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Short communication

Recombinant norovirus GII.g/GII.12 gastroenteritis in children

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ABSTRACT

Recombinant GII.g/GII.12 norovirus (NoV) strains emerged in 2008 in Australia and subsequently have been associated with gastroenteritis outbreaks worldwide. In the winter season 2009–2010 GII.12 strains caused 16% of the NoV outbreaks in the United States. During 2009–2010 we also identified GII.g/GII.12 strains during surveillance of sporadic cases of gastroenteritis in Italian children. Severity scores were calculated for the GII.g/GII.12 NoV infections using the Vesikari scale and in two out of three paediatric cases they exceeded the median value calculated for concomitant GII.4 infections. Upon sequence analysis, the Italian strains were found to be recombinant viruses and displayed different patterns of nucleotide polymorphisms. Phylodynamic analysis with other GII.g/GII.12 recombinants showed a high rate of evolution, comparable to the rates observed for GII.4 viruses. The mechanisms leading to worldwide emergence of GII.12 NoV strains in 2008–2010 are not clear. Monitoring of GII.12 NoV circulation is necessary to understand these mechanisms of evolution.

1. Introduction

Noroviruses (NoVs) belong to the *Caliciviridae* family and are considered as the major cause of acute gastroenteritis in both children and adults. NoVs are responsible for sporadic cases and outbreaks of gastroenteritis in various epidemiological settings, including restaurants, schools, day-care centers, hospitals, nursing homes, and cruise ships. NoV genome includes three open-reading frames (ORFs). ORF1 encodes non-structural proteins including the RNA-dependent RNA polymerase (RdRp), while ORF2 and ORF3 encode the major capsid protein VP1 and minor structural protein VP2, respectively. NoVs are classified into five distinct genogroups (GI through GV) on the basis of the full-length VP1 sequence. GI NoVs infect humans, GII NoVs have been detected in humans and pigs, GIII NoVs circulate in bovines, GIV NoVs infect humans and carnivores, while GV NoVs infect only mice (Green, 2007). Some human and canine NoV strains are not classifiable into any NoV genogroups and could represent a potential novel genogroup (Martella et al., 2009). GI and GII NoV strains have been further classified into at least 8 and 19 genotypes, respectively, but a single genotype, GII.4, has been associated with the vast majority of global outbreaks since the mid-1990s (Bok et al., 2009; Zheng et al., 2006). In the winter season 2009–2010 a novel GII.12 norovirus strain emerged and caused 16% of the norovirus outbreaks in the United States (Vega and Vinje, 2011). Marked increase in the number of non-GII.4 outbreaks was confirmed by the CaliciNet surveillance system, with a GII.12 strain being reported in 14% of the outbreaks reported in 12 states (Vega et al., 2011). Sequence analysis of the US epidemic strain demonstrated its recombinant nature (Vega and Vinje, 2011).

Recombination may create novel chimeric strains bearing ORF1 and ORF2 of different parental origin, thus requiring multiple target (ORF1- and ORF2-based) analysis in order to characterize properly the NoV strains (Ambert-Balay et al., 2005). Epidemiological studies have also revealed heterogeneity in the ORF1 of NoV strains, suggesting that capsid-based classification of NoVs should be implemented with a ORF1-based classification system. Attempts to classify the ORF1 of GII NoVs have been made, in order to designate peculiar NoV strains of epidemiological relevance, although this classification/nomenclature has not been based on precise distance criteria applicable to all NoV strains (Bull et al., 2007; NoroNet, 2011). For consistency with the proposed and widely accepted designation of some recombinant GII NoVs, novel GII ORF1 types have been indicated with letters (a–d) (Bull et al., 2007). The same nomenclature has also been adopted and implemented by the European NoV database (NoroNet, 2011).

