Acute viral gastroenteritis: proportion and clinical relevance of multiple infections in Spanish children

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Dual infections associated with acute infectious diarrhoea and its microbiological, epidemiological and clinical findings have been evaluated in patients selected from a comprehensive survey of children under 4 years old, admitted to hospital emergency rooms from October 1996 to November 1997. A total of 820 children (433 males and 387 females) were enrolled. Stools were tested for rotavirus, adenovirus, astrovirus and bacterial enteropathogens. Patients were grouped according to age, and the seasonality of mixed infections was evaluated. Clinical trends and severity of gastrointestinal disease by Ruuska's score were also analysed. Mixed infections were identified in 39 cases (5%), of which 23 were males and 16 were females. The majority of cases were in the 7–18-month age group (26 cases) and occurred in autumn (67%). Virus–virus co-infections were more frequent (26/39) than virus–bacteria co-infections (13/39). More than two infectious agents were detected in only four cases. The most common viral co-infections were rotavirus–astrovirus (13/26) and rotavirus–adenovirus (10/26). The present report is the first prospective analysis of clinical–epidemiological trends of dual infections in young Spanish children with acute viral gastroenteritis. Our results emphasize the clinical importance of mixed infections as a cause of severe diarrhoea in children.

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INTRODUCTION

Viral intestinal infections are the most common cause of acute infectious diarrhoea in the paediatric population. Worldwide estimates indicate a mean of between 7 and 30 episodes of diarrhoea during the first 5 years of life, and over 11 000 deaths per day throughout the world (Kapikian, 1996; Glass & Kilgore, 1997). In developed countries, acute diarrhoea is a major cause of morbidity in childhood, and substantial medical and healthcare costs are associated with the illness (Glass *et al.*, 1996).

Over the past decade, there have been major advances in our understanding of the causes of viral gastroenteritis. Group A rotavirus is the most prevalent agent responsible for acute diarrhoea in young children worldwide. Other viral agents such as enteric adenovirus, astrovirus, calicivirus and, more recently, torovirus, have also been identi-

fied due to the development of new rapid molecular methods of viral diagnosis (Jamieson *et al.*, 1998; Waters *et al.*, 2000).

To date, the epidemiology of infectious diarrhoeal disease in young children has been widely studied, and the main pathogens responsible have been well-characterized (Lieberman, 1994; Durepaire *et al.*, 1995; Grimwood *et al.*, 1995; Prats *et al.*, 1997). Polymicrobial gastroenteritis has been described previously (Herrmann *et al.*, 1991; Bon *et al.*, 1997; Barnes *et al.*, 1998), but its incidence and clinical characteristics have seldom been reported.

A surveillance study to determine the aetiology of acute infectious diarrhoea in young children, performed for the Paediatric Service of a Spanish reference hospital in collaboration with the Viral Gastrointestinal section of the Spanish National Microbiology Centre, has been conducted. During this study, patients with mixed infections were selected in order to examine the microbiological, epidemiological and clinical findings in this population.

Abbreviations: EIA, enzyme immunoassay; EM, electron microscopy.

METHODS

Patients. The study population included 820 children (433 males and 387 females) younger than 4 years old (mean age, 16 months; range, 2–42 months). All patients were seen in the Emergency Department for acute diarrhoea in Severo Ochoa Hospital between October 1996 and September 1997, and guardians of children were asked for permission to enrol the patients in the study. The clinical centre is the reference hospital of Health Care Area IX in Madrid (Spain), with a population of 350 000 inhabitants. Acute diarrhoea was defined as three or more liquid stools over a 24 h period.

Demographical and epidemiological data were documented in all cases. Patients were classified into 6-month age groups. Date of hospitalization was noted for evaluation of seasonal occurrence. Uniform clinical features including duration of diarrhoea and evacuations per day, frequency of vomiting, dehydration, fever (axillar temperature) and treatment were noted in all cases. Severity of gastrointestinal disease was determined by the numerical score of Ruuska & Vesikari (1990), with a maximum severity score of 20 marks.

