Emerging GII.4 Norovirus Variants Affect Children With Diarrhea in Palermo, Italy in 2006

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Although the genetic/antigenic heterogeneity of human noroviruses (NoVs) is impressive, a few genogroup II strains of genotype 4 (GII.4) are dominant worldwide. GII.4 NoVs evolve rapidly and in the last 15 years six epidemic variants have been identified. In 2005–2006, surveillance of sporadic viral gastroenteritis in children in Palermo, Italy, resulted in the detection of NoV strains in 20.9% of the patients admitted to hospital. By restriction fragment length polymorphism (RFLP) and sequence analysis of region A in the RNA-dependent RNA-polymerase (RdRp) gene, 59 NoV strains were successfully characterized. Eighty-one percent of the strains were characterized as GII.4, 14% as GIIb/Hilversum and 5% as GI.1. Phylogenetic analysis of region A and of the ORF1/ORF2 overlapping region of the GII.4 strains recovered in Palermo in the years 2002–2006 revealed the sequential emergence of four variants, GII.4 2002, 2004, 2006a, and 2006b. The variant GII.4 2006a was detected in June and July, 2006, while the variant 2006b first appeared in August, 2006, becoming predominant thereafter. Based on these findings, the dynamics of replacement and circulation of the GII.4 NoV variants in Italy in 2005–2006 appear to have matched the temporal pattern observed in Europe during the same period. J. Med. Virol. 81:139–145, 2009.

KEY WORDS: norovirus; GII.4 genotype; gastroenteritis; children; Italy

INTRODUCTION

Noroviruses (NoVs) belong to the genus Norovirus in the family Caliciviridae and are the causative agents of epidemic gastroenteritis in humans of all age groups and of acute sporadic gastroenteritis in infants and young children. The introduction of reliable and sensitive diagnostic molecular techniques has demonstrated the relevance of NoV infection, with prevalence rates ranging from 10% to 79.3% [Phan et al., 2006; Colomba et al., 2007].

Although the genetic/antigenic heterogeneity of human NoVs is extensive, a few NoV strains appear to be predominant worldwide in outbreaks and in sporadic gastroenteritis episodes [Gallimore et al., 2004; Kroneman et al., 2004; Lindell et al., 2005; Blanton et al., 2006; Ike et al., 2006]. Human NoVs have been classified into three separate genetic groups (GGI, GGII, and GGIV), and in at least 30 genotypes by analysis of the capsid gene (ORF2) [Green, 2007]. GII NoVs account for at least 90% of the NoV isolates, with GII.4 strains being responsible for the majority of episodes of diarrhea [Bull et al., 2006; Ramirez et al., 2006; Siebenga et al., 2007a]. The high incidence of NoV infections seems to be related to the sequential appearance and rapid spread of new variants and/or of recombinant NoV strains. Key mutations of the capsid protein VP1 are associated with GII.4 NoV variants emerging consecutively worldwide and named, based on the first year of detection, as: <1996, 1996, 2002, 2004, 2006a, and 2006b [Vinje and Koopmans, 1996; Vinje et al., 1997; Kroneman et al., 2004; Lopman et al., 2004; Gallimore et al., 2007; Siebenga et al., 2007b].

Along with the accumulation of point mutations, recombination is a powerful mechanism driving the evolution of NoVs [Green, 2007]. In recent years, recombinant NoV strains, such as GIIb/Hilversum, have been found to be common agents of gastroenteritis in Europe, mainly in children. GIIb/Hilversum NoV strains share a common polymerase gene and display a
variety of capsid genes [Ambert-Balay et al., 2005; Reuter et al., 2005]. Restriction fragment length polymorphism (RFLP) analysis applied to RT-PCR amplicons (PCR-RFLP) of the RdRp gene (region A) proved useful to recognize GII.4 and GI.1b/Hilversum NoVs as the main cause of sporadic cases of acute gastroenteritis in children admitted to hospital in Italy in 2004 [Colomba et al., 2007].

This study covers the years 2005–2006 of the epidemiological surveillance for sporadic NoV infection in Palermo, Italy, by using RFLP and sequence analysis, and describes the molecular evolution of GI.4 variants circulating in Palermo from 2002 to 2006 based on the sequence analysis of ORF1 and ORF2 genome fragments corresponding, respectively, to regions A and C.

MATERIALS AND METHODS

Samples

The stool specimens of 465 children <5 years age, admitted with acute gastroenteritis to the “G. Di Cristina” Children’s Hospital of Palermo from January 2005 to December 2006, were collected (one sample per patient). Two hundred eighty-five specimens were collected during the year 2005 and 180 during 2006. The stool specimens were collected from patients on the day of admission in order to exclude nosocomial infection. On the basis of the data obtained from parents, the cases appeared to be unrelated epidemiologically to each other. All the samples were aliquoted and stored at −20°C until analysis.

