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ORIGINAL PAPER

Genetic Diversity Among Genogroup II Noroviruses and Progressive Emergence of GII.17 in Wastewaters in Italy (2011–2016) Revealed by Next-Generation and Sanger Sequencing

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Abstract

Noroviruses (NoV) are a major cause of gastroenteritis worldwide. Recently, a novel variant of NoV GII.17 (GII.P17_GII.17 NoV), termed Kawasaki 2014, has been increasingly reported in NoV outbreaks in Asia, and has also been described in Europe and North America. In this study, sewage samples were investigated to study the occurrence and genetic diversity of NoV genogroup II (GII) along a 6-year period. Moreover, the spread of GII.17 strains (first appearance and occurrence along time) was specifically assessed. A total of 122 sewage samples collected from 2011 to 2016 from four wastewater treatment plants in Rome (Italy) were initially tested using real-time RT-(q)PCR for GII NoV. Positive samples were subsequently subjected to genotypic characterization by RT-nested PCRs using broad-range primes targeting the region C of the capsid gene of GII NoV, and specific primers targeting the same region of GII.17 NoV. In total, eight different genotypes were detected with the broad-range assay: GII.1 ($n = 6$), GII.2 ($n = 8$), GII.3 ($n = 3$), GII.4 ($n = 13$), GII.6 ($n = 3$), GII.7 ($n = 2$), GII.13 ($n = 2$), and GII.17 ($n = 3$), with the latter two genotypes detected only in 2016. Specific amplification of GII.17 NoV was successful in 14 out of 110 positive samples, spanned over the years 2013–2016. The amplicons of the broad-range PCR, pooled per year, were further analyzed by next-generation sequencing (NGS) for a deeper analysis of the genotypes circulating in the study period. NGS confirmed the circulation of GII.17 NoV since 2013 and detected, beyond the eight genotypes identified by Sanger sequencing, three additional genotypes regarded as globally uncommon: GII.5, GII.16, and GII.21. This study provides evidence that GII.17 NoV Kawasaki has been circulating in the Italian population before its appearance and identification in clinical cases, and has become a major genotype in 2016. Our results confirm the usefulness of wastewater surveillance coupled with NGS to study the molecular epidemiology of NoV and to monitor the emergence of NoV strains.

Keywords Norovirus · GII.17 · Sewage · NGS · Sequencing · PCR

Introduction

Noroviruses (NoV) are a leading cause of sporadic cases and outbreaks of acute gastroenteritis in children and adults worldwide (Bartnicki et al. 2017; Patel et al. 2008). They

The original version of this article was revised: The original version of this article unfortunately contained a mistake. The presentation of Table 1 was incorrect. The corrected table is given below.

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are small round non-enveloped viruses with a 7.5 kb single-stranded positive-sense RNA which contains three open reading frames (ORFs). ORF1 encodes for a large polyprotein that is cleaved into non-structural proteins, including the RNA-dependent RNA polymerase; ORF2 encodes for the major capsid protein VP1; and ORF3 encodes for the minor viral capsid protein VP2 (Green 2013).

NoV belong to the *Norovirus* genus in the Caliciviridae family. They are classified into six distinct genogroups (GI–GVI), further subdivided into different genotypes (Kroneman et al. 2013). Only genogroups GI, GII, and GIV have been identified in humans. Despite the broad genetic diversity, NoV GII.4 has predominated during the last decade (Pringle et al. 2015). Different variants of GII.4 have been detected in outbreaks and sporadic cases of acute gastroenteritis worldwide, with new GII.4 variants emerging every 2–3 years (Eden et al. 2013; Vinje 2015).

In the winter of 2014, a novel GII.17 NoV variant, named Kawasaki 2014, emerged and replaced the previous dominant GII.4 Sydney 2012 variant in several countries in Southern Asia (Chan et al. 2015; Chen et al. 2015; da Silva et al. 2017; Lee et al. 2015; Lu et al. 2015; Matsushima et al. 2015) and it has been hypothesized that this could represent the end of the GII.4 era (de Graaf et al. 2015). The novel strain Kawasaki 2014 has also been reported sporadically in other countries outside Asia, including Italy (da Silva et al. 2017; Degiuseppe et al. 2016; Dinu et al. 2016; LeBlanc et al. 2016; Medici et al. 2015; Parra and Green 2015). Globally, GII.17 Kawasaki viruses were found in 13 countries: Canada, China, Germany, Hungary, Italy, Japan, The Netherlands, New Zealand, Russia, Slovenia, South Korea, Thailand, and the United States (Chan et al. 2017).

