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

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Key words
G-genotyping • Rotavirus • RT-PCR

Summary
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.

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.
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 MgCl2, 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 MgCl2. 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 MgCl2. 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). Thirty-five 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-
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 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 considered 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 to 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**


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