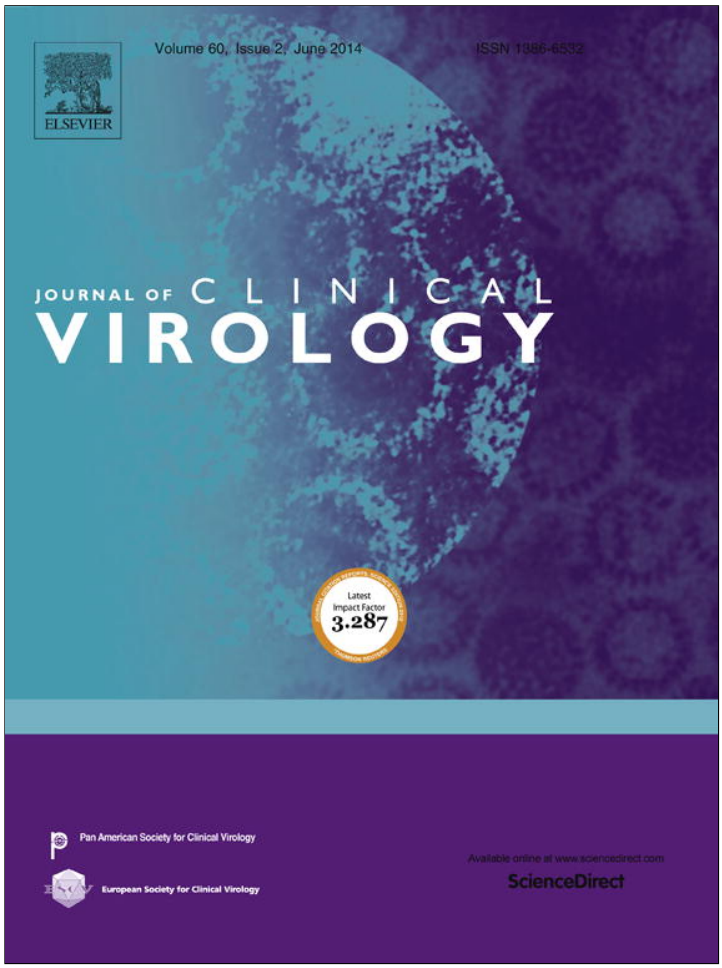

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Molecular and clinical epidemiology of norovirus outbreaks in Spain during the emergence of GII.4 2012 variant

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ABSTRACT

Background: Norovirus (NoV) is the most common cause of acute nonbacterial gastroenteritis outbreaks worldwide, but the impact of NoV infections in Spain remains underestimated.
Objectives: This study aimed to determine the prevalence and genetic diversity of NoVs causing outbreaks of acute gastroenteritis in Northeastern Spain (Catalonia) during 2010–2012, and to compare clinical features and levels of viral shedding of the most prevalent GII.4 2012 variant with its predecessor.
Study design: NoVs were screened and genotyped in stools from gastroenteritis outbreaks. Genetic diversity over a region covering 50% of VP1, and viral loads were analyzed in stools belonging to GII.4 2009 and 2012 variants.
Results: More than 50% of outbreaks were caused by genotype GII.4, although outbreaks caused by multiple strains, GII.6 and GII.1 were also prevalent. During 2012, GII.4 2012 strains clearly replaced GII.4 2009 strains. The first 2012 strain was detected in February 2011, representing the earliest isolate reported worldwide. Epidemiological features of GII.4 2012 and GII.4 2009 outbreaks were comparable, as well as levels of viral shedding in stools. Finally, analysis of the capsid gene showed a higher amino acid variability and diversification in GII.4 2012, affecting sites located at the P2 domain, but also in the shell domain.
Conclusions: Clinical features of outbreaks caused by different genotypes circulating in Spain, including outbreaks caused by GII.4 2012 and GII.4 2009 strains, were comparable. Although shed at similar levels than GII.4 2009 strains, GII.4 2012 strains have clearly replaced the previous predominant strain.

1. Background

Noroviruses (NoVs) are the leading cause of acute non-bacterial gastroenteritis as well as the principal cause of foodborne disease worldwide, infecting all age groups [1,2]. Although NoVs can cause sporadic cases of viral gastroenteritis, they are highly infectious and cause large gastroenteritis outbreaks [3]. NoVs are a genetically and antigenically diverse genus in the *Caliciviridae* family. They are non-enveloped icosahedral small viruses with a 7.5–7.7 kb positive-sense single-stranded RNA genome. NoVs are divided into five genogroups and further classified into a total of at least 30 genotypes [4,5]. During the last decade strains belonging to genogroup II, genotype 4 (GII.4) have been primarily responsible for the majority of the cases and outbreaks throughout the world. Although the reason(s) that would explain why GII.4 strains evolve faster and spread more rapidly throughout the globe have not been fully elucidated [6], it is clear that pandemic GII.4 variants have successively

Abbreviations: NoV, norovirus; GI, genogroup I; GII, genogroup II; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ORF, open reading frame.