A number of studies have reported the detection of GII.17 strains in water environments since 2010, with the first identification in Ireland in influents from wastewater treatment plants (Rajko-Nenow et al. 2013). In Japan, GII.17 was identified in sewage water during the 2012–2013 seasons (Kazama et al. 2017). Later on, GII.17 NoVs were detected in sewage samples from China, Uruguay, and Africa (Mabasa et al. 2017; Victoria et al. 2016; Zhou et al. 2016) and in wastewater treatment plant effluents in France (Prevost et al. 2015). Moreover, GII.17 has been detected in surface water sources in Kenya (Kiulia et al. 2014), in river water in Thailand (Boonchan et al. 2017), and in estuary water in Korea (Kim et al. 2016). Waterborne outbreaks caused by GII.17 NoV have also been described in Russia (Maletskaya et al. 2016) and in China (Qin et al. 2016). In Italy, no nationwide surveillance system exists for human NoV, and epidemiological data on this virus are limited; molecular epidemiology data rely on voluntary networks of the Italian Study Group for Enteric Viruses (ISGEV; https://isgev.net/), which monitors the epidemiology of enteric viruses in children through hospital-based surveillance in Italy.

The present study aimed to expand the data regarding the occurrence and genetic diversity of GII NoV circulating in Italy during a 6-year period, between 2011 and 2016, with a particular focus on GII.17 NoV.

To this purpose, urban sewages were analyzed using conventional (Sanger) and next-generation sequencing methods, in order to provide a more complete picture of NoV types in urban wastewaters. As a matter of fact, wastewaters receive NoV excreted in feces or vomitus from all of the infected individuals in a catchment area, including asymptomatic individuals and those with mild disease, and, in recent years, sewage surveillance has been proved to be a powerful approach to study the epidemiology and the distribution of the enteric viruses in the population (Cowger et al. 2017; Hellmer et al. 2014; Iaconelli et al. 2016, 2017; Kokkinos et al. 2011; La Rosa et al. 2014; Myrmel et al. 2015).

Materials and Methods

Sample Collection and Treatment

A total of 122 raw sewage sample were collected over 6 years (2011–2016) from four Wastewater Treatment Plants (WTPs) in Rome: 19 in 2011, 25 in 2012, 30 in 2013, 15 in 2014, 16 in 2015, and 17 in 2016.

Samples were handled and analyzed as previously described (La Rosa et al. 2014). Briefly, 20 ml of untreated wastewater samples were treated with 2 ml of 2.5 M glycine pH 9.5. After incubation in ice for 30 min, 1/10 vol chloroform was added and samples were centrifuged at 10,000×*g* at 4 °C. The upper aqueous layer was then transferred to a fresh tube and viral nucleic acids were extracted from 5 ml, using the NucliSENS MiniMag semi-automated extraction system (BioMerieux), according to the manufacturer's instructions. Eluates (100 µl each) were divided into small aliquots and subsequently frozen at − 70 °C until analysis. A murine norovirus (MNV1) was used as process control as described elsewhere (La Rosa et al. 2014).

Real-Time RT-(q)PCR

Samples were analyzed for presence of GII NoV using the real-time RT-(q)PCR described in ISO 15216-1:2017 (International Organization for Standardization (ISO), 2017). The amplification reactions were carried out on a ABI Prism 7700 SDS system (Applied Biosystems) using the conditions, primers, probes, and reagents (RNA UltraSense™ One-Step Quantitative RT-PCR System, Life Technologies) reported in the annexes of ISO 15216–1:2017. A list of the primers used in this study is reported in Table 1. The

