322, 2,028, and 564 bp).

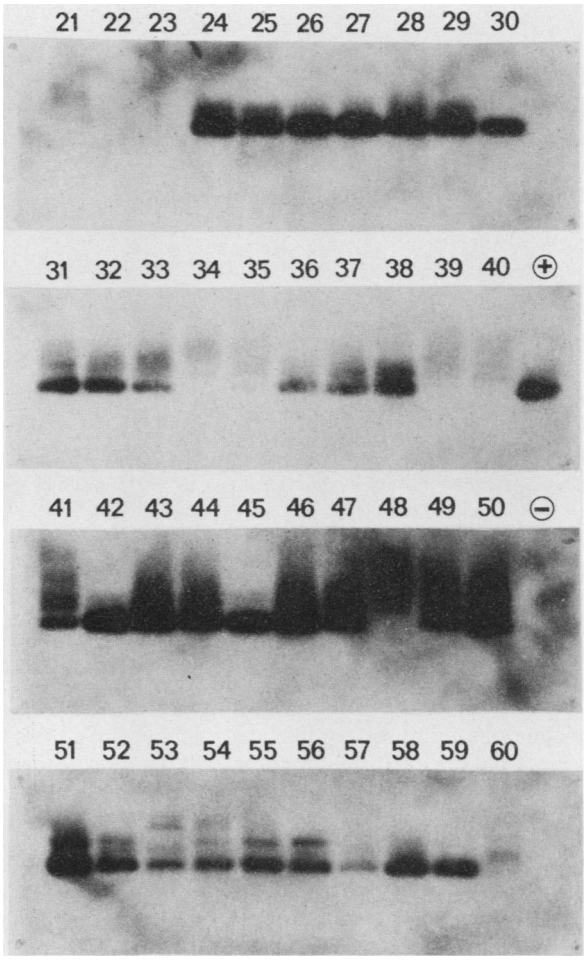

FIG. 9. PCR amplification (35 cycles) of adenovirus DNA extracted from 293 cells inoculated with samples 21 to 60 by use of the general hexon primers. Reaction products were analyzed by Southern transfer of 1% agarose gels and hybridized with a mixture of α-^{32}P-labeled adenovirus DNAs representing all six subgenera. Ten picograms of DNA representing the entire genome of Ad2 was used as a positive control.

27, 28, 42, 51, 57, and 58, showed amplification by PCR (Table 3). Results of Southern blot analysis with an Ad41 probe were the same as those of agarose gel analysis (data not shown). The 40 specimens were finally tested with the Ad40-specific primers. None of the samples were positive either in the analysis of amplified fragments on agarose gels or in Southern blot hybridizations with an Ad40-specific probe (data not shown).

(iii) PCR performed directly on diluted stool samples. All 60 stool samples were pretreated by the NaOH method, diluted 10- and 100-fold, and used in PCR amplification with the hexon primers hexAA1885 and hexAA1913. Thirteen of the 20 Adenolex-positive samples were found positive by PCR (Fig. 10). The 10- and 100-fold dilutions gave the same results, as did Southern blot hybridization analysis. When we tested the EAd-specific primers 41AA142 and 41AA358 with the 20 Adenolex-positive stool samples diluted 100-fold, only the 4 samples that displayed Ad41 restriction fragment profiles (Fig. 6) had a 2,000-bp amplimer (Fig. 11).

The 40 samples that were agglutination negative after tissue culture were also negative in PCR amplification performed directly on NaOH-treated stools (Table 3). Lowered

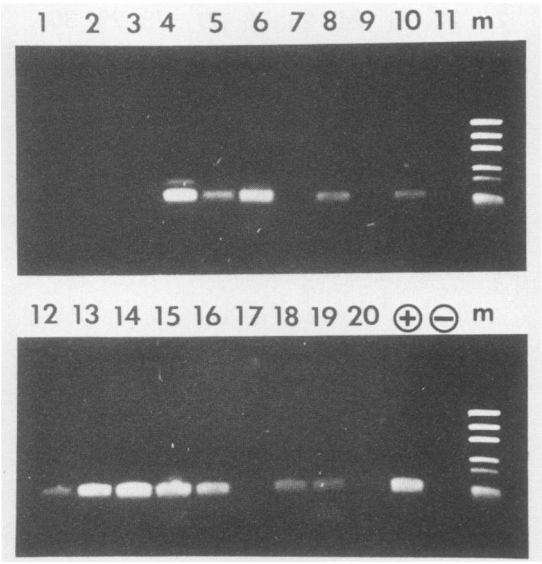

FIG. 10. PCR amplification (30 cycles) of adenovirus DNA in stools by use of the general hexon primers. NaOH-extracted stool specimens 1 through 20 were diluted 10-fold prior to the reaction. Lanes m, *Hae*III-digested ϕX174 DNA size markers.

