Acute Infantile Gastroenteritis Associated with Human Enteric Viruses in Tunisia[▽]

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This prospective study, conducted from January 2003 to June 2005, investigated the incidence and the clinical role of various enteric viruses responsible for infantile gastroenteritis in 632 Tunisian children presenting in dispensaries (380 children) or hospitalized (252 children) for acute diarrhea. At least one enteric virus was found in each of 276 samples (43.7%). A single pathogen was observed in 234 samples, and mixed infections were found in 42 samples. In terms of frequency, rotavirus and norovirus were detected in 22.5 and 17.4% of the samples, respectively, followed by astrovirus (4.1%), Aichi virus (3.5%), adenovirus types 40 and 41 (2.7%), and sapovirus (1.0%). The seasonal distribution of viral gastroenteritis showed a winter peak but also an unusual peak from May to September. The severity of the diarrhea was evaluated for hospitalized infants. No significant differences were observed between rotavirus and norovirus infections with regard to the incidence and the clinical severity of the disease, especially in dehydration.

Diarrhea is one of the major causes of morbidity and mortality among infants throughout the world, especially in developing countries, where malnutrition and poor local health service are factors responsible for the increased severity of the diarrhea. In infants, group A rotavirus (RV) is the major etiologic agent of viral gastroenteritis and is responsible for 29 to 45% of hospitalizations, depending on the income level of the country (32, 33). Other enteric viruses such as human norovirus (NoV), sapovirus, astrovirus (HAsV), adenovirus (HAdV) types 40 and 41, and Aichi virus are also associated with acute gastroenteritis, and their relative importance in high-income countries has been reported previously (2, 4).

Human NoVs, which are members of the *Caliciviridae* family, are found in all age groups (7, 10) and are a major cause of food- and water-related outbreaks (11, 22). Recent work has showed that NoVs are the second most frequent etiologic agents of viral gastroenteritis (16). Sapovirus, another genus of the family *Caliciviridae*, HAsV (41), and enteric type 40 and 41 HAdVs have also been associated with diarrhea. Aichi virus (42) has recently been classified into the *Kobuvirus* genus in the *Picornaviridae* family and has been associated with oyster consumption.

Although the role of these viruses in outbreaks of gastroenteritis in industrialized countries has been established (7, 29), little is known about their contribution to outbreaks in developing coun-

tries and few data are currently available (25). In Tunisia, a middle-income Mediterranean country, earlier studies showed the contribution of RVs (8, 38) and more recently that of HAsVs and HAdVs (12) in cases of childhood diarrhea. However, the nature of the contribution of NoVs and Aichi viruses to outbreaks of gastroenteritis in Tunisia remains unknown.

The aim of this study was to determine the incidence of these enteric viruses and their contribution to diarrheal diseases in Tunisian children. We conducted a prospective 2-year study of children who were hospitalized in or presented to the dispensaries of Monastir, Tunisia.

MATERIALS AND METHODS

Study design. After approval by the local ethical committee, this prospective study was conducted from January 2003 to May 2005 and involved 632 children (325 males and 307 females) under 12 years of age who were hospitalized or not hospitalized and who were suffering from acute gastroenteritis.

Acute gastroenteritis was defined as the occurrence of at least three soft or liquid stools or three bouts of vomiting in 24 h or one of the following signs: diarrhea or vomiting accompanied by at least two additional symptoms, including abdominal pain or fever. Cases of nosocomial or chronic diarrheas (lasting for more than 2 weeks) were excluded from the study.

From January 2003 to May 2005, 252 stool samples were collected from children within 48 h following their hospitalization for acute gastroenteritis in Monastir University Hospital. The samples were screened for routine bacterial agents and then stored at –20°C for further analysis.

From January 2003 to May 2004, 380 samples were also collected from children presenting in the dispensaries for gastrointestinal symptoms.

