JOURNAL OF CLINICAL MICROBIOLOGY, June 1993, p. 1562–1569
0095-1137/93/061562-08$02.00/0

Vol. 31, No. 6

Detection, Typing, and Subtyping of Enteric Adenoviruses 40 and 41 from Fecal Samples and Observation of Changing Incidences of Infections with These Types and Subtypes

JAN C. DE JONG,[1]* KLAAS BIJLSMA,[1] ANTON G. WERMENBOL,[1] MARJA W. VERWEIJ-UIJTERWAAL,[1] HARRIE G. A. M. VAN DER AVOORT,[1] DAVID J. WOOD,[2]† ANDREW S. BAILEY,[2] AND ALBERT D. M. E. OSTERHAUS[3]

Laboratories of Virology[1] and Immunobiology,[3] Rijksinstituut voor Volksgezondheid en Milieuhygiëne, 3720 BA Bilthoven, The Netherlands, and North Manchester Regional Virus Laboratory, Booth Hall Hospital, Manchester, United Kingdom[2]

Received 16 July 1992/Accepted 8 March 1993

Monoclonal antibody (MAb) preparations specific for the enteric adenoviruses of subgenus F (AdF) were generated and evaluated as typing reagents in virus neutralization tests and enzyme-linked immunosorbent assays (ELISAs). A panel of 11 genome types of adenovirus 40 (Ad40), 24 genome types of Ad41, and 47 adenovirus prototype strains was used to determine the specificities of the MAbs in the two assays. In this way two MAbs, MAb 40-1 (anti-Ad40) and MAb 41-1 (anti-Ad41) were selected. These two MAbs showed strict type specificity in both assays. A third MAb reacted in an ELISA with all 47 human adenovirus types. With two other MAbs, three antigenic subtypes of Ad41 could be distinguished by their reactivities in virus neutralization tests and ELISAs. On the basis of the five selected MAbs, a sensitive ELISA system was developed for the direct detection and simultaneous typing and subtyping of Ad40 and Ad41 present in stool specimens. The five MAbs were also used to study the epidemiology of infections with Ad40 and Ad41 in The Netherlands in the period 1981 through 1989. It was shown that there were no significant fluctuations in the annual incidence of the cluster of enteric adenoviruses as a whole. This cluster should therefore be considered to belong to the "endemic" rather than the "epidemic" adenoviruses. The relative incidence of Ad40 infections compared with that of Ad41 infections changed considerably during the period studied; the proportion of Ad41 infections rose from about 30% in 1981 to about 95% in 1986, after which it stabilized at 90 to 95%. The proportion of one of the subtypes of Ad41 (Ad41 subtype M3) increased from about 40 to 80% in the same period.

Among the 47 types and candidate types of human adenoviruses recognized at present (16), the "enteric" or "fastidious" adenoviruses 40 and 41 (Ad40 and Ad41, respectively) constitute subgenus F (AdF) (9, 29, 33). It is more difficult to isolate and type members of this subgenus than the other human adenoviruses (3, 9, 11, 19), and only after the advent of monoclonal antibody (MAb)-based enzyme-linked immunosorbent assays (ELISAs) (14, 15, 26, 27), epidemiological reports on these highly prevalent human pathogens are becoming more frequent (2, 4, 6, 7, 13, 20, 21, 23, 25, 30, 31, 34). Extensive controlled studies by Brandt et al. (3) showed that the presence of AdF, in contrast to the presence of other adenoviruses (Ad non-F), in the stools of infants was associated with the occurrence of diarrhea.

Although Ad40 and Ad41 exhibit extensive cross-reactivity in virus neutralization (VN) assays and cannot be distinguished in hemagglutination inhibition assays with animal antisera (9), they are defined as separate types on the basis of major differences in DNA restriction enzyme (DRE) patterns (9, 29, 33). Since the definition of adenovirus types is primarily based on the antigenic differences found in VN tests (37), we developed, in line with earlier studies (14, 15, 26, 27), neutralizing MAbs for the typing of AdF in VN assays.

In the present study, we evaluated the specificities of the MAbs that we developed with a panel of Ad40 and Ad41 genome types. Part of this evaluation has been documented previously (32). The MAbs were subsequently used for the construction of an ELISA system for the direct detection and typing of AdF in stool specimens and for studies on the epidemiology of infections with Ad40 and Ad41 and its subtypes in The Netherlands from 1981 to 1989.

MATERIALS AND METHODS

Viruses, fecal specimens, and diagnostic procedures. Prototype strains of human Ad1 to Ad36 and Ad39 were obtained from the American Type Culture Collection (ATCC), Rockville, Md. Prototype strains Ad37, Ad38, Ad40, and Ad41 were isolated at the Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM) (8–10). Prototype strains Ad42 to Ad47 were received from R. Wigand, Homburg, Germany. For the determination of the specificities of the various MAbs within subgenus AdF, the panels of 11 genome types of Ad40 and 24 genome types of Ad41 described earlier (32) were used.

For other specificity studies, the following animal adenoviruses were used. Eighteen simian adenovirus prototype strains, canine adenovirus type 1, and mouse adenovirus FL were obtained from ATCC. The simian adenovirus (SA) prototypes included SA17, simian virus prototype 1 (SV1), SV11, SV15, SV17, SV20, SV23, SV25, SV27, SV30, SV31, SV32, SV33, SV34, SV36, SV38, SV39, and V340 (ATCC VR-541). Canine adenovirus type 2 was the vaccine strain Toronto A26/61. The strains were propagated at RIVM in cell cultures originating from the corresponding animal spe-

* Corresponding author.
† Present address: National Institute for Biological Standards and Control, Herts EN6 3QG, United Kingdom.

cies. The guinea pig adenovirus preparation was a sucrose gradient-purified suspension of lung tissue from infected animals.

Between 1981 and 1989, 6,666 fecal specimens from patients with gastroenteritis (mainly children) were sent by physicians to RIVM for diagnostic examination. Fecal specimens found to contain adenoviruses were used in the present study as 10% (vol/vol) fecal suspensions in phosphate-buffered saline (pH 7.2). The patients lived in various parts of The Netherlands, and multiple samples coming from the same household were excluded from the study. Diagnostic procedures carried out with these samples included conventional virus isolation in human embryonic fibroblasts and HEp-2 cell cultures and screening by an adenovirus genus-specific immunofluorescence test with fluorescein isothiocyanate-labeled gamma globulin from horse anti-Ad10 antiserum, after short-term cultivation of the sample in LLC-MK_2D cells for 48 h (SCIF-adeno) (9). The same specimens were also tested for rotavirus by a similar locally developed test (SCIF-rota). After 1987, the SCIF tests were replaced by the ELISAs described below. In total, 265 (4.0%) of the 6,666 fecal specimens were found to be positive for adenovirus in SCIF or ELISA and negative for adenovirus in the conventional virus isolation procedures. By inoculation of Graham 293 cell monolayers, AdF was isolated from all 265 specimens and was subsequently typed in VN assays. During the period 1981 to 1989, 1,453 (21.8%) of the 6,666 fecal samples scored positive in the SCIF-rota or the ELISA for rotavirus detection.

