G2 Rotavirus Infections in an Infantile Population of the South of Italy: Variability of Viral Strains Over Time

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Rotavirus positive samples collected in Palermo, Italy, during 2002–2004 did not react with the G2 type-specific RV5:3 monoclonal antibodies (MAbs) and could be identified as G2 only by RT-PCR genotyping. The genetic variation of VP7 and VP4 antigenic proteins was studied in 14 G2 samples including a selection of both those successfully characterized by serotyping and those failing to be serotyped. The phylogenetic analysis performed on partial VP7 sequences showed a temporal clustering of these strains, with those isolated in Palermo in 2003 belonging to the same lineage of G2 MAbs-unreactive strains identified in UK in 1996–1997 and in Bari, Italy, in 2003–2004. A single amino acid substitution in VP7 antigenic region A, at position 96 (Asp→Asn), was consistently associated with the loss of antigenic reactivity. Five of the G2 strains were further characterized by sequencing of VP4-encoding genes as belonging to the P[4] type, and separate lineages clustering the strains according to a temporal distribution could be described. VP7 and VP4 antigenic proteins analysis provided evidence that over the last 11 years, at least two different populations of G2P[4] rotavirus strains have been infecting the infant population in Palermo. Considering the role of anti-VP7 and anti-VP4 neutralizing antibodies in rotavirus immunity, the emergence of new VP7–VP4 gene combinations might influence rotavirus circulation in the infant population and should be taken into consideration when devising vaccination strategies. J. Med. Virol. 77: 587–594, 2005.

INTRODUCTION

Rotaviruses are the most important etiological agents associated with gastroenteritis in developed and developing countries [Kapikian et al., 2001]. In Italy, rotaviruses are found to be responsible for approximately 25% of episodes of diarrhea in the infantile population [Caprioni et al., 1996]. In Palermo, Southern Italy, an epidemiological surveillance on rotavirus disease in hospitalized children carried out since 1985 revealed peaks of prevalence ranging from 20% to 50% [Arista et al., 1997]. On the basis of the two outer layer proteins, VP4 and VP7, both of which elicit the production of neutralizing antibodies, human rotaviruses are classified into 10 G- (VP7-specific) and 10 P- (VP4-specific) types [Estes, 2001; Santos and Hoshino, 2005]. Strains with G1P[8] specificity are unanimously acknowledged as the most prevalent and ubiquitous; strains with G2P[4], G3P[8], G4P[8] specificity are widely distributed but their incidence varies regionally and temporally [Santos and Hoshino, 2005]. Since 1996, the circulation of strains with G9P[8] or G9P[6] types has been reported widely, while other G types and G/P combinations seem to be sporadic and only relevant locally [Santos and Hoshino, 2005]. Human rotaviruses have also been classified by hybridization analysis into three genogroups, Wa, DS-1, and AU-1 [Nakagomi et al., 1989]. Members of a genogroup share a high degree of genetic relatedness but have significantly less homology with members of other genogroups. G2 rotaviruses belong to DS-1 genogroup and usually show common antigenic

