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ORIGINAL ARTICLE

G-genotyping of rotaviruses in stool samples in Salento, Italy

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Key words

G-genotyping • Rotavirus • RT-PCR

Summary

Rotaviruses are the most common agents of diarrhoeal illness in infants and young children. Gastroenteritis caused by rotaviruses is also more likely to be associated with severe dehydration compared to other viral gastroenteritis.
We determined the G-genotype of rotaviruses circulating in the Salento. During 2004, 144 stool samples were collected from subjects with a positive screening test and stored at -20 °C until confirmation could take place using molecular biology techniques. A reverse transcription-polymerase chain reaction (RT-PCR) assay for the amplification of gene VP7 of rotavirus was developed and used; G-genotype was determined by sequencing and phylogenetic analysis.
A total of 101 stool samples were detected positive by RT-PCR and 35 were sequence analyzed and classified into G1, G2, G4, G9 and G12 type. The overall relative incidence of G-types in Salento is different to that of other developed countries. The most prevalent genotype was G2 while genotype G1 was present at low levels. It is also interesting to note the presence of G9 rotavirus, which is now recognized as the fifth globally important rotavirus genotype.
In this study, therefore, we demonstrate the usefulness of a simple method for correctly determining the G genotypes circulating in a geographic region.

Introduction

Rotaviruses are the major agents of severe gastroenteritis in infants and young children throughout the world. It has been well established that virtually every child becomes infected at least once by 3 years of age [1]. Rotavirus is a member of the *Reoviridae* family which includes seven groups (A-G). A complete virus particle possesses 11 double-stranded (ds) RNA segments surrounded by three concentric protein layers [2]. On the basis of the two outer layer proteins, VP7 and VP4, that elicit the production of neutralizing antibodies, human rotavirus are classified into G- (VP7-specific) and P- (VP4-specific) types [3]. There are 14 G serotype, however, types G1, G2, G3 and G4 are the most common globally and, therefore, are the targets for current vaccine development strategies [4]. In recent years, rotavirus genotyping by RT-PCR has provided valuable information about the diversity of rotaviruses circulating throughout the world, and has revealed much greater strain diversity than that seen in earlier years through the use of serological typing methods [5].
In an epidemiological survey, conducted in 2004, we observed that rotaviruses were detected in 20.8% of children admitted to hospital for enteritis in the Paediatric wards of Hospitals in the province of Lecce. In this study we determined the G-serotype of rotaviruses circulating in the Salento during the same year.

Methods

Rotavirus isolates

A total of 144 stool samples were collected from children under 5 years of age with acute diarrhoea detected during epidemiologic studies in 2004. The diagnosis for rotavirus infection was carried out in the Clinical Virology Laboratories of seven Hospitals in the province of Lecce (Casarano, Copertino, Galatina, Gallipoli, Lecce, Scorrano and Tricase) by means of rapid screening tests currently available on the market, such as latex agglutination, immuno-enzymatic kits and immuno-chromatographic tests.
The specimens were stored at -20 °C until tested by molecular biology techniques.

Viral RNA extraction

The faecal samples were suspended in Phosphate Buffered Saline (PBS) and subsequently decontamination by adding chloroform in a ratio of 1:10 v/v [6].
The double-stranded viral RNA were extracted using the method described in Wilde et al. (1990) [7]. Buffer (Tris-HCl, EDTA, NaCl, SDS and Proteinase-K) was added to the faecal suspension to bring about the digestion of the protein and subsequently the mixture was extracted with phenol-chloroform-isoamyl alcohol. The RNA was then precipitated by means of treatment with ethanol. The precipitated RNA was then collected

Tab. I. Primer oligonucleotides used throughout this study.

Oligonucleotide	Sequence 5'→ 3'	Polarity	Localization
First Primer pair			
RV1	GTCACATCATACAATTCTAATCTAAG	–	1061-1036
RV2	CTTTAAAAGAGAGAATTTCCGTCTG	+	3-27
Second Primer pair			
RV3	TGTATGGTATTGAATATACCAC	+	50-71
RV4 *	ACTGATCCTGTTGGCCAWCC	–	395-376

* Mixed base in degenerated primer: W = A/T

by centrifugation and resuspended in the desired amount of diethyl pyrocarbonate (DEPC)-treated water to be used in RT-PCR.

