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SCREENING ADENOVIRUSES IN STOOL SAMPLES: EVALUATION OF A GENUS-SPECIFIC MONOCLONAL ANTIBODY BASED ENZYME IMMUNOASSAY*

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To screen fecal samples for adenovirus antigens a genus-specific monoclonal antibody based enzyme immunoassay was developed. In a comparative analysis with commercial latex agglutination test, high sensitivity was demonstrated. The assay did not detect other viruses usually found in faeces suggesting its specificity. One hundred and eighty stool samples collected in Baranya County were tested and 13 (7.2%) of them showed reactivity. The application of our immunoassay combined with other, more sophisticated methods may help us to determine the serotype specificity of these adenovirus isolates and assess the importance of adenoviruses in viral gastroenteritis.

Keywords: adenovirus, gastroenteritis, enzyme immunoassay

Introduction

Human adenoviruses (Ads) comprise 51 different serotypes. At least two serotypes, Ad40 and Ad41, also called enteric adenoviruses (EAds), often induce viral gastroenteritis [1]. Most of the adenovirus-associated diarrhoea occur in infants and young children. Seroepidemiological studies indicated that 50% of children develop neutralizing antibodies against these serotypes by four years of age [2, 3]. Although the adenovirus-associated diarrhoea is usually milder than diarrhoea caused by rotavirus, a prolonged hospital treatment has been demonstrated [4, 5]. In addition, enteric adenoviruses are responsible for outbreaks in different settings [6, 7, 8] and may cause severe infections in immunocompromised patients [9] and occasionally death [10].

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The classical diagnostic approach to identify EAd infections by isolation in cell culture has failed for a long time. Although permissive cell lines were later found, this method was not widely used in diagnostic laboratories because of the low level of sensitivity and reproducibility [11]. Therefore, alternative detection methods have been developed. Besides electron microscopy, antigen-based detection methods, for example enzyme immunoassay (EIA) and latex-agglutination (LA) were preferably used in diagnosis of EAd infections, and later nucleic acid-based procedures (e.g. Southern blot and polymerase chain reaction) have also been developed [12, 13, 14, 15]. The major advantage of these techniques is that the adenoviral antigens or DNA can be detected directly from stool specimens.

In the recent years several different commercial EIA systems have been developed for adenovirus diagnostics and some of them can specifically detect enteric adenoviruses. The main reason of our effort to develop a genus-specific enzyme immunoassay, described here, was to set up an inexpensive assay which would allow us to screen large amounts of fecal samples for both enteric and non-enteric adenoviruses. The frequency of some serotypes (e.g. Ad12, Ad18 and Ad31) of non-EAds in the faeces has already addressed the question whether they have the ability to cause gastroenteritis [16, 17, 18]. To investigate this possibility and to determine the epidemiological role of enteric adenoviruses in our country, the use of combination of our assay with nucleic acid-based techniques has recently started for adenovirus serotype determination in fecal samples.

Materials and methods

Viruses

Prototype strain of human adenovirus serotype 40 (HOVI-X) and a strain of adenovirus serotype 41 (R1774/90) were used as antigens in optimisation of MAb-EIA system. The strain HOVI-X was isolated in Finland and kindly provided by Dr. de Jong (National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands). The strain R1774/90 was isolated in Hungary, by Szűcs [19]. These isolates were grown on monolayers of cell lines A549 and Graham 293, respectively. Tissue culture isolated strains of adenovirus, rotavirus, astrovirus as well as positive stool samples for these viruses and calicivirus were also used in the study. Two untyped virus isolates showing enterovirus-like CPE in GMK cell monolayer were also included.

Fecal samples from the paediatric community

Stool specimens were obtained from paediatricians of local hospitals and general practitioners of Baranya County in March 2001. The samples were stored at –20 °C before testing.

Production of the hexon-specific monoclonal antibody 1A3

The monoclonal antibody, 1A3, was prepared against purified human Ad serotype 1 hexon molecule as described previously [20].

Production of hyperimmune rabbit sera against adenovirus serotype 41

The isolate R1774/90 (Ad41) was propagated in confluent monolayer of Graham 293 cells. When complete cytopathic effect appeared, the viruses were harvested from the supernatant and purified by CsCl gradient centrifugation. The fraction with viral particles was desalted using Sephadex-G25 column and subsequently utilised as antigen for immunization. The first three doses of adenovirus antigen with complete and incomplete Freund-adjuvant, respectively, were given subcutaneously, the fourth and fifth doses were given intravenously one month intervals into rabbit, serologically negative for adenovirus. Two weeks after each dose, 2–5 ml of serum was collected and tested by immunfluorescence onto Ad41 infected cells. One month after the fifth immunization the animal was bled, the serum was collected, aliquoted and stored at –20 °C.