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emerged and circulated across all continents [7–9]. Since late 2012, the GII.4 variant named Sydney 2012 has progressively replaced the predecessor GII.4 New Orleans 2009 variant globally [10,13].

Studies on the role of NoV in non-bacterial gastroenteritis outbreaks in Spain indicate increasing prevalences between 2000 and 2006 [14–17]. However, diagnostics of NoV infections is still not performed systematically, and NoV clinical importance in the country remains underestimated. Although strain diversity in the country has been analyzed recently at the environmental level [18], and in mussel samples [19], information on the strains responsible for clinical disease in the population has not been updated since 2006 [15].

2. Objectives

The aim of this study was to determine the prevalence and genetic diversity of NoVs causing outbreaks of acute gastroenteritis in Catalonia, Spain, during 2010–2012, and the especial influence of the recent GII.4 2012 variant and other prevalent genotypes on outbreak dynamics in this territory, which is located in northeastern Spain and has 7.5 million inhabitants. In addition, viral loads in feces from different GII.4 variants were compared to assess whether differences in excretion levels could contribute to explain the higher prevalence of selected new strains over the older ones.

3. Study design

3.1. Epidemiological data, outbreaks, and specimen collection

Outbreaks of acute gastroenteritis were reported to the Public Health Agency of Catalonia. Epidemiological data and 815 stool samples were collected from 169 reported outbreaks with suspected viral origin from January 2010 to December 2012. Stool samples were pre-screened in two laboratories covering different geographic areas (the Laboratory of Microbiology of the Hospital Universitari Vall d'Hebron [HUVH], or the Public Health Agency of Barcelona [ASPB] Laboratory) using standard microbiological tests to rule out bacteria, toxins and parasites. Specimens underwent molecular testing for rotavirus, astrovirus and enteric adenovirus.

3.2. NoV GI and GII screening by quantitative RT-PCR (qRT-PCR), and amplification by semi-nested RT-PCR

Stool samples from outbreaks which were negative for all the above mentioned pathogens were screened for NoV by two different one-step qRT-PCR assays, depending on the laboratory of analysis. Viral RNA was extracted from a 10% stool suspension in phosphate-buffered saline (pH 7.4) and was stored at −80 °C. Samples analyzed at HUVH were extracted using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany) and analyzed by a duplex qRT-PCR assay based on primers and probes described by Kageyama et al. [20]. At the ASPB, samples were extracted using the NucliSENS Lysis Buffer and Magnetic Extraction Reagents and the MiniMag procedure (BioMérieux, France), and analyzed using the MutaREAL Norovirus Real Time RT-PCR Kit (Immundiagnostik, Germany). Semi-nested RT-PCR targeting the ORF1/ORF2 junction region (region C) for amplification and sequencing of 2–3 randomly selected positive samples from each outbreak was performed as previously described [18].

3.3. Sequencing, phylogenetic analysis, and genotype assignment

PCR products were purified and sequenced with the ABI PRISM BigDye® Terminator Cycle Sequencing Ready Reaction Kit V3.1 on an ABI Prism 3700 automatic sequencer (Applied Biosystems). Phylogenetic analysis was performed using the neighbor-joining method (distance calculation by the Kimura-2-parameter correction; pairwise deletion) implemented in the MEGA5 program [21], and results were validated by 1000 bootstrap replicates. Genotypes were assigned based on clustering with reference strains from the sequence database of the European Network (http://www.rivm.nl/en/Topics/Topics/N/NoroNet/Databases) in the phylogenetic tree with >70% bootstrap support, and the Norovirus Genotyping Tool [22] was used to confirm the results. For selected GII.4 samples (those which could not be assigned to a specific variant, the first GII.4 2012 reported outbreak, and four additional randomly selected outbreaks), a larger region of the genome corresponding to the 3′ half of ORF2 was sequenced using primers Set7F (5′CATATTCCAGGCAGTCGTAACT3′) and Set7R (5′GATTAGGGAACCAAGTCCAGAG3′), which span nucleotides 5958–6773 of the genome (positions according to GU445325).