Table 1 Primers used in the present study

Region	Primer name	Primer (5′–3′)	Length (bp)	Cycle	References
Real-time RT-(q)PCR for GII NoV					
ORF1–ORF2 junction	QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA	–	One-step real-time	Kageyama et al. (2003), Loisy et al. (2005)
	COG2R	TCGACGCCATCTTCATTCACA			
	QNIFs	FAM-AGCACGTGGGAGGGCGATCG-TAMRA			
Broad-range GII NoV nested RT-PCR					
ORF2	COG2F	CARGARBCNATGTTYAGRTGGATGAG	398	1st cycle	Kageyama et al. (2003), Kojima et al. (2002)
	G2SKR	CCRCCNGCATRHCCRTTRTACAT			
	G2SKF	CNTGGGAGGGCGATCGCAA	344	2nd round	
	G2SKR	CCRCCNGCATRHCCRTTRTACAT			
Specific GII.17 NoV nested RT-PCR					
ORF2	2063	GCTCCATCTAATGATGGTG	368	1st cycle	La Rosa et al. (2017)
	2062	TCCACTGGGAAATTTGGC			
	2104	TGGTCTCGTACCAGAGGG	310	2nd round	
	2064	GATCTTTCCGGCAGTGAAC			
GII.17 ORF1-ORF2 junction nested RT-PCR					
ORF1–ORF2 junction	GIIPF800 M	GATGCWGAYTAYTCYMGNTGGGA	2354	1st cycle	Koo et al. (2017)
	GIICR1450	ACCCARGMNTCAAAYCTRAART			
	GIIPF750 M	CNGCHHTAGARRTNATGGT	1048	2nd round	
	GII-R1 M	CCRCCNGCATRNCCRTTRTACAT			

Real-time probe labeled at the 5′-end with 6-carboxyfluorescein (FAM) and at the 3′-end with 6-carboxytetramethylrhodamine (TAMRA)

presence of PCR inhibitors was assessed by means of an external RNA amplification control (EC-RNA, ~ 10^4 copies of the target sequence), and results were considered acceptable if amplification efficiency was ≥ 50%. The standard curve for quantification was generated using a linearized plasmid containing the target sequence.

Nested RT-PCR, Sanger Sequencing

GII NoV-positive samples by real-time RT-(q)PCR were subjected to a nested reverse transcription-polymerase chain reaction (nested RT-PCR) with broad-range primers directed at the region C of the capsid gene (ORF2), producing amplicons of 344 bp (Kageyama et al. 2003; Kojima et al. 2002). In parallel, the same samples were tested with an assay specific for GII.17 NoV (La Rosa et al. 2017), also targeting the C region of ORF2 and producing amplicons of 310 bp. Stool specimens containing the GII.17 NoV variant Kawasaki 2014, kindly provided by Dr. Vito Martella of the ISGEV, were used as positive controls.

Samples positive with the GII.17 specific assay were further analyzed to identify the ORF1 (polymerase) genotype and reconfirm the ORF2 genotype, by performing an additional nested RT- PCR analysis for the ORF1-ORF2 junction (1048 bps) (Koo et al. 2017).

Amplifications were performed using MyTaq™ One-Step RT-PCR Kit (Bioline) in a T100 Thermal Cycler (Bio-Rad). PCR reactions were prepared in a 50 µl mixture containing 25 µl of PCR One-Step Mix, 1 µl (10 pmol) of each primer, and 2 µl of extracted RNA. After the first round of PCR amplification, 2.5 µl of the PCR product obtained was used for the second PCR assay, using MyTaq™ Red Mix kit (Bioline).

Products were separated on a 2% agarose gel and visualized using gel red staining. PCR fragments were purified using a Montage PCRm96 Microwell Filter Plate (Millipore) and were subjected to automated sequencing (Bio-Fab Research) in both directions.

Next-Generation Sequencing (NGS)

To further investigate the genome diversity of GII NoV in the samples, the PCR amplicons obtained using the broad-range primers (including those uncharacterized) were combined into six pools, one for each year of sampling, and were subjected to NGS. For each pool, equal amount of purified PCR amplicons were mixed, DNA was precipitated with isopropanol, and pellet was resuspended in nuclease-free water for a final volume of 20 µl. DNA concentration and A260/A280 ratios were evaluated using a Nanodrop 1000 spectrophotometer.