Samples. Stool specimens were collected within 48 h of admission to the hospital for all patients. Samples were obtained by direct deposition in a sterile container and were transported the same day to hospital laboratories, where they were stored at 4 °C until they were processed. Specimens for bacteriological culture were inoculated into appropriate media on the day of collection. Specimens for virus detection were prepared as 10 % homogenates in PBS, pH 7.0, and stored at 4 °C for up to 1 week until they were tested. Diluted and undiluted samples were stored at –70 °C until further testing was required. A single specimen was examined for each patient.

Bacteriology. All specimens were examined for *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Vibrio* spp., *Aeromonas* spp. and *Plesiomonas* spp. by using MacConkey agar, *Salmonella*–*Shigella* (S–S) agar, Cefsulodin–Irgasan–Novobiocin agar and selenite F enrichment broth, incubated in air at 35 °C for 24 h. Selenite F broth cultures were subcultured after 24 h onto S–S agar, and incubated at 35 °C for 24 h. Detection of *Campylobacter* spp. was performed by using *Campylobacter* blood-free selective medium, incubated at 42 °C for 48 h in an atmosphere of 7 % CO₂ and 85 % N₂ (v/v). Isolates were identified by using standard biochemical and serological techniques, as described by Yamashiro *et al.* (1998). Study of diarrhoeagenic *Escherichia coli* was not routinely included because previous surveys have indicated that they are only rare causes of diarrhoea in children in developed countries (Bern & Glass, 1994; Caprioli *et al.*, 1996; Prats *et al.*, 1997). Parasite aetiology was not studied because it is an infrequent cause of acute syndrome in young children with gastroenteritis (Barnes *et al.*, 1998; McIver *et al.*, 2001).

Virology. Rotavirus, adenovirus and astrovirus were detected in faecal samples by specific commercial enzyme immunoassay (EIA) kits, according to the manufacturers' instructions. The IDEIA Rotavirus technique (Dako Diagnostics) uses a microplate-based solid-phase sandwich-type immunoassay with a polyclonal antibody to detect specific group A rotavirus proteins, particularly the internal capsid protein (VP6). This assay is able to detect rotavirus concentrations as low as 7.8×10^5 viral particles ml⁻¹, and shows good correlation, sensitivity and specificity in comparison with electron microscopy (EM) (99.5, 100 and 99.2 %, respectively; Flewett *et al.*, 1989). In our hands, this assay also shows a very good correlation with PCR detection (Wilhelmi *et al.*, 2001). Furthermore, rotavirus-positive specimens were confirmed and typed by sandwich-type EIA using mAbs (Silenus Laboratories) and by the RT-PCR method as described previously (Wilhelmi *et al.*, 1999).

The IDEIA Adenovirus test (Dako Diagnostics) utilizes a mAb in a solid-

phase sandwich EIA to detect a genus-specific hexon epitope of adenovirus (Noel *et al.*, 1994). This assay is able to detect adenovirus concentrations as low as 3.0×10^5 viral particles ml⁻¹, and shows good correlation, sensitivity and specificity in comparison with EM (95.5, 90.1 and 99.0 %, respectively). Furthermore, adenovirus-positive samples were typed by an immunochromatography method using specific monoclonal antibodies (40/41 Adeno-Strip, Coris BioConcept).

The IDEIA Astrovirus test (Dako Diagnostics) utilizes a mAb and a polyclonal antibody in a solid-phase EIA to detect astrovirus antigen. This assay is able to detect astrovirus at dilutions 100-fold greater than EM, and shows excellent correlation, sensitivity and specificity in comparison with EM (100 %; Putzker *et al.*, 2000). Astrovirus-positive samples were further confirmed by RT-PCR, according to the method described by Dalton *et al.* (2002). No tests to detect calicivirus were included in this study.

Statistical analysis. Quantitative variables were expressed as mean, standard deviation and range. Qualitative variables were calculated as frequency and percentage, and were compared by the χ^2 test and Fisher's exact test. Non-parametric Kruskal–Wallis analysis of variance or the Mann–Whitney U-test were used to compare means, when Bartlett's χ^2 test showed that the variances were dissimilar in different samples. All statistical analysis was performed with a significance level of 0.05. Statistical analysis was performed with SPSS software, version 8.0.