3.4. NoV quantification in stool

Fecal virus load was determined using a one-step qRT-PCR assay that has been previously described [18]. For selected stool samples (genotyped samples with available epidemiological data), a 10% stool suspension was prepared adding 10 μl of Mengovirus prior to centrifugation, which is used as a process control virus to monitor virus/nucleic acid extraction efficiency. Enzyme efficiency was monitored by adding an in vitro transcribed RNA as described elsewhere [23], and according to ISO technical specifications (Norovirus and hepatitis A virus analyses from food and animal feed; ISO/TS 15216-1: 2013).

3.5. Nucleotide sequence accession numbers

Nucleotide sequences were submitted to Genbank under the following accession numbers: KF870579–KF870714. Nucleotide sequences from samples RSBS029/2012/J06.3, RCC07/2012/J04.16, and GIR38/2012/Y29.15 are available upon demand.

3.6. Statistical analysis

Statistical differences between categorical variables were determined using the Fischer's exact test, and comparisons between means were performed using the student *t*-test (unpaired) or the ANOVA analysis (DMS method), using the IBM SPSS® Statistics version 20 software (SPSS Inc., Chicago, IL, USA). p values <0.05 were considered statistically significant.

4. Results

4.1. Prevalence and mode of transmission of NoVs in outbreaks of non-bacterial gastroenteritis

A total of 169 outbreaks of non-bacterial acute gastroenteritis were reported between January 2010 and December 2012, affecting a total of 3239 individuals. NoVs were identified as the single agent of gastroenteritis in 128 outbreaks (76%), while 41 outbreaks (24%) remained undiagnosed. Overall, among 122 outbreaks with information regarding genogroup and mode of transmission, 45.1% were foodborne GII outbreaks, 36.1% were person-to-person GII outbreaks, 5.7% were person-to-person GI outbreaks, 4.9% were foodborne GI + GII outbreaks, 2.5% were person-to-person GI + GII outbreaks, 2.5% were waterborne GII outbreaks, 1.6% were foodborne GI outbreaks, 0.8% were waterborne GI outbreaks, and 0.8% were waterborne GI + GII outbreaks. Shellfish accounted for 11 foodborne outbreaks while sandwiches, cake, raw vegetables, durum, meatballs, cured-meat brochettes, and Chinese food