Clinical data involving such disease manifestations as fever, vomiting, abdominal pain, or bloody diarrhea were collected for all patients. Severity criteria, such as duration of the diarrhea, number of stools or bouts of vomiting, range of body temperature, degree of dehydration, capillary refill time (CRT), and the presence of skin blotches, were determined for all hospitalized children.

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Laboratory methods. The stool samples were screened for the presence of group A RVs, NoVs, sapoviruses, HAdVs, type 40 and 41 HAdVs, and Aichi viruses by enzyme immunoassay and/or reverse transcription-PCR (RT-PCR).

Group A RVs, HAdVs, and type 40 and 41 HAdVs were detected with enzyme immunoassay kits (Argene-Biosoft, France, Dako Diagnostic Ltd., United Kingdom, and Meridian Diagnostics Inc., Cincinnati, Ohio, respectively).

All positive samples were confirmed and characterized by molecular biology methods. Viral nucleic acids were extracted from 20% stool suspensions in phosphate-buffered saline with a QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and were then stored at -40°C .

RV-positive samples were systematically tested by RT-PCR using primer set Beg9 and End9 (14) and primer set Con2 and Con3 (13).

Human caliciviruses were detected by RT-PCR using several sets of primers in separate reactions, which allowed the detection of NoVs and sapoviruses. Primer set SR80 and NVP110 (27) and primer set JV12 and JV13 (40) were used to amplify a fragment of the RNA polymerase genes of sapoviruses and NoVs, respectively. Primer set G1SKF and G1SKR and primer set G2SKF and G2SKR (18) were used to detect a fragment of the capsid genes of genogroup I (GGI) and GGII NoVs, respectively. RT-PCRs were performed using a Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

HAdV-positive samples were confirmed by RT-PCR amplification with primers Mon244 and Mon245 (26).

HAdV-positive samples were confirmed by PCR using the primer set Hex1DEG and Hex2DEG (1). Aichi viruses were detected by RT-PCR using primer set Ai6261 and Ai6779 targeting the RNA polymerase gene (43) and a OneStep RT-PCR kit from Qiagen (2).

Genotyping of NoVs, sapoviruses, HAdVs, and Aichi viruses was performed by direct sequencing of the PCR products with the same primers used for amplification by using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit and a 373A DNA sequencing system (both from Applied Biosystems, Foster City, CA).

Sequence analysis was done using Fasta software, version 3, available from the European Bioinformatics Institute. Sequence alignments were carried out using Clustal W software.

Statistical methods. Statistical analyses were performed with SPSS software, version 13. We compared categorical data by the chi-square (χ^2) test. All data are expressed as means $\pm$ standard deviations. Student's *t* test was used to compare means between qualitative data and one-way analysis of variance to compare means between quantitative data. Correlations between qualitative variables were assessed using Spearman's correlation coefficient. Statistical analyses using χ^2 or Fisher's exact test were performed for comparisons of percentages. All tests were two-tailed; *P* values ≤ 0.05 were considered significant.

RESULTS

Population description and virus distribution. Among the 632 children suffering from gastroenteritis, 276 (43.7%) had a positive stool sample for an enteric virus. Of these, 234 (84.8%) were infected by one virus, 37 (13.4%) by two different viruses, and 5 (1.9%) by three different viruses. A total of 140 positive stool samples came from hospitalized children and 136 from nonhospitalized children. Viral infection was more frequent in hospitalized (55.6%) than in nonhospitalized (35.8%) children ($P < 0.001$).

Group A RVs were identified in 142 (22.5%) stool samples, NoVs in 110 (17.4%), sapoviruses in 6 (1.0%), HAdVs in 26 (4.1%), HAdV type 40 or 41 in 17 (2.7%), and Aichi viruses in 22 (3.5%). There was no significant difference between the incidence of RVs and that of NoVs ($P > 0.05$).

Ages of the children ranged from 1 month to 12 years, with a mean of 21.1 ± 19.5 months, and there were no significant differences among results obtained for the six viruses with regard to mean and median ages. The sex ratios determined according to the type of virus were also similar ($P = 0.11$).