RESULTS

Of the 820 stool specimens from children with gastroenteritis, single infections were detected in 492 cases (60 %). Enteropathogenic bacteria were identified in 239 cases (29 %), and viruses in 253 (31 %). Enteric viruses included group A rotavirus in 205 cases (25 %), adenovirus types 40/41 in 25 (3 %) and astrovirus in 23 (3 %). Clinical features of single virus infections are presented in Table 1. Incidences of evacuation and vomiting per day, fever, dehydration and Ruuska score were observed to be greater in single infections by human rotavirus.

Simultaneous infections were detected in 39 cases (5 %), 23 of which were male and 16 female. The mean and median ages were 12.5 months (standard deviation, 9.3 months; range, 2–42 months) and 12 months, respectively.

Mixed infections and their distribution according to age groups are shown in Table 2. Mixed infections were predominantly detected in the 7–18-month age group (26/39 cases), and virus–virus co-infections were significantly more frequent in this age group ($P = 0.05$). Virus–bacteria co-infections were not predominant in any age group. The most frequent mixed infections were rotavirus–astrovirus (13 cases) and rotavirus–adenovirus (10 cases). Detection of more than two infectious agents was infrequent (4 cases).

Viruses associated with co-infection were group A rotavirus (29/39, 74.4 %), astrovirus (23/39, 59 %) and adenovirus (16/39, 41.0 %). Group A rotavirus was the most common agent implicated in mixed virus infections ($P < 0.05$). Serotype G1 rotavirus predominated throughout the study (85 %), with sporadic occurrence of serotype G4; these results have been presented in detail previously (Wilhelmi *et al.*, 1999). All adenovirus infections detected correspond to enteric types 40/41. The enteropathogenic bacteria *Salmo-*

Table 1. Clinical features in single viral infections

Virus	n	Diarrhoea*		Vomiting*		Percentage of cases with:			Ruuska score*
		Events per day	Duration (days)	Events per day	Duration (days)	Fever, >38 °C	Dehydration	Hospitalization	
Rotavirus	205	6 (1–20)	2 (1–7)	3 (0–20)	1 (0–5)	44	41	20	10 (1–16)
Adenovirus	25	5 (1–15)	4 (1–13)	2 (0–5)	2 (0–13)	17	33	24	8 (2–18)
Astrovirus	23	4 (1–15)	2 (1–4)	2 (0–12)	1 (0–3)	39	24	9	8 (4–12)

*Values are means, with range in parentheses.

Table 2. Age distribution of mixed infections in acute viral gastroenteritis

Values are number of cases, with percentage in parentheses.

Agents	Age (months)								Total
	0–6	7–12	13–18	19–24	25–30	31–36	37–42	43–48	
Rotavirus + astrovirus	1	8	3	1					13 (33·3)
Rotavirus + adenovirus		5	3	1	1				10 (25·6)
Rotavirus + astrovirus + adenovirus	1		1						2 (5·1)
Adenovirus + astrovirus							1		1 (2·6)
Astrovirus + <i>Salmonella</i>		1	1		2		1		5 (12·8)
Rotavirus + <i>Campylobacter</i>	1	1							2 (5·1)
Adenovirus + <i>Campylobacter</i>							2		2 (5·1)
Rotavirus + astrovirus + <i>Campylobacter</i>			1						1 (2·6)
Rotavirus + <i>Salmonella</i>		1							1 (2·6)
Adenovirus + <i>Salmonella</i>	1								1 (2·6)
Astrovirus + <i>Campylobacter</i> + <i>Yersinia</i>			1						1 (2·6)
Total	4	16	10	2	3		4		39 (100)

nella (non-*typhi*) (7/39, 17.9%), *Campylobacter jejuni* (6/39, 15.4%) and *Yersinia enterocolitica* (1/39, 2.6%) were detected.

Most of the cases of mixed infection occurred in autumn (26 cases in autumn, five in winter, six in spring but only two cases in summer), and no seasonal differences were detected between the different co-infections.