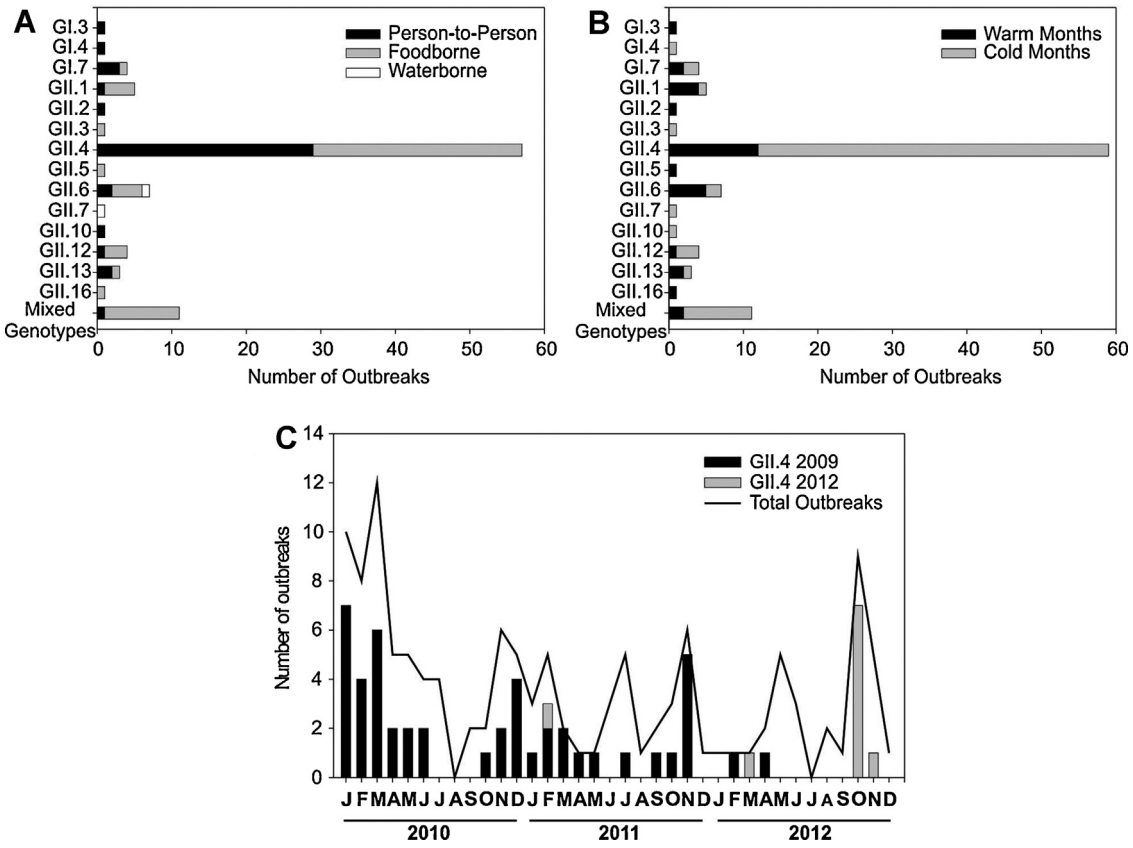

Fig. 1. Major NoV genotypes identified in outbreaks of gastroenteritis in Catalonia between January 2010 and December 2012, according to the mode of transmission (A) and the season in which they occurred (B). Warm months (April–September), cold months (October–March). (C) Monthly distribution of NoV outbreaks occurred in Catalonia during the 3-year period. The solid line indicates the total number of outbreaks over time, and bars indicate the number of outbreaks caused by the 2 most predominant GII.4 strains (2009 and 2012).

(rice and chicken with almonds) were each associated with one outbreak.

4.2. Prevalence and temporal distribution of NoV genotypes identified in outbreaks

Genotype information could be obtained for 103 out of the 128 NoV outbreaks (80.5%), and 3 GI genotypes (GI.3, GI.4, and GI.7) and 12 GII genotypes (GII.1, GII.2, GII.3, GII.4, GII.5, GII.6, GII.7, GII.10, GII.12, GII.13, GII.16, and GII.21) were detected. Genotype GII.4 was the predominant type, causing 58.2% of all genotyped outbreaks (Fig. 1A and B), and genotypes GI.4, GI.7, GII.1, GII.2, GII.4, GII.6, GII.7, GII.10, GII.12, GII.13, and GII.21 were isolated in outbreaks caused by multiple strains. GII.4 was isolated in 54% of these outbreaks in combination with one, two of even four other genotypes. Outbreaks caused by multiple genotypes were significantly associated to foodborne transmission (90.9%, $p = 0.042$) (Fig. 1A).

Regarding the temporal distribution of different genotypes, only GII.4 outbreaks were significantly more frequently reported during cold months (83.3%, $p = 0.010$) (Fig. 1B). Only genotypes GII.1, GII.4, GII.6, and GII.7 were regularly detected every year. The monthly distribution of total NoV outbreaks and outbreaks caused by variants 2009 and 2012 of genotype GII.4 throughout the study period is shown in Fig. 1C. During 2010, a good correlation was observed between the total number of outbreaks and the outbreaks caused by GII.4 2009 strain, but in 2011 and 2012, several outbreaks caused by genotypes other than GII.4 occurred also during June–July and May–June, respectively. A clear replacement of the GII.4 2009 strain by the 2012 strain was observed in 2012. Importantly, the first GII.4 2012 strain was detected as early as February 2011 in a person-to-person outbreak which occurred in a nursing home (outbreak GIR12/2011), being the earliest GII.4 2012 isolate reported worldwide up to date.

4.3. Epidemiological features of outbreaks caused by different genotypes

The epidemiologic characteristics of the outbreaks according to genotype are summarized in Table 1. The average attack rate between GII.13 outbreaks and the one in outbreaks caused by multiple genotypes was significantly different ($p = 0.022$), but there were no other significant correlations. Within GII.4 outbreaks, the average age of the affected individuals, the average attack rate and the average duration of symptoms were not statistically different between 2009 and 2012 variants (60.7 years vs 55.6, 45.7% vs 51.1%, and 2.1 days vs 2.6 days, respectively).

Regarding the distribution of genotypes by age group, the highest diversity was observed in individuals younger than 15 years old, with 10 different genotypes, and decreased with age (Fig. 2). The only genotypes that were detected in all age groups were GII.4 and GII.6.

4.4. Phylogenetic analysis of NoV strains

Genotype information inferred from region C phylogenetic analysis correlated with genotype assignment performed using the Noronet Typing Tool (Figs. 3A,B and 4B). Using this region, all GII.4 strains could be typed at the variant level, with the exception of outbreaks CP84/2011, GIR75/2011, GIR75/2012 and RSBS76/2012 (Fig. 4A, indicated in italics). The use of other

Table 1
Epidemiological features of outbreaks caused by different genotypes.