Monoclonal antibody-based enzyme immunoassay (MAb-EIA)

Culture adapted adenovirus strains, HOVI-X and R1774/90, were used as controls. Stool specimens were prepared in sample diluent reagent of Adenolex® kit (Orion Diagnostica, Finland) or in phosphate buffered saline (PBS).

Polystyrene microtitration plates (Greiner Labortechnik, Germany) were coated with 60 µl of genus-specific monoclonal antibody 1A3 diluted in PBS (1 in 20,000). The plates were incubated overnight at 4 ºC and then washed five times with PBS-Tween 20. Blocking step was carried out using 300 µl of 5% Blotto. After one hour incubation at 37 ºC, the blocking agent was aspirated. The specimens were added in a volume of 25 µl to 25 µl of 2% Blotto and incubated for two hours at 37 ºC. The plates were washed (5×) and incubated with 50 µl of anti-adenovirus rabbit sera for one hour at 37 ºC. After washing again (5×), the rabbit sera were detected using 50 µl anti-rabbit

goat IgG conjugated to horseradish peroxidase (GIBCO BRL, Great Britain) at a dilution of 1 in 10,000. The plates were incubated for one hour at 37 ºC and then washed again (6×). The colorimetric reaction was developed using 100 µl of tetramethyl benzidine (Organon Diagnostika, The Netherlands), and subsequently stopped using 25 µl of 1 M sulfuric acid. The absorbance was measured at 450 nm (Reader 530, Organon Diagnostika, The Netherlands).

Samples were considered to be positive if the absorbance value was ≥ 0.2 and three times higher than the mean value of negative control wells containing PBS.

Latex agglutination (LA)

Commercial latex agglutination test (Adenolex, Orion Diagnostica, Finland; Lot No. BL 4) was carried out as the manufacturer recommended, except that the reactions were performed in a total volume of 50 µl instead of 100 µl.

Results

Optimisation of test parameters

The monoclonal antibody 1A3 was tested in dilutions of 1 in 1,000, 10,000, 100,000 and 1,000,000, respectively. Each MAb dilution was tested with 10-fold dilution of hyperimmune rabbit sera between 500 and 50,000 in the presence of undiluted cell culture supernatant of adenovirus prototype strain HOVI-X and isolate R1774/90, respectively (Figure 1).

In general, the sample R1774/90 (Ad41) gave higher absorbance values among all conditions than the HOVI-X (Ad40) sample. A considerable decrease of absorbance values was observed between dilutions of 10,000 and 100,000 of MAb 1A3 for both samples. Further analysis demonstrated that a dilution of 1 in 20,000 might be optimal (data not shown).

The dilution of hyperimmune sera at 1 in 500 reacted relatively well with the two viruses, however, a considerable decrease of reactivity was observed for HOVI-X at 1 in 5,000, and for both samples at 1 in 50,000. Since the hyperimmune rabbit serum was raised against R1774/90, it is possible that the serotype differences might have influenced the reactivity and/or the strain HOVI-X was grown at a lower titer in the tissue culture A549.

