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Importance of Enteric Adenoviruses 40 and 41 in Acute Gastroenteritis in Infants and Young Children

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In a prospective 1-year study of acute infantile gastroenteritis, adenoviruses were detected in the stools or by seroconversions, or both, in 56 of 416 (13.5%) ill children. By use of DNA restriction enzyme analysis, enzyme immunoassay, and culture techniques, 33 of 56 (59%) adenovirus specimens were identified as enteric adenoviruses 40 and 41 (Ad40 and Ad41). They were found as the sole recognizable cause of diarrhea in 30 of 416 (7.2%) ill children and in 0 of 200 controls. Three additional ill children had enteric adenoviruses as a part of a dual infection. Evidence for established adenoviruses (Ad1 through Ad39) in gastroenteritis was found in 15 of 416 (3.6%) ill children but also in 3 of 200 (1.5%) controls. Eight adenovirus specimens remained untyped. Seroconversions were demonstrated in 17 of 18 (94%) paired serum samples from patients shedding enteric adenoviruses. The predominant symptom of infections with enteric adenoviruses was diarrhea, with a mean duration of 8.6 days (Ad40) and 12.2 days (Ad41). One-third of the children with Ad41 infections had prolonged symptoms (≥14 days). The frequency of respiratory symptoms was low (21%). The established adenoviruses presented a different clinical picture, characterized by diarrhea of shorter duration, higher fever, and significantly increased occurrence of respiratory symptoms (79%). In conclusion, enteric adenoviruses appear to be an important cause of acute infantile gastroenteritis, second only to rotaviruses in this study.

Several investigations have been undertaken to elucidate the role of adenoviruses in acute infantile gastroenteritis, and they have yielded conflicting results. Significantly higher adenovirus isolation rates have been demonstrated for children with diarrhea than for asymptomatic children (20, 23). Other investigators have found no difference in such rates between children with and without diarrhea (11, 47). Furthermore, Fox et al. have shown that adenovirus 1 (Ad1), Ad2, and Ad5 (subgenus C) can be excreted from the gut without any symptoms for at least 2 years after the primary infection (6).

In 1975, previously unrecognized adenoviruses were detected by electron microscopy (EM) in stool specimens from infants with diarrhea (5, 31). These adenovirus species have been designated enteric adenoviruses (EAds). They are fastidious and cannot be cultivated in cell cultures by conventional means. In several studies EAds have been found in 3.9 to 12% of the stool specimens from infants and young children with acute gastroenteritis (1, 2, 15–17, 19, 24, 37). Hospital outbreaks of diarrhea caused by EAds have also been described (3, 5, 25). The importance of EAds in infantile diarrhea in developing countries has been less well studied. Antibodies against EAds have recently been shown to be common and widespread throughout the world (13).

The inability of EAds to grow in vitro initially hampered conventional classification. Recently, however, EAds have been propagated in selected cell cultures, namely, 293 cells (34), Chang conjunctival cells (14), and cynomolgous monkey kidney (CMK) cells (4). Furthermore, EAds have been characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of virion polypeptides (40) and DNA restriction site analysis of the genome (43). Two distinct species of EAds, Ad40 and Ad41 (4, 36), representing two new subgenera, F and G, respectively (36, 43), have now been identified.

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This communication presents a prospective 1-year study of the role of EAds in acute gastroenteritis in infants and young children and describes the clinical features of Ad40 and Ad41 in comparison with the established adenovirus species. It is demonstrated that EAds are causally related to acute infantile diarrhea.

MATERIALS AND METHODS

Subjects. The study population consisted of children up to 15 years of age with acute gastroenteritis who sought medical advice at the Department of Pediatrics of the University Hospital of Uppsala between 1 January 1981 and 31 December 1981. The definition of diarrhea was three or more loose or watery stools daily lasting for at least 1 day and for no longer than 14 days before admittance. The definition of upper respiratory symptoms was the occurrence of either tonsillitis, pharyngitis, otitis, coryza, or cough in association with diarrheal symptoms. The study comprised 416 patients, of whom 144 were hospitalized and 272 remained outpatients. Fifty-five percent of the children were males. The youngest patient was 3 weeks old and the oldest was 13 years old. Of the children, 38% were less than 12 months old, 33% were between 1 and 2 years old, 19% were between 2 and 5 years old, and 10% were more than 5 years old. The control group consisted of 200 children, matched for age and season, admitted to the clinic during the same period for reasons other than gastrointestinal illnesses, such as urinary infections, upper and lower respiratory infections, and malignant diseases. Of those children, 93 were hospitalized and 107 remained outpatients. A detailed history, including information on feeding habits, contact, and past history, was collected on admission and again about 4 weeks later. All children were examined by the same clinician (I.U.).