Epidemiological and clinical features are summarized in Table 3. Diarrhoea was detected in all cases. Mean duration was 3 days, with a mean of six evacuations per day. Diarrhoea was more severe and prolonged in cases of adenovirus–*Campylobacter* spp. co-infections (8 days duration and 12 evacuations per day), but the small number of cases ($n = 2$) did not allow us to perform statistical analysis.

Vomiting was observed in 30 patients, 22 of which had dual viral infection and eight of which had mixed viral and bacterial infection. Fever was detected in 15 cases, and was slightly more common in virus–bacteria co-infections, especially when *Salmonella* was the bacterial agent impli-

cated. Dehydration was diagnosed in 21 patients and was more frequent in virus–bacteria (9/13 cases, 69%) than virus–virus (12/26 cases, 46%) co-infections.

Mean clinical severity of gastrointestinal disease by the Ruuska scale was moderate (11 marks), for both virus–virus and virus–bacteria co-infections. The percentage of hospitalized patients was also similar in both types of mixed infection (21%).

DISCUSSION

Viral and bacterial intestinal infections are common during early childhood and have been the object of numerous studies, but dual infections are not expected and are often misdiagnosed. In fact, there are relatively few comprehensive studies of the infectious aetiology of severe diarrhoea in children admitted to hospital in developed countries (Barnes *et al.*, 1998). The majority of studies are retrospective, and prevalence rates are very dependent on study design. Furthermore, viral gastroenteritis is not a disease of obligatory notification, and it is not an object of specific surveillance for sentinel sanitary systems. Therefore, data about

Table 3. Clinical features in mixed infections

Agents	<i>n</i>	Diarrhoea*		Vomiting*		Number (percentage) of cases with:			Ruuska score*
		Events per day	Duration (days)	Events per day	Duration (days)	Fever, >38 °C	Dehydration	Hospitalization	
Rotavirus + astrovirus	13	6 (2–15)	3 (1–7)	4 (0–5)	2 (0–5)	4	6 (46)	3 (23)	11 (6–16)
Rotavirus + adenovirus	10	5 (1–9)	3 (1–14)	5 (0–15)	1 (0–2)	5	3 (30)	2 (20)	10 (3–13)
Astrovirus + <i>Salmonella</i>	5	9 (5–12)	4 (1–9)	1 (0–3)	1 (0–4)	3	4 (80)	1 (20)	13 (11–17)
Adenovirus + <i>Campylobacter</i>	2	12 (7–16)	8 (2–14)	0	0	0	1	0	8
Rotavirus + adenovirus + astrovirus	2	3·5 (2–5)	3·5 (2–5)	3 (0–5)	1 (0–2)	1	1	0	10·5 (10–11)
Rotavirus + <i>Campylobacter</i>	2	4 (3–5)	1·5 (1–2)	3 (2–4)	1·5 (1–2)	0	0	0	7 (5–9)
Rotavirus + <i>Salmonella</i>	1	4	4	2	4	0	1	0	10
Rotavirus + astrovirus + <i>Campylobacter</i>	1	4	3	1	3	0	1	1	9
Adenovirus + astrovirus	1	4	1	2	1	0	1	0	9
Adenovirus + <i>Salmonella</i>	1	6	1	0	0	1	1	1	10
Astrovirus + <i>Campylobacter</i> + <i>Yersinia</i>	1	8	2	0	0	1	0	0	12
Total virus–virus	26	5 (1–15)	3 (1–14)	4 (0–15)	2 (0–5)	10	12 (46)	5 (19)	11 (3–16)
Total virus–bacteria	13	8 (3–16)	4 (1–14)	1 (0–4)	1 (0–4)	5	9 (69)	3 (23)	11 (5–17)
Total	39	6 (1–16)	3 (1–14)	3 (0–15)	1 (0–5)	15 (38)	21 (54)	8 (21)	11 (3–17)

*Values are means, with range in parentheses.

concomitant infections in gastrointestinal disease are very limited worldwide. Most studies show that mixed infections are less frequent than mono-infections, but the rate of double infections varies widely in the literature.

