Detection and molecular characterisation of adenovirus in children under 5 years old with diarrhoea

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Background/aim: To determine the frequency, genotype distribution, and genetic relatedness of adenoviruses in children under 5 years old with diarrhoea and to investigate their distribution according to clinical findings, age, months, and seasons.

Materials and methods: Stool samples were collected from 180 children with acute gastroenteritis who presented from July 2007 through June 2011 at the Ankara Training and Education Hospital. Stool samples were analysed by immune chromatographic test (ICT), enzyme immunoassay (EIA), and polymerase chain reaction (PCR). All adenovirus types were determined by nucleotide sequence analysis. A phylogenetic tree was constructed by Mega 6.0 using the neighbour-joining method.

Results: Five percent of the samples were positive for adenovirus (9/180) by ICT, 6.1% (11/180) by EIA, and 13.9% (25/180) by PCR. Adenovirus gastroenteritis did not show any differences in age group, sex, month, or season. In this study, 16 (64%) of the PCR positive samples were AdV41, 6 (24%) were AdV40, 2 (8%) were AdV31, and 1 (4%) was AdV7, as determined by nucleotide sequencing.

Conclusion: AdV31 and AdV7 were associated with gastroenteritis. Adenovirus serotypes showed a similarity of 80% (20/25) and 20% (5/25) with Asian and American serotypes, respectively.

Key words: Adenovirus, children, PCR, gastroenteritis, genotype, diarrhoea, phylogenetic analysis

1. Introduction
Diarrhoea is the second most prevalent cause of death in children worldwide. More than 1 billion diarrhoea cases occur every year, and about 700,000 children under 5 years old die (1). Viruses are the major aetiological agents of acute gastroenteritis in children under 5 years of age. In developing countries, enteric viruses such as rotaviruses, noroviruses, human enteric adenoviruses (HAdV), human astrovirus, and sapoviruses are the major cause of childhood diarrhoea (2,3). Bocavirus, aichivirus, and human parechovirus have recently been described in patients with diarrhoea, but their association with acute gastroenteritis has not been established yet (4,5).

Human adenovirus causes acute gastroenteritis sporadically, as well as in outbreaks (6). Besides acute gastroenteritis, adenoviruses cause respiratory diseases, conjunctivitis, and haemorrhagic cystitis (7). Adenovirus belongs to the family Adenoviridae and the genus Mastadenovirus. There are 58 types of adenoviruses identified to date, which are grouped into 7 species, A to G. They are grouped based on neutralisation tests or genome analysis (8).

The adenovirus diseases are associated with species and while a single serotype causes many clinical pictures, a clinic picture may be caused by more than one serotype. Adenovirus species F, which includes types 40 and 41, is associated with gastroenteritis, and thus is called an enteric adenovirus. Types 40 and 41 are responsible for 1%–20% cases of diarrhoea in young children. Other species such as A (types 12, 18, and 31), C (types 1, 2, and 5) and D (types 28, 29, 30, 32, 37, 43–46) have also been associated with diarrhoea (9–11).

This study was aimed to determine the frequency of adenovirus in children under 5 years old with diarrhoea and to investigate the distribution according to clinical findings, age groups, months, and seasons. The methods

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were compared with each other to determine which method used in the diagnosis of adenovirus is best. The study also determined molecular characterisation of the detected adenoviruses to identify adenovirus types associated with gastroenteritis outside 40/41 and to perform a genotyping and phylogenetic analysis of enteric adenoviruses. Hence, genotyping and phylogenetic analysis of enteric adenoviruses have been performed for the first time in Turkey.

2. Materials and methods

2.1. Study population
The duration of the study was from July 2007 through June 2011. Stool samples were obtained from 180 children under 5 years old (90 male and 90 female) with acute diarrhoea that presented to the Ankara Training and Research Hospital.

2.2. Inclusion and exclusion criteria
Children presenting with acute diarrhoea in the diarrhoea wards were included in this study. Diarrhoea was defined as the passing of watery stool more than three times within 24 h. Acute diarrhoea was defined as diarrhoea with a duration between 24 h and less than 14 days. Nondiarrhoeic stool samples were excluded from the study. The stool samples that included other gastroenteritis agents such as bacteria, parasites, norovirus, rotavirus, and bocavirus were excluded from the study.

2.3. Collection of stool samples
The diarrhoeic children's clinical and demographic information, including date of birth (age), sex, and sample collection date, was recorded. Consistency of stool and duration of diarrhoea and the presence and the number of episodes of vomiting was also recorded. Of each stool sample, an aliquot was stored at −80 °C until analysis.

2.4. Adenovirus detection
Stool samples were analysed by rapid chromatographic immune diagnostic test, enzyme immune assay (EIA), and polymerase chain reaction (PCR).