In this article, we describe the detection in sporadic cases of diarrhea in Italian children of strains showing the same ORF1/ORF2 combination as the novel recombinant GII.12 strain described in the United States. Upon multi-target sequence

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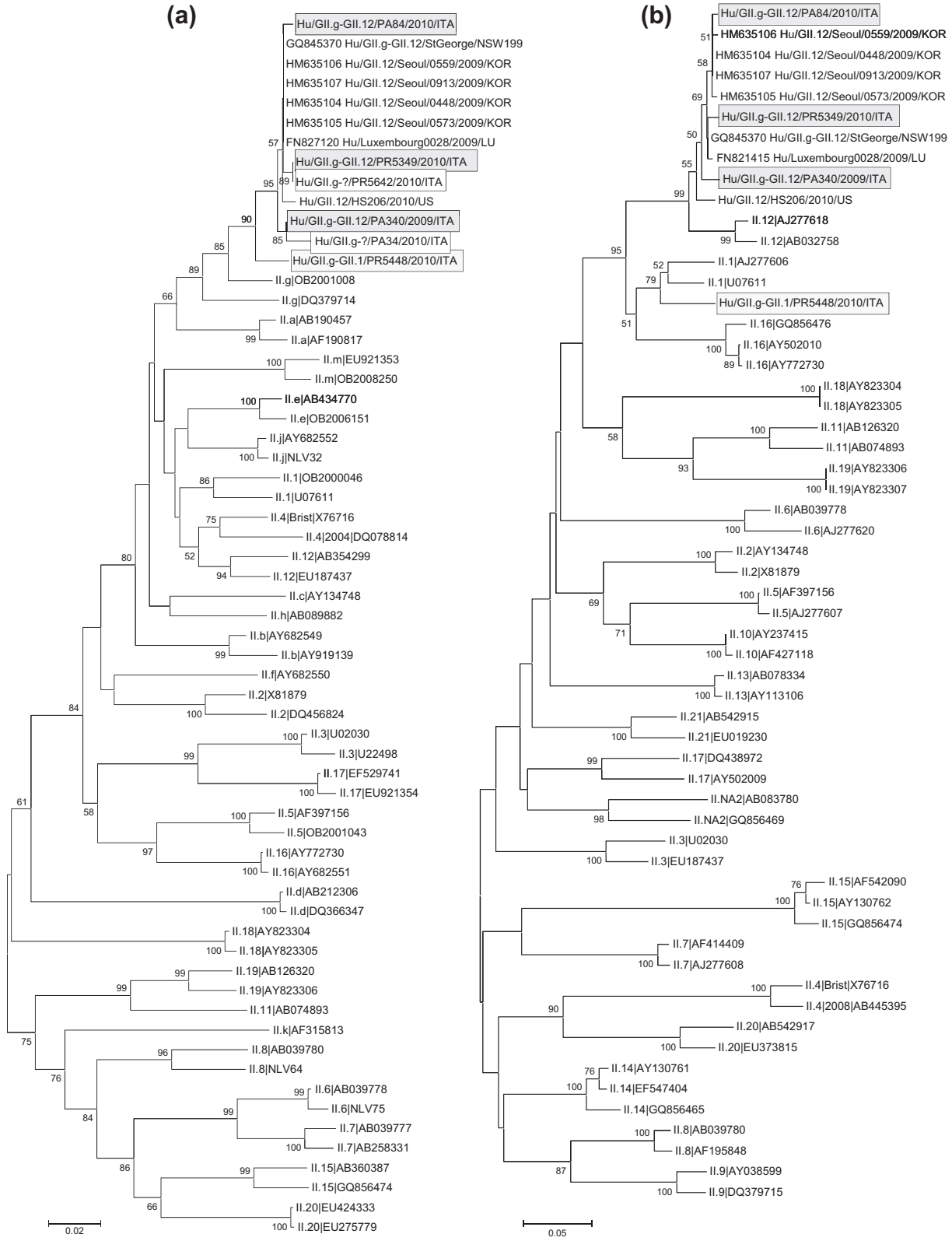