Primers

To identify the genome of viruses belonging to the Rotavirus genus, the nested-PCR used primers specific to the region of the VP7 gene, thus indicating group A Rotaviruses.
The RNA was retro-transcribed and amplified by PCR using the method described in Gilgen et al. (1997) [8] in which were used the two primer pairs RV1/RV2 and RV3/RV4 (Tab. I). The use of the first primer pair for RT-PCR produced an expected size of 1,059 bp, while the use of the second primer pair a fragment of 346 bp.

Reverse transcription

Ten μl of RNA were reverse transcribed by incubation for 60 min at 41 °C followed by 5 min at 95 °C. In a final volume of 20 μl, reaction conditions were 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl, 10 mM DTT, 0.5 mM each dNTP, 1.25 mM antisense primer (RV1), 10 U RNasin (Promega, Madison, WI) and 100 U M-MLV reverse transcriptase (Promega). RNA used for rotavirus RT was denatured for four min at 94 °C in the presence of 5% DMSO and chilled on ice for ten min.

Polymerase chain reaction

The completed RT reaction (20 μl) was mixed with 80 μl of PCR mixture (final concentration: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton-X-100, 2 mg ml BSA, 200 mM each dNTP, 2 U of *Taq* Polymerase (Promega), 0.25 mM of the first primer pair (RV1/RV2) and 1.5 mM of $MgCl_2$. Two μl of the first reaction were used as a template for the second round of PCR, using 0.5 mM of the second primer pair (RV3/RV4) and 3.5 mM of $MgCl_2$. Cycling was done on a Mastercycler (Eppendorf, Hamburg, Germany) with one min at 94 °C, followed by 25 cycles at 94 °C for 30 sec, annealing at 55 °C for 60 sec, 72 °C for 60 sec for the first round and 40 cycles with the same cycling profile for the second nested PCR. In addition, an end extension step was performed at 72 °C for three min after the first PCR and ten min after the second PCR.

All the RT-PCRs were performed with viral RNA extracted from reference samples as positive controls and water as negative control.
Four separate rooms, for RNA extraction, first amplification, second amplification, and gel analysis, were used to avoid cross-contamination of samples.

Electrophoresis

RT-PCR fragments were analyzed by gel electrophoresis. Twenty μl of the amplification products were analyzed on 2% agarose gels in Tris-borate-EDTA buffer with a 100-bp DNA ladder (PROMEGA). After electrophoresis at 120 V for 50 min, the gel was photographed under UV light.

Sequencing

Templates for sequencing were prepared by purification of the amplified products (1059 or 346 bp), randomly selected, by using a JETQUICK Kit (Genomed) based on spin column technique and sequenced automatically using the dideoxynucleotide chain terminator method in an automated sequencer (3130, Applied Biosystems, Inc. Foster City, Calif.). The cycling conditions followed product manufacturer instructions.

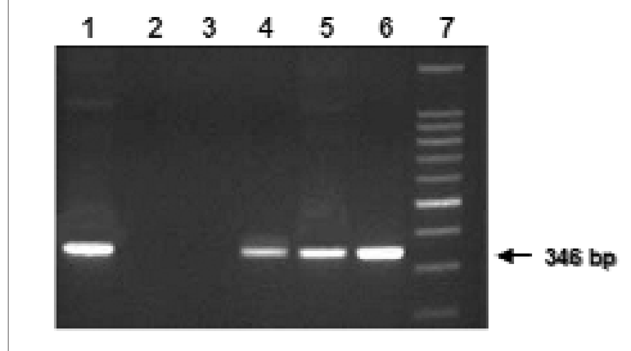
Phylogenetic analyses

The nucleotide sequences obtained were aligned with the reference sequences available in the GenBank database by using BLAST (NCBI) and CLUSTAL W (EBI) programs. Phylogenetic tree was constructed by the neighbor-joining method. Phylogenetic tree was drawn using the program TreeView for Windows version 1.6.6 (Roderic D.M. Page).