Additional AdF strains were part of earlier studies. Seven were from Glasgow, United Kingdom (19), four were from Atlanta, Ga. (11), and seven were from Sandringham, Republic of South Africa (17, 18). Seven fecal samples from the study of Scott-Taylor et al. (24) were kindly provided by those authors. A collection of 57 fecal specimens were selected by negative-contrast electron microscopy (EM), immune EM (IEM), or DRE mapping in Manchester, United Kingdom (39).

Virus isolation, VN tests, DRE analysis, and negative-contrast EM techniques. Ad40 and Ad41 were cultivated in Graham 293 or tertiary cynomolgus monkey kidney (tMK) cells maintained in Eagle's minimal essential medium supplemented with 0.5 μg of crystallized trypsin (Merck, Darmstadt, Federal Republic of Germany) per ml, as described previously (9, 10). Preparation of animal antisera, VN tests, and DRE analysis were also performed as described earlier (9, 32). Strains of AdF were typed in VN tests either with polyclonal animal antisera or with the type-specific MAbs from the present study. The MAb-based VN assays were performed without prior virus isolation. Three aliquots of a 10% fecal suspension were incubated with equal volumes of a fixed dilution (usually 1:500) of MAb 40-1 (anti-Ad40), a fixed dilution (usually 1:100) of MAb 41-1 (anti-Ad41), or dilution buffer, respectively. After 30 min at room temperature, the mixtures were used to inoculate tMK cell monolayers in tubes which were subsequently incubated in roller drums at 37°C. Usually, the type could be read after 2 or 3 days. EM and IEM techniques have been described previously (39).

Development of MAbs. (i) MAbs to Ad40. Ad40 strain Dugan (ATCC VR-931) was propagated in tMK cells. About 350 ml of the supernatant of an infected cell culture showing complete cytopathic changes was concentrated 20-fold by ultrafiltration, and the virus was purified in a CsCl gradient, which yielded about 10^{12} physical virus particles (about 0.1 mg). This preparation was used for immunization of BALB/c mice. The methods used for hyperimmunization of mice, fusion of spleen cells with P3/X-63 Ag 8-653 myeloma cells, and selective growth of hybridoma cells and production of ascites have been described previously (22). Supernatants of hybridoma cell cultures were screened by VN tests with an amount of Ad40 capable of producing a cytopathic effect in 25% of tMK cells after incubation at 37°C for 3 days. The supernatants were also screened by ELISA (see below). Selected hybridomas were cloned twice by the limiting dilution method.

(ii) MAbs to Ad41. Prototype Ad41 strain Tak (ATCC VR-930) was propagated in HEp-2 cells and was concentrated, purified, and used for the generation of MAbs by the procedure described above.

ELISA. The wells of high-binding-capacity microplates (Nunc, Roskilde, Denmark) were coated with gamma globulin. The gamma globulin was obtained by caprylic acid precipitation from horse antiserum to Ad10 and was resolved in a carbonate buffer (pH 9.6) to a concentration of 20 μg/ml. The coating was performed at pH 9.6 for 2 h at 37°C or overnight at 4°C. The wells were washed with phosphate-buffered saline (pH 7.2) containing 0.05% (vol/vol) Tween 20, 100 μl of a 1 or 10% (vol/vol) fecal suspension was added to each well, and the plates were incubated at 37°C for 2 h. The wells were washed again as described above and the plates were incubated for 1 h at 37°C with 100 μl of dilutions of MAbs specific either for adenovirus genus antigen, Ad40, or Ad41, with each MAb being applied in a different well. The MAbs were purified by sodium sulfate precipitation, labeled with horseradish peroxidase (Nordic, Tilburg, The Netherlands), and diluted in phosphate-buffered saline (pH 7.2) containing 0.5% (vol/vol) Tween 20 and 0.5% (wt/vol) bovine serum albumin. For each conjugated MAb, the dilution in use was attuned to the least reactive genome types, e.g., Ad41/M3 subtype for MA41-1 (see Table 4). In practice, 2 μg of MAb 40-1 per ml and 4 μg of MAb 41-1 per ml was used. After washing, 3,3',5,5'-tetramethylbenzidine (Sigma, St. Louis, Mo.) was added as a substrate. The enzyme reaction was stopped with sulfuric acid after 10 min, and the extinction coefficient measured at 450 nm.

Samples were considered positive when the extinction coefficient was reproducibly 0.100 units greater than the value of the control well without sample. This cutoff value is based on the results obtained with a series of 144 fecal specimens which did not test positive for adenovirus in virus culture assays on Graham 293 cells. Their extinction coefficients did not exceed the control value by more than 0.040. If the extinction coefficient found with MAb d-g1 (antigenus) was greater than 1.000 and those produced by the AdF-specific MAbs were less than 0.100, the specimen was considered to contain Ad non-F. ELISA titers were defined as the reverse of the highest dilution of antigen- or antibody-containing preparations which showed, after correction for the control value, an extinction coefficient of greater than 0.100. In the present report, the term "typing-ELISA" refers to the ELISAs with conjugated MAb 40-1, MAb 40-2, and MAb d-g1 described in this paragraph, whereas the term "genus-specific ELISA" refers to the ELISA with MAb d-g1 only. All positive and borderline-negative samples were repeat tested and checked by virus culture when the diagnosis of patients was involved. All viruses isolated were typed by the VN test. The presence of Ad40 or Ad41 was also confirmed in the ELISA in a blocking test. Equal volumes of fecal specimen and polyclonal anti-Ad40 or anti-Ad41 antiserum were incubated at 37°C for 1 h. Reduc-

