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VP7 and VP4 genotypes among rotavirus strains recovered from children with gastroenteritis over a 3-year period in Valencia, Spain

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Abstract. Between September 1996 and May 1999, the incidence and distribution of the main human rotavirus G genotypes (VP7 associated: G1–G4) and P genotypes (VP4 associated: P[8], P[4], P[6] and P[9]) among children with rotavirus gastroenteritis were determined using reverse transcription and polymerase chain reaction (RT-PCR)-based genotyping methods. From a total of 145 rotavirus strains examined, we identified the G type in 131 (90.3%) and the P type in 127 (87.5%) of the samples. An overall predominance of genotypes P[8] G1 (42.7%) and P[8] G4 (32.4%) was found during the period of study, with much lower incidence of genotypes P[4] G2 (5.5%) and P[8] G3 (2%). P[6] and P[9] types were not detected, neither were unusual combinations of P and G types. A significant genotypic shift was observed: whereas P[8] G4 was the most prevalent genotype during the first year of the study (60%), the genotype P[8] G1 gradually increased to account for 62.3% of the strains analysed in the following winter season. Mixed G types revealing dual infections G1/G4 and G3/G4 were found at low frequency (2%).

Key words: Genotypes, Reverse transcription and polymerase chain reaction (RT-PCR), Rotavirus, VP4, VP7

Introduction

Group A rotavirus is the major cause of infectious diarrhoea in children worldwide [1]. Vaccination would be the most effective way to control morbidity and mortality due to rotavirus infections [2]. However, the only vaccine licensed so far had to be withdrawn due to a significant number of cases of intussusception among vaccinated children [3]. The distribution of rotavirus types varies between distinct geographic and socioeconomic regions of the world [1]. It is therefore necessary to ascertain the P and G genotypes of circulating strains in different areas and communities during the course of a number of years. Rotavirus serotype and genotype distributions have a direct influence on the predicted efficiency that a rotavirus vaccine may have for a given region. Genotyping is a recognized epidemiological tool for examining strain diversity, and the correlation between genotypes and serotypes has been well established [4, 5]. Fourteen G serotypes have been described, and 10 have been recovered from humans [6]. In developed countries the most prevalent G serotypes have been found to be G1 to G4 [7–10]. However, it has been shown that in different regions of the world other G and P serotypes are circulating, such as G5 strains in Brazil [11], G8 strains in Malawi [12] and G9 strains in India [13]. In the USA significant numbers of G9 strains have also been detected (7.2% in several midwestern States) [14], and their epidemiological importance has yet to be determined. Based on the sequence variations of the VP4 gene, group A rotaviruses can be differentiated into at least 20 P genotypes, although four P types are by far the most common among human strains: P[8], P[4], P[6], and P[9] [6, 15]. As has been pointed out previously, the typing of gene 4 alleles by reverse transcription and polymerase chain reaction (RT-PCR) may be used as a proxy method for VP4 (P) serotyping in conjunction with the widely used G-typing of VP7-gene alleles [6].

The aim of this study was to perform a retrospective and prospective genotyping study on 145 rotavirus samples recovered from children with acute gastroenteritis from September 1996 to May 1999, which may reflect the prevalence of the main circulating rotavirus genotypes in the area of Valencia, Spain.

Materials and methods

Specimen collection and rotavirus detection in fecal samples

A total of 145 rotavirus-positive fecal samples were obtained from children under 5 years of age with acute gastroenteritis. All the cases were community-acquired infections. A commercial solid phase enzyme immunoassay (Primer Rotaclone, Meridian Diagnostics Inc., Cincinnati, Oh, USA) was used to

detect rotavirus antigen in fecal specimens following the procedure described by the manufacturer. The Rotaclone test utilizes a monoclonal antibody directed against rotavirus VP6 protein as the capture antibody. Positive specimens were frozen at −70 °C until further testing.

Viral RNA extraction

Viral RNA was extracted from the fecal samples by the RNAID kit (BIO101, Vista, Ca, USA) as previously described [16]. Briefly, for each sample 250 µl of 6 M guanidinium thiocyanate (Fluka) was mixed with an equal volume of stool suspension. A volume of 12 µl of the RNAID glass powder was added and the mixture was vortexed and rotated at room temperature for 10 min. After centrifugation for 30 sec at 650 ×*g* in a Beckman microcentrifuge, the supernatant was removed, the pellet washed twice with 400 µl of the RNAID kit wash buffer and recentrifuged at 850 ×*g*. After a third wash in the same buffer, the sample was finally centrifuged at 10,000 ×*g* for 60 sec, dried under vaccum, redissolved in 25 µl of deionized water and incubated for 10 min at 65 °C. The sample was centrifuged at 10,000 ×*g* for 60 sec, and the supernatant stored at −20 °C until required for the RT reaction and PCR. Just before use, samples were heated at 56 °C for 5 min and centrifuged at 10,000 ×*g* for 10 sec to pellet any residual RNAID matrix.