Genotype	Number of outbreaks	Setting (number of outbreaks)	Total number of affected individuals	Total number of individuals who used health services (%)	Total number of hospitalizations (%)	Average attack rate (%)[a]	Average duration of symptoms (days)[a]
GI.3	1	Private home	ND	ND	ND	ND	ND
GI.4	1	School	9	0 (0)	0 (0)	36	5.0
GI.7	4	Restaurant (1), Healthcare institution (1), Youth hostel/Campground (1), School (1)	52[c]	0 (0)[c]	0 (0)[c]	56.1 ± 17.9	2.5 ± 2.1
GII.1	6	Youth hostel/Campground (2), Foodservice/Catering (1), Nursing home (1), School (1), ND (1)	128[d]	33 (25.8)[d]	0 (0)[d]	57.7 ± 4.4	1.4 ± 0.5
GII.2	1	School	ND	ND	ND	57.5	ND
GII.3	1	Private home	ND	ND	ND	ND	ND
GII.4	60	Nursing home (12), Restaurant (12), Healthcare institution (10), Hotel (8), Foodservice/Catering (4), Private home (4), Community-based group residence (4), Youth hostel/Campground (1), School (1), Others[b] (1), ND (3)	1,759[e]	265 (15.1)[e]	16 (0.9)[e]	47.6 ± 29.2	2.1 ± 0.9
GII.5	1	Private home	3	3 (100)	0 (0)	60	ND
GII.6	7	Nursing home (2), Restaurant (1), Foodservice/Catering (1), School (1), Others (2)	160[d]	27 (16.9)[d]	7 (4.4)[d]	36.4 ± 22.1	2.0 ± 0.9
GII.7	1	School	103	7 (6.8)	0 (0)	21.1	1.3
GII.10	1	Nursing home	ND	ND	ND	ND	ND
GII.12	4	School (2), Healthcare institution (1), Restaurant (1)	150	1 (0.7)	1 (0.7)	37.8 ± 15.9	1.3 ± 0.7
GII.13	3	Youth hostel/Campground (2), Restaurant (1)	41	13 (31.7)	0 (0)	21.7 ± 12.0	ND
GII.16	1	Youth hostel/Campground	19	12 (63.2)	0 (0)	48.7	ND
Multiple genotypes	11	Restaurant (6), Private home (2), Foodservice/Catering (1), Hotel (1), Nursing home (1)	122	20 (16.4)	0 (0)	64.2 ± 26.1	2.4 ± 1.4
Total	103		2546	381 (15.0)	24 (0.9)	47.5 ± 27.0	2.1 ± 1.0

ND: no data.
[a] Results are expressed as the mean ± the standard deviation of the mean.
[b] Travel, community events, workplace.
[c] Missing information for 1 outbreak.
[d] Missing information for 2 outbreaks.
[e] Missing information for 4 outbreaks.