Specimens. Stool specimens were obtained from all patients and controls as soon as possible after admission to the hospital, and also from one-third of them at a later stage, to investigate the duration of excretion of the enteropathogenic

agents. Specimens for bacteriological and parasitic examination were collected by standard techniques. For viral studies stool suspensions of 10 to 20% were prepared by shaking feces in phosphate-buffered saline containing penicillin (100 U/ml) and streptomycin (50 μg/ml). The suspensions were clarified by centrifugation at 3,000 rpm. The specimens were tested immediately or stored at −70°C until tested. Paired acute- and convalescent-phase serum specimens were available from 50% of the patients. Clinical laboratory tests such as erythrocyte sedimentation rate, complete blood counts, serum electrolytes, and urinalysis were performed initially whenever possible. Nasopharyngeal secretions for viral studies were not taken.

Examination of stool specimens. All stool specimens were examined for viral, bacterial, and parasitic pathogens. The stools were analyzed for *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, *Yersinia enterocolitica*, enteropathogenic *Escherichia coli*, and enterotoxigenic *E. coli*. All samples were also tested for heat-labile enterotoxin production by the Y1 mouse adrenal-cell culture assay (28). The Formalin-ether concentration method was used for parasitic studies. All stool specimens were examined by EM without prior ultracentrifugation. The pellets were prepared by the method of Madeley et al. (17). Each sample was scanned for 5 min at a magnification of ×28,000 (Philips 200). All stools were tested by enzyme-linked immunosorbent assays (ELISAs) for genus-specific antigens of rotaviruses (46) and adenoviruses (8). Specimens positive for adenovirus were further characterized by species-specific ELISA (8), by DNA restriction analysis (43), and for their capacity to grow in cell cultures (4), as described below.

Virus isolation and restriction endonuclease analysis of viral DNA. Stool suspensions (10%) were inoculated into A549 (provided by W. A. Nelson-Rees, Berkeley, Calif.) and 293 cells (7) in stationary tissue culture tubes as described by de Jong et al. (4). Complete virus replication in A549 cells was considered to be a criterion for the established adenovirus species (Ad1 through Ad39). This conclusion was based on DNA restriction site analysis of all strains that could be propagated in A549 or 293 cells. The primary isolation cultures of adenoviruses were harvested 3 to 5 days postinfection, sonicated, and inoculated into 150-cm^2 bottles by adsorption for 90 min at 37°C on a rocking platform (Bellco Glass, Inc.). The cells were lysed with sodium dodecyl sulfate (0.6% [wt/vol]) at 2 to 4 days postinfection, and intracellular viral DNA was extracted as described (41).

Viral DNA was digested with restriction endonucleases *Bam*HI, *Sma*I, and *Hin*dIII (New England Biolabs, Inc.), under the conditions described by the manufacturer and analyzed by electrophoresis in horizontal submerged 1.2% agarose slab gels (high gelling temperature; Sea-Kem) as described previously (41).

ELISA for adenovirus and rotavirus antigen. The ELISAs were performed by a combination of the methods of Yolken et al. (46) and Voller et al. (38). The polystyrene microtitration plates (M29AR, Dynatech, Ltd.) were coated with capture antibody and then incubated with stool specimens, indicator antibody, and anti-immunoglobulin G conjugate (alkaline phosphatase-conjugated anti-immunoglobulin G [Orion, Ltd.]), and finally substrate solutions (*p*-nitrophenyl substrate [1 mg/ml; Sigma 104]) were added. The reaction product (yellow coloration) was measured at 405 nm in a Titertek Multiscan spectrophotometer (Flow Laboratories, Ltd.). All reagents were added in volumes of 100 μl, and all rinses were carried out with 0.15 M saline containing 0.05% (vol/vol) Tween 20.

Two ELISA techniques for adenoviruses, a genus-specific one that detected all adenovirus species and a species-specific one that detected Ad40, which have been described in detail previously (8), were used. Briefly, the capture antibody was genus specific and of human origin, and the indicator antibody, either genus or species specific, was of rabbit origin. The antiserum against Ad40 was obtained by affinity bead immunization (10). The serum was rendered species specific by passing it through immunosorbent columns containing soluble virus components of Ad1, Ad7, Ad15, Ad16, and Ad31 (8).

An Ad41-specific ELISA technique was developed and applied in this study. A rabbit antiserum against Ad41 was produced by immunization with purified virions. Heterotypic antiadenovirus reactivity was eliminated by passing the serum through immunosorbent columns containing soluble virus components of adenovirus species representing subgenera B through F (9). The serum, diluted 1:500, was used as an Ad41-specific indicator antibody in an ELISA analogous to that described for Ad40 (8).

The ELISA for detection of rotavirus was performed with anti-bovine rotavirus serum (Wellcome Research Laboratories, Ltd.) as capture antibody and rabbit anti-human rotavirus serum (Dakopatts) as indicator antibody.

