Detection of the Norovirus Variants GGII.4 Hunter and GGIIb/Hilversum in Italian Children With Gastroenteritis

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Noroviruses (NoVs) are important enteric pathogens of humans. Although they exhibit an impressive genetic diversity, few NoV strains appear to predominate worldwide. Limited epidemiological data are available on NoV gastroenteritis in Italy. In this study, we assessed the prevalence of human NoV in Italian children with gastroenteritis by using a reverse-transcription nested polymerase chain reaction (RT-PCR) assay specific for the RNA-dependent RNA polymerase (RdRp) on faecal samples collected throughout the 2004 surveillance activity in Palermo, Italy. NoVs were detected in 47% of the stool samples obtained from children <5 years age, admitted to hospital with acute non-bacterial gastroenteritis. A selection of strains was further analyzed by partial sequence analysis of the RdRp gene. The strains were characterized as genogroup (GG) II and clustered into two distinct virus populations that resembled the emerging European GGIIb/Hilversum strains and the Australian Hunter GGII.4 strains. A temporal pattern of distribution of the two NoV strains was observed which was consistent with an independent circulation of two separate strains in the local population. Based on this 1-year study we concluded that NoVs were a diffuse cause of sporadic cases of acute childhood gastroenteritis and that strains of global epidemiological relevance were circulating in Palermo, Italy in 2004. J. Med. Virol. 78:1656–1662, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: norovirus; genotyping; enteritis; children; Italy

INTRODUCTION

Noroviruses (NoVs) are regarded as the most common cause of outbreaks of acute gastroenteritis worldwide and they have frequently been reported to be the etiological agents of sporadic cases in children [Fankhauser et al., 1998; Vinje et al., 2003]. NoVs were first discovered by electron microscopy in 1972 [Kapikian et al., 1972] and for many years their detection was restricted to few specialized laboratories endowed with the proper equipment. Prior to the development and large-scale application of new and sensitive diagnostic techniques specific for NoVs, only rotaviruses and enteric adenoviruses were included in routine diagnostic protocols for non-bacterial gastroenteritis and the aetiology of a large portion of gastroenteric cases remained unknown. In an epidemiological survey of the Italian infantile population in 1996, enteric pathogens (rotavirus, salmonella, and campylobacter) were identified only in 59% of the gastroenteritis episodes [Caprioli et al., 1996]. However, the introduction of diagnostic molecular techniques completely modified this scenario by providing evidence that NoVs are implicated in most gastroenteritis episodes that were formerly considered of uncertain or unknown aetiology [Atmar and Estes, 2001; Simpson et al., 2003; Pang et al., 2004].

Based on sequence information obtained from either the RdRp gene (ORF1) or the viral protein (VP1) gene (ORF2), NoVs have been subdivided into five separate genetic groups (GGI to GGV), and in a number of genetic clusters or genotypes. Due to their impressive genetic diversity and to the lack of a standardized nomenclature and/or classification, designation of NoV genotypes, notably within the major genetic groups I and II, is controversial or ambiguous [Vinje et al., 2003; Wang et al., 2005; Zheng et al., 2006]. According to a

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Accepted 26 July 2006
DOI 10.1002/jmv.20751
Published online in Wiley InterScience (www.interscience.wiley.com)
comprehensive classification scheme proposed recently [Zheng et al., 2006], at least 29 genotypes may be distinguished. GGI includes 8 human genotypes and GGII 16 human genotypes, while GGOV only 1 human genotype [Vinje et al., 2004]. Two other genogroups (GGs), GGGIII and GGV, include bovine and murine strains and have 2 and 1 genotypes, respectively [van Der Poel et al., 2000; Karst et al., 2003], while additional 3 GGII genotypes (GGII.11, GGII.18, and GGII.19) have been detected in pigs [Wang et al., 2005]. The geographical distribution of human NoV genotypes is various and multiple NoV genotypes circulate or co-circulate in the human population. However, a few NoV strains have dominantly emerged worldwide and are of epidemiological relevance [Lindell et al., 2005]. Investigations in several countries have revealed that NoVs are involved in 14–44% of sporadic cases of pediatric gastroenteritis, with GGII strains being predominant [Buesa et al., 2002; Oh et al., 2003; Lindell et al., 2005; Sanchez-Fauquier et al., 2005; Bull et al., 2006]. Epidemiological investigations in the mid 1990s revealed that NoV strains belonging to the GGII.4 cluster, Lordsdale-like, accounted for the majority of gastroenteritis cases [Noel et al., 1999; Hale et al., 2000; Koopmans et al., 2000; Schreier et al., 2000]. After 2000, the emergence and subsequent spread of new GGII.4 variants was documented and a 6-nucleotide-long motif localized within the RdRp gene has been used to identify temporal variations within GGII.4 strains [Lopman et al., 2004; Lindell et al., 2005]. In addition, as of 2000 a novel NoV strain, GGIb/Hilversum, highly prone to recombinational events, emerged in Europe [Ambert-Balay et al., 2005; Reuter et al., 2005]. GGIb/Hilversum was detected more frequently in children than in adults and it was thus assumed that it had a peculiar role in gastroenteritis of children [Lindell et al., 2005]. Few epidemiological data are available regarding NoV gastroenteritis in Italy [Pelosi et al., 1999; Medici et al., 2005]. In the present study, a reverse-transcription nested polymerase chain reaction (RT-PCR) assay specific for the RdRp gene was used to assess the prevalence of human NoVs in Italian children with gastroenteritis during a 1-year surveillance activity in Palermo, Italy. Some of the RdRp amplicons were sequenced partially to investigate the molecular epidemiology of Italian NoVs.

