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Rotavirus Genotypes in Sewage Treatment Plants and in Children Hospitalized with Acute Diarrhea in Italy in 2010 and 2011

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Although the molecular surveillance network RotaNet-Italy provides useful nationwide data on rotaviruses causing severe acute gastroenteritis in children in Italy, scarce information is available on rotavirus circulation in the general Italian population, including adults with mild or asymptomatic infection. We investigated the genotypes of rotaviruses present in urban wastewaters and compared them with those of viral strains from clinical pediatric cases. During 2010 and 2011, 285 sewage samples from 4 Italian cities were tested by reverse transcription-PCRs (RT-PCRs) specific for rotavirus VP7 and VP4 genes. Rotavirus was detected in 172 (60.4%) samples, 26 of which contained multiple rotavirus G (VP7 gene) genotypes, for a total of 198 G types. Thirty-two samples also contained multiple P (VP4 gene) genotypes, yielding 204 P types in 172 samples. Genotype G1 accounted for 65.6% of rotaviruses typed, followed by genotypes G2 (20.2%), G9 (7.6%), G4 (4.6%), G6 (1.0%), G3 (0.5%), and G26 (0.5%). VP4 genotype P[8] accounted for 75.0% of strains, genotype P[4] accounted for 23.0% of strains, and the uncommon genotypes P[6], P[9], P[14], and P[19] accounted for 2.0% of strains altogether. These rotavirus genotypes were also found in pediatric patients hospitalized in the same areas and years but in different proportions. Specifically, genotypes G2, G9, and P[4] were more prevalent in sewage samples than among samples from patients, which suggests either a larger circulation of the latter strains through the general population not requiring medical care or their greater survival in wastewaters. A high level of nucleotide identity in the G1, G2, and G6 VP7 sequences was observed between strains from the environment and those from patients.

Human group A rotaviruses (RVA) are responsible for severe gastroenteritis in children worldwide and cause ~450,000 deaths annually, mostly in developing countries (1). Rotavirus remains a common cause of morbidity with a significant economic burden in developed countries (2). Although reinfection may occur during life, most clinically relevant cases involve children <5 years old (3–5).

Rotaviruses are characterized by a double-stranded RNA (dsRNA) genome with 11 segments, which encode six structural proteins (VPs) and five or six nonstructural proteins (NSPs) (4). RVA are commonly classified based on genes encoding the outer capsid proteins, defining G (glycoprotein) (for VP7) and P (protease-cleaved protein) (for VP4) genotypes. Currently, 27 G and 37 P genotypes in humans and animals have been reported (6, 7). However, a limited number of GxP[y] genotype combinations are common in humans, such as G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] (7–9). Uncommon combinations, such as G8P[4], G12P[8], G12P[6], and others, have also been reported recently (10–13).

Rotavirus is transmitted through the fecal-oral route, directly from person to person, or by water and food contaminated with human or animal feces. After replication in the gastrointestinal tract, viruses are shed at very high concentrations (up to 10^{10} viruses/g) in feces and can persist in the environment for a long time (14–16). Like other enteric viruses, rotavirus is highly resistant to processes used in wastewater treatment plants (WWTPs), which can favor their spread into the environment (17–19), particularly in surface waters. However, RVA have been implicated in waterborne gastroenteritis outbreaks only sporadically (15, 20, 21).

Sewage contains enteric viruses shed by individuals with either overt disease or asymptomatic infection (22, 23), and molecular virus surveillance of urban sewage is therefore useful to assess potential threatening viruses circulating in the population, independent of subjects' age and disease severity.

Two live oral rotavirus vaccines have been used since 2006 in >100 countries worldwide (24), i.e., Rotarix (monovalent G1P[8] vaccine; GlaxoSmithKline) and RotaTeq (pentavalent, containing the G1 to G4 and P[8] genotypes; Sanofi-Pasteur MSD). Surveillance of RVA genotypes in patients is useful to identify viral strains causing residual cases in populations that use mass vaccination, particularly for possible emerging strains of zoonotic origin or imported strains. In Italy, molecular surveillance of RVA gastro-

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enteritis in hospitalized children (RotaNet-Italy) has been conducted since 2007 (25), as part of the EuroRotaNet network (9).