Assays for adenovirus antibodies. (i) CF test. Complement fixation tests were performed as described earlier (21), with an antigen pool of equal amounts of adenovirus-soluble components from Ad7b (subgenus B), Ad5 (subgenus C), Ad20 (subgenus D), Ad4 (subgenus E), and Ad41 (subgenus G). Adenovirus-soluble components were obtained after centrifugation on discontinous CsCl gradients as described (42).

(ii) HI test. A hemagglutination inhibition (HI) assay specific for Ad40 and Ad41 was developed and applied in this study. This test did not discriminate between the two species. A penton-specific antiserum (anti-7b dodecons) was incubated with unfractionated Ad41 virus material for 30 min at room temperature to combine monovalent Ad41 pentons to form complete hemagglutinins (39). Rat erythrocytes were then added, and incubation was continued at room temperature. HI was performed with two units of hemagglutinin as described previously (39).

(iii) ELISA. The microtiter wells were first coated with antigen and then incubated with patient sera, anti-immunoglobulin G conjugate, and finally substrate solutions. Sonicated cell harvests of Ad41-infected 293 cells were clarified by centrifugation at 1,000 rpm (Beckman TJ6 centrifuge) at 4°C. Supernatant fluids were diluted 1:100 in 50 mM carbonate buffer (pH 9.6) and distributed to each well of flat-bottomed microtiter plates (Nunc Immuno plate 1-96F) and incubated overnight at 20°C. Harvests of uninfected 293 cells treated in the same way were added to the wells and used as controls. The plates were washed three times, and test sera diluted 1:300 and 1:3,000 in phosphate-buffered saline (pH 7.4) containing 0.05% (vol/vol) Tween 20 and 0.5% bovine serum albumin were incubated in the antigen-coated wells for 3 h at 37°C. Alkaline phosphatase-conjugated antibody against human immunoglobulin G (Orion) at a dilution of 1:100 was added after washing, and the plates were incubated for 1 h at 37°C. After a final rinse, *p*-nitrophenyl phosphate substrate (1 mg/ml; Sigma 104) in 50 mM $NaHCO_3$ (pH 9.6) containing 1 mM $MgCl_2$ was added. The plates were incubated for 30 min at 20°C. The reaction was stopped by the addition of 50 μl of 3 M NaOH. The absorbances in each well were measured at 405 nm in a Titertek Multiscan spectrophotometer (Flow Laboratories). All reagents were

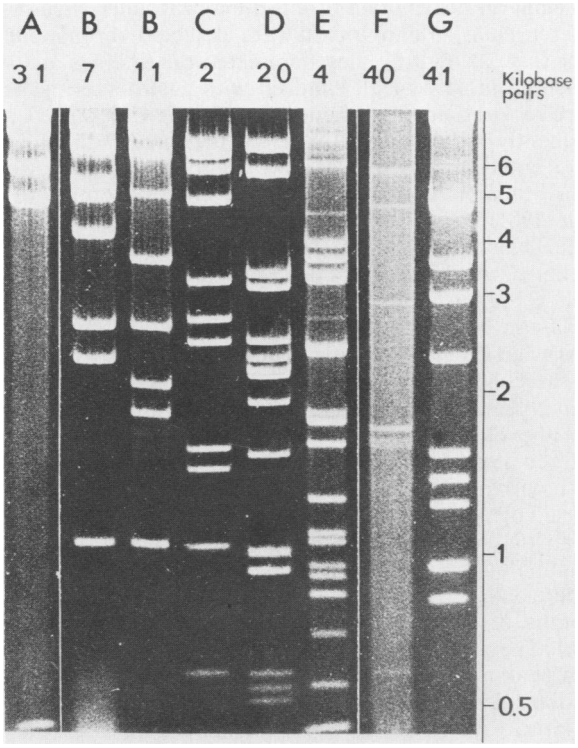

FIG. 1. DNA restriction fragment patterns obtained after digestion of DNA from adenovirus species representing subgenera A to G with *Sma*I (from the Symposium on Standardization of Immunological Procedures—Diarrhoeal Diseases of Man and Animals, Dublin, Ireland, 1982. With permission from S. Karger AG, Basel) (36).

added in volumes of 100 μl, and all rinses were carried out with phosphate-buffered saline–Tween 20.

Calculation of antibody titers in ELISA. The relative antibody content in the tested sera (x) was determined from the following formula as described by Roggendorf et al. (27): x values = [(absorbance sample − absorbance-negative control)/(absorbance standard − absorbance-negative control)] × 100. Two serum samples with high titers obtained in the convalescent phase after infection with EAd were used as standards. A difference of 15 between the antibody values obtained for convalescent- and acute-phase sera was taken as a criterion for an acute adenovirus infection.

Statistical analyses were performed with Fisher's exact test or the Mann-Whitney U test where appropriate.

