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CLINICAL AND EPIDEMIOLOGICAL STUDY

G2 as an emerging rotavirus strain in pediatric gastroenteritis in southern Italy

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Abstract
Background Human rotaviruses (HRVs) represent a major cause of acute gastroenteritis in children worldwide. It is estimated that they are responsible for a large number of diarrhea-associated hospitalizations in childhood each year. In Italy, limited data are available on the patterns of distribution of HRV G and P types. We report here the results of 2 years of rotavirus strain surveillance among children with severe gastroenteritis diagnosed in the town of Portici, Campania, southern Italy.
Methods A total of 421 stool specimens from children between 6 months and 5 years of age and presenting acute diarrhea were collected and tested by routine diagnostic tests for HRV, adenovirus, astrovirus, norovirus, and common bacterial pathogens.
Results The laboratory results showed that 110 of the 225 (26.1%) virus-positive samples contained HRVs. The different G and P rotavirus genotypes were analyzed by polymerase chain reaction (PCR). Among the VP7 genotypes identified, G1 and G2 were predominant, with percentages of 48.2 and 30.9%, respectively. G4, G9, and G10 were detected in a minority of cases. Among the VP4 genotypes, P[8] occurred the most frequently (56.4%), followed by P[4] (31.8%), and only a few P[10] and P[11] at percentages of 1.8 and 0.9%, respectively.
Conclusion Our epidemiological data of HRV strains will contribute to assessing the magnitude of the problem of HRV in the south of Italy.

Keywords Human rotavirus · Gastroenteritis · Rotavirus serotypes/genotypes · Epidemiology

Introduction

Pediatric diarrhea remains one of the major causes of death among infants. This is especially true in Asia, Africa, and Latin America, where it causes millions of deaths in the 0–5 years age group. The immediate causes are often of an infectious nature and include a variety of pathogenic microorganisms. Several different groups of viruses are responsible for the high rate of acute viral diarrhea condition among children during their first years of life.

Human rotaviruses (HRVs) represent the main cause of gastroenteritis in children worldwide [1]. It is estimated that they are responsible for a large number of diarrhea-associated hospitalizations in childhood each year [2].

These infective agents are ubiquitous and about 95% of children worldwide are infected before 3–5 years of age [3]. During the first 12 months of life, the risk of severe forms of infection, which require hospitalization, is particularly high, due to the high risk of dehydration in infants and the difficulty in restoring their electrolyte balance. HRV infections can affect adults, often in sub-clinical

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forms, and occasionally determine clinically evident cases in immunodeficient patients, in the elderly, and in travelers who visit developing countries [4].

HRVs are members of a distinct genus of the *Reoviridae* family. They are non-enveloped triple-layered particles, with a double capsid, a core containing the viral genome, and two surface proteins, VP4 and VP7. HRVs are classified into seven serogroups (A–G) on the basis of the antigenic properties of shared epitopes on the major structural protein, VP6 [5].

Groups A–C are found in both humans and animals, whereas groups D–G have only been found in animals. Group A rotaviruses have clearly been established as causing significant diarrheal disease in infants and in the young of various mammalian and avian species.

Within group A, rotaviruses are classified by a binary system into at least 23 G types (VP7) and 31 P types (VP4) [6, 7].

For G types (known as "G types" for "glycoprotein"), the serotypes and genotypes are generally concordant, so G types are usually referred to only by their serotype (e.g., G1). A lack of readily available typing serum or monoclonal antibodies to different VP4 types, however, has hampered the classification of VP4 (or "P types" for "protease-sensitive protein"). Instead, the properties of VP4 have been studied primarily by sequence analysis. P types are generally referred to by their genotype number, which is denoted in brackets (e.g., P[8]) [8].

The introduction of molecular typing methods has enhanced our understanding of the diversity of HRV strains, which affects the development of rotavirus vaccines.

Worldwide surveillance of HRV strains has demonstrated G1 to G4 serotypes, with P[8] and P[4] genotypes to be the most commonly circulating HRV [9, 10]. In addition, infections due to G9 have become more prevalent in recent years [5, 11, 12]. HRV strains of the G1, G3, G4, and G9 serotypes are preferentially associated with the P[8] genotype, whereas G2 serotype strains are most frequently associated with the P[4] genotype. It has been impossible to predict which rotavirus type will infect children in any season or country, because the seasonal and geographic distributions of rotavirus serotypes have been unpredictable [10, 12].