Fig. 1. Phylogenetic analysis based on the ORF1 (a) and ORF2 (b) of recombinant GII.g/GII.12 NoV strains detected in Italy. Partial nucleotide sequences of the ORF1 (263 nt of region A) and ORF2 (244 nt of region C) were used for the analysis. The reference sequences were retrieved from the NoroNet database. The Italian strains are highlighted in boxes: GII.g/GII.12 strains are in gray boxes; GII.g/GII.1 strain is in light gray box; GII.g strains whose capsid could not be typed are in white boxes. The trees were generated using the neighbor-joining method with ClustalW program. Bootstrap values above 50%, estimated with 1000 pseudoreplicate data sets, are indicated at each node.

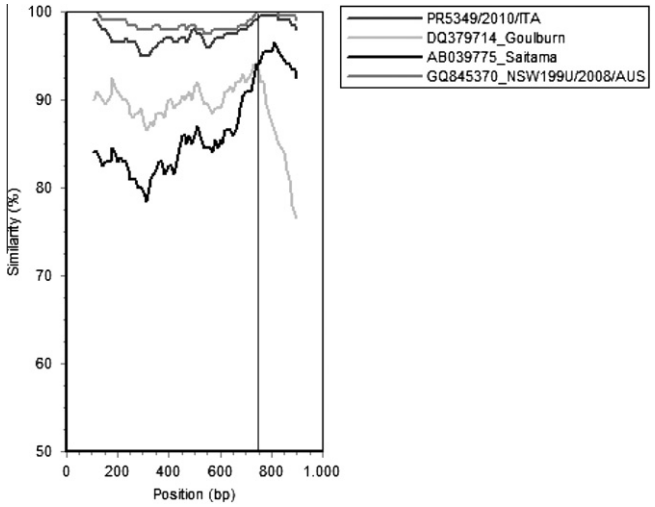

Fig. 2. SimPlot analysis of partial RdRp and capsid gene sequences of GII.g/GII.12 recombinant strains. Window size, 200 bp; step, 10 bp. The vertical axis indicates the nucleotide identities between the query strain, Hu/GIIg-GII12/PA84/2010/ITA, and the reference strains, expressed as percentages. The horizontal axis indicates the nucleotide positions (in base pairs; corresponding to base pairs 4335–5334 of the Hawaii strain with GenBank accession number U07611). Vertical line indicates the beginning of ORF2 (base pair 5085). Dark gray line, Hu/GII.g-GII.12/PR5349/2010/ITA detected in Parma, Italy, in this study; Gray line, Hu/GII.g-GII.12/StGeorge/NSW199U/2008/AU, ancestor of GII.g/GII.12 recombinants (GQ845370); light gray line, GII.g reference strain Hu/GII/GoulburnValleyG5175B/1983/AUS (DQ379714); black line, GII.12 reference strain SaitamaU1 (AB039775).

analysis, the Italian strains were found to have a recombinant nature and to be related to other GII.g/GII.12 NoVs.

2. Materials and methods

2.1. Samples

A total of 1374 faecal specimens were collected from hospitalized children from January 2009 to March 2010. Of these specimens, 615 were collected in Palermo, South of Italy, from children less than 5 years old and 759 in Parma, North of Italy, from children less than 14 years old. Faecal samples were stored at −80 °C upon collection and until use.

2.2. Real-time PCR detection

Viral RNA was extracted from 140 μl 10% faecal suspensions using the QIAamp Viral RNA kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). NoV-positive samples were detected by a real-time reverse transcription (RT)-PCR assay that allows to differentiate between GI and GII NoVs (Kageyama et al., 2003).

2.3. RT-PCR and sequence analysis

Amplification of a RdRp fragment was accomplished by RT-PCR with specific primer pair JV12a and JV13b (Vinje and Koopmans, 1996). A 5′ region of ORF2 of NoV GII was also amplified to characterize firmly the NoV capsid genotype and to identify recombinant strains using primers G2FB–G2SKR (Kageyama et al., 2003; Kojima et al., 2002). Products were sequenced directly and sequence analysis was performed using CLUSTALW and MEGA 5.0 (Tamura et al., 2011).