As an aid to clinical surveillance, monitoring of RVA in sewage ahead of WWTPs may provide an additional means to assess genotypes that also circulate in the normal population (26). However, detection and genotyping of RVA in sewage may be affected by the simultaneous presence of several common or uncommon strains, and the segmented nature of the rotavirus genome may preclude the definite identification of the full genome constellation of RVA detected. Possible RNA inhibitors in environmental samples may also interfere with molecular detection (27, 28).

In this study, we investigated the RVA genotypes present in sewage before entry into 10 WWTPs serving 4 cities located in different areas of Italy, comparing environmental strain genotypes with those detected in pediatric gastroenteritis cases occurring in the same cities and years.

MATERIALS AND METHODS

Virus and cell cultures. Control G1P[8] Wa rotavirus was grown in MA104 cells and titrated in 96-well microcultures after 24 h of infection and immunostaining with a rabbit antirotavirus hyperimmune serum and a peroxidase-labeled anti-rabbit antibody (Bio-Rad, Segrate, Italy), essentially as described previously (29).

Sewage samples. Inlet sewage was sampled at 10 urban wastewater treatment plants located in four different cities in Italy (Naples, Bari, Palermo, and Sassari), where environmental control is particularly considered due to the high magnitude of either tourism or immigration. The WWTPs of Bari and Naples started to collect samples in the last part of 2010, whereas the WWTPs of Palermo and Sassari arranged sample collection only in 2011. All WWTPs monitored are conventional activated sludge plants, receiving waters from urban areas. Daily flows range from 4,800 to 750,000 m^3, with design capacities of 70,000 to 1,000,000 population equivalents. In addition to human waste, Naples WWTPs also treat industrial wastewater occasionally (F. Pennino, University of Naples, personal communication). Sewage samples (1 liter) were taken by using sterile plastic bottles, correlating the number of samples with the sewer-linked population: 1 sample every 15 days for WWTPs serving >300,000 inhabitants and 1 sample every month for populations of <300,000 (Table 1). This sampling schedule was respected in most cases, but fewer samples were collected in some months due to logistic problems.

An automated 24-h sampling system was present in Palermo and Naples (Naples Est and Cuma), where a timer-operated valve allows collection of the total sample volume at regular intervals during 24 h. In Sassari, Bari, and Naples (San Giovanni a Teduccio) WWTPs, manual sampling was performed at selected sites during peak hours. Samples were kept at −20°C until processing was performed.

Rotavirus concentration in sewage. Sixty-five milliliters of sewage was clarified by centrifugation at 1,200 × *g* for 20 min at 4°C, and supernatants were centrifuged in a Beckman L8-80M ultracentrifuge using a Ti45 rotor at 126,000 × *g* for 2 h at 4°C, as described previously by Fumian and coworkers (30). Pellets were suspended in 1 ml of phosphate-buffered saline (PBS), and 140 μl was used for RNA extraction by using the Viral RNeasy minikit (Qiagen, Milan, Italy). Extracted RNAs were eluted in 50 μl of RNase-free water and stored at −80°C.

Rotavirus detection and G and P genotyping. Rotavirus RNA was amplified by reverse transcription-PCR (RT-PCR) using primers GEN_VP6_F and GEN_VP6_R (31). To increase sensitivity, a nested PCR was occasionally performed by using internal primers VP6-F and VP6-R (32). VP6-positive samples were genotyped for both VP7 and VP4 by nested PCR, as described previously (33, 34). The molecular size of genotyping PCR products was determined by agarose gel electrophoresis, using a Gel Doc XR molecular imager with Quantity-One software (Bio-Rad, Segrate, Italy).