NGS was carried out on MiSeq II sequencer (Illumina) according to the manufacturer indications. For the library preparation, 5 ng of PCR amplicons was simultaneously fragmented and tagged with sequencing adapters (Nextera XT DNA Library Preparation Kit and Nextera XT Index Kit). The following run associated with paired-end sequencing, cluster generation, and sequencing by synthesis was set using MiSeq Reagent Kit v3 (600-cycle, extend read lengths up to 2 × 300 bp). Base calls generated on-instrument were firstly processed by Real-Time Analysis software (RTA, Illumina) to assess quality scores and then passed through MiSeq Reporter (MSR) Software to produce the first rough alignment, structural variants, and contig assemblies for each sample (Resequencing pipeline). A no-template control (NTC) was included in the Nextera XT Library Procedures to verify the absence of contaminant amplifications. A V3 PhiX library was also included in each MiSeq run for sequencing quality control.

The software Geneious® version 10.1.3 (Biomatters Ltd) was used for the subsequent bioinformatics analysis. Paired-end reads were combined and then filtered and aligned using a custom workflow with the following parameters: primers G2SKF and G2SKR were trimmed from 5 to 3 ends; reads were trimmed to have an average error rate < 0.1%, no bases with a quality of < Q30, and no ambiguities. Following trimming, duplicate reads and sequences < 50 nucleotides in length were removed. The remaining sequences were then mapped to a set of 44 NoV references, with a minimum overlap identity of 95%, maximum 5% mismatches per read, word length of 14, and index word length of 12. Only consensus sequences over 200 nucleotides in size were considered to determine a virus type in the sample. An additional De Novo assembly of the trimmed readings was carried out to obtain 10,000 sequences which were mapped to the same set of reference strains. The consensus sequences obtained after using both mapping and the De Novo assembly were compared to check whether different assembly methods produced different consensus sequences.

Phylogenetic Analysis

Genotypes were assigned using the Norovirus Genotyping Tool (http://www.rivm.nl/mpf/norovirus/typingtool; Kroneman et al. 2011), and phylogenetic analysis of aligned sequences was carried out using Molecular Evolutionary Genetics Analysis (MEGA) 6 software (Kumar et al. 2008). The phylogenetic relationships of the NoV capsid region C were determined using the neighbor–joining method with 1000 bootstrap replicates. The evolutionary distances were computed using the Kimura-2 parameter model. The reliability of the phylogenetic tree was assessed by bootstrap sampling of 1000 replicates.

Accession Numbers

The sequences obtained in this study were deposited in the GenBank database with the following accession numbers: MF796265-MF796315.

Results

Table 2 summarizes the results of the molecular analyses. A total of 110 samples (90.2%) tested positive by real-time RT-(q)PCR with GII NoV concentrations up to 7.3×10^2 genome copies/ml of wastewater and an average concentration of 1.1×10^2 genome copies/ml (data not shown).

The region C of the capsid gene (ORF2) of NoV was successfully amplified in 60 out of 110 real-time RT-(q)PCR positive samples using the broad-range nested PCR. Positive and negative controls yielded the expected results. Forty virus amplicons were further characterized using Sanger sequencing in eight different genotypes: GII.1, GII.2, GII.3, GII.4, GII.6, GII.7, GII.13, and GII.17. The amplicons from 20 samples could not be characterized because of mixed electropherograms, suggesting the presence of more than one genotype in the same sample.

The most prevalent NoV genotype was GII.4 ($n = 13$ samples), detected in all years from 2012 to 2016; all samples were characterized as variant Sidney_2012, except three unassigned. The second most common NoV genotype was GII.2 ($n = 8$), followed by GII.1 ($n = 6$), GII.3, GII.6, and GII.17 ($n = 3$), and GII.7 and GII.13 ($n = 2$). GII.13 and GII.17 NoV were detected only in 2016.

The GII.17-specific nested PCR confirmed the three positive samples detected by the broad-range assay and detected GII.17 in 11 additional samples. In total, 14 GII.17 positive samples were detected: 3 in 2013 (3/30 = 10%), 1 in 2014 (1/15 = 7%), 3 in 2015 (3/16 = 19%), and 7 in 2016 (7/17 = 41%). Two of the GII.17 positive samples were also successfully amplified with the long-RT-PCR for the ORF1-ORF2 junction, and sequence analysis of the amplicon demonstrated that both sequences were related to the GII.P17-GII.17 strains.