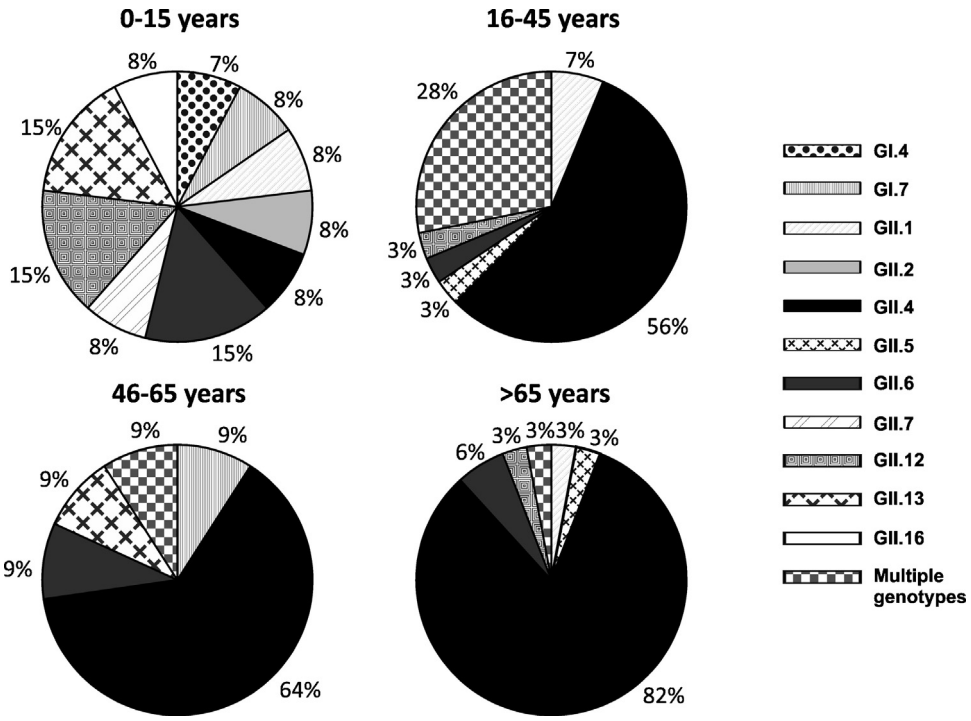

Fig. 2. Prevalence of NoV genotypes in different age groups characterized in Catalonia during the 3-year study period.

algorithms including the HKY851 method was attempted, but similar inconclusive results were obtained (data not shown). These isolates, together with outbreak GIR12/2011 (first GII.4 Sydney 2012 variant isolated in this study period), and 4 additional outbreaks, which were randomly selected (ASPB74/2010, RCC12/2010, RCC15/2010, and TAR02/2012), were chosen to analyze a larger portion of ORF2 including most of the P2 domain (amino acids 307–540 of VP1). Phylogenetic analysis of the 3′ end of ORF2 at the nucleotide level indicated that outbreaks ASPB74/2010, RCC12/2010, and RCC15/2010, as well as CP84/2011 and TAR02/2012, were caused by GII.4 2009 variants, while GIR12/2011, GIR75/2012 and RSBS76/2012 outbreaks were caused by GII.4 2012 strains (sequencing of GIR75/2011 outbreak was unsuccessful) (Fig. 4B).

Compared with the corresponding GII.4 reference strains, amino acid changes were detected in some of the two sequenced VP1 coding regions (Fig. 4C and D). All but one of these sites corresponded to previously identified informative sites subject to selective pressure [24–26]. Most of these non-synonymous mutations are located in the VP1 protruding region, but substitutions inside the VP1 shell have also been described [26]. Most of these sites would not be under pressure due to antibody recognition but to the role of the RNA secondary structure for efficient translation [26]. In this report, we found one previously undescribed mutation in residue 8 (Fig. 4D).

Table 2
Analysis of viral load in diarrheal specimens belonging to GII.4 2009 and 2012 predominant strains.

	GII.4 2009	GII.4 2012
Mean viral load (RNA copies/g)	5.57×10^8	2.12×10^9
Range (RNA copies/g)	1.75×10^7–9.05×10^9	8.84×10^7–1.94×10^{10}
Number of tested samples	12	7
Age of individuals (range in years)	28–48	3–76
Time of stool collection after the onset of symptoms (range in days)	2–39	2–9

4.5. Analysis of viral load in GII.4 2009 and GII.4 2012 diarrheal specimens

Quantification levels for NoV shedding were compared between samples belonging to GII.4 2009 and GII.4 2012 strains (Table 2), but no statistical differences were observed.

5. Discussion

Data presented in this study refer the molecular characterization of NoV strains that caused outbreaks of gastroenteritis in Catalonia, Spain, between January 2010 and December 2012. Foodborne and person-to-person outbreaks caused by GII strains accounted for 81.2% of all reported NoV outbreaks. As reported elsewhere, GII.4 was the most commonly found genotype [15,27–29]. It was the single isolated genotype in 58.2% of all genotyped outbreaks and it was also present in 6/11 multi-strain outbreaks. GII.4 outbreaks occurred preferentially during cold months and were never waterborne outbreaks. Multi-strain outbreaks were the second most common outbreaks (10.7%), with a higher occurrence in cold months and a significant association with a foodborne origin. Other genotypes isolated with prevalences higher than 5% were GII.6 and GII.1. Although not significantly, foodborne transmission was more frequent among these outbreaks. GII.6 has been the second most prevalent genotype in Japan and it has been associated with high genetic variation [30]. Association between GII.6 and foodborne transmission has also been reported in the US [31], and it was also commonly detected in the environment [18] and in mussels harvested in Spain [19]. GII.1 has also been detected with increased prevalence in Europe in 2011 [32].