TABLE 1 Detection of rotavirus in sewage samples from four WWTPs in Italy in 2010 and 2011

City	WWTP[a]	No. of inhabitants	No. of samples collected[b]			No. (%) of positive samples
			2010	2011	Total	
Bari	BF	300,000	15	24	39	27 (69.2)
	MdB	300,000	15	24	39	32 (82.1)
	J	300,000	14	24	38	28 (73.7)
	All		44	72	116	87 (75.0)
Palermo	AdC	130,000	NC	24	24	14 (58.3)
	FV	70,000	NC	20	20	13 (65.0)
	JH	70,000	NC	10	10	7 (70.0)
	All		0	54	54	34 (62.9)
Naples	NCu	1,000,000	NC	30	30	12 (40.0)
	NTe	700,000	9	24	33	13 (39.4)
	NE	500,000	8	20	28	7 (25.0)
	All		17	74	91	32 (35.1)
Sassari	SCa	120,000	NC	24	24	19 (79.2)
	All		61	224	285	172 (60.4)

[a] Abbreviations: BF, Bari Fesca; MdB, Mola di Bari; J, Japigia; AdC, Acqua dei Corsari; FD, Fondo Verde; JH, Jolly Hotel; NCu, Naples Cuma; NTed, Naples Teduccio; NE, Naples Est; SCa, Sassari Caniggia.
[b] NC, not collected.

Rotaviruses in clinical samples. A total of 343 fecal samples were collected from children with rotavirus diarrhea admitted to hospitals in Naples, Bari, Palermo, and Sassari in 2011 within the Italian RVA AGE surveillance program. Rotavirus infection was diagnosed by using a commercial immunochromatographic enzyme-linked immunosorbent assay (ELISA) or latex agglutination methods in use in each hospital. For strain characterization, stool samples were diluted 10% in distilled water, and rotavirus RNA was extracted with the Viral RNeasy minikit and directly subjected to G and P genotyping, performed as described above for sewage samples (33, 34). All samples failing both G and P genotyping were confirmed to be rotavirus positive by RT-PCR amplification of the VP6 gene (32).

Sequencing and phylogenetic analysis. A subgroup of samples was selected randomly for each city, and the respective G and P amplicons were characterized by nucleotide sequencing using PCR primers at Macrogen Inc. (Seoul, South Korea). Chromatograms were analyzed with Chromas Pro 2.23 (Tecnelysium) and aligned with SeqMan II (DNAstar). The phylogenetic dendrograms were constructed with the neighbor-joining method, using the Kimura two-parameter model with MEGA5.1 software (35). The robustness of each node was assessed by 1,000 bootstrap replications. All relevant sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) were used in comparisons. RVA VP7 and VP4 genotypes were defined according to guidelines of the Rotavirus Classification Working Group (RCWG) (31).

Evaluation of rotavirus concentration protocols. To control the efficiency of ultracentrifugation for concentrating rotaviruses from sewage, virus recovery was determined by testing 65-ml sewage samples spiked with 1 ml of a viral stock suspension containing 3×10^5 focus-forming units (FFU)/ml of human Wa rotavirus (~1×10^4 genome PCR units/ml). Ultracentrifugation pellets were serially diluted (1:1.5 and 1:2 steps) and analyzed by VP6 RT-PCR using primers VP6-F and VP6-R (32). The last dilution yielding a discernible PCR-amplified DNA band was considered to contain a rotavirus genome PCR unit. The ratios between molecular titers before and those after the concentration procedure were used to calculate the recovery efficiency.