RESULTS

Infection with EAds and established adenoviruses. EAds were found as the sole recognizable cause of diarrhea in 30 of 416 (7.2%) ill children and in 0 of 200 controls. Three additional ill children had EAds but only as a part of a dual infection (rotavirus [one case], astrovirus [one case], and *C. jejuni* [one case]). Evidence for established adenoviruses in gastroenteritis was found in 15 of 416 (3.6%) ill children but also in 3 of 200 (1.5%) controls. As well as established adenoviruses, rotaviruses (two cases), *C. jejuni* (one case), and enteropathogenic *E. coli* (one case) were found among ill children as a part of dual infections. Eight adenovirus specimens remained untyped, including two cases of dual infection with rotaviruses.

Two distinct species of EAd, Ad40 and Ad41, were identified. Each species displayed a unique DNA restriction pattern (Fig. 1) and was quite distinct in the two species-specific ELISAs. A difference was also observed in culture characteristics; thus, Ad40 grew better in tertiary CMK cells, whereas Ad41 grew better in 293 cells. By these typing methods the 33 EAds could be classified, 14 as Ad40 and 19 as AD41.

Fifteen adenoviruses were isolated in A549 cells and were typed by DNA restriction enzyme analysis as subgroup C (nine cases), Ad7 (three cases), Ad18 (one case), and Ad31 (two cases). A further eight adenoviruses were detected by genus-specific ELISA or EM (four cases), or both, or by seroconversions in the CF test (four cases). These eight infections remained untyped.

Table 1 shows a comparison of the sensitivity of the methods in detecting adenoviruses in stool specimens.

Stool specimens taken in the convalescent phase were available from 26 patients with EAd infections and from 11 patients with established adenovirus infections. No viral particles were observed 4 to 6 weeks after the onset of the diarrheal illness. In 9 of 10 patients studied, EAds were excreted in stool samples up to 8 to 13 days after the onset of disease; in the remaining patient virus was demonstrable for up to 23 days.

It was possible to obtain acute- and convalescent-phase serum specimens from 35 (63%) of the 56 children with adenovirus infections. Sera were analyzed by ELISA, HI, and the CF test. Of 35 paired serum specimens, 34 displayed a significant increase in antibody to adenovirus with one or more of the three techniques. In Table 2 the three techniques are compared with respect to their sensitivity in detecting adenovirus infections. Only patients with EAds developed a fourfold or greater increase in HI antibody. The test was specific for EAd infections but could not discriminate between Ad40 and Ad41. ELISA and HI were of equal sensitivity and detected significant seroresponses in 12

TABLE 1. Comparison of EM, ELISA, virus isolation, and DNA restriction site analysis for detection of adenoviruses in stool specimens

Species	Total no. of viruses	No. detected by:						
		EM	ELISA			Virus isolation		DNA restriction site analysis
			Genus specific	Ad40 specific	Ad41 specific	A549	293 cells	
Ad40	14	14	14	14	0	0	13	13
Ad41	19	18	18	0	18	0	18	18
Established	15	8	5	0	0	15	15	15
Untyped[a]	4	4	3	0	0	0	0	0

[a] Excludes four patients with untyped adenovirus infections detected only by serological responses.

TABLE 2. Comparison of the ELISA, the HI test, and the CF test for detection of serological responses in infections with adenovirus

Type of infection	No. of paired serum samples tested	No. of significant rises in titer by:		
		ELISA	HI	CF
Ad40	5	4	4[b]	2
Ad41	13	8	8[b]	3
Established adenovirus	11	7[a]	0[b]	7
Untyped adenovirus	6	5	0	4

[a] Two paired serum specimens are missing.
[b] Significant difference between the number of rises shown for Ad40 and Ad41 (12) and that shown for established adenovirus (0). $P < 0.001$ by one-tailed Fischer's exact test.

(67%) of 18 children infected with EAds. The detection rate of the CF test was 28% for EAds but 64% for established adenoviruses. ELISA was as efficient as CF for detecting seroresponses in patients infected with established adenoviruses.

The CF test was less efficient in the detection of immune responses in young children. Only 8 (36%) of 22 children below 2 years of age developed significant rises in CF antibodies, compared with 8 (62%) of 13 older children.

An additional 164 paired serum samples from study patients with diarrhea due to other enteropathogens were available. None of these demonstrated a significant increase in CF antibody to adenovirus.

Infections with other enteropathogenic agents. An enteropathogenic agent was detected in 278 (67%) of the 416 patients with acute gastroenteritis. Other agents found by EM, ELISA (for rotaviruses) bacterial culture, and parasitic examination are presented in Table 3. Twenty-seven (6.5%) of the patients had mixed infections, with more than one pathogen being found in the stools.

Infection with enteropathogenic agents among control patients. Only 1 of the 200 control patients shed virus particles (small round virus particles) in the stool, as observed by EM. The ELISAs for common antigens of adenoviruses and rotaviruses in stool samples were all negative. Of 110 paired serum specimens, 3 from the control group demonstrated a fourfold or greater increase in CF antibody to adenovirus. These three children were admitted to the hospital because of urinary infection, otitis, and tonsillitis, respectively. Virus isolation and DNA restriction enzyme analysis revealed adenoviruses in the stool, all members of subgenus C. The bacteria recovered from stools were enteropathogenic *E. coli* (four cases), enterotoxigenic *E. coli* (one case) and *C. jejuni* (one case).

