Detection of types 40 and 41 adenoviruses in stool samples of diarrheal children by solid phase PCR

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Abstract
Adenoviruses (AdVs) types 40 and 41 are the causative agents of diarrhea in children. Hence, rapid sensitive and specific detection of these viruses are of clinical importance. The customary methods such as propagation of virus in cell culture suffer from limitations. Detection of immobilized amplified products in a one phase system (DIAPOPS) method has the potential to overcome these problems. A DIAPOPS method for detection of AdV types 40 and 41 was designed. Forward primers were covalently linked to the Nucleolink surface. After amplification of a 745 bp sequence of DNA binding protein gene, the amplified product was hybridized with the biotinylated probe. The hybrids were detected by the antibody-peroxidase conjugate. After optimization of the DIAPOPS conditions, 80 stool samples from children with clinical manifestation of viral diarrhea were tested. Their DIAPOPS results were compared with those of the conventional polymerase chain reaction (PCR) assay. Positive results were obtained in 11 samples. The comparison between conventional PCR and DIAPOPS showed a significant increase in sensitivity of the DIAPOPS test, 6 samples shown to be negative by conventional PCR, were demonstrated positive by DIAPOPS (p=0.00). The DIAPOPS assay presented in this study can provide a rapid, sensitive, specific and economic method for detection of viral infections. The assay can be performed for numerous samples simultaneously in a day. This DIAPOPS method can provide a practical and reliable tool for diagnosis of enteric adenoviruses. In addition, the risk of contamination in this assay is low.
Keywords: DIAPOPS; PCR; Children diarrhea; Adenoviruses types 40 and 41

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INTRODUCTION

Acute gastroenteritis is one of the most common diseases in humans, and continues to be a significant cause of morbidity and mortality worldwide (Glass and Kilgore, 1997). Children under 5 years of age are particularly prone, and it is calculated that, in this group, there are more than 700 million cases of acute diarrhea every year (Snyder and Merson, 1982; Shinozaki *et al*., 1987; Jarecki-Khan *et al*., 1993). The mortality associated with gastroenteritis has been estimated to be 3-5 million cases per year, the majority of them occurring in developing countries (Bern and Glass, 1994; Guerrant *et al*., 1990; Warren, 1990). In Iran, enteric Adenoviruses are responsible for 6.7% of the diarrhea that leads to hospitalization (Saderi *et al*., 2002).

In the 1970s, the causes of acute non-bacterial gastroenteritis were identified and several viruses associated with the stools of patients suffering from gastroenteritis correlated with their clinical disease. These viruses included rotaviruses, enteric adenoviruses, Norwalk and Norwalk-like viruses (NLVs). Among these, rotaviruses are the most common cause of viral gastroenteritis in infants and young children, followed by enteric adenoviruses (Blacklow *et al*., 1991; Barnes *et al*., 1998; Garthright *et al*., 1998; Benko *et al*., 1999; Caeiro *et al*., 1999).

There are presently various methods, which can be used for diagnosis of Adenoviruses, although each of them has some limitations. For example, the propagation of samples in cell culture is expensive and time-consuming. Direct adenovirus antigen testing with commercially available antigen detection enzyme

immunoassay is also currently used (Glass *et al*., 2000; McIver *et al*., 2000; Moore *et al*., 2000). This method often yields variable results because of insufficient sensitivity and/or specificity and requires confirmation by PCR assay or virus isolation (Allard *et al*., 1990; Atmar and Estes, 2001; McIver *et al*., 2001). The use of PCR based techniques provides better results than conventional methods. The PCR test is performed after cell culture to increase the sensitivity (Scot-Taylor *et al*., 1997), which has its drawbacks. Some methods such as nested PCR are used to augment the sensitivity. The disadvantage, however, is the increased risk of the contamination, and laboratories must take extra precautionary measures to avoid contamination (Tafreshi *et al*., 2005). In addition, interpretation of the results of the PCR assay is fairly subjective and normally requires agarose gel electrophoresis as well as handling of toxic materials such as ethidium bromide. DIAPOPS is a PCR- ELISA like technique in which PCR product captured by a solid support via one of the primers (technically named solid phase primer). The detection procedure is performed by labeled probe. If biotin is used as the label, then conjugated streptavidine-horse radish peroxidase could be used, and blue color will develop after the addition of 3, 3′, 5, 5′, tetramethylbenzidine (TMB) as the substrate. The color is measured by ELISA reader as optical density (O. D.) value.