TABLE 1. Reactivities of MAbs in VN tests and the ELISA

MAb[a]	Isotype	Reactivities in VN tests			Reactivities in ELISA		
		Ad40 (n = 11[b])	Ad41 (n = 24[b])	Non-F (n = 45[c])	Ad40 (n = 11[b])	Ad41 (n = 24[b])	Non-F (n = 45[c])
MA40-1	IgA	11	–[d]	–	11	–	–
MA40-2	IgG1	11	–	NT[e]	10	–	–
MA40-3	IgG2a	–	–	NT	11	–	–
MA40-4	IgG2a	–	–	NT	10	–	–
MA41-1	IgG2a	–	24	–	–	24	–
MA41-2	IgG3	–	12	NT	–	12	–
MA41-3	IgG2b	–	6	NT	–	6	–
MAd-g1	IgG2a	–	–	–	11	24	45
MAd-g2	IgG1	NT	NT	NT	11	24	45
MAd-g3	IgG1	NT	NT	NT	11	24	31
MAd-g4	IgG2b	NT	NT	NT	–	18	9

[a] MAbs MA40-1 and MA41-1 have been used in earlier work (32, 36, 40, 41) under the designations 5-8 and 5-15, respectively, and MAbs MA41-2, and MA41-3 have been applied in a previous study (32) under the designations 1-23 and 7-14.
[b] Number of genome types (DNA variants).
[c] Number of serotypes.
[d] No reactivity with any of the genome types or serotypes: titer <10.
[e] Not tested.

tion of the extinction coefficient by 80% or more was considered proof of the presence of the virus concerned.

MAb 40-1 and MAb 41-1 also formed the basis of an experimental kit for commercial distribution (40). Because of substantial differences in design, the performances of this assay cannot be considered equal to those of the ELISA described here. The commercial kit was not available to us for comparison and has not yet been marketed.

For detection of rotavirus by ELISA, a sandwich-type assay based on locally prepared polyclonal anti-SA-11 antiserum was used.

RESULTS

Generation of MAbs against AdF. Hybridomas producing MAbs that were reactive with AdF were generated from BALB/c mice immunized with Ad40 or Ad41. Fifteen hybridomas were selected on the basis of their specificity profiles in VN tests or ELISAs with the aid of the panel of 11 genome types of Ad40, 24 genome types of Ad41, and 45 Ad non-F prototypes. The following specificities were found for these MAbs (Table 1).

(i) MAbs specific for Ad40. Four hybridomas obtained from mice immunized with Ad40 produced MAbs (MAb 40-1 to MAb 40-4) that recognized Ad40 but not any of the other adenoviruses, including Ad41. All MAbs reacted in the ELISA (titers of ascitic fluids, 10^4 to 10^5), and two MAbs (MAb 40-1 and MAb 40-3) also reacted in VN tests (titers of ascitic fluids, 10^5 to 10^6) with all or most of the 11 genome types of Ad40 but with none of the 24 genome types of Ad41 or 45 Ad non-F prototypes (Tables 2 and 3).

(ii) MAbs specific for Ad41. Three of the hybridomas obtained from Ad41-immunized mice were selected on the basis of the reactivities of their MAbs (MAb 41-1, MAb 41-2, and MAb 41-3) in both VN tests and ELISA, with all or part of the 24 genome types of Ad41 but not with any of the other adenoviruses, including Ad40 (Tables 2 and 4). Three antigenic subtypes of Ad41 (Ad41/M1, Ad41/M2, and Ad41/M3) were distinguished among the 24 genome types on the basis of the specificities of the three selected MAbs in VN tests (Table 2). Ad41/M1 was recognized by all three MAbs, Ad41/M2 was recognized by MAb 41-1 and MAb 41-2, and Ad41/M3 was recognized only by MAb 41-1. The same

TABLE 2. Neutralization titers of MAbs (mouse ascitic fluids) and rabbit antiserum reactive with Ad40 and Ad41

Groups of genome types	Subtype	Median $\log_{10}$ neutralization titer[a] of MAb or RA[b]						DNA site[c]	
		MA40-1	MA40-2	MA41-1	MA41-2	MA41-3	RA[b]	E_1	H_3
Ad40/D1-11 except D8		5.4–6.3	6.0–7.2	—[d]	—	—	1.4 - 2.4		
Ad40/D8		5.6	3.6	—	—	—	1.6		
Ad41/D1,3,6,7,8,17	M1	—	—	5.0–5.7	3.0–3.9	4.5–5.1	2.4–3.0	NS	NS
Ad41/D2,5,9,10,14,21	M2	—	—	5.1–6.3	3.2–3.8	—	2.2–3.2	+	NS
Ad41/D4,11,12,15,16,18, 19,20,22,23,24	M3	—	—	3.8–4.5	—	—	2.2–3.4	NS	+
Ad41/D13	M3	—	—	5.7	—	—	3.2	NS	+
Non-F prototypes Ad1-47		—[e]		—[e]			—		

[a] Antibody preparations were titrated in twofold dilutions in duplicate tests against each of 11 genome types of Ad40 and each of 24 genome types of Ad41. Standard error of the mean, 0.15.
[b] RA, rabbit antiserum to Ad41/D1/Tak/Netherlands/73 (M1).
[c] DNA restriction enzyme sites for *Eco*RI (E_1) and *Hin*dIII (H_3), respectively. NS, no site.
[d] —, titer <1.0.
[e] MA40-1 and MA41-1 did not react to non-F prototypes Ad1-47.

TABLE 3. ELISA titers of MAbs (mouse ascitic fluids) reactive with Ad40 only or with both AdF and non-F adenoviruses (indirect tests)

Genome types	Median $\log_{10}$ ELISA titer of MAb[a]							
	40-1	40-2	40-3	40-4	MAd-g1	MAd-g2	MAd-g3	MAd-g4
Ad40/D1-11 except D7,8	4–5	2–3	4–5	4–5	3–5	5–6	4–5	—[b]
Ad40/D7	2.5	1.5	1.5	0.5	3	3	4.5	—
Ad40/D8	4	0.5	3.5	4	3.5	3.5	4.5	—
Ad41	—	—	—	—	4–5	5–6	4–5	var[c]
Non-F prototypes Ad1-47	—	—	—	—	3–5	5–6	2–5	var

[a] MAbs were titrated in 10-fold dilutions in duplicate tests against each of 11 genome types of Ad40 and each of 24 genome types of Ad41. Standard error of the mean, 0.5.
[b] —, titer <10.
[c] Various reactivities, see text.

specificities of these MAbs were found in the ELISA (Table 4).

From both theoretical and practical points of view, it is interesting that quantitative differences in the reactivities of some of the MAbs were observed with different subtypes of Ad41; the titers of MAb 41-1 against subtypes Ad41/M1 and Ad41/M2 were 40- to 100-fold higher than the titers against subtype Ad41/M3 in both VN tests and ELISA (Tables 2 and 4). Ad41 subtypes M1, M2, and M3 could not be distinguished in VN assays with hyperimmune rabbit antiserum to Ad41/D1 strain Tak (Table 2).