VP4 and VP7 cDNA synthesis and amplification

Reverse transcription was used to synthesize the cDNA corresponding to genomic segments encoding VP7 and VP4, following the procedures previously described [4, 5] with some modifications. The following RT-PCR method was used: 1 µl of dimethyl sulfoxide was added to 9 µl of the extracted RNA and heated at 97 °C for 5 min to denature nucleic acids, cooled on ice and the reaction buffer (6 µl 5X AMV buffer), 0.25 µl RNasin (Promega, Madison, Wi, USA), 3 µl of 10 mM solutions of each of the deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 2.5 µM each consensus primer (primers Beg9 and End9 for G typing; primers con3 and con2 to amplify gene 4 sequence), and 10 units AMV reverse transcriptase (Promega) were added. Reactions were incubated at room temperature for 10 min and subsequently at 42 °C for 35 min, ending with 92 °C for 2 min to inactivate the reverse transcriptase.

The cDNA amplification reactions were carried out in 50 µl 1X PCR buffer (10 mM Tris–HCl, pH 8.8) containing 1 µl of the RT reaction, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 2 mM of each dNTP, 1 µM each primer and 1 unit of *Taq* DNA polymerase (Life Technologies, Paisley, Scotland, UK). Reaction mixtures were overlaid with 50 µl of light mineral oil (Sigma Chemical, St Louis, Mo, USA) and PCR was conducted in a thermal cycler for 35 cycles (94 °C for 1 min, 47 °C for 2 min, and 72 °C for 2 min) with a final 7-min incubation at 72 °C. Negative and positive controls were included in every RT-PCR assay. The positive control was viral RNA from SA11 rotavirus cultivated in MA-104 cells and extracted as described [15].

VP4 and VP7 genotyping

Characterization of the VP7 genotype was performed according to the system previously described [4]. The G-typing PCR incorporated the common primer RVG9 and the G-type specific primers aBT1 (G1-specific), aCT2 (G2-specific), aET3 (G3-specific) and aDT4 (G4-specific). The sizes of the G type-specific PCR products were 749 bp (G1), 652 bp (G2), 374 bp (G3) and 583 bp (G4). The VP4 genotyping method was performed as previously described [5]. RT-PCR reactions were carried out with primers con3 and con2 to amplify the gene 4 sequence; semi-nested second amplifications were performed with primer con3 and the P type-specific primers 1T-1, 2T-1, 3T-1 and 4T-1. The sizes of the P type-specific PCR products were expected to be 345 bp (P[8]), 483 bp (P[4]), 267 bp (P[6]) and 391 bp (P[9]) [4]. A negative control was included during each experiment to monitor cross-contamination and confirm the validity of the genotyping results. Four cell culture-adapted rotavirus strains were used as controls during the genotyping work, Wa (P1A[8] G1), DS1 (P1B[4] G2), SA11 (P5B[2] G3) and VA70 (P1A[8] G4). Their RNAs were extracted from infected MA104 cells using the RNAID method.

PAGE analysis of rotavirus RNA

The PAGE technique for rotavirus RNA detection and analysis was performed by 10% polyacrylamide gel electrophoresis without SDS, and visualized after silver nitrate staining as previously described [17, 18].

Results

VP4 and VP7 genotyping

The G type was successfully identified in 131 (90.3%) of the clinical specimens, and the P type in 127 (87.5%) (Table 1). Predominance of type P[8] G1 (42.7%) and of type P[8] G4 (32.4%) was found throughout the period analysed. As a whole, P[8] was the most common P type, followed by P[4]. The overall prevalence for G-typing was G1 (42.7%), G2 (5.5%), G3 (2%), G4 (32.4%), and mixed G types (2%). The prevalence of each type differed from year to year, and G1 and G4 constituted 80% of all strains analysed. No unusual combinations of G and P types were detected (Figure 1). Mixed G types reflecting dual infections were detected at low frequency (n = 3; 2%). Two of them were G1 + G4, and one case was a mixture of G3 + G4. No mixed P types were found.

The prevalence and temporal distribution of full P and G types that could be determined are shown in

Table 1. Frequency of rotavirus G and P types in Valencia, Spain, from 1996 to 1999

G-type	No. of P-types (%)					
	P[8]	P[4]	P[6]	P[9]	NT[a]	Total
G1	62				2	64 (44.1)
G2		8				8 (5.5)
G3	3					3 (2)
G4	47				6	53 (36.5)
Mixed[b]	3					3 (2)
NT[a]	4				10	14 (9.6)
Total	119 (82)	8 (5.5)	0	0	18 (12.4)	145

[a] NT, not typeable.
[b] Mixed infections included two G1 + G4 samples and one case of a G3 + G4 mixture.