According to our study, DIAPOPS is both a rapid and sensitive method for enteric adenovirus detection. Therefore it may be appropriate for clinical application.

MATERIALS AND METHODS

Patients and clinical specimen: A total of 80 stool samples were collected from neonate to ten years old children at the Imam Khomeini Hospital, Children's Ward of Shahid Hassan Ahari. The samples were selected against bacterial infection and the rest of samples were selected as having viral infection (Table 1). Stool samples were vortexed vigorously, centrifuged at 1500 g for 10 min, and the supernatants were then stored in phosphate buffer saline (PBS) buffer at -20ºC for DNA extraction.

DNA extraction and positive controls: Viral DNA was extracted from viruses and stool samples by a commercial viral DNA extraction kit as described by the manufacturers (NucleoSpin Blood, MN, Germany).

Prototype strains from AdV type 40 and 41 were used as positive controls, and prototype strain from the AdV type 5 was used for the specificity control.

Table 1. The characteristics of the samples (gender vs. age).

Gender \ Age	0-1	1-4	4-10	total
m	11	19	5	35
f	15	27	3	45

From all 80 collected samples, 35 were males and 45 were females. 26 patients were between 0-1 year of age; of these 11 were males. 46 patients were between 1-4 years old; of these 19 were males, and 8 patients were between 4-10 years old; of these 5 were males.

Sensitivity and Specificity test: To test the specificity of our designed primers and probe with other viruses, they were checked by other viruses as follows: AdV type 5, Human Papilloma virus type 16/18 (HPV-16/18), Hepatitis B virus (HBV), and Herpes simplex virus type1 (HSV-1) (data not shown).

Primers and Probe: The Gene Bank accession number for the DNA sequences used, were as follows: *AdV -40*: L19443 (for its complete sequence); *AdV-41*: M19540 (for its DNA binding protein gene). Highly-conserved region within the DNA-binding protein gene of AdV types 40 and 41 was selected for designing of the primers. All primers were designed from regions with least homology to other adenoviruses. Designing of the primers was performed with the Gene Runner Software for Windows version 3.05. The primer and the probe sequences were as follows: Forward PCR primer: 5′-CGACTACTTACTCCCAACGAG-3′ Reverse PCR primer: 5′-GTTTGTCAAACGTGCCCAG-3′ DIAPOPS probe: 5′-CTTGGTCATGTTACATTGAGCCAC-3′.

The probe was designed from the reverse strand. The sequences of forward and reverse primers for DIAPOPS were the same as PCR except that the DIAPOPS solid phase primer had ten thymidine residues and one phosphate group at its 5′ end.

All the sequences were checked for the hairpin loop and dimer formation. The accuracy of the designed primers was confirmed by the Blast program (http://www.ncbi.nlm.nih.gov/Blast/). All the oligonucleotides were synthesized at the Bioneer Company (South Korea).

PCR Amplification: PCR was performed in 25 µl reaction mixtures, containing 100 µg of Bovine Serum Albumin (BSA) per ml as enhancer, and *Taq* DNA polymerase (CinnaGen, Iran).

Thermal cycling of the amplification mixture was performed in a Techne Touchgene gradient PCR for a

total of 30-35 cycles. The cycles involves denaturation for 45s at 94ºC, annealing for 45s at 55ºC, followed by primer extension for 45s at 72ºC. In the first cycle, the denaturing step continued for 5 min at 94ºC.

Agarose and PAGE detection of PCR products: PCR product detection was performed on 1% (w/v) agarose gel electrophoresis. The amplified fragment was verified with Gene Ruler DNA ladder (Fermentas, Lithuania). DIAPOPS positive samples that could not be detected on agarose gel were tested with 6% (w/v) polyacrylamide gel for further confirmation.