Testing for inhibitors of RT-PCR and rotavirus in sewage. Preliminary experiments were conducted to investigate the possible presence of

TABLE 2 Distribution of rotavirus G and P genotypes in inlet wastewater in four Italian cities in 2010 and 2011

Genotype	No. (%) of samples								
	2010		2011				2010 total	2011 total	2010–2011 total
	Naples	Bari	Naples	Bari	Palermo	Sassari			
G types									
G1	8 (88.8)	40 (81.6)	17 (56.7)	28 (53.8)	19 (50.0)	18 (90.0)	48 (82.7)	82 (58.6)	130 (65.6)
G2	1 (11.2)	6 (12.2)	6 (20.0)	14 (26.9)	12 (31.6)	1 (10.0)	7 (12.1)	33 (23.6)	40 (20.2)
G3	0	0	1 (3.3)	0	0	0	0	1 (0.7)	1 (0.5)
G4	0	3 (6.2)	3 (10.0)	2 (3.9)	1 (2.6)	0	3 (5.2)	6 (4.3)	9 (4.6)
G9	0	0	2 (6.7)	6 (11.5)	6 (15.8)	1 (10.0)	0	15 (10.7)	15 (7.6)
G6	0	0	0	2 (3.9)	0	0	0	2 (1.4)	2 (1.0)
G26	0	0	1 (3.3)	0	0	0	0	1 (0.7)	1 (0.5)
Total	9	49	30	52	38	20	58	140	198
P types									
P[8]	8 (72.7)	41 (87.2)	20 (74.0)	35 (63.6)	30 (66.7)	19 (100.0)	49 (84.4)	104 (71.2)	153 (75.0)
P[4]	3 (27.3)	6 (12.8)	5 (18.5)	18 (32.8)	15 (33.3)	0	9 (15.6)	38 (26.0)	47 (23.0)
P[6]	0	0	1 (3.7)	0	0	0	0	1 (0.7)	1 (0.5)
P[9]	0	0	0	1 (1.8)	0	0	0	1 (0.7)	1 (0.5)
P[14]	0	0	0	1 (1.8)	0	0	0	1 (0.7)	1 (0.5)
P[19]	0	0	1 (3.7)	0	0	0	0	1 (0.7)	1 (0.5)
Total	11	47	27	55	45	19	58	146	204

chemical inhibitors of RT-PCR in sewage. RNA extracts from concentrated sewage samples that were negative by RT-PCR were spiked with serial dilutions of RNA extracted from RVA-positive fecal samples and retested by VP6-specific RT-PCR. The results of the test were compared with results for control RNA samples diluted in RNase-free water.

Separate experiments were also performed to exclude possible rotavirus damage following protracted presence in sewage. To simulate field conditions, untreated samples of rotavirus-negative sewage from the different plants or distilled water were spiked with 3×10^5 FFU/ml of human Wa rotavirus and left to stand at room temperature (RT) for 24 h. Titration of residual rotavirus persisting in each sample was then performed by endpoint VP6-specific RT-PCR (see above), and the ratios between viral titers in sewage and those in clean water were calculated.

Statistical assays. The Z-test (36), Fisher's exact test (http://www.langsrud.com/fisher.htm), and Pearson's correlation and Lin's correlation concordance coefficients (37, 38) were used to evaluate possible differences and correlations between the distributions of RVA genotypes in sewage and clinical samples.

Nucleotide sequence accession numbers. The nucleotide sequence data obtained in this study have been submitted to GenBank under accession numbers KF414532 to KF414624.

RESULTS

Efficiency of rotavirus recovery by ultracentrifugation. To evaluate the efficiency of ultracentrifugation in recovery of virus, 20 sewage samples were collected from each WWTP, at the start and at the end of the sampling period, and were spiked with Wa rotavirus. After analysis of serial dilutions of the ultracentrifugation pellets by VP6 RT-PCR, it was calculated that between ≥30 and <67% of spike virus RNA was recovered from the different samples.

Since the sewage sample was eventually concentrated 65-fold, a 21-fold virus concentration or higher was attained throughout the process.

Rotavirus detection and distribution of G and P genotypes in sewage samples. In 2010 and 2011, 285 sewage samples were collected from the WWTPs of the four cities monitored (Table 1). Altogether, 172 (60.2%) samples tested positive by rotavirus VP6 RT-PCR. The proportion of RVA-positive samples ranged between 58.3 and 82.1% in 7 WWTPs serving three cities and was lower (25.0 to 40.0%) for the three WWTPs of Naples.