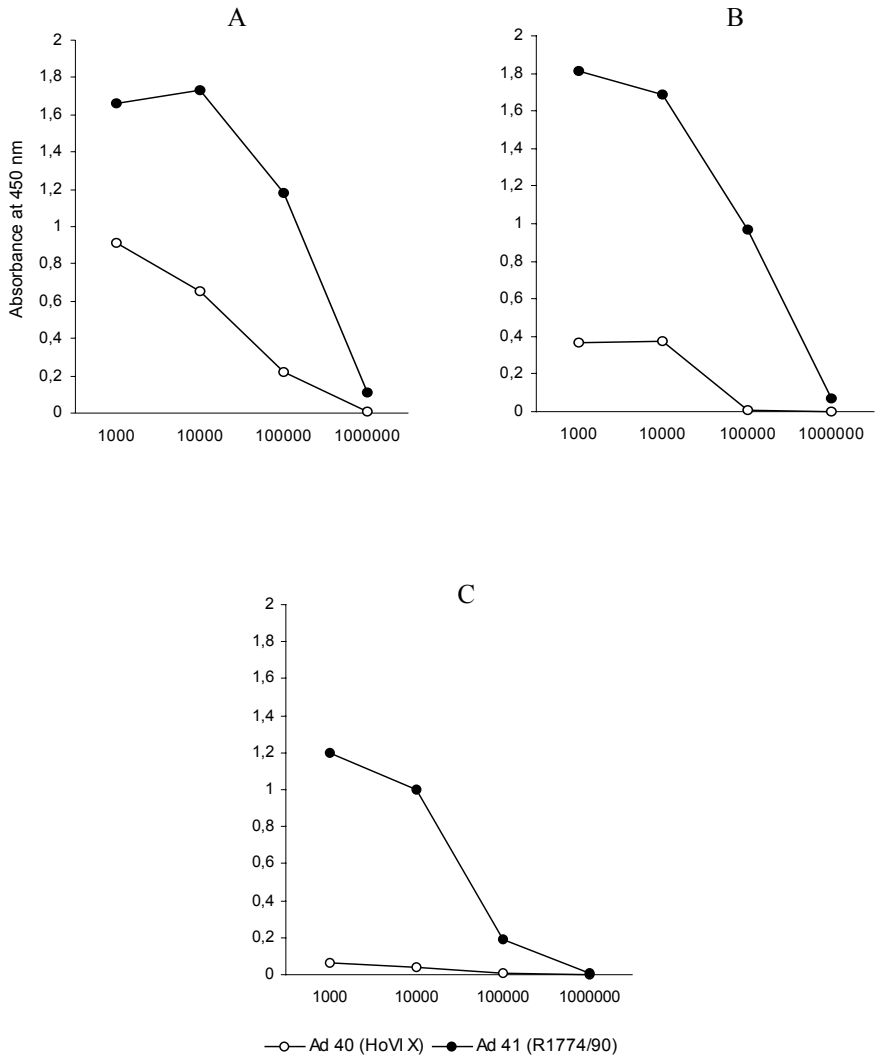

Fig. 1. Reactivity of EAd40 (HOVI-X) and EAd41 (R1774/90) were tested with the genus-specific MAb 1A3 at 10-fold dilutions from 1,000 to 1,000,000 and with the hyperimmune rabbit serum at dilutions of 1:500 (A), 1:5,000 (B) and 1:50,000 (C). The reactivity is expressed in absorbance values at 450 nm

Based on these results, further experiments were carried out using dilutions of 1 in 20,000 for MAb 1A3 and 1 in 500 for rabbit hyperimmune sera.

Specificity

Stool specimens (positive for calicivirus or astrovirus) and tissue culture adapted viruses (adenovirus, rotavirus, astrovirus and enterovirus-like agent) were used to determine the specificity of the assay. Phosphate buffered saline (10 mM) was used as negative control. The specimens, positive for adenovirus (n=6) gave high OD values (1.7 to 2.1) in this assay, and specimens, positive for rotavirus (n=12), calicivirus (n=12), astrovirus (n=6), and enterovirus-like agents (n=2), respectively, were not reactive with only one exception. One of the samples containing enterovirus-like agents gave high absorbance value in the assay. (According to our preliminary results this virus might be an Aichi virus [family Picornaviridae, genus Kobuvirus]. Reuter, personal communication.) However, an immunofluorescence assay confirmed the presence of infectious adenovirus and supported the simultaneous presence of the two viruses in the faeces.

Sensitivity

The sensitivity of the assay was assessed by three-fold serial dilutions (10 to 590,490) of stool suspensions and tissue culture supernatants, respectively. The sensitivity of our assay and a commercial latex-agglutination assay was compared (Table I). This comparative experiment revealed that our MAb-EIA was 3 to 243 times more sensitive than the latex-agglutination test.

Table I

Comparison of sensitivity of MAb-EIA[a] with LA[b]

Sample		Reciprocal of highest dilution of stool specimen found positive by		Serotype by RFLP[c]
Designation	Source	MAb-EIA (home made)	LA (commercial)	
HOVI X	Culture	30	ND[d]	40
R1774/90	Culture	21,870	ND	41
R41/91	Culture	65,610	270	41
B10051/01	Stool	810	90	ND
B9578/01	Stool	196,830	810	ND
B10325/01	Stool	810	270	ND

[a]Monoclonal antibody-based enzyme immunoassay.
[b]Latex agglutination test.
[c]Restriction fragment length polymorphism. This assay was carried out previously by Szűcs et al. [19].
[d]Not done.

Clinical specimens

One hundred eighty stool samples were screened for adenovirus antigen. Based on their reactivity, 13 samples (7.2%) were positive in the assay (Figure 2).

