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Comparison of Detection Methods for Adenovirus from Enteric Clinical Specimens

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Fecal samples submitted for virus examination over July 1990 to June 1991 from children <3 years of age were examined by electron microscopy (EM), virus culture (VC), and enzyme immunoassay [EIA, group-reactive and adenovirus (Ad) 40/41 specific; Cambridge BioScience] to compare the detection rate of adenovirus from pediatric fecal specimens. Ad isolates of serotypes 1–7 grown in HEp-2 or primary rhesus monkey kidney cells were identified by neutralization. Graham 293 cell cultures were used only when specimens were found to be positive for Ad by EM, type-specific Ad40/41 EIA, and for isolates not identified by neutralization. Ads grown in 293 cells were identified by DNA restriction endonuclease analysis. Of the 1187 specimens examined, 105 (9%) were found to be positive for Ad. VC detected 93, while 12 additional positives were detected by EM or EIA. The relative sensitivity of VC, EIA, and EM for the 105 specimens was 89% (93), 45% (47), and 35% (37), respectively. Among the 105 positive specimens, enteric Ad, nonenteric Ad, and untypeable Ad were 28% (29), 65% (68), and 7% (8), respectively. Of 37 EM positives, 62% (23) were enteric Ad; 27% (10) were nonenteric including serotypes 2, 3, 4, 5, 12, and 31, with 4, 1, 1, 2, 1, and 1 isolates of each type positive, respectively; and 11% (4) were detectable only by EM. Five isolates were identified as variant of Ad 2(3), Ad 3(1) and Ad 31(1). Over a 1-year period, a single Ad41 variant strain was the most frequently detected enteric Ad in Winnipeg, Manitoba, Canada. For maximum detection rates of Ad viruses in pediatric fecal specimens, a combination of EM, VC, and EIA is required, but group-reactive EIA, or EM followed by Ad40/41-specific EIA of initial positives, are the most direct and efficient methods for enteric Ad detection.

INTRODUCTION

Adenoviruses (Ads) are currently classified as 42 defined types with a further five unique isolates from AIDS patients recently identified (Hierholzer et al., 1988). Many of the various Ad types have been isolated from stool specimens of children with gastroenteritis and are presumed by frequency of association to be causative agents of disease (Christensen, 1989; Retter et al., 1979). However, some Ads, usually of low-numbered types, have been found to be excreted, sometimes for extensive periods, without clinical symptoms (Brandt et al., 1979; Fox et al., 1977; Kidd et al., 1982). The isolation of Ad from healthy children in some studies has made it difficult to assert that an Ad discovered in the stool of a sick child was definitively the cause of disease. In the absence of a suitable laboratory model of Ad gastroenteritis, causation of disease by these low-numbered or conventional Ad types cannot be proven and should be distinguished from enteric types.

Ad types 40 and 41 (Ad40 and Ad41), on the other hand, have only rarely been isolated from patients without gastroenteritis. These types can cause severe diarrhea and have been isolated from fatal cases of gastroenteritis and observed replicating in the nuclei of gut epithelial cells (Whitelaw et al., 1977). Ad40 and Ad41 are referred to as enteric Ads to denote their specific association with the alimentary tract (Petric et al., 1982) and their association

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with disease is widely accepted. Enteric Ads have fastidious culture characteristics and present a particular challenge in the diagnostic laboratory. This diagnostic difficulty has promoted considerable effort to enhance detection of enteric Ads, producing improved methods for the diagnosis of Ad40 and Ad41, including enzyme immunoassay (EIA) (August and Warford, 1987; Herrmann et al., 1987; Mortensson-Egnund and Kjeldsberg, 1986; Singh-Naz et al., 1988), dot-blot hybridization (Hammond et al., 1987; Kidd et al., 1985), and immune electron microscopy (EM) (Wood et al., 1989). Commercial monoclonal antibody-based enzyme immunoassay tests for enteric and all adenoviruses have become available for routine diagnosis of these viruses in diagnostic laboratories.