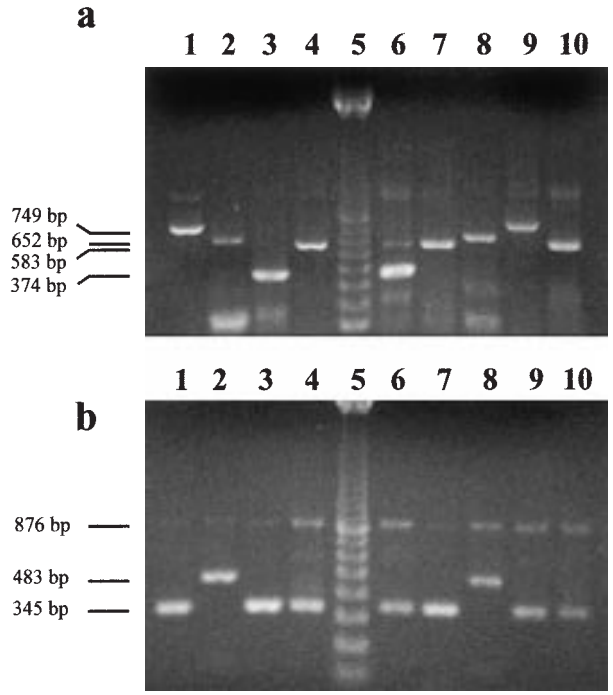

Figure 1. G-typing (a) and P-typing (b) of rotavirus strains by RT-PCR. PCR was carried out to determine both the G and P types of rotavirus strains. (a) The sizes of the G type-specific PCR products were 749 bp (type G1), 652 bp (type G2), 374 bp (type G3) and 583 bp (type G4). Lane 6 shows a mixed G type (G3 + G4). (b) P-typing of rotavirus strains run in the same order as in panel a. The sizes of the P type-specific PCR products were 345 bp (type P[8]) and 483 bp (type P[4]). An amplification product of 876 bp, corresponding to the first-step PCR full length amplicon generated by con3 and con2 primers, is visible in all lanes. Lane 5, 100 bp ladder (Pharmacia Biotech).

Table 2. Strains typed as P[8] G4 were highly predominant during the season 1996–1997 (60%), whereas type P[8] G1 strains were less common (9%). The number of G4 type strains dropped to 10 (16%) in 1997–1998 while G1 types increased to 38 (62.3%). This is a remarkable shift in the predominant genotype, occurring in only 1 year. During the winter season 1998–1999 the same distribution was observed: predominance of the G1 type (65%) and lower prevalence of G4 strains (13.7%).

Electropherotypes

Representative electropherotypes detected in Valencia during the period studied are shown in Figure 2. It was noted that the RNA profiles did not confirm a particular genotype. Mixed electropherotypes were detected in those cases that showed mixed genotype G1/G4 or G3/G4 by RT-PCR. In addition, some other mixed RNA patterns were found. Eight short patterns were observed, all of which corresponded to G2 strains.

Discussion

Rotavirus vaccination is expected to protect against severe diarrhoea and hence reduce the number of hospital admissions for this reason in children under 5 years of age. In spite of the withdrawal of rotavirus vaccine RRV-TV due to a significant number of cases of intussusception among vaccinated children, other vaccine formulations or strategies may be effective and safe in the near future. It has been estimated that rotavirus infections accounted for 25.3% of hospitalizations for gastroenteritis in Spain in 1 year [20], with a seasonal pattern of incidence during the cooler months of the year. To our knowledge, there are very limited data available on rotavirus G and P types circulating in Spain. In a previous survey that we performed from 1989 to 1990 in Valencia we found a clear predominance of type G1 (76%) over G4 (8%) [21]. It has also been reported that 88% of strains detected in Guipuzcoa (Basque Country) from 1989 to 1995 were G1 and G4 serotypes [22]. Our results confirm that all the rotavirus strains analysed belonged to the most common G types G1–G4, the same being observed in most European countries [9, 10, 23, 24]. These data are quite similar to those recently found in a survey performed in Ireland [19]. In addition, our study emphasizes the high frequency of P[8] type strains circulating in our area, as has also been reported in other European countries [19, 24]. We did not find any P[6] or P[9] type strains. Nor were these P types detected in Ireland during a 3-year survey, whereas a genotypic shift from type G1 to G2 and G4 was observed [19]. Although no reference strains for the P[6] and P[9] primers were included in our study, the high specificity of these primers has been noted by others [5, 7]. During the 1997–1998 period high prevalence of type G4 was found in Paris [25]. In the same period of time, the prevalence of G4 strains decreased in Valencia, while G1 strains prevalence rose. Studies on the variability and temporal distribution of rotavirus serotypes and genotypes in a

Table 2. Distribution of rotavirus G and P types over 3 years in Valencia

Winter season	P[8] G1	P[4] G2	P[8] G3	P[8] G4	Mixed types[a]	Not typeable[b]	Total
1996/1997	5	5	1	33	1	10	55
1997/1998	38	0	0	10	2	11	61
1998/1999	19	3	2	4	0	1	29
Total	62 (42.7%)	8 (5.5%)	3 (2%)	47 (32.4%)	3 (2%)	22 (15.1%)	145

[a] Mixed G types reflecting dual infections (2 cases G1 + G4 and one case G3 + G4). No mixed P types were found.
[b] Not typeable strains, with either both P and G types unassigned (10 samples) or with only the P or G type assigned (12 samples).