2.4.1. Immune-chromatographic test
Adenovirus antigen was detected using the commercially available immune-chromatographic test VIKIA Rota-Adeno (bioMérieux, France). The specificity and sensitivity of the VIKIA Rota-Adeno kit (bioMérieux, France) were 100% and 97.6%, respectively. All reagents and stool samples were allowed to reach room temperature before use. Fifty microlitres of homogenised stool sample was added into the buffer and shaken vigorously in order to suspend it. Two drops of dilute sample were added to the test card sample well. The test card was incubated at room temperature and read after 5–10 min. The immune chromatographic stick was coated with polyclonal antibodies specific for all adenovirus hexon antigens. If adenovirus was present in the sample, immunoreaction appeared as a red line. A total absence of colour indicated procedure error or that the test reagent had deteriorated. In that case, the test was repeated with a new strip.

2.4.2. Enzyme immuno-assay
All samples were analysed with EIA Kit, Adenoscreen EIA (Microgen Bioproduct, UK), designed to detect all HAdV serotypes. Both specificity and sensitivity were 98% for the Adenoscreen EIA kit (Microgen Bioproduct, UK) and no crossreaction occurred. The test was performed with 10% faecal suspensions according to the manufacturer's instructions. Fifty microlitres of faecal samples, the positive control, and the negative control were dispensed into respective wells. One drop of conjugate was dispensed into each well, the side of the plate was tapped gently to mix, and the samples were then incubated at room temperature for 30 min. After incubation, the plate was washed with wash buffer. Two drops of substrate were dispensed into all wells and incubated at room temperature again for 20 min. To stop the chromogenic reaction, drops of stop solution were added into each well. The positive sample's colour in the wells changed from blue to a uniform yellow. Microplates were read with a spectrophotometer (das Plate Reader, Italy) using a wavelength of 450 nm. Dilute suspension containing inactivated adenovirus antigen was used as a positive control, and sample diluent was used as a negative control. According to the manufacturer's instruction, the OD of negative control at <0.15 and of positive control >0.6 indicated that the test performance was within accepted limits. Cut-off value was calculated as negative control OD + 0.1. The samples with OD of 10% and above the cut-off value were considered positive and 10% and less than the cut-off value were considered negative.

2.4.3. DNA extraction
DNA was extracted using a QIAamp Viral RNA Kit (QIAGEN, Germany) with the spin column method according to the manufacturer's instructions. Faecal suspensions were prepared and vortexed. Total nucleic acid was recovered in 60 μL of nuclease-free water and stored at −80 °C until analysis.

2.4.4. Polymerase chain reaction (PCR)
Samples were analysed by PCR with hexon-gene–specific primers designed to detect all HAdV types. The primers used for PCR were AdV F: 5’GCCACCGTGCGGTTCCTAAACTT3’ and AdV R: 5’GCCCCCAGTGCTTACATGCACATC3’, which produced 131-bp products. PCR was carried out in a 25-μL final reaction volume containing 1 μL of DNA, adenovirus forward primer (100 nM final concentration), adenovirus reverse primer (100 nM final concentration), PCR master mix (Taq Polymerase, dNTP, MgCl2 and reaction buffer), and PCR-grade water. All amplifications were performed using a Thermal Cycler (Applied Biosystems, Foster City,
Amplification conditions were as follows: 1 cycle at 94 °C for 5 min, 1 cycle at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, and 1 cycle at 72 °C for 5 min. PCR products were analysed by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light.

2.4.5. Determination of nucleotide sequences
PCR amplicons were purified using a PCR purification kit (Performa Short Plate, Edge Bio, USA). Products were sequenced in both directions using the same primers used in PCR. The product was sequenced using a Big Dye DNA sequencing kit in an automated DNA sequencer, an ABI Prism 3130 XL Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

2.4.6. Phylogenetic analysis
The obtained nucleotide sequences were compared with the NCBI GenBank database to determine the adenovirus serotypes. Nucleotide sequence alignment was performed using Clustal W multiple alignment. A phylogenetic tree was constructed by Mega 6.0 using neighbour-joining methods (12). For the construction of phylogenetic trees, nucleotide sequences were used. Bootstrap analysis of 1000 replicates was done to find the significance of the branching of the constructed tree.

2.5. Statistical analysis
The data were analysed using Statistical Package for the Social Sciences (SPSS for IBM-PC, release 20.0; SPSS Inc., Chicago, IL, USA). The prevalence and the median age of adenovirus infection and significant associations between adenovirus positivity seasons and demographic or clinical characteristics in diarrhoeic children were compared using a Mann–Whitney U test and chi-square ($\chi^2$) test. A cut-off P-value of <0.05 was considered significant.