This study was undertaken to examine the individual adenovirus types from fecal specimens in children under 3 years of age in Manitoba, Canada, and to compare the methods of detection by virus culture (VC), EM with concentration by direct ultracentrifugation (Hammond et al., 1981), and EIA (Cambridge Bioscience).

MATERIALS AND METHODS

Specimens

During July 1990–1991, 1187 fecal samples from children <3 years of age were collected at clinics and hospitals and submitted to the Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada, for the diagnosis of virus infection. A portion of untreated stool specimen or a 10% suspension in H_2O stored at $-70°C$ prior to testing by EIA.

Diagnostic Tests

Enzyme Immunoassays

For the detection of Ad by EIA, all pediatric stool specimens were screened by group Ad EIA. To detect the presence of enteric Ad, only EIA screen positives were tested further by species-specific Ad40/41 EIA. Ad-positive specimens in culture were identified by neutralization with antisera to Ad species 1-7. Ads not identified by neutralization were further characterized by restriction analysis of viral DNA fragments cleaved with enzymes and compared with the restriction pattern of prototype strains (Adrian et al., 1986). An EIA for rotavirus detection (Rotaclone Cambridge Biosciences) was used on a seasonal basis from December to March.

Electron Microscopy

All specimens were examined by negative-stain EM after concentration by direct ultracentrifugation (Hammond et al., 1981) and identified by specific morphology. An EIA rotavirus (Rotaclove, Cambridge Biosciences) was used on a seasonal basis from December to March.

Virus Culture

For all specimens, a 10% emulsified suspension of each stool was clarified by centrifugation (12,000 *g*) in RC-5B Sorvall centrifuge (DuPont), and an antibiotic-treated specimen was inoculated into each of the monolayer tubes of human epidermoid carcinoma cells [HEp-2; American Type Culture Collection (ATCC), Bethesda, MD, USA] and primary rhesus monkey kidney cells (Connaught Laboratory, Willowdale, Ontario, Canada). Additionally, 25-cm^2 flasks of Graham 293 cells (ATCC) were inoculated when specimens were found to be positive by EM or Ad40/41 type-specific EIA. Ad-positive cultures were identified by neutralization with antisera (National Institute of Allergy and Infectious Diseases, Bethesda) to Ad species 1–7. Unidentified Ads by neutralization were further characterized by restriction analysis of DNA from 4- to 7-day-old cultures from 25-cm^2 flask, which were extracted by the Hirt procedure (Hirt, 1967) with proteinase K.

Restriction Enzyme Analysis

The suspension of viral DNA was digested with at least two enzymes (Bam HI, Hind III, Bgl II, or Sma 1; Boehringer Mannheim) and electrophoresed as described previously (Scott-Taylor et al., 1990).

Enzyme Immunoassay

Two monoclonal antibody EIA tests, Ad group-specific and Ad type-specific 40/41, were performed in accordance with the manufacturer's instructions. A 100-μl aliquot of 10% stool suspension was added to a microwell coated with the antibody directed against Ad group-specific hexon. A 100-μl aliquot of horseradish-peroxidase-conjugated monoclonal antibody directed against the type-specific hexon portion of the subgroup F (Ad40 and Ad41)-specific epitope, or group-reactive monoclonal antibody for the group-specific Ad EIA, was added to each well and incubated at room temperature for 1 h. After washing the wells with deionized H_2O, substrate A and B (urea peroxidase and tetramethylbenzidine) were added and the reaction was stopped after 10 min with 100 μl of 1 N sulfuric acid. The plates were read in a spectrophotometer at 450 nm. Specimens with an optical density (OD) of >0.15 were considered positive.