In Italy, limited data are available on the patterns of distribution of HRV G and P types [13–21].

We report here the results of 2 years of rotavirus strain surveillance among children with severe gastroenteritis diagnosed in the town of Portici in the Italian region of Campania. Routine diagnostic tests for HRV, adenovirus (AdV), astrovirus (HAstV), norovirus (NoV), and common bacterial pathogens were carried out on all samples.

The aim of this study was to survey enteric viral infections, with particular regard to HRV in comparison with other viral agents [22].

Materials and methods

Specimen collection and storage

From January 2007 to December 2008, a total of 421 stool specimens were collected from children ranging from 6 months to 5 years of age investigated for gastroenteric symptoms in the province of Naples, specifically, in Portici, at the Diagnostic Centre Sanciro srl, National Laboratory of Research. The children selected in our study had sought medical assistance after several episodes of loose or watery stools, in some cases associated with vomiting. The stool samples were collected using wide-mouthed sterile plastic containers and stored at −20°C until the time of assay. All samples underwent only one cycle of thawing and freezing prior to characterization.

Viral and bacterial diagnostic screening

All specimens were examined for *Salmonella* spp. and *Shigella* spp. using MacConkey agar and Salmonella–Shigella agar (BD Diagnostic Systems Europe, Becton–Dickinson France SA, Le Pont de Claix, France) and an incubation of 37°C for 18 h. Detection of *Campylobacter* spp. was performed on feces within a few hours of emission using the filter membrane method, and samples were plated on non-selective blood agar and incubated at 42°C for 48 h in an atmosphere of 7% CO_2 and 85% N_2 (BBL Campy Pouch; BD Diagnostics Systems, Sparks, MD, USA). Isolates were identified by using standard biochemical and serological techniques. *Yersinia* spp. selective isolation were obtained using the Cefsulodin–Irgason–Novobiocin (CIN) Agar formulation of Schiemann (BD Diagnostic Systems Europe, Becton–Dickinson France SA, Le Pont de Claix, France) after incubation at a temperature of 30 ± 2°C and observed after 1–24 h or at 22–25°C and observed after 48 h.

Stool specimens were screened for the presence of HRV, AdV, HAstV, and NoV by enzyme-linked immunosorbent assay (ELISA) kits distributed by Dako Cytomation Ltd. (UK): Idea K6020 for rotavirus, Idea K6042 for astrovirus, Idea K6021 for adenovirus, Idea K6043 and K6044 for NoV were used for identification in fecal samples according to the manufacturer's instructions. The results were visually read and confirmed by absorbance measurements.

Rotavirus strain characterization

All samples were mixed 1:10 with Eagle's minimum essential medium, and 10% stool suspensions were used for nucleic acid extraction using the guanidinium isothiocyanate silica particle method [23]. Reverse transcription with random primers and rotavirus G and P genotyping semi-nested type-specific multiplex polymerase chain reaction (PCR) assays were performed as described previously [24, 25]. The PCR assays detect eight G types (G1, G2, G3, G4, G8, G9, G10, and G12) and six P types (P[4], P[6], P[8], P[9], P[10], and P[11]), respectively. Amplicons were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed under ultraviolet light. The different G and P genotypes were analyzed by comparing the size of the second-round PCR products with a 100-base-pair DNA ladder and PCR products of known rotavirus strains. Rotavirus ELISA-positive samples that were negative by the semi-nested type-specific multiplex PCR assays were analyzed further using a single-step VP6-specific reverse transcriptase PCR (RT-PCR) according to the protocol of Iturriza-Gómara et al. [26]. Samples that were positive by the VP6 RT-PCR were interpreted as non-typeable rotavirus-positive samples. Water was used as the negative control and rotavirus-positive samples, including the five common rotavirus strains verified by sequencing, were used as the positive controls in each RT-PCR run.