(iii) MAbs reactive with both AdF and Ad non-F. Four hybridomas were obtained from Ad41-immunized mice; the hybridomas produced MAbs (MAb d-g1, MAb d-g2, MAb d-g3, and MAb d-g4) that bound in the ELISA to all or part of the AdF genome types but also to Ad non-F (Table 3). MAb d-g1 did not exhibit activity in the VN test. MAb d-g1 and MAb d-g2 reacted with all of the adenoviruses tested, including the 11 genome types of Ad40 and the 24 genome types of Ad41. MAb d-g3 bound to all 35 genome types of AdF and also to all Ad non-F prototypes, although up to 1,000-fold differences in titers were found with the various Ad non-F types. MAb d-g4 reacted with adenovirus types 3, 7, 9, 11, 14, 15, 16, 21, 34, 35, 41, and 47. However, of Ad41, only subtypes Ad41/M1 and Ad41/M3 were recognized by this MAb, which offered an extra opportunity to discriminate between the three subtypes. As was also demonstrated for the reactivities of MAb 41-1 to the Ad41 subtypes, some of the genus-specific MAbs showed quantitative differences in their reactivities with some of the genome types of Ad40 and some of the Ad non-F prototypes (Table 3).

TABLE 4. ELISA titers of MAbs (mouse ascitic fluids) reactive with Ad41 (indirect tests)

Genome types	Subtype	Median $\log_{10}$ ELISA titer of MAb[a]			
		41-1	41-2	41-3	MAd-g4
Ad40/D1-11		—[b]	—	—	—
Ad41/D1,3,6,7,8,17	M1	4–5	3–4	3–4	4.0
Ad41/D2,5,9,10,14,21	M2	4–5	3–4	—	—
Ad41/D4,11,12,13,15,16, 18,19,20,22,23,24	M3	2–3	—	—	4.0
Non-F prototypes Ad1-47		—	—	—	var[c]

[a] MAbs were titrated in 10-fold dilutions in duplicate tests against each of 11 genome types of Ad40 and each of 24 genome types of Ad41. Standard error of the mean, 0.5.
[b] —, titer <10.
[c] Various reactivities, see text.

MAb-based typing ELISA for Ad40 and Ad41. MAbs 40-1, 41-1, and d-g1, which reacted with Ad40, Ad41, and all human adenoviruses, respectively, were incorporated in an ELISA system for the detection and typing of AdF in clinical specimens. The sensitivity of this typing ELISA system was compared with those of virus detection by SCIF, EM, and virus isolation procedures by using panels of clinical specimens. The specificity of the ELISA was assessed by conducting VN tests with adenovirus type-specific antisera and by using reference preparations of other enteric viruses.

(ii) Comparison of the typing ELISA with virus isolation and EM. Among 159 fecal specimens collected from the same number of epidemiologically unrelated diarrheic children, 8 scored positive in both the typing ELISA and virus isolation tests in Graham 293 cells (Table 5). Of these samples, six contained AdF and two contained Ad non-F. In addition, four specimens scored positive only in the virus isolation assay. All four isolates from these specimens proved to be Ad non-F, however. Among 853 fecal samples of similar origin, 41 scored positive in the genus-specific ELISA. Virus isolation showed that 10 of these samples and 20 ELISA-negative specimens contained Ad non-F. Thus, as expected, the sensitivity of the typing ELISA for the detection of Ad non-F is lower than that of virus isolation.

All of the 44 fecal samples which scored positive for adenovirus by EM and containing AdF, as confirmed by VN assays, were also positive in the typing ELISA. Thirty-nine of 43 fecal samples in which AdF was demonstrated by the typing ELISA and virus isolation also scored positive by EM.

By using another approach for assessing the sensitivity of the ELISA, three pools of five fecal samples each were prepared; these fecal samples contained the most prevalent genome types of Ad40 and Ad41 in The Netherlands, i.e., Ad40/D1, Ad41/D2, and Ad41/D12, respectively (32). Serial threefold dilutions of these pools were tested in the typing ELISA and by the EM and virus isolation procedures. For

TABLE 5. Comparison of the typing ELISA with virus isolation tests with fecal specimens from diarrheic children

ELISA result	No. of specimens with the following result by virus isolation tests			Total
	+ (AdF)	+ (Ad non-F)	−	
+	6	2	0	8
−	0	4	147	151
Total	6	6	147	159

TABLE 6. Comparison of the typing ELISA with a genus-specific immunofluorescence test (SCIF-adeno) with fecal specimens from diarrheic children

ELISA result	No. of specimens with the following result by SCIF-adeno			Total
	+ (AdF)	+ (Ad non-F)	−	
+	18	4	0	22
−	1	5	472	478
Total	19	9	472	500

each pool, the ELISA was still positive at dilutions of between $10^{-4.3}$ and $10^{-4.8}$ and EM was still positive at dilutions of 10^{-3}, whereas virus was still isolated at dilutions of up to $10^{-5.8}$ (Ad40/D1) and $10^{-7.3}$ (Ad41/D2 and Ad41/D12).

In order to determine the smallest quantity of viral antigen that could be detected by the newly developed ELISA, Ad40 strain Dugan and Ad41 strain Tak were purified in CsCl gradients. Serial threefold dilutions of these purified virus preparations were tested in the typing ELISA. MAb 40-1 proved to be able to detect 0.01 μg of Ad40, MAb 41-1 detected 0.05 μg of Ad41, MAb 41-2 detected 0.09 μg of Ad41, MAb 41-3 detected 0.05 μg of Ad41, and MAb d-g1 detected 0.04 μg of Ad40 and 0.13 μg of Ad41 antigen per ml.

(ii) Comparison of the typing ELISA with SCIF-adeno. Among 500 fecal samples from diarrheic children, 22 scored positive in both assays (Table 6). Upon virus isolation, 18 of these specimens yielded AdF and 4 yielded Ad non-F. No sample was positive only in the typing ELISA. Six were positive only in the SCIF, and only one of the corresponding six isolates belonged to subgenus F. In order to compare the sensitivities of the ELISA and the SCIF more accurately, 116 SCIF-positive stool samples containing Ad40 or Ad41 were tested in the ELISA. A total of 113 of these specimens were positive by the ELISA.