Etiology of acute gastroenteritis. Among the 234 monoinfections, RVs were detected in 107 stool samples (45.7%),

TABLE 1. Distribution of mixed and single-virus infections^a

Agent	No. of infections					
	RV	NoV	Sapovirus	HAdV	AdV type 40 or 41	Aichi virus
RV	107	19 ^b	2	12 ^{b,c}	3	4 ^c
NoV		85	0	7 ^b	1	1
Sapovirus			4	0	0	0
HAdV				9	0	3 ^c
AdV type 40 or 41					13	0
Aichi virus						16

^a Mixed infections were found in 42 (15.2%) of the 276 positive-testing samples.

^b Three samples were positive for a combination of RV, NoV, and HAdV.

^c Two samples were positive for a combination of RV, HAdV, and Aichi virus.

NoVs in 85 (36.3%), sapoviruses in 4 (1.7%), HAdVs in 9 (3.8%), HAdV type 40 or 41 in 13 (5.6%), and Aichi viruses in 16 (6.8%) (Table 1). RVs and NoVs were the most frequently detected viruses in both populations studied (Fig. 1) and were significantly more frequent in hospitalized than in nonhospitalized children ($P = 0.002$ and $P < 0.001$, respectively). However, no significant differences were observed in comparisons of the results with respect to the incidence of the two viruses in each population.

Forty-two mixed infections were detected, of which 18 were found in hospitalized children and 24 in nonhospitalized children (Fig. 1). As shown in Table 1, combinations of RV with one of the other five viruses were the most frequently detected mixed infections (83.3%).

Virus diversity. Rotavirus genotype combination G3P[8] was predominant with 51.5% of strains. This was followed by G1P[8] (28.9%), G9P[8] (5.6%), G3P[4] (1.4%), and G1P[6] (0.7%). The genotyping of NoVs revealed that the GGII strains were the most frequent (10% and 90% for GI and GII, respectively), with GGII.4 the predominant genotype (62.7%).

Viral identification according to age groups. Patients were grouped into the three following age ranges: ≤ 24 months (75.8% [$n = 479$]), 25 to 60 months (19.0% [$n = 120$]), and > 60 months (5.2% [$n = 33$]) (Fig. 2). RVs were detected in 18.4% of stool samples from children under 24 months old, 15.0% of those from children 25 to 60 months old, and 3.0% of those from children over 60 months old. RVs were significantly less frequent in children over 60 months old than in the two younger groups. In contrast, NoVs had similar distribution levels in these three age groups (13.6% of stool specimens from children under 24 months old, 12.5% of those from children 25 to 60 months old, and 15.2% of those from children over 60 months old [$P = 0.90$]). Similarly, no significant differences were reported in the distribution of the other viruses among the three age groups. In children less than 24 months of age, RVs were significantly more frequent than NoVs ($P = 0.042$), whereas no significant differences were observed for children 25 to 60 months old and for children over 60 months old ($P = 0.50$ and $P = 0.10$, respectively).

Monthly distribution of viral infections. Although enteric viruses were detected throughout the year in samples from children suffering from diarrhea, most of the RV and NoV infections occurred in winter and from June to September

FIG. 1. Distribution of monoinfections and mixed infections in hospitalized and nonhospitalized children. RVs and NoVs were significantly more frequent in hospitalized than in nonhospitalized children, as shown by χ^2 or Fisher's exact test results (*, $P = 0.002$; **, $P < 0.001$). No significant differences were observed in comparisons of the rates of incidence of RVs and NoV infections in each population by analysis of percentages with χ^2 or Fisher's exact test. AdV 40/41, type 40 and 41 HAstV.

(Fig. 3). Indeed, these two peaks of incidence together accounted for 80.5% and 62.2% of RV and NoV infections, respectively.