Temporal distribution of infections with adenoviruses and other agents. Adenoviruses were detected throughout the year (Fig. 2). In July they dominated the findings and were observed in 44% of all children with gastroenteritis; EAds were most common. Strains of subgenus C occurred more frequently in late winter, whereas the three Ad7 infections were noted in July and August. Rotavirus-associated diarrhea was most common during the winter months. In that year (1981), however, there was an unexpected peak in May, which was a very warm month. Infections with bacterial agents occurred all through the year, although infections with *C. jejuni* and *Y. enterocolitica* were more frequent during the autumn and early winter.

Age and sex distribution in relation to adenovirus infections. Of the children with adenovirus infections, 70% were less than 2 years of age. The youngest patient was 4 months old and the oldest was 12.5 years old. The age distributions differed among infections with Ad40, Ad41, and established adenoviruses (Fig. 3). The highest incidence of diarrhea caused by Ad40 was in children younger than 12 months; the mean age was 15.2 months and the median age was 12 months. Patients with Ad41, on the other hand, were significantly older, with a mean and median age of 28.3 and 19 months, respectively ($z = 1.803$, $P < 0.05$; Mann-Whitney U test). The number of established adenovirus infections rose to a peak at the ages of 6 to 12 months, and the mean and median ages were 23.2 and 15 months, respectively. Of nine children with subgenus C infections, six were less than 24 months old. Ad7 infections occurred in older children. Of eight children with untyped adenoviruses, five were less than 1 year old, and three were older than 30 months. Mean and median ages of this group were 32.9 and 9 months, respectively.

There were 27 (48%) boys and 29 (52%) girls with adenovirus infections. Regarding the sex ratios among patients with Ad40, Ad41, established adenoviruses, and untyped adenoviruses, 43, 63, 53, and 38%, respectively, were girls; these differences were not statistically significant.

Clinical features of adenovirus infections. Diarrhea was the most prominent symptom. It was sufficiently severe and persistent for the parents to seek medical advice after a median duration of the symptoms of 4 days. Of the 56 children 21 (38%) were hospitalized: 10 of them had EAd infections, 6 had established adenovirus infections, and 5 had untyped adenoviruses, and the mean lengths of their stays in the hospital were 3.1, 3.3, and 2.2 days, respectively.

The clinical findings for 55 patients with adenovirus gastroenteritis are summarized in Table 4. Clinical data on one patient with established adenovirus infection were not available. Diarrhea lasted for 4 to 23 days (mean, 8.6 days) in

TABLE 3. Occurrence of enteric pathogens in children with acute gastroenteritis and in controls

Study patients	No. tested	No. (%) with indicated infection:								
		Virus			Enteropathogenic bacteria				Parasite (*Giardia lamblia*)	No. of negative samples
		Rotavirus	Adenovirus	Other	*E. coli*	*C. jejuni*	*Y. enterocolitica*	*Salmonella* and *Shigella* spp.		
Children with gastroenteritis[a]	416	187 (45.0)	56 (13.5)	2 (0.5)[b]	21 (5.0)	20 (4.8)	12 (2.9)	10 (2.4)	4 (1.0)	138 (33.2)
Controls	200	0 (0)	3 (1.5)	1 (0.5)[c]	5 (2.5)[c]	1 (0.5)	0 (0)	0 (0)	2 (1.0)	188 (94.0)

[a] Dual infections account for mathematic discrepancies.
[b] Calicivirus (one case) and astrovirus (one case).
[c] Small round virus particles.

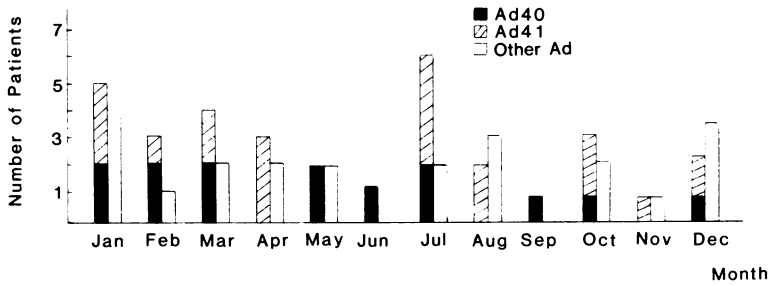

FIG. 2. Monthly distribution of gastrointestinal infections associated with Ad40, Ad41, and other adenoviruses (untyped and established).