DIAPOPS: Detection of Immobilized Amplified Products in a One Phase System

Covalent binding of the soluble phase primer: DIAPOPS was performed according to the Nunc Tech Note with some modifications. The solid phase primer was coated onto the NucleoLink strips (Nunc, Germany), which are suitable for covalent binding of oligonucleotides. Coating was performed with 100 µl fresh coating buffer (100 nM solid phase primer, 10 mM carbadiamide, 10 mM imidazole; pH 7) for 24 h at 50ºC for each well. Plates were washed three times with washing buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20; pH 7.5) and deionized water.

Amplification of target DNA: The reaction was carried out in the coated plates. PCR was performed in a 25 µl mixture composed of template DNA, PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl), 2 pmol reverse primer, 2.8 pmol forward primer, 0.2 mM of each dNTP, 2U *Taq* DNA polymerase and 1mg/ml BSA (Fluka, Switzerland).

After PCR, the liquid phase was removed and stored at 4°C for further analysis such as electrophoresis the emptied wells were washed three times with freshly made 0.2 M NaOH and 0.1% Tween 20.

Optimization of detection conditions: In the detection procedure, three factors are important: 1) hybridization temperature, 2) incubation time of the probe and 3) incubation time of streptavidine. These factors can affect the accuracy of the results, and the overall time the technique consumers. We used three temperatures: 50, 55 and 60ºC as the hybridization temperature. For all the above temperatures, samples were incubated for times 1 h, 3h, 6h and overnight representing probe incubation times.

After finding the best probe incubation time and temperature, the optimization of the streptavidine incubation time was performed from 20 min to 1h with 10 min intervals.

Detection of product by biotinylated probe: *The detection procedure*: The hybridization buffer (50 nM biotinylated probe, 5X sodium citrate, sodium chloride (SSC) contains 43.75 g NaCl, 22.05 g sodium citrate, adjusted to 1 liter with water; pH 7, 0.1% Tween 20, 0.5% BSA) was added to each well, the wells were sealed, and incubated for 1h at 60ºC, according to our optimization results. Then the wells were washed five times with a buffer composed of 0.5X SSC and 0.1% Tween 20, each time for 5 min at room temperature, except for the third round that was performed at 50ºC, for 15 min. The detection buffer [1/5000 diluted horse radish peroxidase (HRP), 100 mM Tris-HCl pH 7.5, 0.1% Tween 20, 150 mM NaCl, 0.5% BSA] was added to each well, and the wells were sealed and incubated at 50ºC for 30 min, according to our optimization results. After washing three times with washing buffer, 100 µl of ready to use TMB (CinnaGen, Iran) mixture was added to each well.

Before the production of strong background color in the negative controls, the reaction was blocked by 0.1 M H_2SO_4. The results were read on ELISA reader (SCO diagnostic, RS-232 Germany).

Cut-off value of probability: Forty negative samples were used to determine cut-off value of probability for DIAPOPS, and results were read by the same ELISA-reader that was used for clinical samples. The cut-off value of probability for the DIAPOPS was determined according to the equation below for a 100% probability: cut-off value of probability= $X+Z_{(\alpha/2)}.\sigma/\sqrt{N}$, where, $Z_{(\alpha/2)}= 3$, Average (X) = 0.26057, standard deviation (σ) = 0.70914 and N= 40, positive cut-off is as calculated below: Cut-off. = $0.26057 + 3 * 0.70914/\sqrt{40} = 0.596$

An assay was considered positive, if optical density of the sample was greater than the cut-off value of probability.

Statistical analyses: The reproducibility of each method was calculated by the kappa test, and the significance of the difference between the results of each test was calculated by the Chi square test. The latter was performed with the SPSS, version 11.5.