(iii) Specificity of the typing ELISA. Sixty fecal samples from which Ad40 was isolated and 139 fecal samples from which Ad41 was isolated were tested in the typing ELISA. All scored positive. In 197 cases (99%), the typing results agreed with the results of the VN assays. In two samples which yielded only Ad41 by the virus isolation method, the ELISA demonstrated the presence of both Ad40 and Ad41. Seven fecal samples originating from Canada that contained Ad41 and that had escaped detection by a commercial MAb-based ELISA (24) scored positive for Ad41 in the typing ELISA.

All 18 simian adenovirus prototype strains tested proved to react with MAb d-g1 and MAb d-g2 in the genus-specific ELISA. The same MAbs in the same assay, however, produced negative results with adenoviruses from other animals, including mice, guinea pigs, and dogs.

All 12 fecal samples shown to contain rotavirus, astrovirus, calicivirus, or Norwalk virus (three samples for each virus) by EM scored negative in the typing ELISA.

Epidemiological studies concerning Ad40 and Ad41 infections. (i) Age distributions of individuals with symptomatic Ad40 and Ad41 infections. On the basis of the results of diagnostic testing of 6,666 fecal specimens from diarrheic patients in The Netherlands in the period 1981 to 1989, the age distributions for diarrhea caused by Ad40 and Ad41 proved to be similar to those for gastroenteritis caused by rotavirus (Fig. 1). It is interesting that the adenovirus infections occurred most frequently in the first half year of life, whereas the rotavirus-associated diarrhea was most prevalent in the second half of the first year of life.

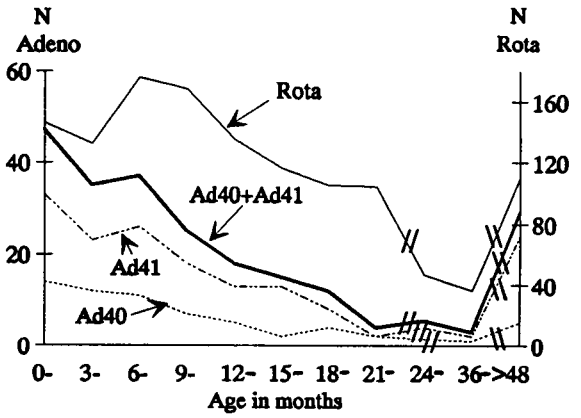

FIG. 1. Age distributions of infections with Ad40, Ad41, and rotavirus in the period 1981 through 1989. The curve marked "Ad40 + Ad41" represents the combined numbers of infections with Ad40 and Ad41. On the abscissa, 0 means 0 through 2 months of age, 3 means 3 through 5 months of age, and so on.

(ii) Monthly distributions of Ad40 and Ad41 infections. Data for the period 1981 through 1989 demonstrate that Ad40 and Ad41 infections are not subject to major seasonal influences, although they seemed to occur slightly more frequently in winter and early autumn months (Fig. 2). For comparison, the monthly incidences, over the same period, of infections with the subgenus C viruses Ad1 and Ad2 and with the subgenus B viruses Ad3 and Ad7 are presented in Fig. 3.

(iii) Annual distributions of Ad40 and Ad41 infections. The incidence of AdF infections did not vary greatly from year to year during the study period 1981 through 1989 (Fig. 4). The distributions of infections with Ad1, Ad7, and rotavirus, diagnosed during the period 1961 through 1989, are shown for reference purposes in Fig. 4.

(iv) Changing relative incidence of Ad40 infections compared with that of Ad41 infections. Although the total yearly number of AdF isolations did not show major variations between 1981 and 1989, the ratio of the frequency of isolation of Ad40 compared with that of Ad41 gradually changed

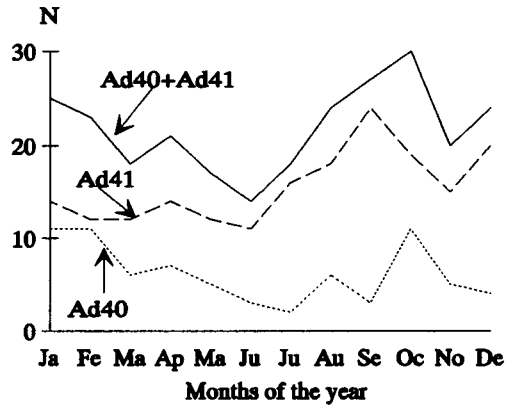

FIG. 2. Monthly distributions of infections with Ad40 and Ad41 in the period 1981 through 1989. The designation "Ad40 + Ad41" is the same as that described in the legend to Fig. 1.

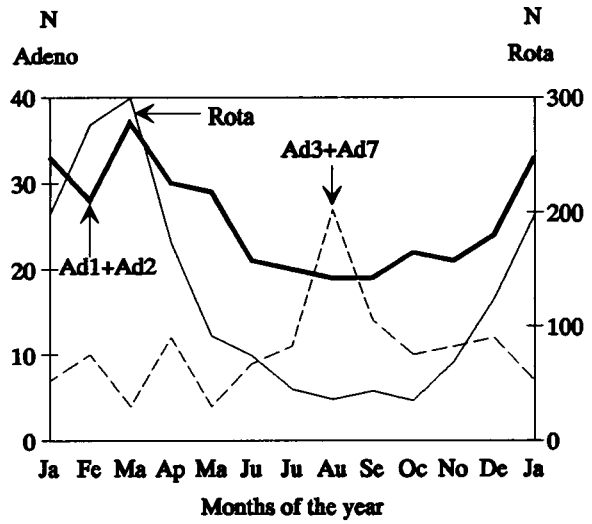

FIG. 3. Monthly distributions of infections with nonenteric adenoviruses and rotavirus in the period 1981 through 1989. The designations "Ad1 + Ad2" and "Ad3 + Ad7" have meanings analogous to that for "Ad40 + Ad41" described in the legend to Fig. 1.