2.4. Recombination breakpoint analysis

In order to assess recombination and to map the breakpoint recombination site, GII.g/GII.12 amplicons encompassing ORF1–ORF2 junction region were obtained by the primer pair 1421f–G2SKR (Nakamura et al., 2009). The cross-over sites of recombinant strains were determined using the program SimPlot provided by SCRoftware website (Lole et al., 1999).

2.5. Nucleotide sequence accession numbers

The GenBank accession numbers for the ORF1 and ORF2 nucleotide sequences of the Italian GII.g/GII.12 strains are JN206641–JN206644.

3. Results and discussion

Out of 615 faecal specimens tested in Palermo, 69 (11.2%) NoV-positive samples were identified by the real time PCR. Of these 43 (62.3%) could be amplified by the JV primers for sequence analysis and characterization of the RdRp region. In Parma, 147 of 759 specimens (19.4%) were positive for NoV by real time PCR and 66 (44.9%) were characterized by RdRp sequence analysis. Upon analysis of the RdRp sequences with the automated NoV genotype prediction server Norovirus Genotyping Tool Version 1.0, National Institute of Public health and the Environment, The Netherlands (http://www.rivm.nl/mpf/norovirus/typingtool), most strains were characterized as GII.4 (89/109; 81.7%), followed by GII.b (8/109; 7.3%), and GII.g (6/109; 5.5%). Other genotypes (GII.1, GII.2, GII.6 and GII.e) were also detected but sporadically. Only two GI strains were detected in Palermo in 2009 but they were untypeable. GII.g strains had never been detected before 2009 in NoV surveillance studies in Italy (Ramirez et al., 2006; Ramirez et al., 2008). GII.g polymerase was originally associated to GII.13 capsid type in the recombinant strain Goulburn Valley G5175 B/1983/AUS (DQ379714) (Symes et al., 2007). ORF1-based characterization through sequence analysis of RdRp fragments may predict the capsid (ORF2) genotype. However, as recombination may create novel chimeric strains bearing ORF1 and ORF2 of different parental origin, multiple target (ORF1- and ORF2-based) analysis is required in order to characterize NoV strains properly (Ambert-Balay et al., 2005). Therefore, we further characterized the GII.g strains by sequencing the 5' region of ORF2 with primers G2FB–G2SKR (Kageyama et al., 2003). The ORF2 of four such NoV strains could be amplified and sequenced, allowing us to classify three of these viruses as GII.12 and one as GII.1. It was not possible to generate an ORF2 sequence for two GII.g strains, which remained untyped. The ORF1 and ORF2 characterization of the GII.g and GII.12 viruses is shown in Fig. 1. In order to rule out possible mixed NoV infections and to map the recombination site, amplicons encompassing the highly recombination-prone ORF1–ORF2 junction region (Bull et al., 2005) were generated with the primer pair 1421f–G2SKR. As, expected, by comparing the GII.g/GII.12 Italian strains with parental NoV strains (SaitamaU1/1997/JP, GII.4/GII.12; StGeorge/NWS199U/2008/AUS, GII.g/GII.12; Goulburn ValleyG5175B/1983/AUS, GII.g/GII.13), using the program SimPlot [26], the cross-over site was mapped to the ORF1–ORF2 junction region (Fig. 2).

GII.g/GII.12 is a novel GII recombinant strain, first detected in Australia in 2008 (strain NSW199U/2008) from a sporadic case of NoV gastroenteritis. This recombinant virus, had also been isolated in oyster-associated NoV outbreaks in New Zealand during 2008, although it was designated by Eden et al. as GII.e/GII.12 (Eden et al., 2010).