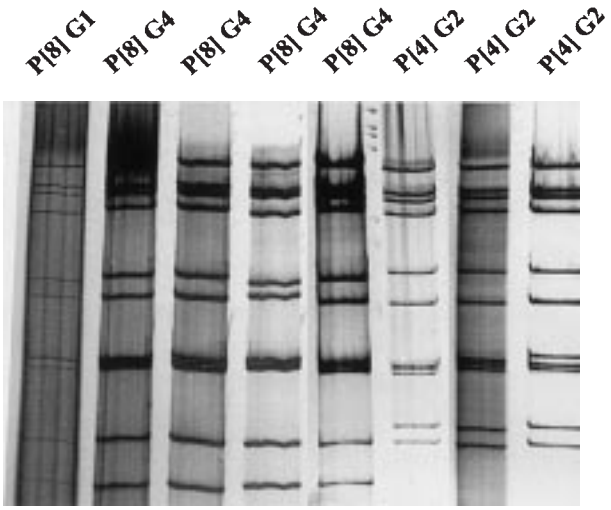

Figure 2. Representative electrophoretic migration patterns of the genomic dsRNA of rotavirus strains (electropherotypes) in relation to their P and G types. Different long RNA profiles were observed within the same P[8] G4 genotype. Every short electropherotype detected corresponded to a P[4] G2 strain.

variety of countries have identified important regional variations and temporal changes in G and P types [11, 13, 26]. The present study and similar observations made by others [8, 9] show that major shifts in the predominant serotype can occur.

Several samples were found to be untypeable in our study (18 samples by P-genotyping and 14 samples by G-typing). This failure could be due to the presence of inhibitors or low amounts of viral RNA in the 10 specimens for which we could not assign a P or a G type (Table 1). Some of these samples were also negative when analysed by PAGE. In four P[8] strains a G type could not be assigned, and two G1 and six G4 strains could not be P-typed despite several attempts to re-extract the RNA and perform the RT-PCR procedure for both genotypes. This partial genotyping (either G or P types obtained) for 12 strains (8.3%) has also been reported in other studies [19, 25, 26] and could be due to the accumulation of point mutations in primer binding sites which would prevent primer elongation or drastically reduce the efficiency of the genotyping PCR [27] in some strains. Failure to type due to natural variation in primer binding sites has also been reported for G8 strains [28]. This is a likely explanation for the untypeable strains in our study. However, we cannot formally exclude the possibility of G and P types other than those identified in our survey and for this reason we consider that these strains deserve further investigation.

Despite its limitations, RT-PCR is a sensitive and specific technique for the determination of P and G types for human rotaviruses [29], and more affordable for most laboratories than serotyping by enzyme immunoassay using monoclonal antibodies. A modified approach for genotyping group A rotaviruses has recently been described employing a single RT reaction with random primers for both G- and P-typing [30]. This method reduces the appearance of non-specific amplification products, although sensitivity has been found to be slightly lower than RT-PCR with VP4- and VP7-specific primers.

Analysis of RNA migration patterns by PAGE is a useful technique for virus detection and also for molecular epidemiological studies [31], although the RNA profiles do not correlate with the serotype and/or the genotype of a given viral strain [15, 32]. It is well known that analyses based merely on the RNA profiles do not provide conclusive evidence on the identical nature of two rotavirus strains [33]. However, it has been suggested that electropherotype analysis could help in identifying unusual strains that may arise be reassortment [24, 34].

Reassortants may be responsible for epidemics or outbreaks of gastroenteritis within a population, as has been demonstrated by sequence analysis of the VP1 and VP7 genes [32]. On the basis of our findings it is possible to speculate that the shift of the predominant genotype P[8] G4 to P[8] G1 may be a consequence of reassortments occurring during the course of mixed infections by G1 + G4 under the selective pressure of neutralizing antibodies, or the result of progressive antigenic variation due to selection of viral strains by immunological determinants.

The factors that determine the diversity of rotavirus genotypes in circulation among the human population, as well as the year-to-year variations they present, deserve more intense investigation that should lead to a clearer understanding of the epidemiology or rotaviruses.

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