Results

Of the total of 144 stool samples collected from children with acute gastroenteritis, 101 (70%) turned out to be rotavirus positive by RT-PCR (Fig. 1).
Thirtyfive positive samples were sequence analyzed and classified in G genotype. Results of alignment and phylogenetic analysis of both fragments (1059-bp and 346-bp) were in agreement, allowing correct G genoty-

Fig. 1. Typical PCR results from samples containing rotaviruses. Lane 1: PCR positive control; lane 2: PCR negative control; lane 3: sample negative for rotavirus; lane 4-6: samples positive for rotavirus showing the expected amplicon of 346 bp; lane 7: molecular weight marker (100 bp ladder).



ping of all sequences by examination of only the 346-bp PCR product.
The most prevalent G genotype was G2 (37.1%), followed by G4 (34.3%), G9 (14.3%), G1 (8.6%) and G12 (5.7%) (Tab. II).
The results of the phylogenetic sequence analysis of some positive samples are displayed in Figure 2.
Phylogenetic analysis of the nucleotide sequences revealed that the CO04-30 clustered with G2 reference strains, CO04-15 with G4, CO04-07 with G1, CA04-14 with G12 and CA04-21 with G9 and were separated from G3, G5, G6, G8 and G10 strains.

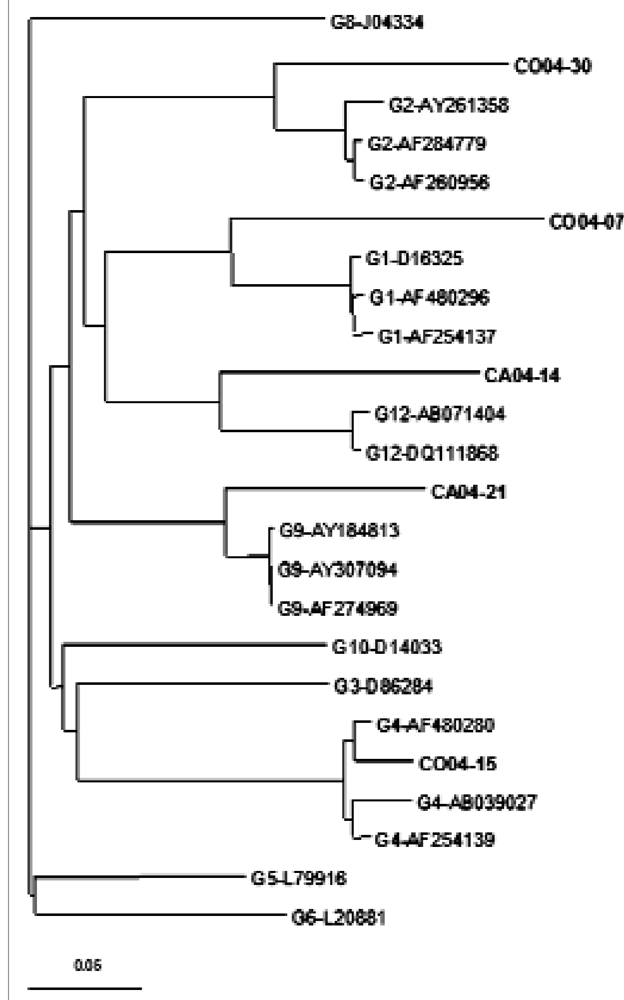
Discussion

Our research provides data on enteritis caused by rotavirus in children admitted to hospital for acute diarrhoea in the Salento, thus contributing to the understanding of the epidemiological framework of this disease in Italy. In general, rotaviruses were detected by means of rapid screening tests in 20.8% of children admitted to hospital for enteritis in the Paediatric wards of Hospitals in the province of Lecce. Data obtained are in line with the results observed in

Tab. II. Results of the characterisation of the G-types, reference strains used for comparison with GenBank accession numbers.