Results

A total of 421 stool specimens from children between 6 months and 5 years of age and presenting acute diarrhea were collected and tested for enteric viruses and common enteric bacteria. A pathogen etiology was confirmed in 287 (68.2%) cases. Monobacterial infections were detected in 65 (15.4%) patients, namely: *Salmonella* spp. in 37 (56.9% of bacterial infections), *Shigella* spp. in 18 (27.7%), *Campylobacter* spp. in 6 (9.2%), and *Yersinia* spp. in 4 (6.2%) (Table 1). Viral infections were found in 225 (53.4%) patients (Table 1). Viral bacterial coinfection was present in 3 (0.7%) analyzed samples, while viral coinfection (Table 1) was present in 68 (16.2%). Bacterial–viral coinfection was rare and, specifically, two samples showed a mixed-infection NoV-*Salmonella* spp. and one showed an HRV–*Salmonella* spp.

Laboratory investigation revealed that 110 of the 225 (26.1%) virus-positive samples contained rotaviruses. Other viruses identified in the present study were NoV in 134 (31.8%) samples, HAstV in 20 (4.7%) samples, and AdV in 29 (6.9%) samples (Table 1).

Mixed viral infections were quite frequent, accounting for 30.2% of the total samples where a viral pathogen was detected, but the majority were due to coinfections with NoV and HRV. These two viruses were found coinfecting the same patient in 54 cases out of 68 mixed viral–viral infections (79.4%). Other viral–viral mixed infections detected were: norovirus–adenovirus (10.3%), norovirus–astrovirus (5.9%), adenovirus–rotavirus (2.9%), and astrovirus–rotavirus (1.5%). In Table 1, data are reported on the total number of 421 analyzed samples.

Therefore, HRVs were responsible for 26.1% of all of the acute diarrhea cases diagnosed by the Microbiology Unit of the Diagnostic Centre Sanciro of Portici, Naples, in the 2-year period from January 2007 to December 2008. The major epidemic periods of HRV infection were in

Table 1 Incidence of pathogens detected in 421 stool samples

	No. of cases (%)			Mixed-infection type	
Pathogens	287 (68.2)	**Viruses**	No. of cases (%)	**Viral–viral coinfections**	No. of cases
		HRV	110 (26.1)	NoV–HRV	54
		AdV	29 (6.9)	NoV–AdV	7
		HAstV	20 (4.7)	NoV–HAstV	4
		NoV	134 (31.8)	AdV–HRV	2
				AdV–HAstV	0
				HAstV–HRV	1
		Bacteria	No. of cases (% of bacterial infection)	**Viral–bacterial coinfections**	No. of cases
		Salmonella spp.	37 (56.9)	NoV–*Salmonella* spp.	2
		Shigella spp.	18 (27.7)	HRV–*Salmonella* spp.	1
		Campylobacter spp.	6 (9.2)		
		Yersinia spp.	4 (6.2)		
No pathogen	134 (31.8)				

Among the 421 stool specimens analyzed, 71 coinfections were found (68 viral–viral and three viral–bacterial)

March–May 2007 (45 cases out of a total of 69 HRV-positive samples in the relative year) and February–June 2008 (34 cases out of a total of 41 HRV-positive samples in the relative year). HRV infection was almost absent in the period August–October of both of the years considered in the present study (Fig. 1).

All HRVs were G and P genotyped. The overall results are shown in Table 2. Among the VP7 genotypes identified, G1 and G2 were predominant, with percentages of 48.2 and 30.9%, respectively. G4, G9, and G10 were detected in a minority of cases.

Among the VP4 genotypes, P[8] occurred the most frequently (56.4%), followed by P[4] (31.8%) and only a few P[10] and P[11] at percentages of 1.8 and 0.9%, respectively. The G or P genotype could not be assigned to 7.3 and 9.1% of cases, respectively.

Among the rotavirus strains identified in the present study, a number of different G and P combinations were detected, some of which were very frequent, while others occurred rarely. The most common combinations were G1P[8] and G2P[4], which together accounted for 58.2% of the total strains. These were followed by G2P[8] and G1P[4] both at 7.3%, while the remaining combinations, namely, G2P[10], G4P[4], G4P[8], G9P[8], G9P[11], and G10P[8] accounted for 14.4% altogether (Fig. 2).