The RVA G and P genotypes identified in sewage samples are shown in Table 2. The VP7 genotype detected more frequently was G1 (130/172 sewage samples), followed by G2 (40 cases), G9 (15 cases), G4 (9 cases), and G3 (1 case). Genotypes G6 and G26 were detected in 2 samples and 1 sample, respectively. In 32 cases, 2 different genotypes were detected in the same sample. These genotypes were mostly G1 with either G2 or G4 (26 and 3 cases, respectively). Genotypes P[8] and P[4] were found in 153 and 47 sewage samples, respectively. Multiple P genotypes were detected in 38 samples, 4 of which contained the uncommon P[6]+P[8], P[9]+P[4], P[14], and P[19] genotypes, respectively. Twelve samples contained G1+G2-P[4]+P[8] virus.

Absence of RT-PCR inhibitors and rotavirus inactivation in sewage samples. To verify if the lower RVA detection rate obtained consistently in the WWTPs of Naples could be due to RT-PCR inhibitors being present in sewage, negative RNA samples extracted from all WWTPs were retested by RT-PCR after spiking with rotavirus RNA. No difference between spiked sewage and RNase-free water samples was observed (see Table S1 in the supplemental material).

Possible rotavirus destruction by a prolonged stay in sewage was also investigated by spiking two raw sewage samples that had tested RVA negative in each WWTP with RVA Wa. The residual virus after 24 h of incubation at RT was titrated by endpoint VP6 RT-PCR. No decrease in virus titer was observed for any sample, excluding the occurrence of significant rotavirus damage in any of the WWTP samples investigated (see Table S2 in the supplemental material).

Prevalence of rotavirus G and P genotypes in clinical samples. Three hundred forty-three rotaviruses from stool samples of

TABLE 3 Rotavirus G/P genotypes in samples from children hospitalized with acute gastroenteritis in four Italian cities in 2011

Genotype	No. (%) of samples				
	Naples	Bari	Palermo	Sassari	Total
Common					
G1P[8]	37 (56.1)	77 (79.5)	62 (68.1)	79 (88.8)	255 (74.4)
G2P[4]	5 (7.6)	5 (5.1)	10 (11.0)	3 (3.4)	23 (6.7)
G3P[8]	4 (6.1)	2 (2.1)	0	0	6 (1.7)
G4P[8]	0	8 (8.2)	0	0	8 (2.3)
G9P[8]	10 (15.2)	2 (2.1)	0	2 (2.2)	14 (4.1)
Total common	56 (84.8)	94 (97.0)	72 (79.1)	84 (94.4)	306 (89.2)
Uncommon					
G3P[9]	0	0	1 (1.1)	0	1 (0.3)
G10P[8]	0	0	1 (1.1)	0	1 (0.3)
G12P[8]	0	1 (1.0)	0	0	1 (0.3)
G8P[4]	0	0	0	1 (1.1)	1 (0.3)
G1P[4]	0	0	1 (1.1)	0	1 (0.3)
Total uncommon	0	1 (1.0)	3 (3.3)	1 (1.1)	5 (1.4)
Mixed types					
G1,G2P[4,8]	2 (3.0)	0	0	4 (4.5)	6 (1.7)
G2,G9P[4,8]	4 (6.1)	0	0	0	4 (1.2)
G2,G9P[8]	0	1 (1.0)	0	0	1 (0.3)
G1,G4P[8]	0	1 (1.0)	0	0	1 (0.3)
G1,G9P[8]	2 (3.0)	0	0	0	2 (0.6)
G1,G2P[4]	0	0	2 (2.2)	0	2 (0.6)
Total mixed types	8 (12.1)	2 (2.0)	2 (2.2)	4 (4.5)	16 (4.7)
Untypeable					
GNtP[8]	1 (1.5)	0	7 (7.7)	0	8 (2.3)
GNtP[4]	0	0	4 (4.4)	0	4 (1.2)
GNtP[9]	0	0	1 (1.1)	0	1 (0.3)
G1P[Nt]	0	0	1 (1.1)	0	1 (0.3)
G2P[Nt]	1 (1.5)	0	0	0	1 (0.3)
GNtP[Nt]	0	0	1 (1.1)	0	1 (0.3)
Total untypeable	0	0	14 (15.4)	0	16 (4.7)
Total	66 (100.0)	97 (100.0)	91 (100.0)	89 (100.0)	343 (100.0)