KEY WORDS: rotavirus G2; genetic variation; VP7 protein; VP4 protein; phylogenetic analysis
and genetic characteristics, such as P[4] type, VP6 subgroup I, NSP4 type A, and short RNA profile [Kapikian et al., 2001]. Recently, the identification of a porcine rotavirus strain related genetically in the VP7 to human G2 strains has been reported providing additional evidence for a relationship between human and animal rotaviruses [Martella et al., 2005]. In Europe, epidemiological studies reported low circulation rates of rotavirus with such type specificity [Zao et al., 1999; Iturriza-Gomara et al., 2001, Page and Steele, 2004a]. Lack of reactivity with the G2-specific monoclonal antibodies (MAbs) of rotavirus strains has been reported in many occasions [Ward et al., 1991; Unicomb et al., 1999; Iturriza-Gomara et al., 2001, Page and Steele, 2004b]. Infections with G2 rotaviruses have been associated in some studies with greater disease severity [Barn et al., 1992; Cascio et al., 2001]. However, other investigations have demonstrated lack of correlation between serotype/genotype and clinical symptoms [Barnes et al., 1992; Polanco-Marin et al., 2003]. It is thought that homotypic immune response to rotavirus correlated with protection against severe diarrhea, and heterotypic antibodies within a genogroup may also be important for protection from secondary infections [Bishop et al., 1983; Offit, 1996; Velazquez et al., 1996]. Primary infections with G1 type followed by symptomatic infection with G2 type have been described by Linhares et al. [1988] and symptomatic G2 infections in adults have also been reported [Timenetsky et al., 1996; Griffin et al., 2002]. In a study in South Africa, it was demonstrated that a temporal distribution with peaks in the prevalence of G2 strains after 10 years could be observed [Page and Steele, 2004a]. Genetic and evolutionary relationships among G2 rotavirus circulating in different countries have been demonstrated [Zao et al., 1999; Iturriza-Gomara et al., 2001; Page and Steele, 2004a].

The aims of this study were: (i) to investigate, through genotyping, the presence of G2-type strains among those that could not be G-serotyped from 1985 to 2004; (ii) to characterize by sequence analysis G2 strains circulating in the pediatric population of Palermo over the years; and (iii) to compare them to G2 rotavirus circulating in other geographical areas and to reference strains included in rotaviral vaccine preparations.

**MATERIALS AND METHODS**

**Samples**

In the period 1985–2004, 221 out of a total of 1,151 rotavirus-positive samples, obtained from children of less than 5 years of age, hospitalized with acute gastroenteritis at the “G. Di Cristina” Children’s Hospital of Palermo, could not be serotyped with type-specific neutralizing MAb. One hundred eight of these rotavirus-positive fecal samples that could not be serotyped, were selected for genotyping according to the availability of fecal material. A further selection of 4 HRV strains typed as G2 by genotyping and of 10 rotavirus strains successfully serotyped using G2-specific MAb RV5:3 were subjected to sequence analysis.

**Electropherotyping**

Electropherotyping of viral RNA was performed on 10% polyacrylamide gels, as described elsewhere [Arista et al., 1990].

**Determination of Serotype and Subgroup**

G typing was carried out according to previously published methods [Arista et al., 1990] using MAbs RV4:2, RV5:3, RV3:1, and ST3:3, respectively, reactive with G1–G4-specific viral protein VP7 [Coulson et al., 1987]. Subgrouping was performed with subgroup I- and subgroup II-specific MAbs (255/60 and 631/9) reactive with viral protein VP6, as described elsewhere [Arista et al., 1990].

**RNA Extraction and RT-PCR**

Rotavirus RNA was extracted from 10% fecal suspensions according to the method described by Boom et al. [1990]. The extracted RNA was resuspended in RNase-free sterile water and used for reverse transcription (RT)-PCR with random primers [Iturriza-Gomara et al., 1999]. To determine the G and P genotypes, specimens were analyzed with type-specific primers by semi-nested PCR strategy. In a first round PCR, the whole-length or a 881-nt long fragment of the gene encoding the VP7 was amplified using a generic oligonucleotide primer pair (Beg9/End9 or VP7F/VP7R, respectively) [Gouvea et al., 1990; Iturriza-Gomara et al., 2004], and the G types were subsequently predicted in a second round PCR using a pool of internal primers specific for the G1, G2, G3, G4, G9, and G10 genotypes in combination with the reverse consensus primer [Gouvea et al., 1990; Iturriza-Gomara et al., 2004]. Similarly, a 876 bp fragment of VP4 was amplified using the generic primers, Con3 and Con2 and P genotype was carried out using internal primers specific for the P[4], P[6], P[8], P[9], and P[11] genotypes [Gentsch et al., 1992; Iturriza-Gomara et al., 2004]. The PCR mixes were prepared and thermal-cycling was performed as previously described [Iturriza-Gomara et al., 2004]. Amplicons were analyzed by electrophoresis using a 2% NuSieve 3:1 (Cambrex, BioScience Rockland Inc., Rockland, ME) agarose gel in TBE at 7–8 V/cm for 75–90 min.