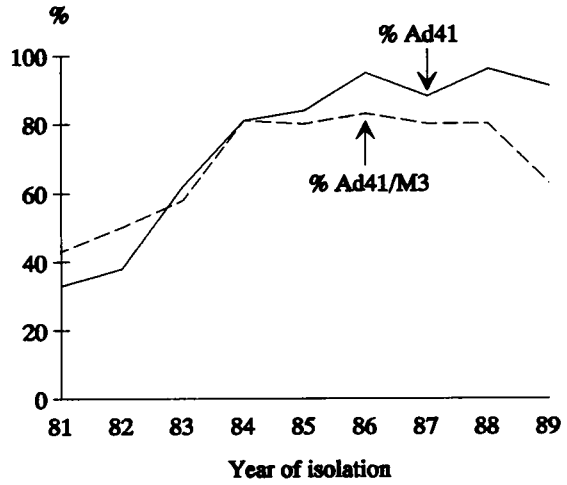

FIG. 5. Proportions of Ad41 and Ad41/M3 infections in the period 1981 through 1989. The percentages of the number of Ad41 infections relative to the total number of AdF infections and the percentages of the number of Ad41/M3 infections relative to the total number of Ad41 infections per year are plotted.

from about 2:1 in 1981 to about 1:20 in 1986 and thereafter (Fig. 5). The changing ratio between the frequencies of isolation of Ad40 and Ad41 coincided roughly with a shift in the proportions of the incidences of the three subtypes (Ad41/M1 to Ad41/M3) within Ad41, which we described above. Before 1984, only Ad41 strains of subtype M1 were found, whereas Ad41/M2 strains accounted for about half of the Ad41 strains isolated before 1983 and gradually declined in number thereafter. The increase in the proportion of Ad41/M3 strains among the Ad41 strains paralleled the rise in the proportion of Ad41 strains among AdF as a whole (Fig. 5). Ad41/M3 strains made up about 40% of the Ad41 strains isolated in 1981, and this increased to about 80% from 1984 onward.

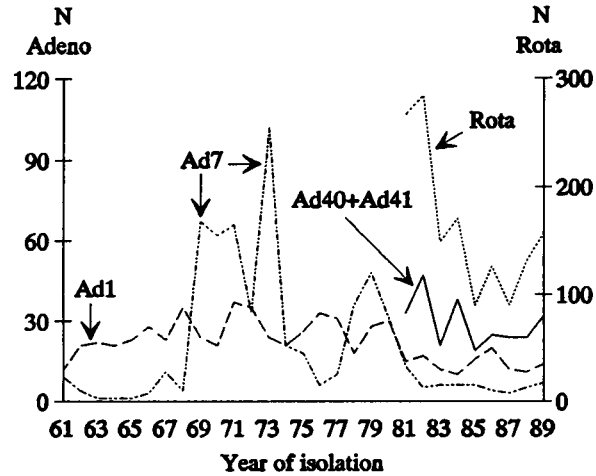

FIG. 4. Annual distributions of Ad1 and Ad7 infections in the period 1961 through 1989 and annual distributions of AdF and rotavirus infections in the period 1981 through 1989. The meaning of the designation "Ad40 + Ad41" is the same as that described in the legend to Fig. 1.

DISCUSSION

In the present report and in line with earlier studies (14, 15, 26, 27), we described the development of a panel of MAbs that can be used for the rapid detection and typing of Ad40 and Ad41. Some of these MAbs were also used in previous studies (32, 36, 40, 41) (Table 1). In view of the close antigenic relationship between Ad40 and Ad41 in VN assays and the marked antigenic variability within these two types (9), there is a risk that anti-AdF MAbs would react with certain strains of both types or, alternatively, that they may not react with all the strains of the type concerned. Unlike earlier studies (14, 15, 26, 27), our study reduced this risk by assessing the specificities of the anti-AdF MAbs against a panel of 35 genome types of Ad40 and Ad41. The relevance of this problem was illustrated by the finding of Scott-Taylor et al. (24) that a commercial MAb-based ELISA failed to demonstrate the presence of a highly prevalent genome type in Canada. This variant is closely related or identical to Ad41/D12, which is highly prevalent in The Netherlands. As expected, seven fecal specimens from the Canadian study proved to be positive in our typing ELISA.

Evaluation of the specificities of the newly developed MAbs showed that some of the MAbs indeed failed to react with a proportion of the strains within the type involved (Tables 1–4). Even when they did recognize all the genome types within one type, they sometimes displayed different levels of reactivity to these variants (MA41-1 in Tables 2 and 4). Three MAbs were selected for the detection and typing of AdF strains. Type-specific MAb 40-1 and MAb 41-1 could be used in a type-specific ELISA, and genus-specific MAb d-g1 could be used in a genus-specific ELISA. These ELISAs were used to detect the presence of Ad40 or Ad41, and of adenoviruses in general, directly in stool samples.

The possibility of the existence of Ad40 or Ad41 strains lacking the epitopes recognized by MAb 40-1 and MAb 41-1 or even novel types of AdF should be considered. Such variants would, however, still be detected by the genus-specific ELISA. Subsequent typing with polyclonal antisera in VN tests would then identify any deviant variant of Ad40

or Ad41. The reactivities of the genus-specific MAbs d-g1 and d-g2 were shown to be limited to the adenoviruses that infect primates. They failed to detect adenoviruses from nonprimate animals, including dogs, guinea pigs, and mice. In contrast, MAb 2Hx-2 described by Cepko et al. (5) reacted with adenoviruses from primates as well as with canine, bovine, porcine, and murine adenoviruses.

The sensitivity of the typing ELISA proved to be greater than that of EM and roughly the same as that of virus isolation in Graham cells. However, the ELISA is more rapid and less laborious than the two other techniques. Moreover, several fecal samples scored positive by the typing ELISA and EM but were negative by the virus isolation procedure (data not shown).

On the basis of these results and by using the same MAbs, an ELISA for the detection and typing of AdF was designed; this ELISA will soon become commercially available (40). The same MAbs were also applied successfully by others in typing enteric adenoviruses for diagnostic purposes (36) and in IEM analyses of fecal samples (41).

From a clinical point of view, a major advantage of MAb-based ELISAs over tests with polyclonal antibodies (12) is their ability to differentiate between enteric and nonenteric adenoviruses. This is of diagnostic importance because, in contrast to AdF, the presence of nonenteric adenoviruses in infant stools is not associated with diarrhea (2, 3). Although the polyclonal antibody-based tests generally do not detect nonenteric adenoviruses in stool specimens because the levels of virus excretion are too low, a certain proportion of such samples does score positive (unpublished data), which may lead to erroneous conclusions. Another diagnostic advantage of the MAb-based ELISA system is the ability to discriminate between Ad40 and Ad41 infections. Although the direct clinical implications of this differentiation seem limited (29), the present study demonstrates its special relevance for epidemiological investigations.