2.6. Ethical review of the proposal and the consent
The research proposal was approved by the ethical review board of the Faculty of Medicine, Gazi University (25901600-43). The verbal consent of the mother or the guardian of the child was obtained prior to the sample collection.

3. Results
A total of 180 patients were enrolled in this study for stool sample collection. There were 90 males and 90 females. The samples were found to be positive in 5% (9/180) of samples by ICT, 6.1% (11/180) by EIA, and 13.9% (25/180) by PCR. The patients’ ages ranged from 0 to 59 months; 58.8% (106/180) were under 24 months, and 41.2% (74/180) over 24 months old. Of the patients, 2 (1.1%) of 0–2 months, 3 (1.7%) of 3–5 months, 5 (2.8%) of 6–11 months, 4 (2.2%) of 12–23 months, 5 (2.8%) of 24–35 months, 3 (1.7%) of 36–47 months, and 3 (1.7%) of 48–59 months old were found to be adenovirus-positive by PCR.

Age group distribution of adenovirus-positive diarrhoea did not show statistically significant differences among the three methods (Figure 1).

Among the adenovirus-positive cases, 52% (13/25) were male and 48% (12/25) were female. Sex distribution of adenovirus-positive diarrhoea did not show any statistically significant differences among the three methods (Figure 2).

Adenovirus positivity by ICT, EIA, and PCR was investigated by month of occurrence. Adenovirus was identified throughout the year; two peaks were observed, in May (28.5%) and January (23%). Monthly distribution of adenovirus-positive diarrhoea did not show any

![Figure 1](attachment:fig1.png)  
**Figure 1.** The number of patients with adenovirus diarrhoea distributed according to age.
statistically significant differences among the three methods (Figure 3).

Adenovirus diarrhoea was highest in winter. Seasonal distribution of adenovirus-positive diarrhoea did not show any statistically significant differences among the three methods (Figure 4).

We identified that vomiting in adenovirus gastroenteritis is an important feature, as 77 patients (42.8%) had vomiting and 103 (57.2%) were not vomiting, but a statistically significant relationship between the presence of adenovirus and vomiting was absent.

In 48% (12/25) of adenovirus-positive patients, 6 times and higher number of daily diarrhoea was observed. Only the presence of adenovirus and the number of daily episodes of diarrhoea showed a statistically significant difference (P < 0.05) (Table 1).

The samples were found to be positive in 5% of cases (9/180) by ICT, 6.1% (11/180) by EIA, and 13.9% (25/180) by PCR. Two samples that could not be tested by ICT were determined by EIA (Table 2).

Compared with PCR results (Figure 5), the sensitivity of ICT with 36% specificity was 100%, PPV was 100%, and NPV was 90.6%. Again, compared with PCR results, EIA test sensitivity has been identified as 44%, specificity 100%, PPV 100%, and NPV 91.7% (Table 3).

Twenty-five PCR-positive samples were sequenced, and four different adenovirus serotypes were determined by using BLAST. Sixteen samples (64%) were positive for AdV41, 6 (24%) for AdV40, 2 (8%) for AdV31, and 1 (4%) for AdV7 (Figure 6).

In the phylogenetic tree, AdV41 strains showed two clusters. The Turkish strains AHP195, AHH233, AHP239, AHP308, AHP530, AHP535, AHP166, AHP179, AHP353, AHP544, AHP563, AHP643, and AHP694 formed a cluster with strains from China (GenBank accession number: KC953653 and JX412892). Turkish strains AHH237, AHP331, and AHP469 formed another cluster with a strain from the USA (KF303071) (Figure 7).

Turkish strains AHH126, AHP273, AHP381, AHP424, AHP433, and AHP734 of AdV40 formed a cluster with strains from Thailand and Japan (KC632648 and AB330121) (Figure 7).

Turkish strains AHP582 and AHP753 of AdV31 formed a cluster with a strain from the USA (KF268119) (Figure 7). Turkish strain AHP497 of AdV7 formed a cluster with a strain from China (JF713007) (Figure 7).
4. Discussion

Diarrhoeal diseases are a major health problem for children in Turkey, as in other developing countries in the world. One billion diarrhoea cases occur every year and about 700,000 children die. The most common viral agents of childhood gastroenteritis in Turkish children are rotavirus, norovirus, and adenovirus (13).

Although adenoviruses cause disease in all age groups, disease is more common in the above 2 years old age group (13–15). In the present study, more than half of adenovirus infections occurred in the above 2 years old age group, but it did not show statistical significance with other age groups. It may be associated with the ages of 0–24 months because in this age group children start walking, and can therefore come in contact with contaminated surfaces and the environment.

As in other studies, we could not find any relationship between adenovirus positivity and sex (16,17–21).