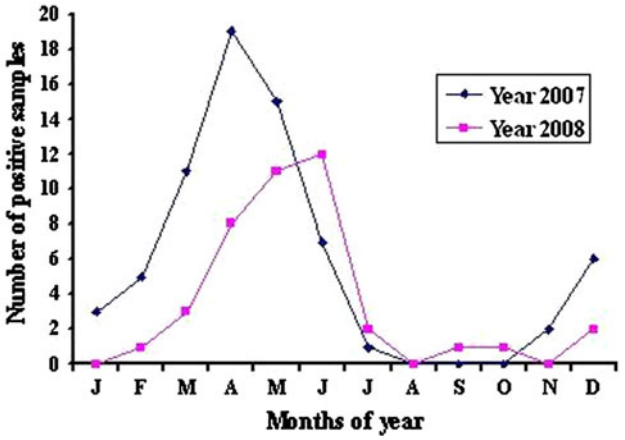

Fig. 1 Monthly distribution of human rotaviruses (HRVs). Distribution by month of HRVs from January 2007 to December 2008

Table 2 Distribution of rotavirus G and P genotypes detected during a 2-year period

G or P type	No. of cases in 2007	No. of cases in 2008	Total (%)
G1	35	18	48.2
G2	20	14	30.9
G4	5	3	7.3
G9	2	1	2.7
G10	1	3	3.6
Gnt[a]	6	2	7.3
P[4]	24	11	31.8
P[8]	34	28	56.4
P[10]	2	0	1.8
P[11]	1	0	0.9
Pnt[a]	8	2	9.1

[a] *nt* non-typeable

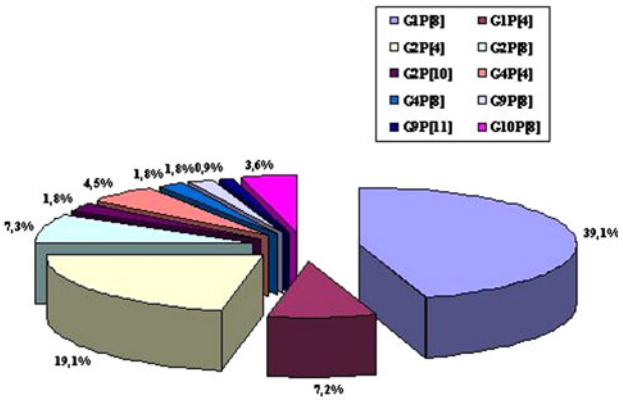

Fig. 2 HRV G/P combinations detected during the period 2007–2008. The percentage of isolation of predominant HRV G/P combinations are shown

Discussion

During a 2-year (January 2007–December 2008) surveillance of enteric infections children, we found a prevalence rate of HRV infections of 26.1%, in comparison with 4.7% of single HAstV and 6.9% of AdV infection, while NoV gastroenteritis had the highest incidence (31.8%) in the age group selected (from 6 months to 5 years). It is apparent that norovirus infections were more frequent than rotavirus infections and this is an interesting finding, confirming data from the Netherlands, Japan, and Austria [27–29]. Pathogens were detected in 68.2% of samples, of which 53.4% were represented by viruses. Therefore, this result is in accordance with other reports of similar studies in Europe [29–31]. A bacterial pathogen was detected in 15.4% of the analyzed stools, with a great prevalence (56.9%) of *Salmonella* spp. In the remaining samples, we could not find any pathogen. These data confirm the importance of HRV as one of the main viral pathogens of enteric disease in pediatric patients. Dual infections are generally not expected and often misdiagnosed. In our analyses, virus–virus coinfections were detected in 16.1% (68 cases) and HRV–NoV was the most frequently observed viral coinfection (12.8%, 54 cases). We could not observe a consistent statistical difference in clinical symptoms between single and mixed infections. It is difficult to define the significance of single or mixed viruses in gastrointestinal

infections, and it may reflect the consequence of earlier infections; in fact, HRVs can be detected for a long time after infection, and, in our study, we could detect a high prevalence of NoV. Thus, HRVs are one of the leading causes of acute gastroenteritis among young children and infants [32]. As described in other temperate climate countries, HRV infection presented a seasonal pattern, with a major incidence in winter (28.2%, 31 cases) and spring (65.4%, 72 cases), and with a few cases also being observed during the summer months.