Blocking Assay

A blocking test was performed on specimens with discordant results; only specimens positive for enteric Ad by type-specific 40/41 EIA and noncultivable by cell culture on 293 cells were tested. For the blocking assay, monoclonal antibodies specific for Ad40 and Ad41 were supplied by Dr. de Jong (The

Netherlands). A total of 100 μl of each specimen was added to each of three tubes. A 10-μl aliquot of buffer was added to one tube (control), 10 μl of Ad40 monoclonal antibody to the second tube, and 10 μl of Ad41-specific monoclonal antibody to the third tube. After mixing, the tubes were incubated at room temperature for 1 h and the Ad40/41 EIA was performed by transferring the treated specimen into three separate microwells. Reference antigens for Ad40, Ad41 (ATCC), and the local Ad41 variant were used as blocking controls. The specimen was considered to be a confirmed positive for Ad if the OD of the specimen with hyperimmune serum was 50% less than the OD reading of the specimen with buffer control.

RESULTS

A total of 1187 stool specimens were examined during the period of study. Viruses were detected in 336 specimens (28.3%). Ad was found more frequently than other viruses, being detected in 105 (8.8%) of specimens compared with 83 (7%) rotavirus (38 by EIA and 45 by EM). EM also detected eight (0.7%) Norwalk virus, seven (0.6%) astrovirus, six (0.5%) calici viruses, four (0.3%) corona viruses, and 35 (2.9%) undetermined small round viruses. Virus culture yielded 88 (7.4%) enterovirus isolates.

A comparison of the number of Ad isolates detected in stool specimens by tissue culture (TC), EIA, and EM, and typing by restriction enzyme analysis is shown in Table 1. The sensitivities of TC, EIA, and EM were 89% (93 of 105), 45% (47), and 35% (37), respectively. The relative percentage (and number) of enteric and conventional Ad types among the 105 positive specimens were 28% (29), and 65% (68) respectively, with 7% (8) of positive specimens proving untypeable.

A total of 93 specimens yielded Ads in cell culture, 21 of which were enteric types and 68 conventional isolates. Four culture-positive Ads grown on HEp-2 cells could not be repassed for serotyping. The serotypes of nonenteric Ad are shown in Table 2: 27% (10 of 37) of the EM positives were found to be nonenteric Ad, including Ad serotype numbers 2, 3, 4, 5, 12, and 31 with 4, 1, 1, 2, 1, and 1 positives, respectively. Five culture-positive Ads were identified as variants of Ad2 (3), Ad3 (1), and Ad31 (1) by restriction enzyme analysis as they could not be identified by serum neutralization.

A total of 29 (62%) of the 47 specimens that reacted with the group-reactive EIA were also positive with the Ad40- and Ad41-specific EIA. Similarly, 23 of 37 specimens or 62% of EM-positive specimens were identified as containing enteric adenovirus. Four of 37 EM positive Ad or 11% were only detectable by EM. Both the EIA and EM results suggest that the enteric Ads are more often present in amounts that are detectable by these techniques. Of the EIA-positive specimens, 21 demonstrated restriction patterns of a variant strain of Ad41 (Hammond et al., 1985). The remaining eight Ad40/41 type-specific enteric EIA-positive specimens could not be cultured in 293 cells. The blocking assay was performed on five of these eight specimens and the results are shown in Table 3. The reference strains of Ad40 (Dugan) and Ad41 (TAK) showed >50% reduction in OD with homologous Ad40 and Ad41 monoclonal antibody. With the variant strain, a partial reduction in OD (37%) of Ad41 variant and four Ad-positive specimens (varying from 12% to 33%) was seen with monoclonal antibody against Ad41. These data suggest that these culture-negative specimens may represent serotypes other than 40 or 41, or were variant enteric serotypes. The EM-negative stools and the stools containing other viruses were

TABLE 1 Detection of Adenovirus (Ad) in Stool by Electron Microscopy (EM), Enzyme Immunoassay (EIA), and Tissue Culture

Species	Total No. of +ve Spec (%)	EM	EIA		Virus Isolation	
			Ad Group Reactive	40/41 Type Specific	HEp-2	293[d]
Enteric Ad	29 (28)	23[b]	29	29[c]	1	20
Nonenteric Ad	68 (65)	10	18	0	68	10[e]
Untyped Ad	8 (7)	4	0	0	4	0
Total	105[a]	37 (35)	47 (45)		93	(89)

[a]9% (105 of 1187) positive for Ad.
[b]62% (23 of 37) found to be enteric Ad.
[c]21 of 29 were Ad 41 variant by DNA restriction analysis.
[d]Only Ad-positive specimens by EM were cultured in 293.
[e]These 10 specimens were also positive on HEp-2 cells.