Clinical symptoms and severity of viral diarrhea. With regard to clinical features such as fever (70.0% incidence), vomiting (50.3%), abdominal pains (5.3%), and bloody diarrhea (1.8%), there were no significant differences between infected and noninfected children or between the six viral infection categories ($P > 0.005$), especially between RV- and NoV-positive patient results. Comparison of the results seen with mixed-infection symptoms showed no significant differences

among the 42 patients ($P > 0.05$) with mixed infections or compared to monoinfection results ($P > 0.05$).

Among the 632 patients, 92.7% had one or more episodes of watery stools (the mean duration of diarrhea was estimated at 4.0 ± 8.8 days), and vomiting was reported for 209 children (33.1%).

Correlations between the severity of the disease and the presence of each virus in stool samples from hospitalized patients under 24 months old and infected by a single virus are reported in Table 2. In this age group, 101 of the 196 stool samples were positive for one of the five viruses: 49 (25.0%)

FIG. 2. Distribution of positive samples of the enteric viruses detected in Tunisian children for the following three age groups: ≤ 24 months, 25 to 60 months, and > 60 months. RVs were significantly more frequent than NoVs (*, $P = 0.042$) in children under 24 months old, whereas no significant differences were observed in the other 25-to-60-month and > 60 -month age groups ($P = 0.50$ and $P = 0.10$, respectively). RV grA, group A RV; AdV 40/41, type 40 and 41 HAstV.

FIG. 3. Monthly distribution of viral infections in hospitalized children between January 2003 and May 2005 in the district of Monastir, Tunisia. Most of the RV and NoV infections occurred in winter and from June to September, and these two peaks of incidence together accounted for 80.5% and 62.2% of RV and NoV infections, respectively. HAdV, type 40 and 41 HAdV. Reading left to right across the bottom of the figure, the letters J, F, M, A, M, J, J, A, S, O, N, and D represent the months January, February, March, April, May, June, July, August, September, October, November, and December, respectively.

stool samples were positive for RVs, 39 (19.9%) for NoVs, 1 (0.5%) for HAstV, 4 (2.0%) for enteric HAdVs, and 8 (4.1%) for Aichi viruses, but no sapovirus infections were found. There were no significant differences in symptoms among the five viral infection categories ($P > 0.05$).

For RV- and NoV-positive patients, the results with respect to duration of the diarrhea and numbers of stools and bouts of vomiting per 24 hours were not significantly different. The degree of dehydration of children infected by RV was moderate in 32.7% and severe in 10.2% of cases, whereas for those infected by NoV, the degree of dehydration was moderate in 38.5% and severe in only 5.1% of cases. With regard to the severity of the symptoms, including the degree of dehydration and state of shock of the patients as estimated by CAT recovery results and the presence or absence of blotches, there were no significant differences between results for RV and NoV infections or among other virus infections ($P > 0.05$).

Nevertheless, the results representing delays of medical care were significantly different between RV and NoV infections, with a mean period between the onset of the diarrhea and hospitalization of 1.5 and 2.9 days, respectively ($P < 0.0001$).

DISCUSSION

The present study was designed to investigate the role of viruses in acute diarrhea in Tunisian children. This was the first study to investigate the role of currently recognized viruses, especially NoVs, sapoviruses, and Aichi viruses, in infections of children. As was the case in a previous study (36), this two-year surveillance also showed that the vast majority (75.8%) of the children with gastroenteritis were less than 2 years old. Almost half of the infections (43.7%) were due to one or several viral agents, mainly to RVs and NoVs.

The importance of viruses in infantile gastroenteritis has been underlined in several reports. For most of them (5, 9, 28,

31, 37), the incidence rates range from 45% to 60%, which is compatible with our results. The conditions of the studies, such as the season of sampling, the socioeconomic level of the population, and sampling methods, can explain these between-study differences in detection rates. For example, in our study, the detection rate for viral infections was significantly higher for hospitalized children than for those attending outpatient clinics ($P < 0.001$), a finding which can modify the incidence rates. This difference has already been described (20) and is explained by the comparatively easy detection of the severity of the gastroenteritis in hospitalized children with higher levels of viral excretions.