**Sequence and Phylogenetic Analysis**

First round amplicons of the gene encoding VP7 and VP4 were directly sequenced using primers VP7F/VP7R and Con2/Con3, respectively. Amplicons were purified using the GeneClean purification spin-columns (Q-biogene, Cambridge, UK) prior to sequencing using the CEQ2000 Dye Terminator Cycle Sequencing Kit (Beckman Coulter). Electropherograms were analyzed with the Geneious software 11.1.7 (Biomatters Inc., New Zealand).
Quick Start Kit (Beckman-Coulter, Fullerton, CA). All methods were carried out following the manufacturers’ instructions. Sequences were resolved using an automated sequencer (CEQ, Beckman-Coulter). The DNA sequences produced in this study have been submitted to the GenBank database and assigned the following accession numbers: DQ172838 through DQ172842 and DQ172843 through DQ172856, for VP4 and VP7 sequences, respectively. Sequence alignment was performed using CLUSTAL W [Thompson et al., 1994]. Phylogenetic analysis was carried out using the MEGA software version 3.0 [Kumar et al., 2004], using the Kimura 2-parameter model as a method of substitution and the neighbor joining method to construct the phylogenetic tree. The statistical significance of the phylogenies inferred was estimated by bootstrap analysis with 1,000 pseudoreplicate data sets.

Phylogenetic analysis was performed on partial VP7 and VP4 nt sequences and on the deduced amino acid sequences (aa 10–266 for VP7 and aa 10–290 for VP4). The sequences were aligned with and compared to additional sequences of G2 and P[4] rotavirus genotypes, respectively, obtained from the sequence collection of the Centre for Infections, Health Protection Agency, London, from the literature and from online databases.

**RESULTS**

Genotyping of 108 rotavirus untypeable strains identified them as belonging to the following G types: 44 (40.7%) G1, 17 (15.7%) G2, 7 (6.5%) G3, 4 (3.7%) G4, and 36 (33.3%) G9. The majority of the 17 G2 strains revealed by genotyping were collected in 2003 (76.5%) and the remaining in 2002 (5.9%) and 2004 (17.6%). The G2 strains genotyped in 2003 corresponded to more than half (56.5%) of the 23 not typeable isolates collected in Palermo during that year. All the strains identified by sero- and geno-typing exhibited subgroup I specificity and short electropherotype. All but one was of P[4] type, with the only exception being P[8].

A selection of 14 G2 rotavirus strains based on year of isolation and including strains characterized successfully by serotyping (years 1993–1997) and strains that failed to be serotyped (years 2003–2004), was subjected to further characterization by sequencing the VP7 encoding gene. The phylogenetic analysis performed on partial VP7 sequences (766 bp) showed a temporal clustering of G2 strains (Fig. 1), with the sequences from strains failing to be serotyped isolated in Palermo in 2003–2004 clustering in lineage II according to the nomenclature of Page and Steele [2004a]. Strains identified as G2 in UK in 1996–1997 [Iturriza-Gomara et al., 2001] and in Bari, Italy, in 2003–2004, which also could not be serotyped, belonged to the same cluster. The VP7 sequences of eight MAb-reactive strains recovered in Palermo from 1996 to 1997 were in lineage III as well as one of the two strains from 1993. The other G2 strain from 1993 clustered in lineage II, but in a separate branch from the strains isolated in 2003–2004. Nucleotide diversity between the strains in lineages II and III recovered in Palermo was 8%. Lineage I clustered the G2 prototype DS-1 and a number of G2 strains from different geographical areas dating back to 1976–1985.

