

Identification of Viral Agents Causing Diarrhea Among Children in the Eastern Center of Tunisia

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Viral diarrhea remains a major cause of childhood morbidity and mortality worldwide. In Tunisia, no comprehensive studies of all viral agents related to diarrhea in children have yet been conducted. The present study was performed to investigate the role of enteric viruses in acute diarrhea in the country. Six hundred thirty-eight stool samples were collected from children under 5 years of age seeking medical care for acute diarrhea between October 2003 and September 2005 in hospitals from the Eastern-Center Tunisia. All samples were tested for rotavirus, astrovirus, and adenovirus using commercial antigen enzyme immunoassays (EIAs). Positive samples for rotavirus and astrovirus were confirmed by an "in-house" reverse transcriptase-polymerase chain reaction (RT-PCR). Samples positive for adenovirus antigen were subjected to further EIA screening for species F enteric adenovirus types 40 and 41. At least one viral agent was found in 30% of the specimens. The frequency of rotavirus, astrovirus, and adenovirus was 20%, 7%, and 6%, respectively. Of the stool samples containing adenovirus, 57% (20/35) were found to be positive for species F adenovirus types 40/41. Dual infections were found in 9% (17/191) of the positive samples. Enteric viruses appear to play an important role in pediatric diarrhea in Tunisia. The introduction of affordable viral diagnosis in pediatric hospitals will improve patient care by reducing the unnecessary use of antibiotics. **J. Med. Virol. 78:1198–1203, 2006.** © 2006 Wiley-Liss, Inc.

KEY WORDS: diarrhea; rotavirus; astrovirus; adenovirus

INTRODUCTION

Diarrheal disease is a major cause of childhood morbidity and mortality, especially in developing countries [Bern et al., 1992]. In the year 1990, it was estimated that 2.9 million deaths worldwide could be attributable to diarrheal disease [Murray and Lopez, 1997].

Several different groups of viruses have been shown to be responsible for the high incidence of acute viral diarrhea among children during their first years of life. Three major categories of viruses are now recognized as clinically important including rotavirus, astrovirus, and adenovirus [Wilhelmi et al., 2003]. Rotavirus is the single most important etiological agent in severe dehydrating diarrhea. They comprise a genus within the family *Reoviridae* characterized by non-enveloped triple-layered viral particles with a viral genome composed by 11 segments of double-stranded RNA (dsRNA) [Parreño et al., 2004]. Each year, group A rotavirus causes approximately 111 million episodes of gastroenteritis requiring only home care, 25 million clinic visits, 2 million hospitalizations, and 440,000 deaths in children under 5 years of age [Parashar et al., 2003]. Astrovirus has only recently been recognized as a common cause of diarrhea in children. Astroviruses are single stranded, positive sense RNA viruses and are

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members of the family *Astroviridae* [Cunliffe et al., 2002]. Currently, eight serotypes of astrovirus have been reported. They are responsible for between 2.5% and 9% of cases hospitalized with diarrhea [Glass et al., 1996]. A limited number of adenovirus strains have been associated with childhood diarrhea, types 40 and 41. Adenoviruses are double-stranded DNA viruses, the 47 types characterized are classified in six subgroups A–F. Each adenovirus type is defined as a unique serotype by specific neutralization and hemagglutination inhibition reactions [Scott-Taylor et al., 1995]. Enteric adenovirus types 40/41 have been identified up to 7.9% cases of diarrhea in children [Grimwood et al., 1995; Bon et al., 1999; Qiao et al., 1999; Giordano et al., 2001; Simpson et al., 2003].

In Tunisia, according to data from the “sentinel system” (Ministry of Health), the incidence of diarrhea in the public health sector in children younger than 5 years of age is 7%, and in two-thirds of cases, a viral cause is clinically presumed [Soltani et al., 1999]. While the role of rotaviruses has been established previously in the country [Trabelsi et al., 2000], little is known about the frequency of other gastrointestinal viruses such as astroviruses and adenovirus types 40/41. Defining the viral agents related to diarrhea will assist in providing an accurate estimate of disease burden within the country. This will allow for a proper assessment of the contribution of each virus to morbidity. The availability of accurate information on the etiology of viral diarrhea will also be useful when assessing the impact of vaccinations when they become available.

The present study was undertaken to determine the relative frequency of rotavirus, astrovirus, and adenovirus infection in young children from the Eastern-Center of Tunisia, using improved diagnostic methods, and to broaden our knowledge on the epidemiology of diarrhea disease in young Tunisian children.