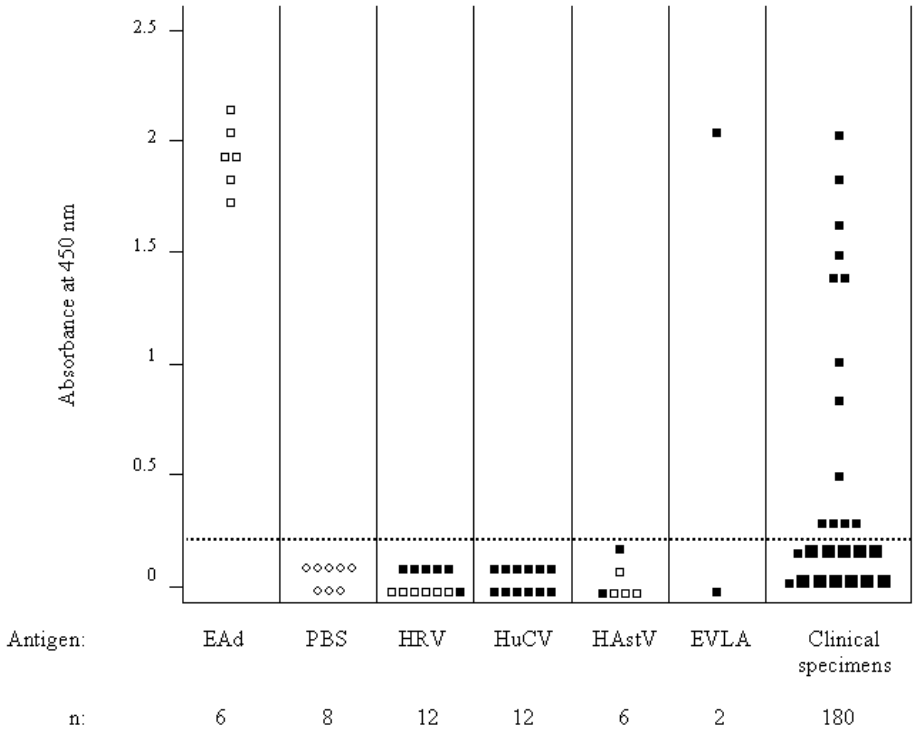

Fig. 2. Specificity of the MAb-EIA. Tissue culture adapted viruses (□) and stool samples (■) positive for different enteric viruses were used to assess the specificity of the assay. Previously identified viruses such as enteric adenoviruses (EAd), human rotaviruses (HRV), human caliciviruses (HuCV); human astroviruses (HAstV) and enterovirus-like agents (EVLA) were included. Phosphate buffered saline (PBS; symbol: ○) was used as negative control. One hundred and eighty stool samples collected in Baranya County were also tested in the assay. A small square indicates one sample, a large square indicates 15 samples. Dotted line indicates the cut-off value

Discussion

Adenoviruses are significant agents of viral gastroenteritis in children, and cause sporadic cases and outbreaks [1]. To investigate their epidemiological role in Hungary, we developed a monoclonal antibody-based sandwich enzyme immunoassay.

The hexon-specific monoclonal antibody 1A3 was selected because studies on adenovirus hexon epitopes confirmed its reactivity with a genus-specific epitope [20, 21]. This raised the possibility of its application as diagnostic reagent for adenovirus infections. Lengyel et al. developed a latex agglutination assay which was specific and sensitive enough to detect 100 ng/ml of adenovirus antigen [22]. The clinical application of latex agglutination is preferable, however, for epidemiological investigations, 96-well format EIA systems may provide higher efficacy. As a part of our surveillance for human enteric viruses, we decided to merge the benefits of this genus-specific monoclonal antibody and the 96-well format EIA system. The assay developed was specific and more sensitive than the latex agglutination assay. The difference in the sensitivity in our experiment is consistent with the observation published by others [22, 23]. One possible explanation is that enzyme immunoassay recognizes also soluble viral components, while other antigen detection methods, such as solid-phase immune electronmicroscopy or latex agglutination test depend on the presence of intact viruses or incomplete viral capsids.

A small segment of our sample collection was selected, and tested retrospectively. Of 180, mostly diarrhoeal stool samples 13 (7.2%) were reactive in this assay suggesting that adenovirus antigens were present in these specimens. Since adenoviruses, different from enteric adenoviruses may also be shed in the faeces, further analysis, including molecular biological techniques, has to be performed to discriminate the enteric adenovirus isolates from non-enteric adenoviruses. Combining these methods, our assay may become a sensitive and specific screening tool for epidemiological investigation of human adenoviruses.

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