The alignment of the deduced amino acid sequences of the antigenic regions A (aa 87–101), B (aa 142–152), C (aa 208–221), E (aa 189–191), and F (aa 233–242) of VP7 genes showed three temporally significant substitutions, the first at position 96 (Asp→Asn) of the antigenic region A, the second at position 213 (Ser→Asp) of the antigenic region C, and the third at position 241 (Ile→Val) in the antigenic region F (Fig. 2). Regardless of the year of isolation and MAbs reactivity, all the strains sequenced in Palermo had an amino acid substitution at position 87 (Ala→Thr) with respect to the prototype G2 strain DS-1. No differences from the prototype DS-1 sequence were observed in regions B and E (data not shown). Two strains collected in Bari, Italy, in 2003–2004 and which failed to react with the G2-specific MAbs showed high homology at the nt level and 100% identity in the amino acid sequences of the antigenic regions with respect to the contemporary isolates from Palermo.

Five G2 strains spanning the study period were characterized further through VP4 sequencing. Also the phylogenetic analysis performed on partial VP4 sequences (844 bp) and including reference sequences available from GenBank or the Health Protection Agency, London, showed clustering into lineages (Fig. 3). The strains collected in Palermo in 1993 and 2003–2004 fell within the same lineage with most of the reference sequences, while the strains recovered in 1996–1997 clustered with the L26 strain, exhibiting G12P[4] specificity. Nucleotide sequence divergence between the two lineages ranged from 5% to 7%. The partial VP4 deduced amino acid sequences showed three tentative temporal cluster defining patterns for 1993, 1996–1997, and 2003–2004 sequences, respectively. Nine cluster defining substitutions were identified in the 280 aa of VP4 sequences analyzed (Fig. 4).

**DISCUSSION**

Two significant increases in the number of rotavirus not G-serotypeable strains were detected in Palermo, Italy in the years 1999–2000 [Arista et al., 2004] and 2003 (data not shown). Among the 1999–2000 isolates, 51.8% had been already attributed to the G9 type by genotyping [Arista et al., 2004]. This study allowed to recognize as G2 56.5% of the 2003 rotavirus strains that were not typeable by using monoclonal based methods. Sequencing of the VP7 gene of the newly recognized G2 strains, and comparison with sequences derived from older successfully serotyped G2 rotavirus strains, allowed us to highlight the presence of two different populations of G2 strains in Palermo between 1996 and 2003. In fact, analysis of the antigenic regions of the VP7 protein revealed the presence of significant substitutions at the antigenic regions A, C, and F, at position 96, 213, and 241, respectively. Only the substitution at aa position 96 (Asp→Asn) was associated consistently
with strains that failed to be serotyped with RV5:3 MAb. Iturriza-Gomara et al. [2001] attributed the loss of reactivity of some G2 strains with the specific MAbs S2-2G10, RV5:3, and IC10 to the same substitution at aa 96. However, Page and Steele [2004b] described a G2 strain from Burkina Faso displaying the same aa changes as the British 1996 isolates that reacted with both S2-2G10 and IC10 anti-G2 MAbs.

The amino acid substitution at position 87 was present in both MAbs-reactive and non-reactive strains.
as well as in some South African [Page and Steele, 2004a] and Taiwanese [Zao et al., 1999] strains, and in the prototype G2 strain HN126 [Green et al., 1987]. Previous studies suggested that Asn at aa position 213 was most critical for reaction with neutralizing MAb [Coulson et al., 1996]; in the present work, the substitution of such amino acid both with Asp and Ser did not influence MAb reactivity, nor did the substitution at position 241 which was observed only in serotypable G2 strains from Palermo, but not in the strains from UK regardless of their reactivity with the G2-specific MAb. Accordingly, the binding of MABs to G2 strains might be more complex than previously thought and it might involve other VP7 antigenic regions than A, B, and C. However, the amino acid substitutions observed may have played a role in the re-emergence of G2 strains in the pediatric population of Palermo.