clinical pediatric cases in Bari, Naples, Palermo, and Sassari were genotyped by RT-PCR. Altogether, G1 was the predominant genotype (74.6% of strains investigated), followed by G2 (10.3%), G9 (5.8%), G3 (2.0%), and G4 (2.5%) (Table 3). Uncommon G8, G10, and G12 rotaviruses were observed in a single case each. A G genotype could not be defined for 14 samples, although the presence of RVA was confirmed by either P genotyping or VP6 RT-PCR. The common G types G1, G3, G4, and G9 and the common G type G2 were associated mostly with the common P types P[8] (87.0%) and P[4] (11.5%), respectively (Table 3).

Sixteen (4.7%) and ten samples presented dual G and P genotypes, respectively, indicating mixed rotavirus infection.

Comparison of rotavirus genotypes from sewage samples and samples from gastroenteritis cases. Overall, the relative distribution of the G and P RVA genotypes detected in sewage sample (Table 2) was similar to that detected in acute gastroenteritis (AGE) patients (Table 3), with G1 and P[8] accounting for most of the genotypes identified in both cases. In particular, comparison of the distributions of different genotypes in sewage samples and patient samples by correlative statistic tools yielded high concordance, i.e., Pearson's correlation coefficient of 0.957 and Lin's correlation concordance coefficient of 0.933 (not shown).

Although it is likely that the viruses found in the WWTPs reflect the strains circulating in the population, comparisons of the two sets of data have some limitations. In fact, the detection of particular G and P types may indicate either a higher concentration or a higher stability of these strains in sewage, whereas the data from patients correspond to the actual numbers of children infected with each RVA strain.

The proportions of sewage samples and samples from children where specific RVA genotypes were detected in 2011 presented some differences between cities. In particular, in Bari and Palermo, genotype G1 was detected in 80% and 68% of patients, respectively, whereas this genotype was present in 54% and 50% of sewage samples, respectively. In contrast, G2 RVA infected 5% and 11% of children with AGE in Bari and Palermo, respectively, and was detected in 27% and 32% of the corresponding sewage samples (Tables 2 and 3). Equivalent ratios were also found for