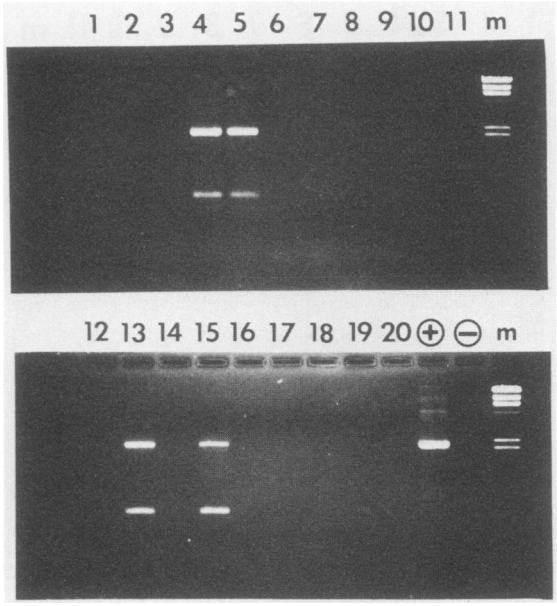

FIG. 11. PCR amplification (30 cycles) of EAd-specific DNA from NaOH-extracted stool specimens 1 to 20 diluted 10-fold. Lanes m, *Hin*dIII-digested λ DNA size markers.

annealing temperatures or extended numbers of cycles did not affect the results. Southern blot hybridization of the gels did not give any indications of amplified products.

DISCUSSION

We have identified a group-specific region for detection of adenoviruses which is partially located in the basal part of the β-barrel-forming P1 domain within the hexon (19). By use of primers flanking this structure, 18 of 18 adenovirus types representing all six subgenera could be detected by PCR. Consequently, they can serve as general primers for human adenoviruses. We also have identified a specific pair of primers which can be used for the detection of the two EAds Ad40 and Ad41 and another pair of primers which can differentiate between them. The four nucleotide sequences used for the selection of EAds are all located within early region E1.

The limit of detection of the PCR assay was 10^2 virus particles when the hexon primers were used and 10^3 virus particles when the EAd primers were used. This result corresponds to the detection of 38 and 380 fg of adenovirus DNA, respectively. The high specificities of the primers used were demonstrated by amplification of DNA representing 18 different adenovirus types tested with the hexon primers (Fig. 2) versus the total absence of amplification of DNA representing nonenteric adenovirus types tested with the two different pairs of EAd-specific primers. To check cross-reactivity with other viruses, we tested plasmids containing human papillomavirus types 6, 16, 18, and 33 with the three sets of primers by PCR. No amplimers were found. The oligonucleotide sequences corresponding to the adenovirus-specific primers were also aligned to the adeno-associated virus type 2 genome (EMBL Database, release 23.0). No sequence with sufficient homology to the three sets of adenovirus primers that would allow amplification could be found (data not shown).

The reliability of the primers was tested on clinical specimens. Sixteen of 60 samples were positive for EAd when PCR analysis was performed on viral DNA from cell-cultured stools. In most cases (15 of 16), the EAd-positive specimens yielded DNA after 30 cycles, both with EAd-specific and with general hexon primers; in no case was 35 cycles required to detect EAd-specific amplification (Table 3). Altogether, 27% of the specimens contained EAd-specific DNA, presumably Ad41. This is a high but not unreasonable figure. It should be pointed out that the 63 rotavirus-positive stools were excluded from the study.

The same four Ad41 specimens were identified by PCR performed directly on stools and by DNA restriction analysis after growth in 293 cells (Table 3).

In 3 of the 12 stools containing nonenteric adenoviruses, as revealed by DNA restriction, EAd-specific DNA was clearly demonstrated by the PCR. It is likely that in the two cases of diarrhea Ad41 rather than Ad2 and Ad3 was responsible for the diarrheal symptoms that served as a criterion for inclusion in the study. Ad41, Ad1, Ad2, Ad3, and Ad5 were the only types identified. This was the expected outcome, since infections with members of subgenus C and Ad3 are characterized by a prolonged intermittent excretion (up to 906 days) and a frequent isolation rate (5, 9, 15). The possible persistent excretion of Ad41 has not been studied. However, in this study Ad41 was always detected already after 30 cycles (Table 3).

Thirteen of 60 samples became positive when general hexon primers were tested directly on diluted stools. Minute amounts of adenovirus particles are frequently shed since, after amplification and through replication in cell cultures, 51 of 60 samples (corresponding to 85%) were positive for adenoviruses in general when PCR analysis was performed on viral DNA from cell-cultured stools. This is a high but not unexpected incidence, since all children were less than 40 months old and were sick. Several adenovirus types can be shed for long periods after the primary infection.

The Adenolex method, which has been evaluated by Grandien et al. (10), was here shown to be an insensitive method for the direct detection of adenoviruses in stools. Cell passage of samples to enhance detection had to be done;

this procedure is not recommended by the manufacturer. PCR performed directly on stool specimens was more sensitive than was Adenolex but failed to detect many samples that became positive after cell culturing. To optimize detection by PCR, we had to inoculate stools on 293 cells to obtain both natural replication and PCR amplification. DNA restriction site analysis can be a useful method for identification. However, in this study it was less sensitive than was PCR, even after growth in a permissive cell line.

In summary, the general primers can be used whenever fastidious adenovirus infections, for example, those of subgenus A, are suspected. The selective primers offer a method for detecting adenoviruses directly in a sample and could be used to determine the degree of fecal contamination in wells, sewage, or seawater as well as for clinical diagnosis.

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