MATERIALS AND METHODS

Samples

One hundred ninety-nine stool samples from children, admitted with acute gastroenteritis to the “G. Di Cristina” Children’s Hospital of Palermo from January to December 2004, were examined. A stool specimen was collected from each patient on the day of admission to exclude nosocomial infection. The cases were epidemiologically unrelated to one other. A panel of 40 stool specimens was included to second as negative and positive controls. Thirty-two NoV-negative specimens included 20 stool samples from asymptomatic newborns (2–15 days) that had tested negative for viral and bacterial enteropathogens and 12 samples from symptomatic children that were positive for viruses other than NoVs. Eight NoV positive specimens, as confirmed by Reverse Line Blot Hybridization (RLBH), were used as positive controls and included 2 GGI strains (Queensmans-GI.4 and Sindlesham-GI.6) and 6 GGII strains (3 strains Lordsdale-GII.4, 1 strain Hilversum-GIIb, and 2 strains GII, not assigned). All the stool samples were aliquoted and stored at −20°C until analysis.

RNA Extraction and RT-PCR

NoV RNA was extracted from 200 μl of 10% faecal suspension in Minimum Essential Medium balanced salt solution (MEM) using guanidinium isothiocyanate/silicae according to the procedure described by Boom et al. [1990]. RNA was eluted in 50 μl H2O DEPC with RNasin (0.2 μg/μl, Promega, Madison, WI) and used in reverse transcriptase (RT)-PCR.

RT-PCR was performed with specific primers, JV12/JV13, targeting the RdRp region (region A) [Vinje and Koopmans, 1996] using Superscript III One step (Invitrogen, Paisley, UK). Samples testing negative in the first PCR step were analyzed by semi-nested PCR with JV13-Ni primers. In particular, 2 μl of PCR reaction mix were added, yielding a total volume of 50 μl, consisting of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.2 mM dNTP, 5 U of AmpliTaq Gold (Applied Biosystems Foster City, CA) and 0.4 μM of each primer. Samples were denatured for 7 min at 95°C and subjected to 35 cycles at 94°C for 30 sec, 38°C for 30 sec, 72°C for 30 sec followed by 72°C for 5 min. Amplified products were analyzed by gel electrophoresis on ethidium bromide-stained agarose. The outer primer set yielded a product of 327 bp and the nested inner pair yielded a product of 110 bp.

Sequence and Phylogenetic Analysis

Amplicons were purified using purification spin-columns (Montage PCR, Millipore, Bedford, MA) and sequenced with JV12 (first step) or Ni (semi-nested) primer (MWG-biotech, Ebersberg, Germany). The first step sequences were aligned using CLUSTAL W [Thompson et al., 1994]. Phylogenetic analysis was carried out using the software MEGA version 3.1 [Kumar et al., 2004] with Kimura 2-parameter model as a method of substitution and the neighbor joining method to reconstruct the phylogenetic tree. The statistical significance of the phylogenies inferred was estimated by bootstrap analysis with 1,000 pseudoreplicate data sets. The sequences were aligned with and compared to a selection of sequences of representatives of the various NoV genotypes that were available in the databases [Fankhauser et al., 1998].

RESULTS

NoVs were detected in 93 (46.7%) of the 199 specimens examined. In particular, 74 (79.6%) samples yielded

positive results in the first PCR step and 19 (20.4%) in the semi-nested-PCR.

Positive control stool samples were correctly recognized, while negative control stool samples were not recognized.

A total of 36 RT-PCR amplicons, 28/74 first step-positive and 8/19 nested-positive, were submitted to sequencing. Comparison by BLAST (NCBI) and FASTA (EMBL) analysis of the sequences allowed all the strains to be characterized as GGII. Assignment to genotypes on the basis of the RdRp partial sequences (327 bp for first step amplicons and 110 bp for second step amplicons) was done following previously defined working criteria: >85% similarity for GGI strains and 90% for GGII strains [Vinje et al., 2000].