TABLE 2 Serotypes of Non-Group-F Adenovirus (Ad) in Stools of Children <3 Years of Age

Species	No Positive	Positive by EM[a]
Ad 1	14	0
Ad 2	34 (3)[b]	4
Ad 3	4 (1)	1
Ad 4	4	1
Ad 5	7	2
Ad 6	2	0
Ad 12	1	1
Ad 31	2 (1)	1
Total	68	10

[a]Ad serotypes 2, 3, 4, 5, 12, and 31 found to be positive by electron microscopy.
[b]Number of variants of prototypes of Ad species by restriction enzyme analysis.

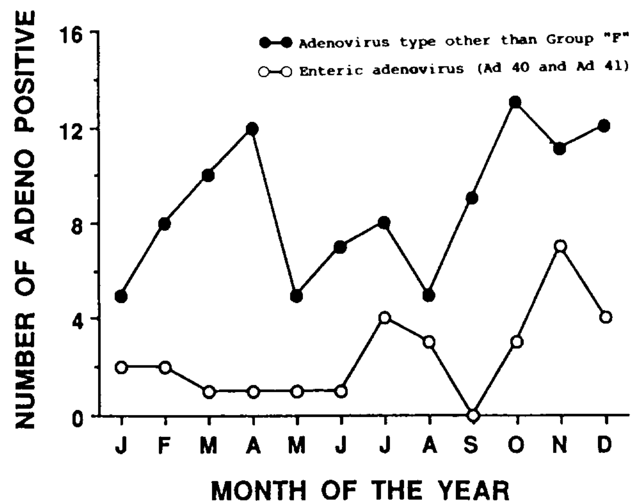

FIGURE 1 Monthly distribution of adenovirus infection.

found to be negative by EIA, which reveals the high specificity of the Ad group EIA test.

Ad was detectable throughout the year. More diagnoses of Ad occurred during spring (April) and early fall (September through November) as shown in Figure 1. Distribution of Ad and rotavirus positives by age is shown in Figures 2 and 3, respectively. Of the Ad-positive children, 73% were found to be <2 years of age.

DISCUSSION

Ad types 40 and 41 are the fastidious viruses that have been established as being associated with gastroenteritis in infants. They have also been implicated in diarrheal outbreaks in hospitals (Chiba et al., 1983; Flewett et al., 1975; Richmond et al., 1979). Ads detectable in stools by EM are frequently associated with gastroenteritis (Brandt et al., 1979; Retter et al., 1979). In our study, both nonenteric and enteric Ads, belonging to subgroups A, B, C, and F, were detectable by EM. Nonenteric Ads detectable by EM included serotypes 2, 3, 4, 5, 12, and 31. Two isolates of Ad31 (one Ad31 variant) were also identified, which have been implicated in diarrheal disease (Brown, 1990; Hammond et al., 1985), which produce clinical symptoms similar to enteric Ad (Krajden et al., 1990).