PATIENTS AND METHODS

Study Design

Stool samples were collected from children under 5 years of age seeking medical care for acute diarrhea between October 2003 and September 2005 in five hospitals from the Eastern-Center of Tunisia: University Hospital Farhat Hached (Sousse), University Hospital Sahloul (Sousse), University Hospital Fatouma Bourguiba (Monastir), University Hospital Tahar Sfar (Mahdia), and University Hospital Hedi Chaker (Sfax). Samples were collected either from children hospitalized in a Pediatric Unit or from children attending the out-patient department of the hospital for acute diarrhea. Samples were stored at -20°C until testing. All samples were tested for rotavirus, astrovirus, and adenovirus presence using commercial antigen enzyme immunoassays (EIAs). Positive samples for rotavirus and/or astrovirus were confirmed by in-house reverse transcription-polymerase chain reactions (RT-PCRs). Samples positive for

adenovirus antigen were subjected to further screening for species F enteric adenovirus types 40 and 41.

Viral RNA Extraction

Viral RNA was extracted and purified from 140 μl of 10% fecal suspensions prepared in distilled water using the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany). This relies upon lysis of specimen with guanidium thiocyanate, followed by nucleic acid purification by binding to a silica membrane. The extracted nucleic acids were eluted in a final volume of 60 μl RNase-free water, and the samples stored at -20°C until use.

Rotavirus Detection and Confirmation

Rotavirus antigen was detected using a commercial EIA kit (IDEIA Rotavirus, DAKO Ltd., Glostrup, Denmark) following the manufacturer’s recommendations and the positive samples were confirmed by RT-PCR using the Beg9 and End9 pair of primers as previously described [Gouvea et al., 1990].

Astrovirus Detection and Confirmation

Astrovirus antigen was detected using a commercial EIA kit (IDEIA Astrovirus, DAKO Ltd.) following the instructions of the manufacturer.

The EIA-positive samples were confirmed by RT-PCR with primers Mon348/Mon340 for an expected size product of 289 nucleotides region within ORF1a [Belliot et al., 1997]. Briefly, 4 μl of RNA template was added to 2 μl of primer Mon348 (10 μM), 5 μl of 10 \times Mg-free buffer (Thermophilic DNA polymerase 10 \times buffer, Promega, Madison, WI), 0.1 μl of RNasin (Promega), 2 μl of 10 mM deoxynucleoside triphosphates mix, 0.5 μl of 0.1 M dithiothreitol (Invitrogen, Burlington, Canada), 3 μl of 25 mM MgCl_2 (Bioline GmbH, Luckenwalde, Germany), 29.9 μl of DEPC-treated water and 0.5 μl of avian myeloblastis virus reverse transcriptase at 24 U/ μl (Roche, Mannheim, Germany). This mixture was incubated at 42°C for 1 hr. After immediate cooling on ice for at least 2 min, the mixture was added to 3 μl of PCR mixture containing 0.1 μl of 10 \times Mg-free buffer, 2 μl of primer Mon340 (10 μM), 0.5 μl of DNA polymerase at 5 U/ μl (BioTaq DNA polymerase, Bioline GmbH, Germany), and 0.4 μl of DEPC-treated water. Thirty amplification cycles were carried out (1 min at 95°C , 1 min at 50°C , and 1 min at 72°C), followed by a final extension at 74°C for 5 min. The PCR products were analyzed by agarose gel electrophoresis (2%) containing 0.5 $\mu\text{g/ml}$ ethidium bromide followed by visualization under ultraviolet light.

Adenovirus Detection

Stool samples were subjected to direct adenovirus antigen detection with the Ridascreen Adenovirus EIA kit (R-Biopharm GmbH, Darmstadt, Germany) following the manufacturer’s recommendations. This test uses monoclonal antibodies directed against

the hexon-antigen of adenoviruses in a solid-phase sandwich-type ELISA.

Adenovirus Types 40 and 41 Detection

Samples positive for adenovirus antigen were subjected to further screening for enteric adenovirus types 40 and 41 with the species F-specific Premier Adeno-clone-Type 40/41 EIA kit (Meridian Bioscience, Nice, France) following the instructions of the manufacturer.

Statistical Analysis

The data were processed utilizing the Statcalc Program Epi Info version 6.0 (Centers for Disease Control and Prevention) to determine if there was significant difference in the detection rates of each virus between inpatients and out-patients. The variables were compared by means of the "One-Way ANOVA" test (Analysis Of Variance). Significance level of $P < 0.05$ was used for all analyses.