NoV Detection by RT-PCR

The presence of NoVs was detected using primers JV12/JV13, targeting the RdRp region A [Vinje and Koopmans, 1996] using Superscript III One step (Invitrogen, Paisley, UK). When the RT-PCR no specific PCR product (327 bp in length) was obtained, a semi-nested PCR with primer pair JV13/Ni was carried out, that amplifies a 110-bp fragment [Ramirez et al., 2006]. The PCR products were visualized by gel electrophoresis on ethidium bromide-stained 2% agarose.

Restriction Analysis

Fifty-nine amplicons were obtained in the first-step RT-PCR and were submitted to restriction analysis [Ramirez et al., 2008]. Briefly, 10 μl of each product was digested in a final volume of 20 μl with XmnI and BstXI (Promega Corporation, Madison, WI), AcuI and AhdI (New England Biolabs, Hitchin, UK) according to the manufacturer’s protocol. The digestion products were electrophoresed on 2% agarose gel stained with ethidium bromide. An additional 38 samples tested positive in the second-round PCR but they could not be used for RFLP analysis due to the small size of the amplicon.

Sequence Analysis

Sequence analysis was undertaken on 39 amplicons (327 bp in length), yielded with primer pair JV12/JV13 that targets the ORF1 RdRp region A. The analysis included all the 14 RFLP-untypeable strains and a selection of 25 RFLP-typed GII.4 strains (11 from 2005 to 2006 and 14 from 2002 to 2004). Sequence analysis was also performed on 23 amplicons (425 bp in length) obtained with primer pair GIISKR/GIIFBN1–2–3, that targets the 3’ end of ORF1 and 5’ end of ORF2 (ORF1/ ORF2 junction region) [Gallimore et al., 2007]. The analysis included the ORF1/ORF2 amplicons of 16 GII.4 strains from 2005 to 2006 (8 RFLP-untypeable and 8 RFLP-typeable) and 7 GII.4 strains from 2002 to 2004. The amplified products were purified using spin-columns (microcon50 Millipore, Bedford, MA) and sequenced directly using primers JV12 and GIISKR (MWG-Biotech, Ebersberg, Germany). Web-based analysis tools, BLAST (http://www.ncbi.nlm.nih.gov) and FASTA (http://www.ebi.ac.uk/fasta33) with default values, were used to find homologous hits in the sequence database. The closest matching sequences were aligned and analyzed using Clustal W [Thompson et al., 1994]. Phylogenetic analysis was carried out using the software MEGA version 4 [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.

Nucleotide Sequences

The nucleotide sequences determined in this study have been deposited in GenBank under the accession numbers EU872452 through EU 872486 (RdRp region), EU876860 through EU876881, and FJ025876 (ORF1-ORF2 overlap).

RESULTS

NoV Prevalence and Typing

NoVs were detected in 97 of 465 (20.9%) patients admitted to hospital during 2005–2006. The prevalence was 15.8% (45/285) during 2005 and 28.9% (52/180) in 2006. The majority (29/45) of the 2005 NoV-positive samples were detected in the second-round PCR, while 43/52 of the positive samples collected in 2006 were identified directly in the initial RT-PCR (Fig. 1). A total of 59 first-step amplicons were analyzed by RFLP analysis, and this allowed us to characterize 37 strains as GII.4 after the specific restriction patterns generated by digestion with XmnI and AcuI and 8 strains as GIIB after digestion with AhdI and BstXI. Fourteen amplicons, which could not be typed unequivocally by PCR-RFLP analysis, were sequenced. Three strains were characterized as GI.1 and 11 strains as GII.4. The GI.1 strains displayed >85% identity to the RdRp sequence of the GI.1 prototype strain Norwalk (M87661), while the GII.4 strains displayed 99.9%–99.0% nucleotide identity to the reference GII.4 strain Sydney532D/04 (DQ078801). Table I shows the combined results of RFLP digestion and sequence analysis of the 11 GII.4
strains that were not typed by RFLP, and shows the nucleotide changes in RdRp region A that affected correct recognition by the RFLP enzymes.