Analysis by NGS of the pooled amplicons confirmed the results obtained by Sanger sequencing. Moreover, for each pool, further genotypes, undetected by Sanger sequencing, were identified: one additional genotype was detected in pools from the years 2011 and 2012, two genotypes in those from years 2014 and 2016, four in year 2015, and seven in year 2013 (Table 2). Overall, three additional genotypes (GII.5, GII.16, and GII.21) were detected by NGS beyond the eight found by conventional sequencing. Regarding GII.17, NGS analysis detected this genotype in all the pools since 2012 although a minimum read depth

Table 2 PCR and sequencing results by year and method

	Years						Period 2011–2016
	2011	2012	2013	2014	2015	2016	
N° of tested samples	19	25	30	15	16	17	122
N° of positive samples by real-time RT-PCR (%)	16 (84%)	22 (88%)	30 (100%)	12 (80%)	14 (88%)	16 (94%)	110 (90.2%)
Genotypes detected by broad-range PCR for GII (N° of samples)	GII.1 (1) GII.2 (2) GII.3 (1) GII.6 (1)	GII.1 (3) GII.3 (2) GII.4 (1) GII.7 (2) Untyp. (1)	GII.1 (2) GII.2 (2) GII.4 (6) GII.6 (1) Untyp. (11)	GII.2 (1) GII.4 (2) GII.6 (1) Untyp. (1)	GII.2 (1) GII.4 (1) Untyp. (3)	GII.2 (2) GII.4 (3) GII.13 (2) GII.17 (3) Untyp. (4)	GII.1 (6) GII.2 (8) GII.3 (3) GII.4 (13) GII.6 (3) GII.7 (2) GII.13 (2) GII.17 (3) Untyp. (20)
Detection of GII.17 by specific PCR (N° of samples)			GII.17 (3)	GII.17 (1)	GII.17 (3)	GII.17 (7)	GII.17 (14)
Genotypes detected by NGS	GII.1 GII.2 GII.3 **GII.4** GII.6	GII.1 GII.3 GII.4 **GII.6** GII.7	GII.1 GII.2 **GII.3** GII.4 **GII.5** GII.6 **GII.7** **GII.13** **GII.16** **GII.17** **GII.21**	GII.2 GII.4 GII.6 **GII.13** **GII.17**	GII.2 GII.4 **GII.6** **GII.7** **GII.13** **GII.17**	GII.2 **GII.3** GII.4 **GII.7** GII.13 GII.17	GII.1 GII.2 GII.3 GII.4 **GII.5** GII.6 GII.7 GII.13 **GII.16** GII.17 **GII.21**

Genotypes detected exclusively by NGS are reported in bold

Untyp Untypable

of 50 for the entire 344 nucleotides of the capsid fragment was obtained only in pools from years 2013 and 2016.

Figure 1 shows the phylogenetic tree of GII NoV capsid sequences obtained in this study. Sequences formed well-supported monophyletic groups in the tree, and are represented with their corresponding prototype strains (see Fig. 1) within each genotype. The GII.17 NoV sequences detected in this study are grouped in two different GII.P17_GII.17 clusters: the "Kawasaki 308" cluster (16 sequences), comprising the four GII.17 NoV sequences detected in clinical samples thus far in Italy (Accession Numbers KX592170, KX592171, KX592172, KT346356) and the "Kawasaki 323" cluster (two sequences, collected in 2013 and 2014). More distant clusters contained the GII.17 NoV sequences identified in years 1978–2005, and characterized as GII.P4_GII.17 (JN699043), and GII.P16_GII.17 (KJ196286 and AY502009). The degree of nucleotide sequence identity within the GII.P17_GII.17 sequences from this study ranged from 96 to 100%.

Discussion

Viruses of the GII.17 genotype have been circulating in the human population since 1978. Sequence JN699043 Norovirus Hu/GII.17/C142/GF/1978 represent the oldest sequence of GII.17 present in the National Center for Biotechnology Information (NCBI) database (Rackoff et al. 2013). Since then, viruses with a GII.17 NoV capsid have been sporadically detected in Africa, Asia, Europe, America, and Australia (de Graaf et al. 2015), associated with different polymerases (GII.P13_GII.17, GII.P4_GII.17, GII.P16_GII.17, and GII.P17_GII.17).