RESULTS

Six hundred thirty-eight stool samples were collected from children with diarrhea. Three hundred eighty-one samples were obtained from inpatients and 257 were collected from out-patients. Among the 638 stool specimens, 191 (30%) contained at least one of the three viruses examined for, whereas no virus could be detected in the 447 (70%) remaining samples. The frequency of each individual agent according to the EIA and PCR results is summarized in Table I. Frequency of detection of rotavirus, astrovirus, and adenovirus was 20%, 7%, and 6%, respectively. Among the 35 samples that were positive for adenovirus, 20 (57%) belonged to species F types 40/41, which represents 3% of the study sample cohort.

Dual infections were found in 17 of the 191 positive samples (9%). Seven of these were combinations of rotavirus with astrovirus, five were combinations of rotavirus with adenovirus, and five were combinations of astrovirus with adenovirus. No stool with three viral agents was identified.

TABLE I. Frequency of Detection of Rotavirus, Astrovirus, Adenovirus, and Adenovirus Types 40/41 Among Children With Diarrhea in the Eastern Center of Tunisia From October 2003 to September 2005

	Number of EIA-tested samples	Number (%) of EIA-positive samples	Number (%) of samples positive by both EIA and PCR
Group A	638	133 (20.8%)	128 (20.1%)
Rotavirus			
Astrovirus	638	45 (7.1%)	45 (7.1%)
Adenovirus	638	35 (5.5%)	ND
Adenovirus types 40/41	35	20 (57.1% ^a)	ND

ND, not determined.

^a57.1% of Adenovirus-positive strains belonged to serotype 40/41. Globally, 3.1% of the 638 tested samples were positive for adenovirus types 40/41.

Among the 381 samples collected from hospitalized children, 151 (40%) were positive for at least one virus: frequency of detection of rotavirus, astrovirus, and adenovirus was 28%, 9%, and 5%, respectively. Among the 257 stool specimens collected from out-patients consulting for diarrhea, 40 (16%) were positive for at least one virus: frequency of detection of rotavirus, astrovirus, and adenovirus was 8%, 5%, and 6%, respectively (Fig. 1). A higher prevalence of rotavirus and astrovirus ($P < 0.001$) was observed in hospitalized children compared to those presenting to the out-patient department of the hospital. For adenovirus, no significant difference was found between hospitalized and consulting patients.

The monthly distribution of the number of total diarrhea cases reported and the number of viral diarrhea in children from October 2003 to September 2005 is shown in Figure 2. The monthly distribution of rotavirus, astrovirus, and adenovirus detection in stool samples is shown in Figure 3. The seasonal trends of rotavirus detection was characterized by a peak incidence during cold months of the year (November to March), whereas astrovirus and adenovirus diarrhea occurred without any obvious seasonality, as no peak incidence was noted.

DISCUSSION

Although the importance of viral diarrhea as a prime cause of morbidity and mortality in developing countries is well recognized, to our knowledge no systematic studies have been conducted to evaluate the role of viral agents in childhood diarrhea in Tunisia. The present study, carried out during 2 years from October 2003 to September 2005 in the Eastern Center of Tunisia, used EIA techniques to screen stool samples for viral agents responsible for diarrhea. Thus, previous studies indicated that EIA, which is easier to perform, faster, and cheaper than RT-PCR, may be sufficiently sensitive and suitable for routine diagnostic work. Also other investigators have shown that although the RT-PCR may be more sensitive than EIA, this may not be relevant in

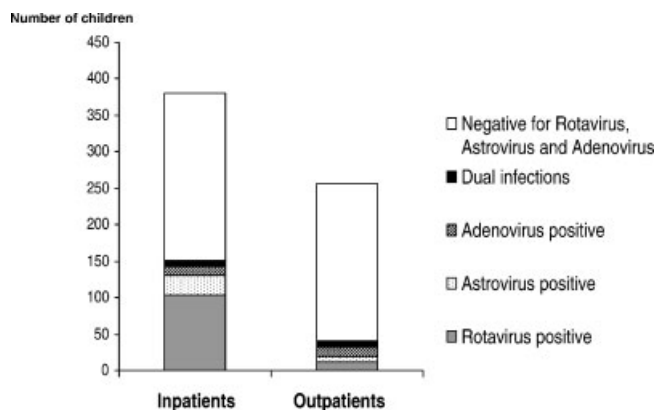


Fig. 1. Frequency of detection of rotavirus, astrovirus, and adenovirus among hospitalized and consulting children with diarrhea in the Eastern Center of Tunisia from October 2003 to September 2005.