Samples	VP7 G type	Reference strains (GenBank accession number)
1	G1	AB081799 - Japan
1	G1	D16325 - Japan
1	G1	AF260950 - Cina
1	G2	AY261346 - Africa
10	G2	AY261349 - Africa
1	G2	AY261358 - Africa
1	G2	AF409087 - India
12	G4	AF480280 - Uruguay
5	G9	AF274969 - Brazil
2	G12	AB071404 - Thailand

Fig. 2. Phylogenetic tree built with partial sequences (346 bp) from prototype strains and from representative samples for each of the G-genotypes obtained in our study (CO04-30, CO04-07, CA04-14, CA04-21, CO04-15). The scale bar represents 5% nucleotide difference.



Spain (24%) [9], Finland (26%) [10] and England (29%) [11].
The successful determination of the G-serotype by sequencing and phylogenetic analysis of a partial fragment has been reported previously in Baggi and Peduzzi (2000) [12]. Their study demonstrated the efficiency of the nucleotide sequence analysis of 189 bp at the 5' end of the VP7 gene for correctly determining the G genotypes. The fragment considerated in our work includes the same nucleotide sequence.
Molecular analysis of the VP7 gene revealed the presence of five different G-genotypes in the Salento. Sequencing results showed G2 genotype be predominant. This doesn't correspond to the usual distribution described in the literature where G1 is described as the most common genotype. The high percentage of G4-genotype, instead, is in agreement to several studies conducted in Europe which have reported high frequency and marked fluctuation of genotype G4 [13-16].
Of note, no G3 rotavirus strains, the most common worldwide after G1 and G2, was detected during the period of observation in the Salento.

Five G9-genotype samples were detected. This result confirms the emergence and wide geographic distribution of this serotype, detected, since 1995, in several countries including India, Italy, the United States, Bangladesh, Malawi, the United Kingdom, Australia, France, Ireland, Japan, Thailand, The Netherlands, Libya, and Kenya and also in Latin American countries, such as Brazil, Argentina, and Cuba [17-21]. This result reinforces the possibility that rotavirus type G9 may represent the fifth globally important serotype to be considered in vaccination programs [20 21].
Detection of two strains with G12 (5.7%) specificity is very significant since is a rare genotype. G12 was first detected in Philippines in 1990 [22] and in the United States [23], Thailand [24] and India [25] in 2002. This observation implies that G12 may be an emerging strain.

Conclusion

In conclusion, the present study, confirmed the usefulness of a simple method for correctly determining the G genotypes circulating in a geographic region of interest, allowing rapid epidemiological surveys of rotaviruses in clinical samples.

References

[1] Kapikian AZ, Hoshino Y, Chanock RM. *Rotaviruses*. In: Knipe DM, Howley PM, Griffin DE, eds. *Fields Virology, 4th ed*. Philadelphia: Lippincott Williams and Wilkins 2001, pp. 1787-1834.

[2] Arias CF, Dector MA, Segovia L, Lopez T, Camacho M, Isa P, et al. *RNA silencing of rotavirus gene expression*. Virus Res 2004;102:43-51.

[3] Desselberger U, Iturriza-Gòmara M, Gray J. *Rotavirus epidemiology and surveillance*. Novartis Symp Ser 2001;238:125-47.

[4] Hoshino Y, Kapikian AZ. *Rotavirus serotypes: classification and importance in rotavirus epidemiology, immunity and vaccine development*. J Health Popul Nutr 2000;18:5-14.

[5] Iturriza-Gòmara M, Kang G, Gray J. *Rotavirus genotyping: keeping up with an evolving population of human rotaviruses*. J Clin Virol 2004;31:259-65.

[6] Maggi F, Fornai C, Zaccaro L, Morrica A, Vatteroni ML, Isola P, et al. *TT Virus (TTV) loads associated with different peripheral blood cell types and evidence for TTV replication in activated mononuclear cells*. J Med Virol 2001;64:190-4.

[7] Wilde J, Eiden J, Yolken R. *Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions*. J Clin Microbiol 1990;28:1300-7.

[8] Gilgen M, Germann D, Luthy J, Hubner P. *Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples*. Int J Food Microbiol 1997;37:189-99.

[9] Calderon SM, Garcia Lopez JL, Alvarez-Dardet Diaz C, Perea Perez EJ. *Incidencia de diarreas en una cohorte de ninos en la ciadad de Sevilla*. An Esp Pediatr 1990;32:114-8.