Between 11 and 30 July 2008, in the Auckland Region 30 separate foodborne illness incidents were notified, affecting 121 people

Table 1
ORF1 and ORF2 nucleotide sequence comparison of the GII.g/GII.12 recombinant strains circulating worldwide. The Italian NoV strains of this study are highlighted in bold.

Strain	Nt position[a]																
	ORF1										ORF2						
	4328	4360	4414	4441	4462	4486	4492	4501	4505	4563	5117	5168	5258	5267	5291	5309	5312
GQ845370 Hu/GII.g-GII.12/StGeorge/NSW199U/2008/AUS	G	A	T	C	A	C	A	T	G	T	T	A	G	T	C	C	A
HM635104 Hu/GII.12/Seoul/0448/2009/KOR	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
HM635105 Hu/GII.12/Seoul/0573/2009/KOR	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
HM635106 Hu/GII.12/Seoul/0559/2009/KOR	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
HM635107 Hu/GII.12/Seoul/0913/2009/KOR	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
Hu/GII.c-GII.12/Luxembourg0028/2009/LU	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hu/GII.g-GII.12/PA340/2009/ITA 29-07-2009	–	**G**	.	.	.	**T**	.	**C**	.	.	.	.	.	.	.	**T**	.
NoNet 93SE1209 32068-1 FRA 24-12-2009	.	.	C	.	G	.	.	.	A	.	.	C	.	.	.		G
NoNet 67SC0110/2 32115-1 FRA XX-01-2010	.	.	C	.	G	.	.	.	.	.	.	T	.	C	.	.	.
NoNet 71RE0110 32116-1 FRA 07-01-2010	.	.	C	.	G	.	.	.	.	.	.	C	.	.	.	.	G
NoNet 67SC0110 32106-1 FRA 11-01-2010	.	.	C	.	G	.	.	.	.	.	.	T	.	C	.	.	.
NoNet 58IM0110 32122-1 FRA 13-01-2010	.	.	.	T	.	.	G	.	.	.	C	.	A	.	.	.	.
NoNet SE2010 1062 32781-1 SWE 13-01-2010	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.	.
NoNet 84VA0110 32344-1 FRA 18-01-2010	–	.	C	.	G	.	.	.	.	.	.	C	.	.	.	.	G
NoNet 66CO0110 32373-1 FRA 21-01-2010	.	.	C	.	G	.	.	.	.	.	.	C	.	G	.	.	G
Hu/GII.g-GII.12/PR5349/2010/ITA 01-02-2010	–	.	**C**	.	**G**	.	.	.	.	.	.	**C**	.	.	.	.	**G**
NoNet 44CR0110 32380-3 FRA 06-02-2010	.	.	C	.	G	.	.	.	A	.	.	C	.	.	.	.	G
NoNet 68MU0210 32393-1 FRA 11-02-2010	.	.	C	.	G	.	.	.	.	C	.	C	.	.	.	.	G
Hu/GII.g-GII.12/PA84/2010/ITA 05-03-2010	–	.	.	.	.	.	.	.	.	**C**	.	.	**A**	.	.	.	.
NoNet 56RI0310 32432-1 FRA 10-03-2010	.	.	.	T	.	.	G	.	.	.	C	.	A	.	.	.	.
NoNet o42/2010 32362-1 HUN 18-03-2010	–	.	C	.	G	.	.	.	.	C	.	C	.	.	.	.	G
NoNet 53LA0310 32445 FRA 19-03-2010	.	.	.	T	.	.	G	.	.	.	C	.	A	.	.	.	.
NoNet 59WA0310 32448 FRA 22-03-2010	A	.	C	.	G	.	.	.	A	.	.	C	.	.	.	.	G
NoNet 35AU0310 32449 FRA 23-03-2010	A	.	C	.	G	.	.	.	A	.	.	C	.	.	.	.	–
NoNet 27CY0310 32452 FRA 26-03-2010	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
NoNet SE2010 1064 32780 SWE 26-03-2010	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
NoNet 38VO0310 32460 FRA XX-04-2010	.	.	C	.	G	.	.	.	.	C	.	C	.	.	.	.	G
NoNet 35CH0610 32597 FRA 14-06-2010	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.	.
NoNet 02VO0810 32784 FRA 01-09-2010	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
Hu/GII.12/HS206/2010/Ohio-US 2010	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	

[a] The nucleotide positions correspond to those of the Hawaii strain with GenBank accession number U07611.