RESULTS

PCR based detection of AdV: Only 5 samples were reported as positive from a total of 80 samples that

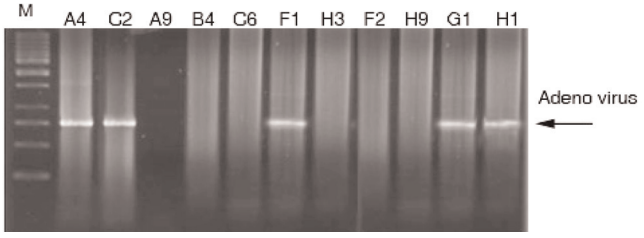

Figure 1. PCR results. Lines A4, C2, F1, G1 and H1 are clinical samples with positive bands. Line A9 is a negative control, and the others are clinical samples that PCR did not detect but DIAPOPS detected them as positive. M is DNA molecular size marker (Fermentas Russia).

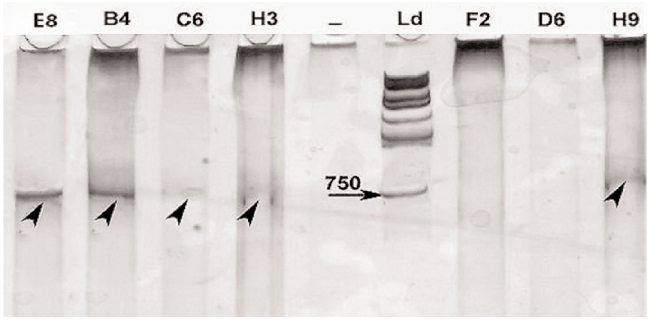

Figure 2. PAGE confirmation of DIAPOPS positive samples with no detectable band on agarose gel after PCR. The arrowheads show the bands that are expected. F2 is the sample that was positive in DIAPOPS but not detectable in PAGE.LD is the DNA molecular size marker (Fermentas, Lithuania), - is the negative control and D6 is one of the samples with an OD value less than the cut-off value of probability.

were tested with the agarose gel electrophoresis method, (Fig. 1). All of them were less than 4 years old, and 2 of them were under 1 year of age. Three of these positive samples were female and others were male. A kappa value of one was obtained that showed excellent reproducibility.

DIAPOPS optimization: The best probe hybridization result was obtained at a temperature of 60ºC and an incubation time of 1h. The best results for the stereptavidin incubation time were obtained at 30 min.

DIAPOPS: DIAPOPS could detect all the samples that were reported as positive in PCR. In addition, DIAPOPS detected 6 further samples as positive. This difference was highly significant (p= 0.00). The optical density of these samples is shown in Table 2. All of them were under 4 years of age, 4 of them were under 1 year of age. Five of these samples were male and the others were female.

PAGE: Those samples which were positive in DIAPOPS but did not produce any band on agarose gel were tested with PAGE. Five of them showed a narrow band on this gel. But one of them, which had the least optical density, did not show any detectable band (Fig. 2). It suggests the higher sensitivity of DIAPOPS than the PAGE procedure. Table 2 compares the results of these three tests.

Specificity: The specificity results showed that PCR and DIAPOPS were highly specific and any other viruses, which were tested, were not detected (data not shown).

DISCUSSION

Propagation of virus on cell culture has been considered as the gold standard for laboratory diagnosis of adenoviruses types 40 and 41, however, detectable replication typically requires upto 3 days to 3 weeks, and depends on the source of specimen and the load of virus in the specimen. Furthermore, this technique requires infective virus. Adenoviruses can be inactivated during sample collection and transportation of the samples, or a prolonged interval between specimen procurement and culture inoculation. Interference with virus isolation can also result from bacterial contamination or toxic effects of the specimen itself. Microscopic methods and detection of AdVs by direct hybridization have proven to be insensitive (Horwits, 1996). Antibody detection has limited value in the diagnosis of primary AdV infec-

Table 2. The summery of PCR product detection on agarose, polyacrylamide gel electrophoresis and DIAPOPS.