**Genetic Analysis of GII.4 NoVs**

To investigate the temporal evolution of the GII.4 NoVs detected in Palermo, 21 2005–2006 GII.4 RdRp sequences (11 obtained from RFLP-untypeable strains and 10 obtained from RFLP-typeable strains) were analyzed together with 14 GII.4 RdRp sequences of NoV strains identified in Palermo in the years 2002–2004 [Ramirez et al., 2006] and with GII.4 reference sequences retrieved from GenBank. As shown in Figure 2, the Italian GII.4 NoV strains grouped in at least three separate lineages. One lineage included three strains recovered in 2002 and 2004 belonging to the variant GII.4 2002. A second lineage included all but one of the GII.4 Italian RFLP-untypeable strains along with strains of variant GII.4 2006b circulating in Germany, Spain, Netherlands, and Japan in 2006–2007. The strains in this lineage exhibited >96.8% RdRp nucleotide identity and possessed a cytosine at position 4537 and an additional 2 substitutions (G → A and T → A/G) at positions 4531 and 4558. Three Italian 2006b strains isolated in November and December 2006 showed a mutation in their region A at position 4543 (Lordsdale numbering, X86557). All the 2005–2006 RFLP-typeable strains clustered in a third lineage, encompassing variant GII.4 2004 strains. This lineage also included strain PA71/06 (RFLP pattern X-) and the majority of the GII.4 strains circulating in Palermo in 2004. The strains within this genetic lineage shared 95–100% nucleotide identity. In this lineage, two well-defined sublineages (bootstrap values >87%) could be distinguished. One sublineage included three Italian strains from 2006 and reference strains of the variant GII.4 2006a, while the other lineage included Italian strains from 2005 and 2006. The three Italian strains (PA133/06, PA118/06, and PA121/06) characterized as 2006a variant displayed two peculiar substitutions, T → C and C → T, at positions 4449 and 4562, respectively, when compared to strain Sydney532D/04 Hunter, representative of the variant GII.4 2004.

In order to characterize in greater detail the Italian GII.4 strains, amplicons of the ORF1/ORF2 overlapping region were produced from 16 2005–2006 NoV strains, that were representative of different RdRp lineages and sublineages, including RFLP-untypeable and -typeable GII.4 strains. The sequences obtained were analyzed in comparison with cognate sequences from GII.4 strains identified in Palermo in 2002–2004 and with ORF1/
Fig. 2. Phylogenetic analysis of partial sequences (327-bp) of the polymerase gene of GII.4 NoV strains. Kimura 2-parameter model was used as a method of substitution and the neighbor joining method allowed us to reconstruct the phylogenetic tree. Bootstrap values above 50%, estimated with 1,000 pseudoreplicate data sets, are indicated at each node. The accession number was reported for the sequences retrieved from GenBank. The month of isolation is indicated for the Italian GII.4 strains detected during 2005 and 2006.
ORF2 sequences representative of the six GII.4 variants recovered in Europe in the last 15 years. The characteristic motifs reported by Gallimore et al. [2007], based on the amino acids at position 6, 9, and 15, were taken into consideration for determination of the variant. This allowed the identification of four NoV variants, 2002, 2004, 2006a, and 2006b, in agreement with the results obtained by RdRp sequence analysis (Fig. 3). The GII.4 2002 variant was present in the years 2002 and 2004. In 2004, the variant GII.4 2004 appeared, persisting throughout the 2006 surveillance period. The variant GII.4 2006a differed from the variant GII.4 2004 in two amino acids, serine at position 6 and asparagine at position 9.

**DISCUSSION**

During 2004 NoVs were the most common viral agents detected in stool specimens of children admitted to hospital with gastroenteritis at the “G. Di Cristina Children Hospital” of Palermo [Colomba et al., 2007]. Indeed, NoV was recognized as the causative agent of disease in 39.2% of the children with gastroenteritis, while rotaviruses were detected in 33.8% of the patients. In the years 2005 and 2006, the prevalence rates of NoV infection were 15.8% and 28.9%, respectively, revealing yearly changes in the prevalence rates in a settled population, as shown elsewhere [Phan et al., 2006]. On the whole, the findings of the present research were consistent with previous studies on NoV epidemiology [Green, 2007], confirming that NoV is a major enteropathogen in infants and children in Italy.

Genotyping of the Italian NoV strains isolated during 2005–2006 revealed the circulation of GII.4 and GIIb/Hilversum strains and sporadic infections by GI.1 strains. GIIb strains accounted for 94.9% of the circulating NoV strains, with GII.4 being predominant (81.4%), and GIIb/Hilversum being detected less frequently (13.6%). The PCR-RFLP assay was able to characterize as GII.4 or GIIb 93.8% of the NoV strains detected in 2005 but only 69.8% of the NoV strains detected in 2006. The majority of the RFLP-untypeable strains detected in 2006 were characterized by sequence analysis as GII.4 genotype. Failure to recognize such GII.4 strains by the RFLP assay was due to a single point mutation in the RdRp region A, disrupting an enzyme recognition site. This finding stresses the need for continual update of diagnostic systems.