In Melbourne, Australia, Barnes *et al.* (1998) detected 1.6 % (62/3785) mixed infections in hospitalized children with acute gastroenteritis; in that study, the main type of association detected was virus–bacteria (80 %, 50/62), with rotavirus (46/62) followed by *Salmonella* (28/62) being the most common pathogens implicated; no attempt to identify astrovirus was made. In Dijon, France, Bon *et al.* (1999) reported 16.7 % (50/299) dual viral infections in children with acute viral gastroenteritis; the majority of these (94 %) were combinations of rotavirus with other viruses (astrovirus, adenovirus 40/41 or calicivirus). In Barcelona, Spain, Prats *et al.* (1997) detected 6.8 % (232/3380) mixed infections in children and adults with enteritis over a 4-year period; neither adenovirus nor astrovirus were studied.

It is important to emphasize that previous studies included all type of associations (virus–virus, virus–bacteria, virus–parasite, bacteria–parasite and/or parasite–parasite), whereas this study focuses on mixed infections in viral gastroenteritis in children. Therefore, comparison of incidences with previous studies is difficult. Other variables such as patient age, standards of hygiene, seasonal peaks or techniques used may also explain the differences detected. The present report is the first prospective analysis on the clinical–epidemiological trends of dual infections, whereas none of the previous studies specifically analysed the clinical data of simultaneous infections. In our hands, mixed infections (virus–virus and/or virus–bacteria) were detected in 5 % of patients.

Group A rotavirus was the viral agent most frequently implicated in co-infections, confirming the importance of rotavirus as the main pathogen of paediatric diarrhoea. The most frequent viral co-infection was rotavirus plus astrovirus, which shows that the high percentage of cases of diarrhoea associated with astrovirus can be described as dual infections (Herrmann *et al.*, 1991). A limitation of the current study is that no specific molecular test for detection of caliciviruses (e.g. Norwalk virus) was performed; the application of molecular techniques for detection of this infectious agent may identify it as a cause of mixed infections in children with diarrhoea.

In this study, the majority of viral co-infections (67 %) were in the 7–18-month age group; in contrast, an increased incidence of bacterial pathogens was not identified in any particular age group. Other authors detected that mixed infections ranged from 1 month to 7.6 years, with 25 months as the mean age (Barnes *et al.*, 1998).

The highest proportion of dual infections was identified in autumn, in contrast to the seasonal occurrence of some mono-infections; infection by group A rotavirus is more frequent in winter and spring, with particular peaks occurring in November and February in Madrid (data not shown).

All children with mixed infections presented with watery diarrhoea, often with vomiting and fever; we could not detect great differences in these symptoms between virus–virus and virus–bacteria co-infections, but the limited number of cases does not allow us to perform statistical estimations.

Statistical comparison of single virus infection with double infections is complex because most of the co-infection groups are small. In our hands, their clinical severity was similar except for adenovirus and astrovirus infections, which had a higher Ruuska score in double infections. However, this may be explained by the fact that most mixed adenovirus and astrovirus infections were adenovirus–rotavirus or astrovirus–rotavirus co-infections, and rotavirus, even as a single infectious agent, causes more severe disease than adenovirus or astrovirus (Table 1).

Finally, viral and bacterial intestinal pathogens could affect either the same or different regions of the gut, and their effects would be enhanced (Di Biase *et al.*, 2000). Currently, we still know little about the exact pathology of human gut infections, and it is useful to report studies which examine both viruses and bacteria. The results of this study emphasize the clinical importance of mixed infections as a cause of severe viral diarrhoea in children, therefore the possibility of dual infection should be investigated more often (Taylor *et al.*, 1997). Systematic detection of the main enteric pathogens associated with gastroenteritis in children allowed us to observe a relatively high percentage of co-infections (5 %), the majority of which were combinations of rotavirus and/or astrovirus with other enteropathogens. Dual infections raise the question of whether a single pathogen is responsible for illness, or whether several pathogens act in synergy. Further studies must be performed in order to obtain a better understanding of these infections.

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