In the winter of 2014, a novel GII.17 NoV variant (GII.P17_GII.17), named Kawasaki 2014, emerged and replaced the previous dominant GII.4 Sydney_2012 variant in several countries in Asia and has also been reported sporadically in other countries outside Asia (da Silva et al. 2017; Degiuseppe et al. 2016; Dinu et al. 2016; LeBlanc et al. 2016; Medici et al. 2015; Parra and Green 2015).

In Italy, GII.17 Kawasaki 2014 variant was identified for the first time in two sporadic cases of acute severe gastroenteritis in young children hospitalized in February and March 2015 in two distinct Italian regions (Medici et al. 2015). Subsequently, in the winter season 2015–2016, GII.17 strains

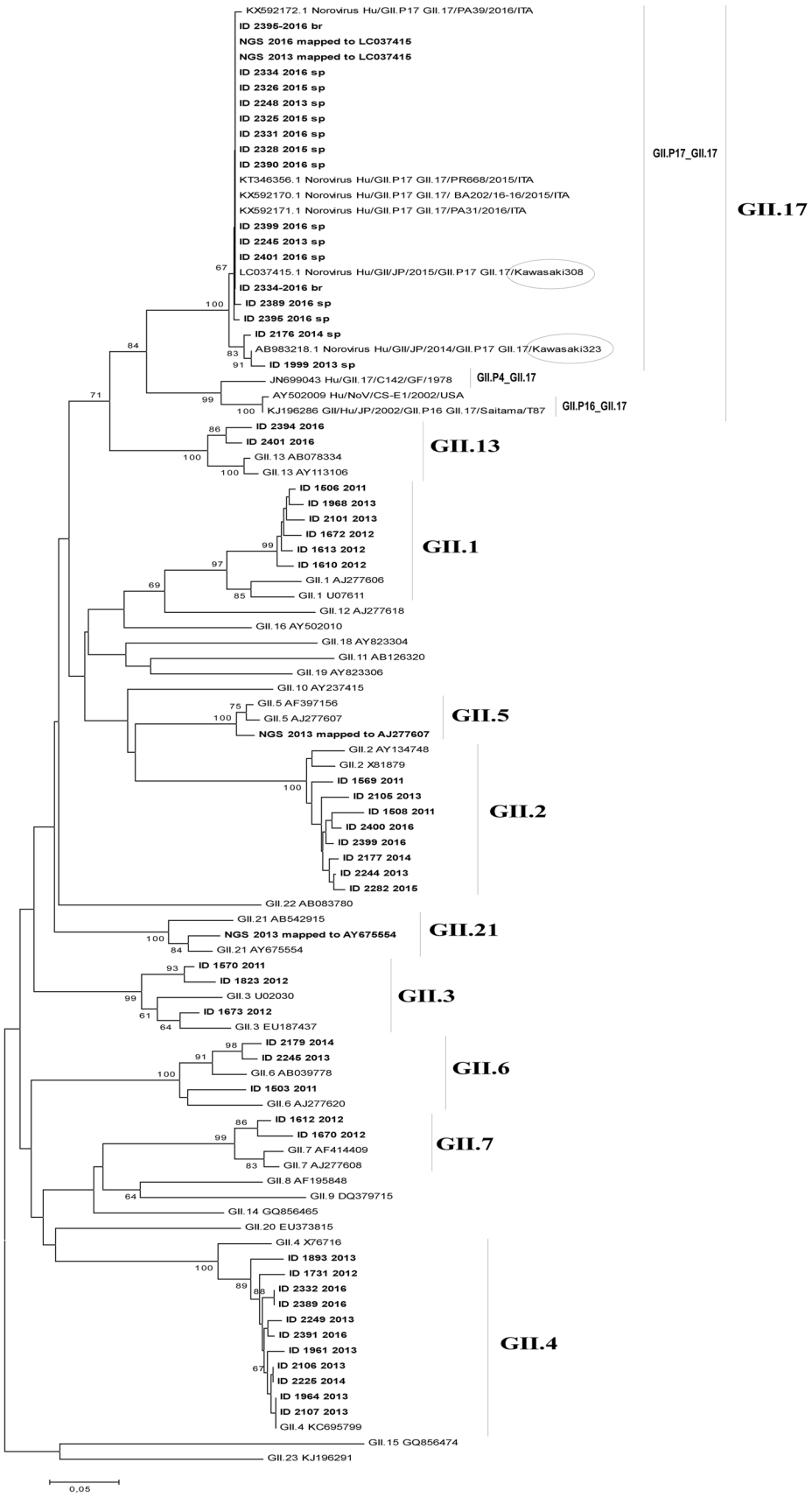

Fig. 1 The phylogenetic tree of GII NoV capsid sequences obtained with the broad-range assay targeting the region C of the capsid gene of GII NoV. The tree includes 40 NoV reference prototypes retrieved from GenBank and 55 GII NoV sequences from this study (in bold). Specifically, these sequences include 37 sequences obtained by the broad-range PCR assay, and characterized as GII.1, GII.2, GII.3, GII.4, GII.6, GII.7, GII.13, and GII.17 by the NoV genotyping tool; four sequences obtained by NGS, characterized as GII.5, GII.17 (two sequences, related to years 2013 and 2016), and GII.21, and, finally, 14 GII.17 sequences, detected by the GII.17 specific PCR assays. Sequences of poor quality or too short were not included in the phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. There were a total of 204 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

have emerged as the third most common NoV in pediatric patients hospitalized with sporadic acute gastroenteritis, suggesting increased circulation of the variant Kawasaki 2014 among children, although GII.4 variants were still predominant (Giammanco et al. 2017).