children with Ad40, for 2 to 47 days (mean, 12.2 days) in children with Ad41, for 2 to 12 days (mean, 6.2 days) in patients with established adenoviruses, and for 1 to 10 days (mean, 4.5 days) in those with untyped adenoviruses. The mean duration of diarrhea differed significantly between children infected with EAds and those infected with untyped adenoviruses ($z = 2.073$, $P < 0.05$). Prolonged diarrhea was common in association with Ad41; one-third of the patients had symptoms for 14 days, and in three children the diarrhea persisted for 1 month. Patients with EAd gastroenteritis experienced a variable frequency of loose stools, although sometimes there was a short early phase of very watery and occasionally profuse diarrhea. The daily frequency varied from 3 to 30. The mean maximum numbers or stools passed daily were 9.7 (Ad40), 6.8 (Ad41), and 5.4 (established adenoviruses). No bloody stools were observed, but in one-fifth of the children mucus strands were found in the stools. Abdominal pain, a symptom difficult to evaluate in young children, was noted at a lower rate for patients infected with Ad40.

Vomiting occurred less frequently in patients infected with established adenoviruses than in those infected with EAds. It started, on an average, 1.3 and 1.4 days, respectively, after the onset of diarrhea. Vomiting was mild and persisted for a median of 2 days in all patients. Fever and higher temperatures were significantly more common in children infected with established adenoviruses than in those infected with EAds, and the mean duration of fever also differed significantly, with a mean of 5 days as compared with 2.4 days ($z = 2.67$, $P < 0.05$; Mann-Whitney U test). The untyped adenoviruses presented a clinical picture similar to that of the EAds.

The frequency of dehydration did not differ between the adenovirus groups. It was mostly mild and of an isotonic nature. Only one child with established adenovirus infection had a sodium level >150 mmol/liter. Three children infected with EAds and two infected with established adenoviruses required intravenous fluid therapy. No child was severely ill, and there was no fatal outcome.

Of the patients with established adenovirus gastroenteritis, 79% showed evidence of upper respiratory infection, compared with 21% of the EAd group ($P < 0.001$, two-tailed Fisher's exact test) and 25% of the untyped adenovirus group ($P < 0.05$, two-tailed Fischer's exact test). The same kind of respiratory symptoms were observed in the different groups, e.g., tonsillitis, pharyngitis, otitis, coryza, and cough.

At follow-up three children were found to have had difficulties in consuming lactose-containing products for 5 to 7 months after the EAd gastroenteritis. In addition, one of them, a 5-month-old formula-fed girl, had not tolerated gluten-containing food for 9 months. No standardized tests for malabsorpation were performed.

One to four months after the adenovirus infection five patients were admitted to the clinic for rotavirus gastroenteritis.

Clinical laboratory test. There were no significant differences between the groups with respect to laboratory results such as erythrocyte sedimentation rate, peripheral leukocyte count, and serum electrolytes. The erythrocyte sedimentation rate was elevated (range, 20 to 51 mm/h) in 15 (40%) of 38 tested patients. A complete blood count was performed for 39 patients. In 22 (56%) of 39 patients the leukocyte count ranged from 3.2×10^9 to 12.0×10^9/liter, and in 44% it ranged from 12.0×10^9 to 26.6×10^9/liter. In 1 of 19 serum specimens tested for serum electrolytes, the sodium level was elevated above 150 mmol/liter. Of 24 tested urine samples, 13 showed acetonuria, as assayed by the dipstick method (Rediatest; Boehringer Mannheim Biochemicals).

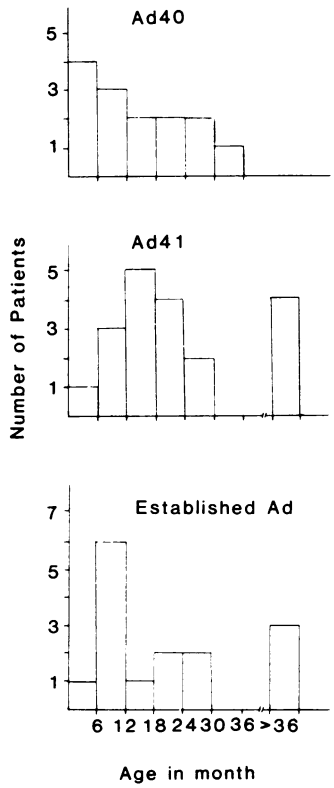

FIG. 3. Age distribution of the patients with adenovirus gastroenteritis.

TABLE 4. Clinical characteristics of 55 children with adenovirus gastroenteritis[a]

Type of infection	Total no. of patients	No. (%) with indicated clinical finding:							
		Diarrhea	Vomiting	Fever		Total no. with fever	Abdominal pain	>5% dehydration	Respiratory symptoms
				37.5–38.9°C	>39°C				
Ad40	14	14 (100)	11 (79)	7 (50)	1 (7)	8 (57)	1 (7)	2 (14)	3 (21)
Ad41	19	18 (95)	15 (79)	7 (37)	1 (6)	8 (42)	7 (37)	3 (16)	4 (21)
Established adenovirus	14	14 (100)	7 (50)	5 (36)	9 (64)[b]	14 (100)[c]	5 (36)	2 (14)	11 (79)[d]
Untyped adenovirus	8	8 (100)	6 (75)	2 (25)	3 (38)	5 (63)	4 (50)	1 (13)	2 (25)

[a] Footnotes denote significant differences between established adenoviruses and EAds (Ad40 and Ad41).
[b] $P < 0.001$ by two-tailed Fischer's exact test.
[c] $P < 0.01$ by two-tailed Fischer's exact test.
[d] $P < 0.001$ by two-tailed Fischer's exact test.