Our epidemiological studies showed that 4.0% of 6,666 fecal samples collected previously from diarrheic children in The Netherlands scored positive in the typing ELISA. Of the same collection of specimens, 21.8% scored positive for rotavirus. Similar percentages have been found in other countries: 4.1% (2), 7.9% (30), and 5.2% (20) for AdF and 45% (30) and 21% (20) for rotavirus. Rotavirus infections peaked markedly in the winter months, whereas AdF appeared to circulate more uniformly throughout the year (Fig. 2 and 3). These findings are in accordance with observations by investigators in Britain and Sweden (23, 29) and show a correspondence with the seasonal pattern of the endemic Ad1 and Ad2 infections rather than with the epidemic Ad3 and Ad7 infections (Fig. 2). Furthermore, AdF infections prevailed in the first half year of life and rotavirus infections prevailed in the second half year of life (Fig. 1), which is in agreement with the trends noted by investigators in the United States and Britain (2, 23, 38).

A comparison of the numbers of isolations of Ad40 and Ad41 made in The Netherlands by our laboratory over the period 1981 through 1989 revealed a decrease in the rate of isolation of Ad40 and a concomitant increase in the rate of isolation of Ad41 (Fig. 1). The total annual number of AdF isolates recovered, however, remained about the same (Fig. 2). If, on the basis of the close antigenic relationship in VN tests, Ad40 and Ad41 are considered one antigenic cluster, the incidence of infections with this cluster is subject to relatively minor variations over the period involved. Although data from only a limited period could be studied, this observation also indicates that AdF should be classified among the endemic group of adenoviruses (28, 37).

The MAbs developed in the present study distinguished three subtypes of Ad41, the antigenic differences of which correlated with the presence or absence of two DNA restriction sites within the hexon gene (Table 2), which is responsible for type-specific reactivity (1, 32).

The three subtypes observed were not evenly distributed over the observation period. Ad41/M3 took over from Ad41/M1 and Ad41/M2 to become the major subtype in The Netherlands since 1983. In this connection it is interesting that subtype Ad41/M3 (genome type Ad41/D4) was already identified in Malaysia in 1979 (17). The proportional increase in the circulation of subtype M3 within Ad41 coincided with the overall rise in the incidence of Ad41 (Fig. 4). It is possible that Ad41/M3 not only superseded Ad41/M1 and Ad41/M2 but also superseded Ad40. The cross-reactivity between the two adenovirus types in VN tests (9) may have resulted in a mutual competition in infecting susceptible individuals.

A similar shift may have occurred in the United Kingdom. Of 10 Ad41-positive fecal specimens collected in Manchester in 1986 and 1987, 4 were shown to contain Ad41/M2 and 6 were shown to contain Ad41/M3 viruses by the MAb-based ELISA. In contrast, 25 of 26 Ad41-positive fecal samples collected in Manchester in 1988 proved to be Ad41/M3 (unpublished data). Recently, the disappearance of Ad40 from the United Kingdom (Newcastle upon Tyne) (38), Canada (Toronto) (4), and Japan (Tokyo) (25) in about 1985 was reported. Therefore, we speculate that a similar shift of the Ad41 subtype has formed the basis for the epidemiological phenomena in all the countries studied.

The serial succession of subtypes of Ad41 described here reveals the change in certain antigenic sites during successive years, a phenomenon reminiscent of the events evolving from interactions between the human immune system and influenza viruses (35).

ACKNOWLEDGMENTS

The excellent technical assistance of Timo P. L. Zomerdijk, Gerrie Verwaaijen, Mieke Snoek, and Heinz Broeders, the gift of simian adenoviruses from G. van Steenis, the supply of fecal specimens and computerized data by Jan Vinjé and Dirk Sanders, and the attention of Nel van Dijk and Jolanda Pathuis to the manuscript are gratefully acknowledged.

The work was supported by grant C6/181/198 from the Diarrhoeal Diseases Control Programme of the World Health Organization.

REFERENCES

1. **Boursnell, M. E. G., and V. Mautner.** 1981. Recombination in adenovirus: crossover sites in intertypic recombinants are located in regions of homology. Virology **112**:198–209.
2. **Brandt, C. D., H. W. Kim, W. J. Rodriguez, J. O. Arrobio, B. C. Jeffries, E. P. Stallings, C. Lewis, A. J. Miles, M. K. Gardner, and R. H. Parrott.** 1985. Adenoviruses and pediatric gastroenteritis. J. Infect. Dis. **151**:437–443.
3. **Brandt, C. D., H. W. Kim, R. H. Yolken, A. Z. Kapikian, J. O. Arrobio, W. J. Rodriguez, R. G. Wyatt, R. M. Chanock, and R. H. Parrott.** 1979. Comparative epidemiology of two rotavirus serotypes and other viral agents associated with pediatric gastroenteritis. Am. J. Epidemiol. **110**:243–254.
4. **Brown, M.** 1990. Laboratory investigation of adenoviruses associated with gastroenteritis in Canada from 1983 to 1986. J. Clin. Microbiol. **28**:1525–1529.
5. **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.