In this study, sewage samples were investigated to study the occurrence and genetic diversity of GII NoV along a 6-year period, using both Sanger and NGS sequencing. Overall, combining the results of the different methods used, 11 NoV genotypes were detected in wastewater samples in the study period. Eight genotypes were detected using the broad-range assay, with GII.4 NoV prevalent and GII.17 detected only in 2016. The use of the specific assay, conversely, demonstrated that GII.17 NoV has already been circulating since 2013 (data confirmed by NGS results), 2 years before it was first detected in gastroenteritis patients in Italy. In 2013, both the Kawasaki 308 and 323-like variants were detected, while all samples collected in 2015–2016 were characterized as Kawasaki 308-like. Furthermore, prevalence of GII.17 NoV in samples showed an increase over time: the genotype, in fact, was detected in less than 10% of samples in years 2013–2014, in 19% of samples in 2015, and reached 41% in 2016. Similarly, the Italian Study Group for Enteric Viruses, which monitors the epidemiology of enteric viruses in children through hospital-based surveillance in Italy, detected norovirus GII.P17_GII.17 Kawasaki 2014 in 3.3% of norovirus positive samples in 2015, but the percentage reached 18.4% in 2016, when it represented the third most common strain in Italy, after GII.P4 New Orleans 2009_GII.4 Sydney 2012 (34.7%) and GII.Pe_GII.4 Sydney 2012 (24.5%) (Giammanco et al. 2017).

Environmental studies documenting the increasing detection of GII.17 worldwide have been published in recent years. In Thailand, GII.17 was detected in river water by ultra-deep sequencing-based analysis as a minor population in 2013, and as a major genotype in 2014 (Boonchan et al. 2017). In Korea, GII.6 was described as the dominant strain in 2014 before it was replaced by GII.17 in 2015 in estuary water (Kim et al. 2016); however, GII.17 remained predominant until September 2015, but was later replaced by NoV GII.4 Sydney 2012 in 2016 (Choi et al. 2017). Recently, environmental surveillance for NoV in South African wastewaters revealed the emergence of the novel GII.17 in 2015–2016 (Mabasa et al. 2017). In Italy, GII.17 NoVs have been recently described in seawater discharges and in bivalve shellfish suggesting the potential for both waterborne and food transmissions (La Rosa et al. 2017).