VP4 sequence analysis allowed the further characterization of the Italian G2 strains and confirmed them as belonging to the P[4] type. Even if such specificity is the second most commonly detected after P[8] [Santos and Hoshino, 2005], among circulating rotavirus strains worldwide very few P[4] VP4 sequences [Gorziglia et al., 1988; Taniguchi et al., 1990; Mahajan and Rao, 1996; Cataloluk et al., 2005] and no phylogenetic analysis have been published to date. In this study, P[4] strains cluster into two different lineages and this clustering might correlate with their temporal evolution. Despite the reduced number of sequences examined, a nucleotide divergence of up to 7% was observed between the two main lineages, similarly to what was found for VP7 coding genes. A nucleotide divergence of up to 13% had been found in a larger study among P[8] rotaviruses in Palermo [Arista et al., 2005]. The greater degree of diversity observed within the P[8] strains may respond to their wider and constant distribution with high prevalence, whereas P[4] strains circulate at significantly lower levels, increasing and decreasing their incidence in a more cyclical fashion [Page and Steele, 2004a].

It has been also hypothesized that the VP4 structure might influence the expression of VP7 epitopes [Dunn et al., 1994]. However, an almost complete nt identity was observed in the P[8]-type VP4 sequences of G4 and G9 cross-reacting strains [Arista et al., 2004]. In this study, the VP4 sequences of MAB-reactive 1996–1997 rotavirus strains and those of the 2003 strains, which failed to be serotyped, were in separate lineages (Fig. 3). Over the last 11 years in Palermo, at least two different populations of G2P[4] rotavirus strains have been infecting the infant population. VP7 sequence analysis indicated that the G2 strains circulating in Palermo in 1996–1997 clustered within the genetic
lineage III alongside strains isolated in South Africa, also identified as monotype 2b in Australia on the basis of the aa sequence at residues 147, 213, and 217 [Coulson et al., 1996]. However, only residue 213 in the aa sequence of the lineage III strains isolated in Palermo in 1996–1997 corresponded to monotype 2b description.

Monotype 2b strains were originally considered to be more likely to cause localized outbreaks [Page and Steele, 2004b], but in Palermo lineage III G2 strains were recovered from sporadic cases of infantile gastroenteritis over a 2-year G2 peak. The 2003 G2 epidemic was caused in Palermo by strains that were different in

**VP4 (aa 10-290)**

![Fig. 3. Phylogenetic analysis of partial VP4 nucleotide sequences (nt 27–871) of type P[4] strains. The phylogenetic tree was constructed using the neighbor joining method and Kimura's 2-parameter model. Type G1P[8] Wa-1974 sequence was used as an outgroup. Percentage bootstrap values above 50% are given at branch nodes.](image)

**Fig. 4.** Partial (positions 10–290) deduced amino acid sequences of the VP4 genes of some representative Italian G2P[4] strains compared to homologous sequences of reference strains from different geographical areas. DS-1 is included as the G2P[4] standard.
Variability of G2 Rotavirus in Italy

both their VP7 and VP4 sequences from those circulating in Palermo in 1996–1997. The new G2P[4] strains were highly homologous to those detected in South Africa and UK in the year 1996–1998 [Iturriza-Gomara et al., 2001; Page and Steele, 2004a] on the basis of their VP7 sequences, which strongly suggests that this lineage is maintained over time.

In conclusion, the recent G2 Italian strains are quite distantly related in both their VP7 and VP4 antigenic proteins to those isolated 10 years ago and no longer circulating. Considering the role of anti-VP7 and anti-VP4 neutralizing antibodies in rotavirus immunity, the need for a continuous surveillance of the rotavirus strains must be stressed in order to anticipate antigenic changes that might affect the effectiveness of the proposed vaccines. However, epidemiological surveys should be based on genetic than serological methods due to the poor ability of MAbs to serotype successfully currently circulating rotavirus strains.

REFERENCES