In the present study, RVs (22.5%) and NoVs (17.4%) were clearly the two major viral agents in hospitalized children (22.6% and 19.4%, respectively) and outpatient children (13.2% and 9.5%, respectively). The rates of incidence of RV and NoV infections were not significantly different between hospitalized and outpatient children, although RV infections are significantly more frequent than NoV infections in children under 24 months old.

RVs are described as the major etiologic agent of gastroenteritis in children, whereas NoVs are considered the leading cause of gastroenteritis outbreaks affecting all age groups in Western countries (10, 11, 22). However, NoVs have also been recognized in recent studies as the second most frequent viral cause of childhood gastroenteritis. Studies in Chile, China, France, and Australia reported NoVs as the etiologic agent in 8 to 15% of children with moderate to severe gastroenteritis who either attended the outpatient clinic or were hospitalized (4, 17, 30). Other studies in Finland (31) and Germany (28) and recently in Vietnam (25) found NoV in 20% to 55% of stool samples, whereas the incidence of RV was found in 31% to 67% of stool samples. Moreover, by coupling fecal detection with serological testing, Parashar et al. (34) found in Peruvian children under 5 years old an incidence of NoVs that reached

TABLE 2. Correlation between disease severity and etiological viral agent for hospitalized children less than 2 years of age

Disease manifestation	No. (%) of infections					<i>P</i> ^a	<i>P</i> ^b
	RV (<i>n</i> = 49)	NoV (<i>n</i> = 39)	HAstV (<i>n</i> = 1)	AdV type 40 or 41 (<i>n</i> = 4)	Aichi virus (<i>n</i> = 8)		
Duration of diarrhea (days)						0.86	0.68
1–3	20 (40.8)	14 (35.9)	0	2	2		
4–6	14 (28.6)	13 (33.3)	0	2	4		
>6	15 (30.6)	12 (30.8)	1	0	2		
No. of bouts of diarrhea (stools/24 h)						0.53	0.25
1–3	14 (28.6)	12 (30.8)	0	1	3		
4–5	15 (30.6)	13 (33.3)	1	2	5		
≥6	20 (40.8)	14 (35.9)	0	1	0		
No. of bouts of vomiting (episodes/24 h)						0.13	0.70
1	6 (12.2)	0 (0.0)	0	0	1		
2–4	30 (61.2)	22 (56.4)	1	4	6		
≥5	2 (4.1)	1 (2.6)	0	0	0		
ND ^c	11 (22.4)	16 (41.0)	0	0	1		
Fever (°C)						0.36	0.71
<37.5	15 (30.6)	13 (33.3)	0	2	5		
37.5–38.5	19 (38.8)	20 (51.3)	1	2	2		
38.6–39	10 (20.4)	1 (2.6)	0	0	0		
>39	5 (10.2)	5 (12.8)	0	0	1		
Dehydration (%)						0.43	0.72
0	18 (36.7)	18 (46.2)	0	2	3		
<5	10 (20.4)	4 (10.3)	1	1	2		
5–10	16 (32.7)	15 (38.5)	0	1	2		
>10	5 (10.2)	2 (5.1)	0	0	1		
CAT ^d (duration in s)						0.88	0.99
<3	41 (83.7)	34 (87.2)	1	4	7		
3	1 (2.0)	1 (2.6)	0	0	0		
4	3 (6.1)	1 (2.6)	0	0	1		
5	4 (8.2)	3 (7.7)	0	0	0		
No. of blotches						0.29	0.72
0	40 (81.6)	35 (89.7)	1	4	7		
1	9 (18.4)	4 (10.3)	0	0	1		

^a Two-tailed χ^2 or Fisher's exact test comparing RVs and NoVs.^b Two-tailed χ^2 or Fisher's exact test comparing all viruses.^c ND, the number of bouts of vomiting was unknown.^d CAT testing is performed on the fingernail to detect the presence of hypovolemic shock.