Table 2
Severity evaluation of gastroenteritis in the three Italian children with GII.g/GII.12 infection compared to GII.4 infections detected in the same time period.

Isolate	Age of patient (months)	Duration of diarrhea (days)	Maximum number of diarrhea stools/ 24 h	Duration of vomiting (days)	Maximum number of vomiting episodes/24 h	Temperature (°C)	Dehydration	Treatment	Vesikari severity score (Ruuska and Vesikari (1990))
Hu/GIIg-GII12/ PA340-09/ 2009/ITA	80	6	6	0	0	38.6	Mild	Hospitalization	12 (severe)
Hu/GIIg-GII12/ PA84-10/ 2010/ITA	39	6	7	3	10	37.4	Moderate	Hospitalization	18 (severe)
Hu/GIIg-GII12/ PR5642/ 2010/ITA	18	3	6	0	0	38.5	No	Hospitalization	8 (non-severe)
GII.4 (13 patients)	17[a]	3[a]	4[a]	2[b]	3[b]	38.1[c]	Mild = 3 Moderate = 4 Severe = 1	Hospitalization	10[a] (non-severe)

[a] Median value for 13 patients.
[b] Median for 9 patients with vomiting.
[c] Mean for 3 patients with temperature >37 °C.

who had consumed raw Pacific oysters (*Crassostrea gigas*), grown and marketed locally (Grey et al., 2009). Both faecal and oyster samples were found positive for a recombinant GII NoV strain, never reported before, and tentatively designated as GII.c/GII.12. These GII.c/II.12 strains were also associated with sporadic cases of gastroenteritis and an outbreak in an infant daycare center in Luxembourg in 2009 (Kremer et al., 2010). By BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST), NoV strains from Korea with 98% nt identity to the Italian GII.g/GII.12 NoVs were also found (Han et al., 2011). The Korean viruses were identified from outbreaks occurred in Seoul from January to December 2009. Increased circulation of GII.12 viruses closely related to the Australian virus strain NSW199U/2008 was also reported in USA in 2010 (Lembke and Cartwright, 2010). Detailed analysis of viruses from an outbreak in Ohio revealed that the GII.12 viruses were recombinant strains with a GII.g ORF1 type (Takanashi et al., 2011).

Search of the NoroNet norovirus molecular epidemiology database (http://www.rivm.nl/pubmpf/norovirus/database#/) revealed a total of twenty NoV GII.g/GII.12 outbreaks that have occurred in France (17 outbreaks), Hungary (1 outbreak) and Sweden (2 outbreaks) between December 2009 and September 2010 and that affected as many as 414 people (NoroNet, 2011). Most of the outbreaks were related to consumption of contaminated food in schools, hospitals, geriatric residential institutions, restaurants, hotels, catering and private houses.