DISCUSSION

This study demonstrated that adenoviruses are strongly associated with acute infantile gastroenteritis. These viruses were found to be the second most frequently detected causative agents, and 13.5% of 416 diarrheal children displayed evidence of adenovirus infection. Approximately three-fifths of the isolates were identified as EAds, and thus these enteropathogens were the most important among the adenoviruses. EAds, represented by two species, Ad40 and Ad41, were recovered in 7.9% of the patients, whereas rotaviruses were found in 45% of the children. Similar detection rates for adenoviruses and rotaviruses have been reported by other Scandinavian workers (35, 37).

An etiological relationship between EAds and acute infantile gastroenteritis has been suggested (2, 25) and also questioned (12, 30). Our investigation strongly supported a causal relationship on the basis of the following: the observation of the shedding of adenoviral particles in association with diarrhea but not during convalescence, exclusion of other causative organisms and toxins, documentation of significant seroresponses, and lack of the same findings in the control group. Furthermore, other investigators (3, 5, 25) have documented hospital outbreaks of acute gastroenteritis caused by EAds, providing additional evidence of causality. A recent outbreak in Japan was due to Ad40 (3). The EAd responsible for the outbreak report by Richmond et al. (25) has since been made available to the present authors and identified as Ad40. Human volunteer studies, to obtain final proof of causation, have not yet been undertaken.

In previous studies we have examined the expressed genetic variability of EAds along two lines. By immunological characterization with ELISAs (8, 36), two different species of EAds, Ad40 and Ad41, have been demonstrated. By biochemical characterization of the polypeptide composition of virions (40a, 43) and DNA restriction site analysis (36, 43), Ad40 and Ad41 have been found to represent two new subgenera, F and G, respectively. On analysis by solid-phase immune electron microscopy (33), we observed a pronounced two-way cross-reaction between the two EAd species. Furthermore, with neutralization tests de Jong et al. (4) found Ad40 and Ad41 to be distinct, but with HI tests they were cross-reactive. The characteristics of Ad40 and Ad41 are summarized in Table 5. So far, there is no additional candidate species of EAd.

Of the patients with EAd infections, 58% shed Ad41 and 42% shed Ad40. The different species of established adenoviruses found, i.e., members of subgenera A, B, and C, were in accordance with observations in previous studies (6, 29). All these subgenera mainly infect infants and are associated with gastrointestinal disease (29, 32). Species of subgenus C have been regarded as endemic and have also been considered to cause respiratory symptoms (6). Similarly, the three patients in our control group with adenovirus infections, confirmed by virus isolation and seroresponses, all shed strains of subgenus C. The eight adenovirus infections that could not be typed included four detected only by seroconversion in CF tests. The remaining adenoviruses were found by EM (four cases) and by genus-specific ELISA (three cases). None of them grew in cell cultures or was detected by species-specific ELISA. These specimens may consist of new species of EAd.

ELISA and EM proved to be of equal sensitivity in the detection of EAds in stool specimens. In contrast, virus isolation was the best method for detecting established adenoviruses. We observed by EM that EAds were excreted in large amounts, whereas established adenoviruses were scanty. These findings may explain the low detection rate of

TABLE 5. Characterization of Ad40 and Ad41

Species	Subgenus	Growth characteristics	Polypeptide composition	DNA-restriction site pattern	Serum neutralization	HI	ELISA	SPIEM[a]
Ad40	F	Chang conjunctival, tCMK[b], and 293 cells	Unique	Unique	Distinct	Cross-reacts	Distinct	Cross-reacts
Ad41	G	Chang conjunctival, tCMK, 293, HEp-2, and HT-29 cells	Unique	Unique	Distinct	Cross-reacts	Distinct	Cross-reacts

[a] Solid-phase immune electron microscopy.
[b] Tertiary CMK.

established adenoviruses obtained with direct methods. It may also reflect different tissue tropism and different pathogenic mechanisms. EAds have been identified in intestinal cells (24, 44) but not in nasopharyngeal secretions (22), suggesting that these viruses are restricted to the gastrointestinal tract. Established adenoviruses persist and replicate in lymphatic tissue and can often be found in the nasopharyngeal tract (18).