Enteric Ad was detectable in 28% (29) of the 105 Ad-positive specimens, 21 of which were confirmed

TABLE 3 Analysis of Noncultivable Enteric Adenovirus (Ad) by Ad 40/41-Blocking Assay

Lab No.	Group EIA	Ad 40/41-Specific EIA	EM[a]	Ad41 Mab % Blocking	Ad40 Mab % Blocking
11365	+	+	+	32	2
10839	+	+	+	18	0
13258	+	+	+	NSQ[b]	
11774	+	+	−	12	0
11861	+	+	−	33	10
7746	+	+	−	0	0
12533	+	+	−	NSQ	
13690	+	+	−	NSQ	0
Controls					
Ad40 (Dugan)	+	+		8	89
AD41 (TAK)	+	+		67	11
Ad41V[d]	+	+		37[c]	—

[a]EM, electron microscopy.
[b]Not sufficient quantity.
[c]Partial blocking of Ad41V against 41 monoclonal antibody (Mab).
[d]Ad41 variant strain.

to be a single variant of Ad41 by DNA restriction analysis, previously reported (Hammond et al., 1985). There was no Ad40 observed, which has been reported to be diminishing in recent years (Brown, 1990; Van der Avoort et al., 1989).

For serotyping of enteric Ad, EIA and immune EM have been developed, but they are not widely available (Wood et al., 1989). Restriction endonuclease of DNA has been found to be a very useful technique for typing virus other than serotypes 1–7 and Ad variants that may not be identified by species-specific antisera.

EIA (Cambridge Bioscience) is the first commercial immunoassay that has been made available to detect Ad directly in stool specimens. The Ad from the stool can be differentiated from the established Ad by Ad40/41 type-specific EIA. The sensitivity of EIA compared with EM was very similar, and the majority of positive specimens by both technologies were enteric Ad. Eight additional enteric Ads (detectable by type-specific Ad40/41 EIA) could not be grown by VC because of the fastidious nature of the virus or due to the absence of viable virus in the stool. Ad40/41 type-specific EIA was found to be a rapid method of differentiating fastidious Ad, which overcomes the problems of VC.

In conclusion, we found that a variant of enteric Ad41 was the only enteric Ad serotype in the pediatric fecal specimens in Manitoba during a 1-year study. Although virus isolation was found to be most sensitive, it is labor intensive and cannot detect nonviable or particularly fastidious virus. Sequential testing by EM or Ad group-reactive EIA, followed by specific rapid detection of enteric Ad directly on stools by using Ad40/41 type-specific EIA, is the most practical laboratory method of enteric Ad detection.

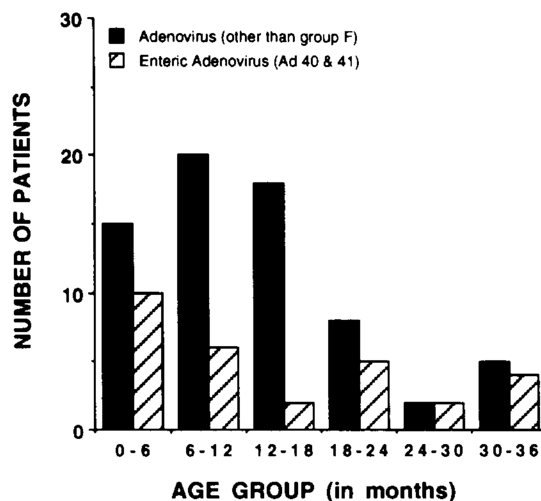

FIGURE 2 Distribution of adenovirus-positive children by age.

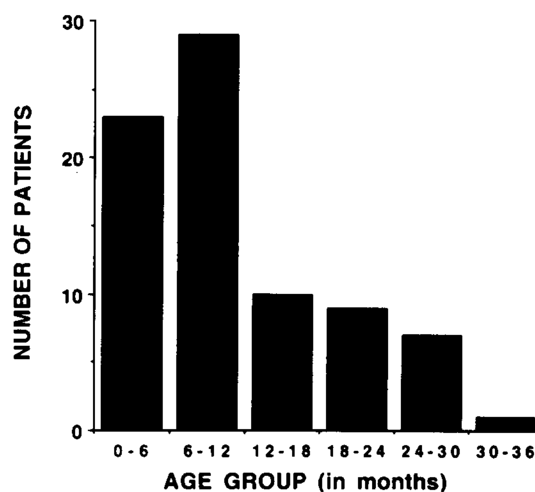

FIGURE 3 Distribution of rotavirus-positive children by age.

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