Several studies from Turkey and other countries have shown that enteric adenoviruses occur year-round (16,19,22–24). Adenovirus incidence was more frequent in winter, but it was not statistically significant in our study. Monthly distribution showed that adenovirus infection was higher in May than in other months; however, this result was also not statistically significant.

We identified that vomiting in adenovirus gastroenteritis is an important symptom, but statistical significance was absent. In about half of the adenovirus-positive patients, at least 6 episodes of diarrhoea per day were observed. This result was statistically significant. Other studies showed similar results; thus, it may be an important point for differential diagnosis of adenovirus diarrhoea (25,26).

Table 1. Adenovirus PCR and numbers of daily diarrhoea occurrences.

<table>
<thead>
<tr>
<th>Adenovirus PCR</th>
<th>Daily diarrhoea</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1–3</td>
<td>4–5</td>
</tr>
<tr>
<td>Positive Number</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>%</td>
<td>16%</td>
<td>36%</td>
</tr>
<tr>
<td>P</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Negative Number</td>
<td>35</td>
<td>73</td>
</tr>
<tr>
<td>%</td>
<td>22.6%</td>
<td>47.1%</td>
</tr>
<tr>
<td>Total Number</td>
<td>39</td>
<td>82</td>
</tr>
<tr>
<td>%</td>
<td>21.6%</td>
<td>45.6%</td>
</tr>
</tbody>
</table>

* Adenovirus presence and 6 times and higher number of occurrences daily of diarrhoea was statistically significant.

Table 2. Result of immune chromatographic method, EIA, and PCR (n: 180).

<table>
<thead>
<tr>
<th>PCR</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Immune chromatographic method</td>
<td>155</td>
<td>0</td>
</tr>
<tr>
<td>EIA</td>
<td>155</td>
<td>0</td>
</tr>
</tbody>
</table>
In Turkey and Europe, 1.16%–11.7% of diarrhoea samples have been found positive for adenovirus by ICT (27–30), which is concordant with our study. However, it should be mentioned that these studies used ICTs from different manufacturers, which may have affected the sensitivity of the test. In the present study, EIA was found to be more sensitive than ICT. Two samples that could not be detected by ICT were determined by EIA. EIA allowed detection of antigens at very low concentrations, there was no crossreaction, it was ready to use, was low cost, and did not require skilled personnel or laboratory equipment. Therefore, EIA can be used for large numbers of samples to detect more positive samples.

In different settings, the proportion of adenovirus-positive samples determined by EIA varied from 2.9%–16.7% (31–34). The proportion of adenovirus positivity that we have identified in Turkey by EIA is concordant with that found in Europe.

Studies have shown that ELISA tests specific for AdV40 and AdV41 detect a lower number of positive samples than ELISA tests that can detect all adenovirus serotypes (25,35). These studies have supported the finding that gastroenteritis is also associated with other serotypes of adenovirus besides AdV40 and AdV41.

The results of adenovirus detection rate by PCR in our study are in concordance with results from other countries (36). There are a few studies on the detection of enteric adenovirus by PCR in Turkey. The Public Health Institute of Turkey Virology Reference Laboratory found that adenovirus is present in 4% of diarrhoeal samples, which is considerably lower than the results found in the present study. The reason behind this difference may possibly lie in the use of two different PCR methods; we used adenovirus specific primers only for detection of adenovirus serotypes, but the reference laboratory used multiplex real-time PCR.
Multiplex real-time PCR includes numerous primers and determines more than one species, and so detecting certain species is difficult with multiplex real-time PCR. Additionally, we selected stool samples that were negative for other gastroenteritis agents such as bacteria, parasites, norovirus, rotavirus, and bocavirus. When ICT and EIA are compared with PCR, PCR was more specific and had higher PPV and NPV.

When Rovida et al. (37) compared immunological methods and molecular methods for diagnosis of viral gastroenteritis, the sensitivity of ICT was in concordance with our study. Several studies revealed that along with serotypes AdV40 and AdV41, serotypes AdV31, AdV18, AdV15, AdV12, AdV7, and AdV5 are associated with adenovirus gastroenteritis (9,25,35,36,38). The frequency of AdV41 was highest in this study, which was concordant with world literature. The phylogenetic analyses showed that our genotypes were in close association with isolates from China, Japan, Thailand, and USA. Adenovirus serotypes

Figure 7. For the construction of phylogenetic tree, nucleotide sequences were used.
that were detected in our study were in concordance with Asian serotypes; however, AdV31 showed similarity with those from the United States. We think that adenovirus serotypes in our continent might have come from the same origin.

The molecular epidemiology, genotyping, and phylogenetic analyses of enteric adenovirus serotypes from Turkey are described for the first time in the present study. Our study reveals that like serotypes AdV40 and AdV41, AdV31 and AdV7 might also be associated with

References