A phylogenetic tree was developed using the first-step sequences and cognate RdRp gene sequences of representatives of the various NoV genotypes, including 8 GGII strains, 5 GGI strains and 1 GGIV strain. In addition, 14 NoV strains responsible for outbreaks or sporadic cases of gastroenteritis in Europe, Asia and Australia in the years 2000–2004 were selected from GenBank. In the phylogenetic tree, the Italian strains segregated into two separate clusters (Fig. 1). Sixteen strains were grouped along with GGIib/Hilversum strains and displayed >97% nucleotide identity to the reference GGII/Hilversum sequivar Gottenburg1 (AF365989). Twelve Italian strains were grouped along with GGII.4 NoVs. Within this branch, a single GGII.4 strain (PA1/04) showed 97.6% identity to the Farmington Hills sequivar (AY502023). The PA1/04 strain exhibited a peculiar 6-nucleotide-long motif (AGTCTG rather than the usual AATCTG) localized within the RdRp gene (starting position 4820 relative to Norwalk virus, M87661). The remaining GGII.4 strains were grouped with the GGII.4 Australian strain Hunter (DQ078801) and displayed >98.7% nucleotide identity to this Australian sequivar and 95.1–96.0% nucleotide identity to the Farmington Hills sequivar. Nucleotide variation within the Italian GGIIib/Hilversum strains was <5.6% and variation within the Italian GGII.4 strains of the Hunter sub-cluster was <2%.

Eight 76 bp-long sequences were determined from the nested PCR amplicons. These sequences were 100% (nt) identical to each other and displayed >97% homology with other Italian GGII.4 sequences. As shown in Figure 2, a temporal pattern of distribution of the NoV strains was observed. GGIib/Hilversum strains were detected mainly between February and March, while they occurred sporadically over the course of the year except for summer when they were not detected at all. Most GGII.4 strains were detected in the autumn-winter period. The distribution of the 57 untyped strains reflected the pattern of the typed strains, with 82% of the NoV infections detected between February and May.

The median age of the children with NoV infection was 12 months. Forty-nine percent of the NoV cases were associated with vomiting, 25% with fever and 21% with dehydration. Hospitalization was required for 3 (range 1–11) days.

DISCUSSION

Systematic application of sensitive molecular techniques, that is, single-step RT-PCR and nested RT-PCR, allowed us to detect a high (46.7%) prevalence of NoV infections in children hospitalized for gastroenteritis in Palermo in 2004. Even when allowances are made for the lower figure of 37.2% of NoV positive cases detected with conventional single-step RT-PCR, NoVs appear to be an important causative agent of infantile diarrhoea in Palermo during that year. In a similar fashion, high rates of NoV infections (15–44%) in children with gastroenteritis were reported between 1997 and 2004 in Sweden, Spain and Australia [Lindell et al., 2005; Sanchez-Fauquier et al., 2005; Bull et al., 2006]. Lopman et al. [2004] observed a marked increase in NoV circulation from 1995 to 2002 in some European countries and ascribed this phenomenon to the onset of new NoV variants. A review of the literature and the data collected over the past decade underscores significant changes that occurred in NoV epidemiology. The NoV GGII.4 Lordsdale-like strains were largely circulating worldwide in the mid 1990s [Koopmans et al., 2000; Foley et al., 2001], while a decrease in their circulation was observed during 2002–2004. Almost in the same years, a new GGII.4 variant, Farmington Hills, emerged in the United States causing a high number of outbreaks and sporadic cases of enteritis in children [Blanton et al., 2006]. Enteritis episodes caused by GII.4 Farmington Hills strains were subsequently documented in England, Wales, Germany, Netherlands, Sweden, and Australia, suggesting the spread of Farmington Hills-like NoVs on a global scale [Fankhauser et al., 1998; Buesa et al., 2002; Lindell et al., 2005; Bull et al., 2006]. This GGII.4 variant was thought to be more virulent or more stable in the environment than other GGII.4 viruses [Lindell et al., 2005]. The GGII.4 Hunter sub-cluster, displaying 4% nt divergence from the Farmington Hills variant in the RdRp region, was identified in Australia in 2002–2004. GGII.4 Hunter NoV was responsible for 42.9% of the outbreaks and 15.6% of the sporadic cases in children hospitalized with enteritis [Bull et al., 2006]. In 2000 another NoV strain, GGIib/Hilversum, emerged in France and became predominant in the following seasons with outbreaks and sporadic cases in Spain, Sweden, and Hungary.
Fig. 1.
All the 2004 NoV strains sequenced in the present study were characterized as GGII NoVs. This finding mirrors the data obtained in a recent study in Parma, Northern Italy, reporting a predominant circulation of GGII strains in sporadic cases of NoV-related infantile gastroenteritis, and is in agreement with the evidence that GGII strains usually out-number GGI strains [Vinje and Koopmans, 1996; Gallimore et al., 2004b].