Interestingly, two of the genotypes most commonly isolated from river and wastewater samples in our previous study performed between 2007 and 2009 (GI.4 and GII.21) [18] were scarcely detected in the studied outbreaks. GI.4 was also commonly detected

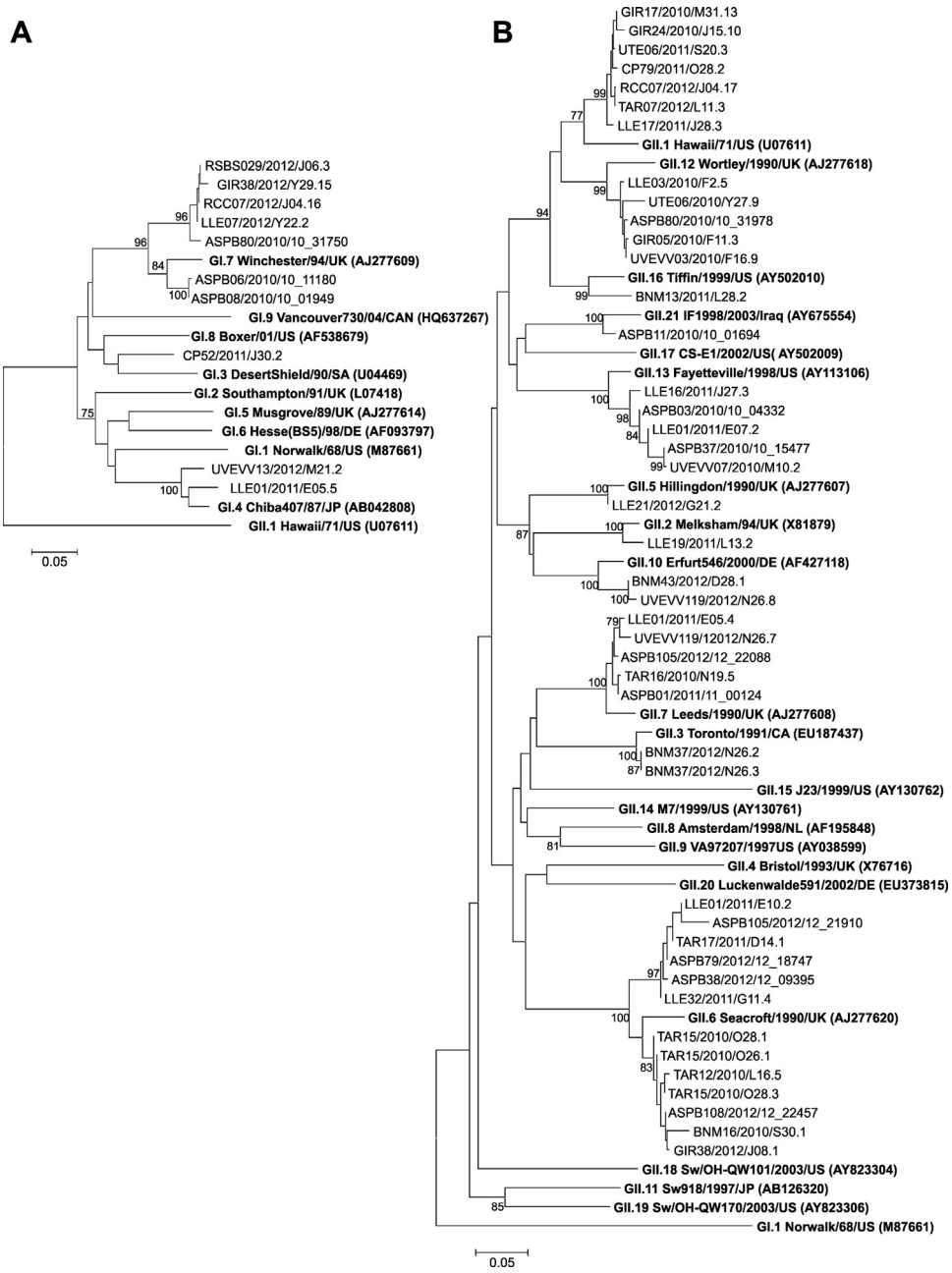

Fig. 3. Phylogenetic analysis of NoVs strains isolated in outbreaks of gastroenteritis (region C). Panel A includes all GI genotypes, and panel B includes all GII genotypes except GII.4. The nucleotide dendrogram was inferred using the neighbor-joining method with distance calculation by the Kimura-2-parameter correction implemented in the MEGA5 software [21]. A bootstrap of 1000 replicates was performed and values above 75 are shown in the figure. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bold indicates reference strains for the respective genotypes. Isolate names are composed of a letter code indicating the geographic region followed by the outbreak number, the year, and the sample name.

in mussels from Spain [19]. These discrepancies may reflect a decrease in prevalence over time, or a combination of high environmental resistance and lower pathogenicity. In contrast, genotypes GI.7, GII.1, GII.5, GII.7, GII.10, GII.12, and GII.13 were isolated in the environment between 2007 and 2009 in less than 2% of samples [18], but have been isolated in a significant number of 2010–2012 outbreaks. These differences might be due to an emerging trend, to a lower persistence in the environment, or to variability in the target region amplified by the qRT-PCR assay, which could improve detection efficiency.