Recent GII.g/GII.12 recombinants display <90% nucleotide (nt) identity to the ancestor ORF1 sequence Goulburn ValleyG5175B/ 1983/AUS (Fig. 2). Sequence alignment allowed comparing the ORF1 and ORF2 fragments of the recently circulating GII.g/GII.12. Ten and seven nt positions could be considered as "hot spots" in ORF1 and ORF2, respectively, since these residues varied in at least two of the sequences studied with respect to the ancestor Hu/GII.g-GII.12/StGeorge/NSW199U/2008/AUS strain (Table 1). Hot spot nts allowed distinguishing eight and nine different patterns in ORF1 and ORF2, respectively, whose combination produced a total of twelve ORF1/ORF2 nt sequence variants from 2009 to September 2010. The majority of these nt substitutions are silent, namely those in the capsid region, and seem to have accumulated in a limited time span. Strain PA340/2009 shared two hot spots with strain HS206/2010 from the United States, while strain PR5349/2010 was quite similar to strains causing outbreaks in France and Hungary in 2010. Strain PA84/2010 showed a peculiar pattern. In order to estimate the evolution rates of these emerging NoVs, bayesian phylogenetic reconstructions were performed on partial ORF2 sequences (region C) of a selection of GII.g/GII.12 (listed in Table 1). The analysis was performed using Markov chain Monte Carlo (MCMC) analysis implemented in BEAST (Drummond and Rambaut, 2007) and an HKY nt substitution model with gamma-distributed rate variation, a lognormal relaxed clock model (Drummond et al., 2006) and a coalescent constant size tree prior. The MCMC analysis was run for 10 million generations and diagnosed using Tracer (http://tree.bio.ed.ac.uk/software/tracer). ORF2 analysis showed an evolutionary rate of 3.7×10^{-3} (SD 7.2×10^{-5}) nt substitutions/site/year.

GII.g/GII.12 infections were detected in children of 80, 39 and 18 months of age (Table 2), and occurred in July 2009 and March 2010 in Palermo and in February 2010 in Parma, respectively. An additional two sporadic cases by GII.g NoVs, untypeable in the ORF2, were detected in Palermo in January 2010 from a 5-month old child and in Parma in March 2010 from a 1-month old child. Severity scores were calculated for the GII.g/GII.12 NoV infections using the Vesikari scale (Ruuska and Vesikari, 1990). For two out of three GII.g/GII.12 infections the scores exceeded the median value (considered as a baseline) calculated using concomitant GII.4 infections (Table 2). Nosocomial transmission could be ruled out since stool samples were collected on the day of admission. Gastro-enteric signs in children's relatives were not reported on the anamnestic data. There was no apparent epidemiological link among the cases, as neither spatial nor temporal relations could be identified and a possible exposure to a common virus source (food/water) was also ruled out.

4. Conclusions

GII.g/GII.12 recombinants were first described in 2008 but they now appear to have reached a worldwide distribution. Assuming that a common ancestor generated all the recently circulating GII.g/GII.12 viruses, in our analysis a high polymorphism (defined as hot-spot patterns) and a high rate of evolution was evidenced in partial ORF2 sequences. A similar evolution rate was obtained for GII.4 NoVs using the full-length ORF2 (Bull et al., 2010). Like influenza virus, GII.4 NoVs are able to generate several variants over a limited time period (Bull et al., 2010) likely under the pressure generated by herd immunity. Significant antigenic differences among GII.4 variants have been demonstrated in binding assays (Lindesmith et al., 2011). Also the emergence of GII.12 NoV strains worldwide in 2008–2010 may be related to either mechanisms of antigenic escape from existing herd immunity against predominant genotypes (i.e. GII.4) or to improved adaptation to host receptors (Glass et al., 2009). Similarities between GII.4 and GII.12 NoVs have been suggested by the comparison of the crystal structures of

their capsid domains which showed virtual identical means of HBGA recognition (Hansman et al., 2011). Emerging GII.12 NoV seems to infect humans regardless of A/B/O blood type (Takanashi et al., 2011). The GII.12 strain HS206 was able to infect pigs suggesting the possibility of interspecies transmission of these strains (Takanashi et al., 2011).

Interestingly, although statistically not significant, in our limited investigation in hospitalized children the clinical scores were apparently severer for GII.g/GII.12 than for GII.4 NoV infections.

Circulation of the GII.g/GII.12 NoV in the infantile population, even at low rates, may provide a good opportunity to recombine with other co-infecting NoVs, thus generating novel strains. Due to their global spread, understanding the mechanisms of evolution of GII.12 NoVs will surely provide valuable information on the evolution pathways of NoVs. Further monitoring of GII.12 NoV circulation is warranted.

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