[10] Ruuska T, Vesikari T. *A prospective study of acute diarrhea in Finnish children from birth to 2.5 years of age: clinical severity, etiology and risk factors*. Acta Paediatr Scand 1991;80:500-7.

[11] Djuretic T, Ramsay M, Gay N, Wall PG, Ryan M, Fleming D. *An estimate of the proportion of diarrhoeal disease episodes seen by general practioners attributable to rotavirus in children under 5 year of age in England and Wales*. Acta Paediatr 1999;426(Suppl):38-41.

[12] Baggi F, Peduzzi R. *Genotyping of rotaviruses in environmental water and stool samples in Southern Switzerland by nucleotide sequence analysis of 189 base pairs at the 5' end of the VP7 gene*. J Clin Microbiol 2000;38:3681-5.

[13] Arista S, Giammanco GM, De Grazia S, Colomba C, Martella V. *Genetic Variability among Serotype G4 Italian Human Rotaviruses*. J Clin Microbiol 2005;43:1420-5.

[14] Bànyai K, Gentsch JR, Glass RI, Uj M, Mihàly I, Szücs G. *Eight-year survey of human rotavirus strains demonstrates circulation of unusual G and P types in Hungary*. J Clin Microbiol 2004;42:393-7.

[15] Ehlken B, Laubereau B, Karmaus W, Petersen G, Rohwedder A, Forster J, et al. *Prospective population-based study on rotavirus disease in Germany*. Acta Paediatr 2002;91:769-75.

[16] Sanchez-Fauquier A, Wilhelmi I, Colomina J, Cubero E, Roman E. *Diversity of group A human rotavirus types circulating over a 4-year period in Madrid, Spain*. J Clin Microbiol 2004;42:1609-13.

[17] Bok K, Castagnaro N, Borsa A, Nates S, Espul C, Fay O, et al. *Surveillance for rotavirus in Argentina*. J Med Virol 2001;65:190-8.

[18] Coluchi N, Munford V, Manzur J, Vazquez C, Escobar M, Weber E, et al. *Detection, Subgroup Specificity, and Genotype Diversity of Rotavirus Strains in Children with Acute Diarrhea in Paraguay*. J Clin Microbiol 2002;40:1709-14.

[19] Cunliffe NA, Dove W, Bunn JE, Ben Ramadam M, Nyangao JW, Riveron RL, et al. *Expanding global distribution of rotavirus serotype G9: detection in Libya, Kenya, and Cuba*. Emerg Infect Dis 2001;7:890-2.

[20] Jain V, Das BK, Bhan MK, Glass RI, Gentsch JR, The Indian Strain Surveillance Collaborating Laboratories. *Great diversity of group A rotavirus strains and high prevalence of mixed rotavirus infections in India*. J Clin Microbiol 2001;39:3524-9.

[21] Santos N, Volotao EM, Soares CC, Albuquerque MC, da Silva FM, de Carvalho TR, et al. *Rotavirus strains bearing genotype G9 or P[9] recovered from Brazilian children with diarrhea from 1997 to 1999*. J Clin Microbiol 2001;39:1157-60.

[22] Taniguchi K, Urasawa T, Kobayashi N, Gorziglia M, Urasawa S. *Nucleotide sequence of VP4 and VP7 genes of human rotaviruses with subgroup I specificity and long RNA pattern: implication for new G serotype specificity*. J Virol 1990;64:5640-4.

[23] Griffin DD, Nakagomi T, Hoshino Y, Nakagomi O, Kirkwood CD, Parashar UD, et al. *Characterization of nontypeable rotavirus strains from the United States: identification of a new rotavirus reassortant strain (P2A[6], G12) and rare P3[9] strains related to bovine rotaviruses*. Virology 2002;294:256-69.

[24] Pongsuwanna Y, Guntapong R, Chiwakul M, Tacharoenmuang R, Onvimala N, Wakuda M, et al. *Detection of a human rotavirus with G12 and P[9] specificity in Thailand*. J Clin Microbiol 2002;40:1390-4.

[25] Soma D, Varghese V, Chaudhury S. *Emergence of Novel Human Group A Rotavirus G12 Strains in India*. J Clin Microbiol 2003;41:2760-2.

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