Sequence and phylogenetic analysis demonstrated that two NoV strains, GGII.4 and GGIIb/Hilversum, co-circulated in Palermo in 2004. GGII.4 appeared to be more frequent in autumn, while the GGIIb/Hilversum strains appeared to circulate throughout the winter season. Accordingly, it is possible to speculate that two distinct and consecutive epidemics of NoVs occurred in the local population. The Italian GGII.4 isolates could be further distinguished into two sub-clusters, Hunter and Farmington Hills.

Lopman et al. [2004] identified a 6-nucleotide-long motif localized within the RdRp gene of GGII.4 strains (starting position 4820 relative to Norwalk virus, M87661) and observed that this nucleotide stretch displays a pattern of temporal variation. The nucleotide motif AACTT G, that was considered to be specific for the GGII.4 strains circulating in Europe before 2002, was found in the Italian GGII.4 Hunter strains. The same motif was also found in GGII.4 strains detected in Stockholm in 2001–2002 [Lindell et al., 2005]. After 2002, a new motif, AATCT G, was predominant not only in Stockholm but also in other European countries and in USA [Lopman et al., 2004; Widdowson et al., 2004; Lindell et al., 2005; Ike et al., 2006]. Curiously, a peculiar motif, AGTCTG, was found in the PA1/04 sequivar that was grouped in the Farmington Hills sub-cluster [Buesa et al., 2002; Reuter et al., 2005]. Based on the analysis of the RdRp gene fragment, the Italian GGIIb strains formed a homogeneous group and appeared to be closely related to the GGIIb strains detected in the other European countries [Buesa et al., 2002; Lindell et al., 2005; Reuter et al., 2005]. Classification of the NoV GGIIb strains is controversial as these strains have a peculiar RdRp gene, GGIIb/Hilversum, while they may display a variety of capsid genotypes [Reuter et al., 2005, 2006], such as Mexico/1989 (GGII.3), Snow Mountain/1976 (GGII.2), Haway/1971 (GGII.1) and Lordsdale/1993 (GGII.4). Hence, analysis of the RdRp gene is a good target for epidemiological tracking and a good proxy to predict the NoV genotype, while exhaustive characterization may be achieved only by analysis of the capsid gene. By contrast, epidemiological studies relying solely on analysis of fragments of the capsid gene may fail to detect recombinant strains such as the emerging GGIIb/Hilversum NoVs. Analysis of the informative six-nucleotide-long motif identified in GGII.4 strains by Lopman et al. [2004], was also applied to the cognate region of GGIIb strains. A highly conserved motif, GATCTG, was found in all GGIIb strains irrespective of their geographic origin and year of detection. This may be suggestive of a recent introduction/emergence of the GGIIb/Hilversum strains in European countries.
Epidemiology of GGII Norovirus in Italy

In conclusion, the results of the present study confirm a significant prevalence of NoV infection in children hospitalized with gastroenteritis during the 2004 surveillance activity in Palermo, Italy. A major problem with molecular detection of NoVs is that no single assay is able to detect all the NoV variants [Vinje et al., 2003; Medici et al., 2005; Blanton et al., 2006]. The development of broadly reactive diagnostic methods to detect NoV infections is indispensable to investigate the exact role of NoVs in gastroenteritis [Vinje et al., 2003; Medici et al., 2005]. All the PCR primers used in this study were selected in the region coding for the RdRp gene, which is highly conserved among NoV strains of both GGs. Nested-PCR provides a 10-fold increase in sensitivity compared to RT-PCR when applied to GGIIb and GGII.7 genotypes, and a 100-fold increase when applied to strains of the GGII.4 genotype [Medici et al., 2005]. In our experience nested-PCR significantly increased the rate of detection of NoV infections compared to conventional one-step PCR. Gallimore et al. [2004a] showed that low-level excretion of GGII.4 NoVs, which can be detected by nested-PCR, may be observed in asymptomatic adults. Two predominant strains were detected in the 2004 surveillance period, GGII.4 Hunter and GGIIb Hilversum. GGII/b/Hilversum is suspected to be closely associated with infections in children and our findings add strength to this hypothesis [Lindell et al., 2005]. Interestingly, for the first time in continental Europe our study detected GGII.4 Hunter strains, which were first identified in Australia [Bull et al., 2006]. These findings underscore the constant threat posed by the first identified in Australia [Bull et al., 2006]. These findings underscore the constant threat posed by the spread of new norovirus variant. Lancet 363:882–888.


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