For routine diagnosis of adenovirus antigen in stool samples, we prefer the genus-specific ELISA. If there are clear-cut respiratory symptoms in addition to diarrhea, a combination of ELISA and virus isolation appears suitable for detecting the established adenoviruses. With access to EM, solid-phase immune electron microscopy can be used to differentiate EAds from established adenoviruses. For discrimination between Ad40 and Ad41, the two species-specific ELISAs are, in our hands, the superior techniques; they are specific, sensitive, and simple to perform. In comparison, DNA restriction site analysis is not yet a routine method, but it can be used as an alternative. de Jong et al. (4) have described neutralization tests and serum neutralization of immunofluorescent foci as methods for typing of Ad40 and Ad41. Since EAds are difficult to grow even in selected cell cultures, these methods are tedious, and their use may therefore be hampered.

In our hands HI and ELISA were the best combination of methods for serological diagnosis of adenovirus infections. Each of the three techniques used, HI, ELISA, and CF, displayed different characteristics and advantages. HI proved to be specific for EAd infections but could not differentiate between Ad40 and Ad41. Besides the HI test, Kidd et al. (13) have described a neutralization test specific for EAds. There is still a need for species-specific ELISA tests to distinguish antibodies against Ad40 and Ad41.

Concerning the genus-specific tests, CF proved to be inefficient in detecting seroresponses in young children, a fact that has been documented from other studies (6). In spite of using an optimal antigen pool containing members of five subgenera, only one-third of the children below 2 years of age were found to have developed a significant rise in CF antibodies. However, CF was more efficient in diagnosing infections caused by established adenoviruses than by EAds. Further investigations are needed to elucidate this difference. On the whole we found ELISA to be the most sensitive technique for detection of a genus-specific adenoviral immunoresponse.

In this study adenoviruses were observed throughout the year and displayed no marked seasonal pattern, in contrast to the winter prevalence of rotaviruses. Ad40 and Ad41 showed similar seasonal distributions, with a small peak in July, when one-third of all gastroenteritis was due to EAds. Other investigations have observed similar peaks of adenoviruses in warmer months (2, 12, 15). However, an even distribution all through the year has also been found (26).

The two EAd species differed in their pattern with age. It was noteworthy that Ad40 infections predominated in patients younger than 12 months of age, whereas Ad41 infections were more common in children above that age. The established adenoviruses are a heterogenous group representing several subgenera, and the age distribution was consequently found to be uneven. Strains of subgenera A and C mainly infected children less than 2 years old, whereas the children with Ad7 infections were older; these findings are supported by other workers (6, 29).

An interesting observation of unknown implication was the discovery that two-thirds of the children with Ad41 infections were girls. This is in contrast to the frequently reported predominance in males among children with diarrhea (29).

The clinical picture of EAds was characterized by persistent diarrhea accompanied by fever and vomiting of short duration. The EAd species differed with regard to the duration of diarrhea. In patients with Ad41 infections the mean duration of diarrhea was 12.2 days, and prolonged symptoms commonly occurred. The diarrheal symptoms in association with Ad40 were more intense at the onset, and lasted for a mean of 8.6 days. The children with established adenoviruses, on the other hand, had diarrhea of shorter duration (mean, 6.2 days) and less frequent vomiting and had fever and higher temperatures significantly more often. The clinical features of untyped adenovirus infections resembled those of EAds, apart from diarrhea of significantly shorter duration (mean, 4.5 days). Significant dehydration was an uncommon feature of adenovirus gastroenteritis, and parenteral therapy was seldom required. Even though the symptoms were relatively mild, one-third of the patients with EAd infections and half of the patients infected with other adenoviruses were hospitalized. Upper respiratory symptoms were relatively uncommon in association with EAds (21%) but occurred significantly more frequently in connection with established adenoviruses (79%).

In a similar study of 22 children with EAd-associated gastroenteritis, Zissis et al. described the diarrhea as being not very acute but of unusually long duration (mean, 10 days) (G. Zissis, J. P. Lambert, and J. L. Fonteyne, Abstr. Int. Symp. Recent Adv. Enteric Infect., Brügge, Belgium, abstr. no. 27, 1981). In hospital outbreaks of gastroenteritis due to EAds the illnesses have been reported as clinically mild, lasting only 2 to 4 days (5), but also as severe and lasting for 7 days (25). In addition, a fatal case of EAd gastroenteritis has been reported (44), and this adenovirus isolate has since been typed by us as Ad41. Recently Yolken et al. (45) detected EAds in 52% of 27 children hospitalized for diarrhea during a 12-week study. Apart from this unusually high rate of gastroenteritis, the EAd was also found to be associated with respiratory symptoms in 93% of the children. These findings are not compatible with our observations; however, their study group consisted of institutionalized children, whereas ours consisted of children who acquired their infections outside the hospital.

In conclusion the present study documents that adenoviruses, especially the two EAd species, are important etiological agents in acute infantile gastroenteritis. Their role in developing countries is not known, and this question requires further study. EAds, particularly Ad41, cause long-lasting diarrhea and could contribute to malnutrition and chronic diarrheal states.

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