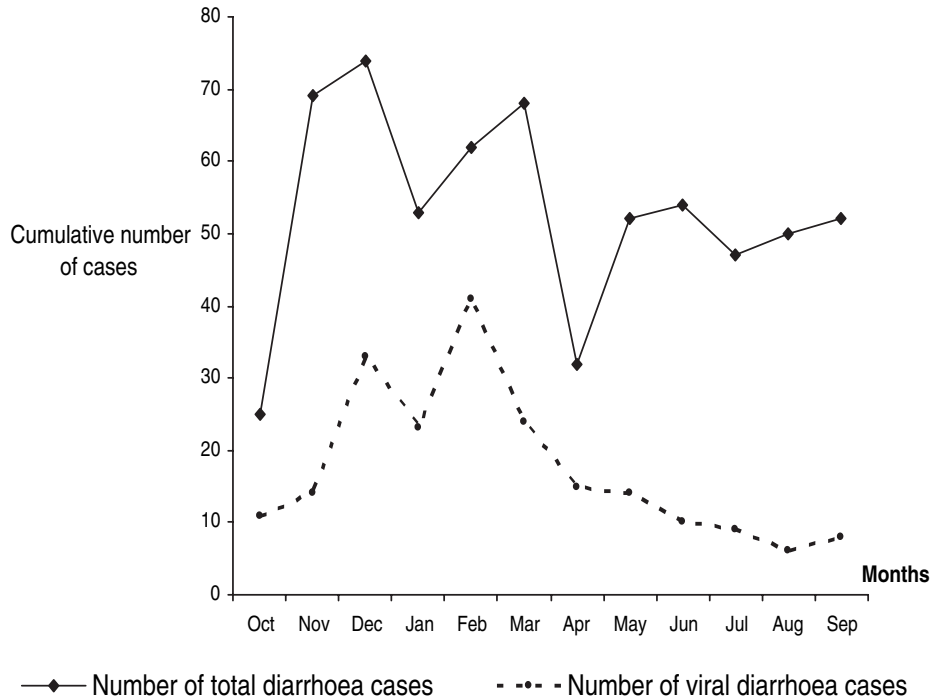


Fig. 2. Monthly distribution of the number of total diarrhoea cases reported and the number of viral diarrhoea among children in the Eastern Center of Tunisia from October 2003 to September 2005.

routine diagnostics since large amounts of viral particles, adequate for antigen detection by EIA, are shed in feces in a virus-induced diarrhoea [Mitchell et al., 1995; Buesa et al., 1996; Taylor et al., 1997; Liu et al., 2005].

The present report revealed that 30% of children less than 5 years old with diarrhoea were infected with at least one viral agent. Similar studies realized in China [Liu et al., 2005] and France [Bon et al., 1999] found

higher detection rates, as they detected at least one of the four viruses in 67% and 72%, respectively. The lower detection rate observed in the present study had at least one evident reason: this report investigated stool samples from the entire year, whereas previous studies were carried out during a winter season, which is the peak season of viral diarrhoea in temperate regions. Indeed, the peak of incidence during the cold months of

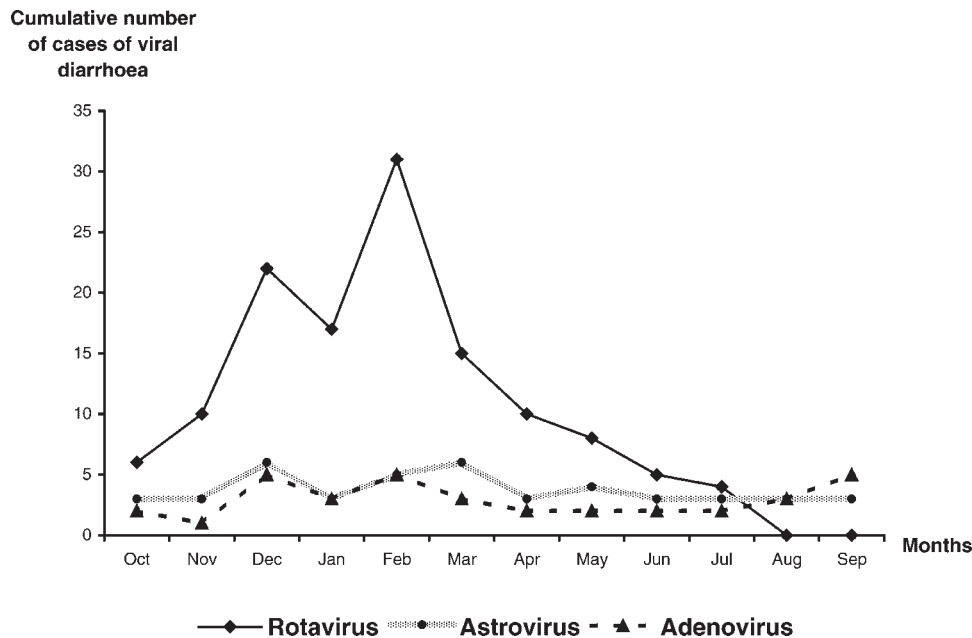


Fig. 3. Monthly distribution of rotavirus, astrovirus, and adenovirus detection in stool specimens collected from children in the Eastern Center of Tunisia from October 2003 to September 2005.