6. **Christensen, M. L.** 1989. Human viral gastroenteritis. Clin. Microbiol. Rev. **2**:51–89.
7. **Cruz, J. R., P. Cáceres, F. Cano, J. Flores, A. Bartlett, and B. Torún.** 1990. Adenovirus types 40 and 41 and rotaviruses associated with diarrhea in children from Guatemala. J. Clin. Microbiol. **28**:1780–1784.
8. **de Jong, J. C., R. Wigand, T. Adrian, J. C. Hierholzer, J. G. Kapsenberg, C. J. Muzerie, and A. G. Wermenbol.** 1984. Adenovirus 38: a new human adenovirus species of subgenus D. Intervirology **22**:164–169.
9. **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: enteric adenoviruses from human infant stool. J. Med. Virol. **11**:215–231.
10. **de Jong, J. C., R. Wigand, G. Wadell, D. Keller, C. J. Muzerie, A. G. Wermenbol, and G. J. P. Schaap.** 1981. Adenovirus 37: identification and characterization of a medically important new adenovirus type of subgroup D. J. Med. Virol. **7**:105–118.
11. **Gary, G. W., Jr., J. C. Hierholzer, and R. E. Black.** 1979. Characteristics of noncultivable adenoviruses associated with diarrhea in infants: a new subgroup of human adenoviruses. J. Clin. Microbiol. **10**:96–103.
12. **Grandien, M., C. A. Pettersson, L. Svensson, and I. Uhnoo.** 1987. Latex agglutination test for adenovirus diagnosis in diarrheal disease. J. Med. Virol. **23**:311–316.
13. **Herrmann, J. E., N. R. Blacklow, D. M. Perron-Henry, E. Clements, D. N. Taylor, and P. Echeverria.** 1988. Incidence of enteric adenoviruses among children in Thailand and the significance of these viruses in gastroenteritis. J. Clin. Microbiol. **26**:1783–1786.
14. **Herrmann, J. E., D. M. Perron-Henry, and N. R. Blacklow.** 1987. Antigen detection with monoclonal antibodies for the diagnosis of adenovirus gastroenteritis. J. Infect. Dis. **155**:1167–1171.
15. **Herrmann, J. E., D. M. Perron-Henry, D. Stobbs-Walro, and N. R. Blacklow.** 1987. Preparation and characterization of monoclonal antibodies to enteric adenovirus types 40 and 41. Arch. Virol. **94**:259–265.
16. **Hierholzer, J. C., R. Wigand, L. J. Anderson, T. Adrian, and J. W. M. Gold.** 1988. Adenoviruses from patients with AIDS: a plethora of serotypes and a description of five new serotypes of subgenus D (types 43-47). J. Infect. Dis. **158**:804–813.
17. **Kidd, A. H.** 1984. Genome variants of adenovirus 41 (subgroup G) from children with diarrhoea in South Africa. J. Med. Virol. **14**:49–59.
18. **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.
19. **Kidd, A. H., and C. R. Madeley.** 1981. In vitro growth of some enteric adenoviruses from stool specimens. J. Clin. Pathol. **34**:213–216.
20. **Kotloff, K. L., G. A. Losonsky, J. G. Morris, S. S. Wasserman, N. Singh-Naz, and M. M. Levine.** 1989. Enteric adenovirus infection and childhood diarrhea: an epidemiological study in three clinical settings. Pediatrics **84**:219–225.
21. **Lew, J. F., C. L. Moe, S. S. Monroe, J. R. Allen, B. M. Harrison, B. D. Forrester, S. E. Stine, P. A. Woods, J. C. Hierholzer, J. E. Herrmann, N. R. Blacklow, A. V. Bartlett, and R. I. Glass.** 1991. Astrovirus and adenovirus associated with diarrhea in children in day care settings. J. Infect. Dis. **164**:673–678.
22. **Osterhaus, A. D. M. E., A. L. van Wezel, G. van Steenis, G. A. Drost, and T. G. Hazendonk.** 1981. Monoclonal antibodies to poliovirus. Intervirology **16**:218–224.
23. **Richmond, S. J., D. J. Wood, and A. S. Bailey.** 1988. Recent respiratory and enteric adenovirus infection in children in the Manchester area. J. R. Soc. Med. **81**:15–18.
24. **Scott-Taylor, T., G. Ahluwalia, B. Klisko, and G. W. Hammond.** 1990. Prevalent enteric adenovirus variant not detected by commercial monoclonal antibody enzyme immunoassay. J. Clin. Microbiol. **28**:2797–2801.
25. **Shinozaki, T., K. Araki, Y. Fujita, M. Kobayashi, T. Tajima, and T. Abe.** 1991. Epidemiology of enteric adenoviruses 40 and 41 in acute gastroenteritis in infants and young children in the Tokyo area. Scand. J. Infect. Dis. **23**:543–547.
26. **Singh-Naz, N., and R. K. Naz.** 1986. Development and application of monoclonal antibodies for specific detection of human enteric adenovirus. J. Clin. Microbiol. **23**:840–842.
27. **Singh-Naz, N., W. J. Rodriguez, A. H. Kidd, and C. D. Brandt.** 1988. Monoclonal antibody enzyme-linked immunosorbent assay for specific identification and typing of subgroup F adenoviruses. J. Clin. Microbiol. **26**:297–300.
28. **Straus, S. E.** 1984. Adenovirus infections in humans, p. 451–496. *In* H. S. Ginsberg (ed.), The adenoviruses. Plenum Press, New York.
29. **Uhnoo, I., G. Wadell, L. Svensson, and M. E. Johansson.** 1984. Importance of enteric adenoviruses 40 and 41 in acute gastroenteritis in infants and young children. J. Clin. Microbiol. **20**:365–372.
30. **Uhnoo, I., G. Wadell, L. Svensson, E. Olding-Stenkvist, E. Ekwall, and R. Mölby.** 1986. Aetiology and epidemiology of acute gastro-enteritis in Swedish children. J. Infect. **13**:73–89.
31. **Van, R., C.-C. Wun, M. L. O'Ryan, D. O. Matson, L. Jackson, and L. K. Pickering.** 1992. Outbreaks of human enteric adenovirus types 40 and 41 in Houston day care centers. J. Pediatr. **120**:516–521.
32. **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 enteric adenovirus types 40 and 41 by DNA restriction enzyme analysis and by neutralizing monoclonal antibodies. Virus Res. **12**:139–158.
33. **van Loon, A. E., T. H. Rozijn, J. C. de Jong, and J. S. Sussenbach.** 1985. Physicochemical properties of the DNAs of the enteric adenovirus species 40 and 41. Virology **140**:197–200.
34. **Wadell, G., A. Allard, M. Johansson, L. Svensson, and I. Uhnoo.** 1987. Enteric adenoviruses. *In* Novel diarrhoea viruses. CIBA Foundation Symposium 128. John Wiley & Sons, Ltd., Chichester, United Kingdom.
35. **Webster, R. G., W. G. Laver, and G. M. Air.** 1983. Antigenic variation among type A influenza viruses, p. 127–168. *In* P. Palese and D. W. Kingsbury (ed.), Genetics of influenza viruses. Springer-Verlag, New York.
36. **Wigand, R.** 1987. Fast identification of adenovirus 40/41 in infants with enteritis. Eur. J. Clin. Microbiol. **6**:606–607.
37. **Wigand, R., and T. Adrian.** 1986. Classification and epidemiology of adenoviruses, p. 409–441. *In* W. Doerfler (ed.), Adenovirus DNA. Martinus Nijhoff, Boston.
38. **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.
39. **Wood, D. J., and A. S. Bailey.** 1987. Detection of adenovirus types 40 and 41 in stool specimens by immune electron microscopy. J. Med. Virol. **21**:191–199.
40. **Wood, D. J., K. Bijlsma, J. C. de Jong, and C. Tonkin.** 1989. Evaluation of a commercial monoclonal antibody-based enzyme immunoassay for detection of adenovirus types 40 and 41 in stool specimens. J. Clin. Microbiol. **27**:1155–1158.
41. **Wood, D. J., J. C. de Jong, K. Bijlsma, and H. G. A. M. van der Avoort.** 1989. Development and evaluation of monoclonal antibody-based immune electron microscopy for diagnosis of adenovirus types 40 and 41. J. Virol. Methods **25**:241–250.