The temporal distribution of outbreaks (Fig. 1C) showed the occurrence of two unexpected peaks in 2011- and 2012-warm months attributable to non-GII.4 strains (GI.3, GI.7, GII.1, GII.2, GII.6 and GII.13). Of note, most of these outbreaks occurred in youth hostels, campgrounds or schools, highlighting the not negligible importance of NoVs in such settings during school vacation activities. Our surveillance also detected the replacement of GII.4 2009 variant by GII.4 2012 during 2012, mirroring what has been reported elsewhere [10–13]. While most reports indicate that GII.4 2012 predominates since the end of 2012, we detected it in February 2011, being the earliest report of this strain worldwide. Although phylogenetic analysis of this early strain showed a strong clustering with other 2012 strains (Fig. 4A and B), a remarkably high number of amino acid substitutions within the P domain was observed (2.5% of the analyzed VP1 region). Although identical sequences are not found in Genbank, GIR12/2011/F23.5 strain

Fig. 4. Phylogenetic analysis of NoVs GII.4 strains isolated in outbreaks of gastroenteritis and amino acid (AA) changes in capsid informative sites. Panel A shows the dendrogram inferred from region C, and panel B shows the dendrogram of selected strains using a 715-nucleotide region of the 3′end of ORF2. Trees were inferred using the neighbor-joining method with distance calculation by the Kimura-2-parameter correction implemented in the MEGA5 software [21]. A bootstrap of 1000 replicates was performed and values above 75 are shown in the figure. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bold indicates reference strains for the respective genotypes. Names written in italics indicate GII.4 isolates that were difficult to type at the variant level. Asterisks indicate isolates that were selected to sequence a larger fragment of ORF2. Isolate names are composed of a letter code indicating the geographic region followed by the outbreak number, the year, and the sample name. Panels C and D show the amino acid changes found in informative sites within studied regions of the VP1 capsid protein: P domain (AA 307–540) (C) and shell domain (AA 1–75) (D). AA numbering is indicated at the top of the alignments. Reference strains are indicated in bold. Residues showing mutations compared to the corresponding reference strain are highlighted in gray boxes.

shares some mutations with strains isolated in France (AGT95928), Australia (AGS08091) and New Zealand (AGS08169). In agreement with previous observations that indicate that GII.4 2012 strains undergo antigenic diversification faster than their predecessor strains [33], our analysis of the VP1 region in GII.4 2009 and 2012 strains also indicate a higher number of variable positions in 2012 strains, especially at sites which may be important in modulating the antigenic profile of the virus (Fig. 4C and D).

Reports analyzing virulence and severity of disease caused by GII.4 2012 strains compared with other variants are diverse [34,35], and some works have reported that GII.4 2012 outbreaks disproportionally affect older persons in healthcare-related settings transmitted from person to person. Clinical features of the GII.4 2012 outbreaks reported here were similar to the previously predominant GII.4 2009 strain. A high percentage of foodborne GII.4 2012 outbreaks was also detected, and the average age of the affected individuals was 55 years old. Our laboratory has also detected GII.4 2012 strains in cases of sporadic NoV infections in children (data not shown). In fact, the variety of strains that cause outbreaks in the young population has been remarkable, suggesting that immune protection acquired from infection at least with some genotypes may last long enough to prevent reinfection. At the same time, recombination between different viruses, at least between non-abundant genotypes, may be more likely to occur in children than in older individuals.

Compared with other genotypes, the higher fitness of GII.4 strains is associated to a higher rate of evolution of the capsid proteins [6], but other factors may contribute to their success as well. Since higher replication rates may result in higher viral loads, and higher shedding could favor transmission, we examined whether differences exist between GII.4 2009 and GII.4 2012 excretion levels. Our results indicate that these viruses are shed at similar levels. Other factors that may also influence transmission such as particle stability outside the host should be explored too. Although shed at similar levels, GII.4 2012 strains have clearly replaced the previous predominant strain.

In conclusion, this study supports the importance of pursuing surveillance of NoV strains circulating in the community.

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Competing interests

None of the authors declares any conflict of interest.

Ethical approval

Ethical approval was not required.

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