the years was noticed for rotavirus infections. On the other hand, the winter seasonality reported by Traore et al. [2000] for astrovirus infections was not observed in the present study.

While rotavirus has been studied in Tunisia, little is known about the frequency of other viral agents of diarrhea. In this report, 20% of children with diarrhea were infected with rotavirus (Table I). The rotavirus detection rate according to a prospective study realized in the same region of Tunisia between 1995 and 1999 among children from 1 to 60 months of age was 17% [Trabelsi et al., 2000]. Generally, the present study shows good agreement with previous studies reporting detection rates of group A rotavirus, astrovirus, and adenovirus in children with gastroenteritis in developing countries [Bern et al., 1992] as well as in industrialized countries [Bon et al., 1999; Chikhi-Brachet et al., 2002]. This result may suggest that, as it is now well known for rotavirus infections, there may be little difference in the prevalence of viral diarrhea in children from developing or industrialized regions.

A limited number of adenovirus strains have been associated with childhood diarrhea. Subgenus F represented by adenoviruses 40 and 41, which are known to be the most common adenovirus serotypes of acute gastroenteritis, were found in 57% of the adenovirus-positive stool samples (Table I). Thus, in 43% of cases, "nonenteric" adenoviruses were detected among children with diarrhea. This result, reported in previous studies investigating both diarrheic and asymptomatic children, underscores that "nonenteric" adenoviruses such as adenovirus 12 may also play an important role in causing acute gastroenteritis in children [Akihara et al., 2005].

The systematic detection of rotavirus, astrovirus, and adenovirus allowed us to observe a high percentage (9%) of dual infections among positive samples. Similar result has been previously described in China [Liu et al., 2005] and France [Bon et al., 1999], where 9% and 17% of dual infections have been reported, respectively. Dual infections raise the question of whether a single virus is responsible for illness or whether two viruses act in synergy to potentiate each other. The main reason for the diarrheal illness remains unclear, but previous reports did not find a statistically significant difference in clinical symptoms between mono-infections and dual infections [Herrmann et al., 1991; Bon et al., 1999; Liu et al., 2005].

The high prevalence of rotavirus and astrovirus ($P < 0.001$) observed in hospitalized children compared to those presenting to the out-patient department of the hospital was expected for rotavirus but surprising for astrovirus. Although astrovirus infection is common in the community, the disease severity is such that patients are often managed without hospitalization [Waters et al., 2000; Ratcliff et al., 2002]. In comparison, rotavirus infection may be more severe, and patients are more likely to require hospitalization. The high prevalence of rotavirus as a cause of severe gastroenteritis is well documented [Hafferjee, 1995; Glass et al., 1996;

Waters et al., 2000; Ratcliff et al., 2002], and the increasing recognition of the role of astrovirus has been reported [Palombo and Bishop, 1996; Inouye et al., 2000; Koopmans et al., 2000; Pang et al., 2000; Ratcliff et al., 2002] and commonly associated with a less severe illness.

In conclusion, this study shows a high frequency of viral agents, mainly rotavirus and astrovirus, in children treated for diarrhea in hospitals from the Eastern Center of Tunisia. The results indicate that, under the assumption of a causal role of group A rotaviruses, astroviruses, and adenoviruses types 40/41, these viruses accounted for an attributable risk of acute diarrhea of 30% for the Tunisian population between October 2003 and September 2005. The high incidence of astroviruses in diarrhea provides important clues as to possible undiagnosed etiology of gastroenteritis in Tunisian children. The introduction of affordable viral diagnosis in pediatric hospitals will improve patient care and reduce unnecessary use of antibiotics. Moreover, considering the high incidence of rotavirus diarrhea in Tunisian children, the introduction of an effective rotavirus vaccine would be a significant benefit to them and reduce the diarrheal disease burden. Other enteric viruses, such as Caliciviruses, group C rotaviruses, sapoviruses, toroviruses, picobirnaviruses, and coronaviruses, were not investigated in the present study and further studies are needed to fully understand the etiology of viral diarrhea in Tunisian children.

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