55%, a rate comparable to that seen with of RVs. As others had found in studies of similar populations (30, 39), they noticed that NoV was detected throughout the year and more commonly during the summer months. This high rate of NoV infection was partly attributed to the poor sanitation of the studied population. In countries with a low or medium socioeconomic level, the commonly observed combination of failure of water treatment and poor hygiene increases the spread of enteric viruses, especially NoVs, and contributes to a high incidence of diarrhea.

In this Tunisian study, two annual gastroenteritis peaks were detected: one peak in winter and another lower peak in June or September. The winter peak comprised mainly RV infections and was comparable to those observed in previous studies of temperate Western countries (4, 19). In contrast, the proportion of NoVs isolated in the peak in June to September was similar to or higher than the proportion of RVs. These NoV summer peaks have also been observed in studies of children in Spain (3), Peru (34), and Brazil (39). This seasonal pattern

could be associated with food- and waterborne outbreaks which occur throughout the year or during the spring and summer months (21, 39).

The severity of the symptoms were evaluated solely with hospitalized children under 2 years old for whom the gastroenteritis episodes were significantly more severe (data not shown). The data on mixed infections were excluded from the analysis. As in previous studies (4, 37), no significant difference in clinical symptoms was observed between single and mixed infections (data not shown). For children younger than 2 years, no significant differences were observed between RV and NoV infections or between these and other virus infections with regard to the severity of the symptoms ($P > 0.05$). Although a study conducted in Vietnam (25) also found similar rates of severity of RV and NoV infections, this result is in contradiction to most of the previous work carried out in developed countries (6, 9, 23, 24, 31), which found lower levels of disease severity with NoVs. In the present study, the delay to hospitalization for NoV-infected children and the lack of medical

care provided at home might have been responsible for the relative level of severity of disease symptoms observed on their admittance to hospitals. Another explanation may be found in the criteria usually used to define severity. In a report of a recent Italian study, Colomba et al. (9) showed conflicting results between the severity scoring and the degree of dehydration. Although most authors used a severity score, few used specific criteria such as dehydration.

Although the G1P[8] genotype combination was the prevalent RV type observed in Tunisia from 1995 to 1999 (38), the G3P[8] was the predominant combination detected during the period of the study. Such variability in the genotype profiles has been shown in previous studies to be dependent on a given place and period of time. In contrast, the high level of prevalence of GGII.4 NoVs detected during this period was similar to that previously reported in many countries in Europe (4, 15, 24).

Like sapoviruses, HAstVs and enteric HAdVs were rarely implicated in gastroenteritis in children. The 26 HAstV-positive samples, for example, were isolated mainly from children with mixed infections (17 samples) and from nonhospitalized children (17 samples). These results remain in accordance with those of several other studies showing that this virus causes asymptomatic infections or mild forms of gastroenteritis which are treated at home (5, 25, 28). More surprising was the result regarding the incidence of Aichi virus monoinfections. They accounted for 2.5% of the total samples and 3.6% of the samples from hospitalized children. The Aichi virus is known as an agent causing gastroenteritis associated with oyster consumption (43). Its involvement in gastroenteritis in children has recently been shown, but this remains a rare event (2, 28, 35). These recent data and our present results support the idea of a role of Aichi virus in cases of gastroenteritis and suggest that oysters may not be the only vector for transmission of the virus.

In conclusion, this report shows that RVs and NoVs are the main causative agents of diarrhea among Tunisian children and are both responsible for severe symptoms in infants. The relative high incidence of NoV infections reported here suggests a possible problem of hygiene, such as water contamination. Therefore, the improvement in the quality of the water distribution network might help to reduce the burden on the healthcare system by lowering the number of infected children. Finally, the high frequency of Aichi virus infections in hospitalized children again raises the issue of its role in viral gastroenteritis.

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