As soon as surveillance and molecular epidemiological studies for NoV have been implemented, several GII.4 genetic variants have been identified [Fankhauser et al., 1998; Noel et al., 1999; Dingle, 2004; Lopman et al., 2004; Widdowson et al., 2004; Siebenga et al., 2007b]. Such variants are probably the result of a selection, driven by mechanisms of antigenic escape, from a pool of co-circulating strains [Green, 2007]. The consecutive spread of new variants may also be accounted for by increased viral fitness due to increased capsid stability and/or increased affinity for the cellular receptors. In Europe, during 2006–2007, the GII.4 2006a and 2006b variants accounted for the majority of outbreaks of viral gastroenteritis [Siebenga et al., 2008]. Different genome fragments, such as region A (part of the RdRp gene), region C (part of the capsid gene), or the region spanning the ORF1 and ORF2, have been used to demonstrate the evolution of GII.4 variants [Gallimore et al., 2007; Siebenga et al., 2007b; Buesa et al., 2008]. In this study, sequence analysis of the region A allowed us to demonstrate the evolution of GII.4 variants [Gallimore et al., 2007; Siebenga et al., 2007b]. Strains belonging to variant GII.4 2006a clustered along with strains of variant GII.4 2004 in the same lineage in the

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phylogenetic tree confirming their close evolutionary relationships [Siebenga et al., 2007b; Buesa et al., 2008]. However, two peculiar substitutions, T → C and C → T, at positions 4436 and 4562, respectively, allowed us to differentiate the variant 2004 from the variant 2006a. All but one of the RFLP-untypable GI.4 strains belonged to the 2006b variant. This variant emerged in 2006 almost contemporaneously in Europe, USA, Japan, and Australia (variant v6, Minerva or Kobe) [Siebenga et al., 2008], and became dominant over the GI.4 2006a variant during the summer and autumn 2007. In Palermo, the GI.4 2006a variant was detected in June and July 2006, while the GI.4 2006b variant started circulating after August 2006. The Italian GI.4 2006b strains isolated in November and December 2006 showed a mutation in their region A (position 4543) that was also described in GI.4 2006b strains detected in outbreaks at the end of the 2006–2007 season [Siebenga et al., 2008].

Sequence analysis of the ORF1/ORF2 region confirmed the characterization based on the RdRp region of the GI.4 NoV strains detected in Italian children, revealing the circulation of distinct GI.4 variants. From 2002 to 2006 in Palermo, several GI.4 NoV variants were responsible for sporadic gastroenteritis in children. The variant GI.4 2002 was detected in the years 2002 and 2004, the variant GI.4 2004 was identified in the years 2004–2006, while the variants GI.4 2006a and 2006b appeared in 2006. Accordingly, the temporal distribution of the NoV variants in Palermo, matched the temporal patterns observed in Europe [Gallimore et al., 2007; Buesa et al., 2008; Reuter et al., 2008; Siebenga et al., 2008].

The PCR-RFLP technique developed for the analysis of the ORF1 RdRp region was able to characterize correctly the GI.4 strains belonging to the variants 2002, 2004, and 2006a and the GIb/Hilversum strains. Sequence analysis was necessary to recognize the GI.4 2006b variant and the rare GI.1 NoV strains. Failure of the PCR-RFLP assay to characterize the 2006b strains was due to a unique mutation, T → C at position 4537, disrupting the restriction site for XmnI. The NoV variant GI.4 2006b was circulating in 2006–2007 in Germany, Spain, Netherland, and Japan [Siebenga et al., 2007b]. In addition to the nucleotide mutation affecting the XmnI site, in the RdRp region of such strains there were two peculiar nucleotide changes and such nucleotide polymorphisms could be used as RdRp markers of the variant 2006b.

In conclusion, the high detection rate of GI.4 NoVs in Italian children with gastroenteritis confirms their prominent role as human enteropathogens. GIb/Hilversum strains were detected at high frequencies in this study, while GI.1 strains were detected sporadically both in 2005 and in 2006. Similar epidemiological data have been reported in other European countries [Buesa et al., 2008]. At least four distinct GI.4 NoV variants appeared in Palermo in the last 5 years, and this pattern mirrored the epidemiological changes observed in other European and non-European countries. Continual surveillance for NoVs is required in order to develop strategies suitable for the control and prevention of NoV infection.

REFERENCES