Samples	A4	C2	F1	G1	H1	E8	B4	C6	H3	H9	F2
Agarose results	+	+	+	+	+	-	-	-	-	-	-
Polyacrylamide results	+	+	+	+	+	+	+	+	+	+	-
DIAPOPS results	+	+	+	+	+	+	+	+	+	+	+

Using agarose method five samples by PAGE method 10 samples and by DIAPOPS method 11 samples were detected as positive... The + symbol shows the positive samples.

tion, because most people have been infected during childhood with one or more AdV types, and in immunosuppressed patients, a diagnostic rise in titer of antibody against AdVs may fail to develop. Also there is a risk that anti-AdF MAbs (monoclonal antibodies against fastidious adenoviruses) may not react with all the strains. So there is a need to use more than one type of MAb for even one type of adenoviruses, to detect all the strains. The relevance of this problem proved as illustrated by the finding that a commercial MAb-based ELISA failed to demonstrate the presence of a highly prevalent serotype in Canada (Scott-Taylor, 1990).

The advantages of detecting viral DNA by PCR include speed, sensitivity, ability to detect non-infective particles, and potential elimination of toxic effects of the specimen or contaminating microorganisms. PCR assays for the detection of enteric Adenoviruses (subgenus F, type 40 or 41) have also proven to be more sensitive and specific than commercially available enzyme immunoassay and AdV isolation by the cell culture technique (Allard *et al*., 1990).

Although there are many PCR methods to detect adenoviruses, they detect all AdV, and restriction analysis is needed for determining the type of the virus. Since only enteric AdVs show clear association with children's gastroenteritis, it is clinically suitable to detect them specifically in the shortest time and with high sensitivity. Because both types have the same clinical characteristics, there is no need to detect them separately. We designed the specific primers from a conserved sequence between Adenoviruses types 40 and 41, so both types were detectable, but not any other viruses and Adenoviruses could not be detected by PCR. The specificity of both tests was the same and none of them detected other viruses. But, as we assumed, PCR does not have enough sensitivity to detect all positive samples. Although this higher sensitivity is the advantage over the PCR, DIAPOPS as described by the Nunc Company, takes too long to be used clinically. Therefore, we modified and optimized this technique for clinical applications. We increased the probe incubation temperature up to 10°C (50 to 60°C), so its time decreased from overnight to 60 minutes. Consequently the overall time of amplification and detection is reduced to just one day. When it is considered that the coating process can be performed beforehand, and the coated plates can be stored at 4ºC for a long time, it is clear that this one day time period has clinical importance for a diagnostic test. In addition, as in clinical conditions, if the number of samples increases, the number of required gels increases too and thus more time would be required. But in DIAPOPS method one could do the detection procedure for many samples simultaneously. Our results show that streptavidine incubation time can be reduced from one hour to 30 min. The reduction of probe and streptavidine incubation time, reduce the risk of false positives (especially in the case of streptavidine incubation time), and increase the accuracy of the method without any deleterious effects on the results. Although PAGE has a reliable sensitivity, it is time consuming and not suitable for clinical samples. PAGE did not detect the DIAPOPS positive sample with the least O. D. value (Fig.2). It suggests the higher sensitivity of DIAPOPS than the PAGE procedure. But it needs more experimental data.

The detection of the PCR product in this procedure requires no electrophoresis apparatus, UV light, or darkroom, and furthermore the use of toxic chemical agents such as ethidium bromide is avoided. Moreover, the technique allows the simultaneous handling of a large number of samples and can be automated, making it very attractive for use in any clinical laboratory (Cherian *et al*., 1998; Venturoli *et al*., 1998; Sails *et al*., 2001). PCR-based assays almost completely obviate the need for direct handling of the pathogen, thus drastically reducing the risk of infection of laboratory personnel (Staszkiewicz *et al*., 1991; Fiori *et al*., 2000; Yagupsky *et al*., 2000). Finally, any sample can be stored at -20ºC until processing, thus enabling it to be collected by any physician and either processed immediately or stored if necessary, safely sent to another laboratory.

In conclusion, our DIAPOPS system for detection of enteric adenoviruses provides a powerful, sensitive and specific test, which can be used clinically.

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