This study extends our knowledge of the circulation of HRVs in a generally underconsidered but densely populated area of south Italy, Campania, where epidemiological studies on viral gastroenteritis have been scarce in the recent past.

The present study also highlights the high prevalence of strains of the G1, G2, and G4 types. These findings are in accordance with the epidemiological data described in other regions of Italy, in other European countries, and mainly among most of the developed countries [10, 12, 33, 34]. This study pointed out a relatively high number of G1P[4] and G2P[8] isolates which are likely to be the natural reassortant of co-circulating rotavirus strains [35]. In the past, in Italy, G3 HRVs have been detected sporadically or at a very low prevalence. However, since 2003, they accounted for a higher percentage of up to 17% of gastroenteritis episodes, thus, acquiring an important epidemiological role. Increased prevalence of infections by G3 HRV in children has also been described in recent years in Ireland and Japan [36, 37].

In our study, limited to 2 years and to only to a single sample collection site, we could not detect any G3 HRV type. This finding is of importance considering the high frequency of G3 detection in Italy, and especially in the south of the country in recent years, and extends our understanding of rotavirus diversity in Italy.

On the other hand, we detected almost 3% of G9 HRV strains. This genotype is a recent presence in Italy; in fact, it was detected for the first time in 1999 [38]. In 2005, G9 represented the most common isolates in Sicily, accounting for 60.8% of HRV infections. Likewise, high rates of detection of G9 strains were reported in Bari, Italy, in 2001–2002 [39], in Hungary in 2002–2003 [40], and in Belgium in 2000–2001 and 2002–2003 [41]. During the last decade, the G9 genotype has emerged as one of the five most common types worldwide [12, 18, 39, 40, 42–45]. A high prevalence of G9 was detected in France (55%) and Italy (84%), whereas a lower prevalence was found in Germany (8%) and the UK (13%) [46]. The surveillance of the G9 genotype is important, because the degree of protection of current vaccines against this emerging genotype is still unknown, and notwithstanding the low rate of detection in Campania, our results confirms its presence in Italy.

Finally, we need to point out a quite high frequency of G10 strains, generally quite rare genotypes, but common in bovines, therefore, considering the partial rural environment surrounding the area selected, it is conceivable to consider the possibility of a zoonotic spread of some virus genotypes.

Our epidemiological data of HRV strains in the south of Italy will contribute to assessing the magnitude of the problem of HRV in different settings and to set priorities for intervention, such as vaccine-based prevention strategies.

Continuous monitoring of the HRV genotype distribution is, therefore, valuable in order to describe the diversity and changes in the circulating strains that might be found in different areas, especially after the introduction of rotavirus vaccination. In Italy, two live, oral rotavirus vaccines, Rotarix™ (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq™ (Merck & Co., Inc., Whitehouse Station, NJ, USA), are now licensed. Rotarix™ is based on a live attenuated human G1P[8] rotavirus strain, while the pentavalent, live-attenuated human–bovine reassortant vaccine, RotaTeq™, is focused on four G types (G1–G4).

Despite the availability of these vaccines, in Italy, the national vaccination program does not include or suggest any of the rotavirus vaccines for newborns. It is difficult to quantify the amount of people vaccinated against rotavirus in Campania, and, overall, it could be considered as non-influential on our epidemiologic study. A significant result of our study is the emergence in southern Italy of the G2 genotype. This result is in line with other reports from other countries in Europe showing an increase of the prevalence of G2 strains circulating among non-vaccinated children.

Since the population under study was naïve for rotavirus vaccination, the high frequency of G2 cannot be explained by the introduction of the vaccination, but might be due to the normal fluctuation of co-circulating rotavirus genotypes. Because diverse rotavirus strains co-circulate in the human population, it is important to maintain updated epidemiological data and, when future vaccines are designed, it should be taken into account the distribution of different genogroups around the world.

Conflict of interest None.

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