As for the molecular methods used, the use of the GII.17 specific assay allowed us to detect more sewage samples positive for GII.17 NoV compared to the broad-range PCR, as already demonstrated previously with marine water and shellfish samples (La Rosa et al. 2017). Indeed, when more than one genotype is present in a sample (frequent condition in food or environmental samples), a broad-range assay may successfully amplify the type predominant in terms of concentration or the type towards which the primers show higher affinity, and, consequently, underestimate the prevalence of some, less common, genotypes. Therefore, a major disadvantage of the conventional amplicon sequencing approach is that only a limited number of representative sequences can be successfully obtained among the multiple viral strains potentially present in the samples. In this study, NGS proved to be an effective strategy for NoV genotyping in wastewater samples, since it confirmed results obtained by the broad-range PCR, and additionally detected three genotypes unidentified by conventional Sanger sequencing. Previous studies have demonstrated the advantages of NGS over classical Sanger sequencing for the study of norovirus diversity in urban wastewaters. Pyrosequencing techniques were successfully used to demonstrate the simultaneous existence and dominance of the GII.17 strains in sewage, oysters, and gastroenteritis cases in Japan (Pu et al. 2016). In another study using pyrosequencing, a total of 14 NoV genotypes, with up to eight genotypes detected per sample, were observed in wastewater in Japan (Kazama et al. 2016).

Of the NoV genotypes detected in this study, GII.1, GII.2, GII.3, GII.4, GII.6, and GII.7 are recognized as common genotypes worldwide, while other genotypes have been less frequently observed. For instance, GII.21 has been occasionally detected in clinical samples in several countries (Alam et al. 2016; Kumthip et al. 2017; Mans et al. 2016; Medici et al. 2014b; Rahman et al. 2016; Rupprom et al. 2017; Supadej et al. 2017; Timurkan et al. 2017) and in shellfish samples in Japan and in the US (Imamura et al. 2016; Woods et al. 2016). Moreover, a recent study on water samples from coastal streams and a neighboring waste water treatment plant in South Korea reported GII.21 NoV as the second highest prevalent genotype after GII.17 (Koo et al. 2017). In Italy, genotype GII.21 has been sporadically described in samples from pediatric patients (Medici et al. 2014a; Giammanco et al. 2017) and in a nosocomial outbreak (Valentini et al. 2017) but it has never been described in sewage or other environmental samples.

GII.13 and GII.16 are other genotypes regarded as globally uncommon (limited circulation both in frequency and geographical locations) (Hoa Tran et al. 2013). Recently, however, there have been an increasing number of reports describing the emergence of these genotypes worldwide (Cannon et al. 2017; Hoa-Tran et al. 2015; Khamrin et al. 2017; Vega et al. 2014). In Italy, GII.13 NoV has been detected in clinical samples (Medici et al. 2014a, b) and, more recently, in shellfish (La Bella et al. 2017). In the present study, GII.13 was constantly detected from 2013 to 2016. To our knowledge, this is the first study reporting

the detection of this genotype in water environments in our country and highlights that this genotype is steadily present in the population of the catchment area.

As for GII.16, this genotype has been sporadically detected worldwide (Alam et al. 2016; El Qazoui et al. 2014; Mans et al. 2016; Nakjarung et al. 2016; Portal et al. 2016; Supadej et al. 2017; Zhirakovskaia et al. 2015), and a small number of outbreaks have been described in association to this genotype (Vega et al. 2014). In Italy, as far as we know, GII.16 capsid sequences have never been described, while GII.P16 RpRd sequences have been obtained previously from clinical samples (Medici et al. 2014a, b).

GII.5 NoV is also relatively uncommon among GII genotypes; indeed, it caused only 0.3% of all norovirus outbreaks reported in the United States between 2009 and 2013 (Vega et al. 2014) and 0.6% in the period 2013–2016 (Cannon et al. 2017). Few studies have reported the identification of GII.5 NoV in clinical samples worldwide (Alam et al. 2016; Brown et al. 2016; Fukuda et al. 2010; Yoon et al. 2008) and recently a GII.5 strain has been associated with an outbreak among river rafters in the Grand Canyon, Arizona (Kitajima et al. 2017). To our knowledge, this is the first report of GII.5 NoV in Italy thus far.

In conclusion, our analyses revealed the circulation of several NoV genotypes in the environment, some of which are uncommon worldwide and detected for the first time in Italy. Moreover, we documented the introduction and progressive spread of GII.17 NoV in Italy, demonstrating that it was circulating in the human population, since 2013, before becoming a major strain in the population.

This study provides novel information on NoV genotypes circulating in Italy and confirms the usefulness of wastewater surveillance coupled with NGS to study the molecular epidemiology of NoV and to monitor the emergence of NoV strains.

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