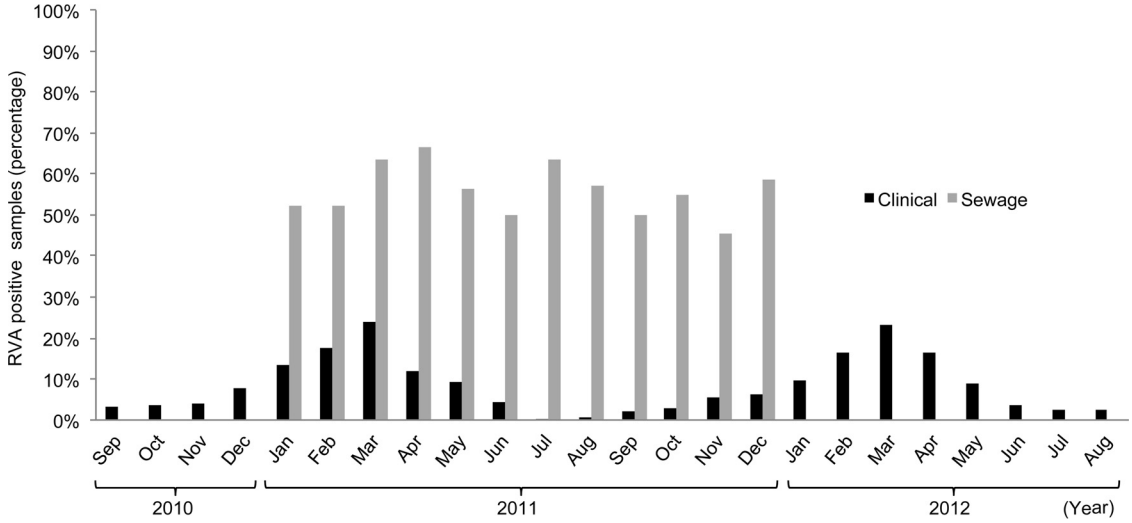

FIG 1 Distribution of RVA-positive sewage samples (2011) and samples from pediatric AGE cases (2010 to 2012) in Naples, Bari, Palermo, and Sassari, by month.

comparison of the P[8] and P[4] genotypes in Bari and Palermo. In the other two cities, the ratios between genotypes G1 and G2 in patient samples were very similar to those determined in sewage samples.

G9 RVA was found for between 7% (Naples) and 16% (Palermo) of sewage samples but was detected in a high percentage (15%) of patient samples only in Naples, being rare elsewhere (<2.2%).

The uncommon G6 genotype was detected in two sewage samples from Bari, one of which also contained the rare P[14] genotype. Other uncommon genotypes included G8 and G10 strains in samples from two patients in Sassari and Palermo, respectively, and three P[9] strains in samples from two patients in Palermo (Table 3) and in one sewage sample from Bari (Table 2).

The two rare P[6] and P[19] genotypes detected in Naples were present only in sewage (1 sample each), despite sewage samples in this city being positive for RVA less frequently than elsewhere. This observation may suggest that these uncommon RVA circulate in the population more extensively than is indicated by patient strain genotyping.

Figure 1 reports the average proportions of RVA-positive sewage samples by month in 2011, when samples were taken from all four cities of the study, showing the occurrence of RVA throughout the year. For a better comparison with the marked seasonal distribution of rotavirus infections, data on pediatric patients were reported for the period from September 2010 to August 2012, including two sequential winter-spring epidemic peaks. Whereas patients presented a marked seasonal distribution, with cases peaking in March and being almost absent during summer, the percentage of RVA-positive sewage samples ranged between 45 and 67% throughout the year, showing no specific seasonal trend.

Phylogenetic analysis of rotavirus G and P genotypes. Seventy-one strains with common G types (G1 to G3 and G9) detected in either wastewater (32) or clinical (39) samples in the cities investigated in 2011 were selected randomly and subjected to VP7 and VP4 gene sequencing and phylogenetic analysis.

All G1 strains sequenced presented high nucleotide identity (97.5 to 100%), regardless of the source and city, except for strain NA11-112, identified in Naples (Fig. 2a), which coclustered with the Wa (G1P[8]) prototype strain. Sewage sample strain NA11-112 also contained a P[8] sequence that coclustered with the P[8] Wa strain separately from the other P[8] sequences analyzed in this study.

The nucleotide identities were also very high (97 to 99%) among the G2 strains, except for strain NA11-15, which presented a >10% difference from all other G2 sequences (Fig. 2b). Similarly, most G9 clinical strains and the G9 sewage strain NA11-152 from Naples coclustered within the G9-III lineage, except for sewage strains J11-06 and F11-11 from Bari (Fig. 2c).

The G3 VP7 gene sequence from a sewage sample from Bari was closely related to a G3P[6] strain reported in Belgium in 2009 (Fig. 2c). A second strain formerly genotyped as G3 by PCR was eventually assigned to the rare G26 genotype after sequencing, showing strict relatedness to swine G26 strain TJ4-1, detected in Japan in 2010 (Fig. 2c). The generation of a G3-sized amplicon was likely due to the 74% sequence identity found between nucleotides 250 and 268 of the G26 gene and the G3 primer.

The uncommon G6 strains M11-07 and M11-12 detected in the Bari WWTP in April and June (Fig. 2d) were identical and presented a 96% similarity to human G6P[14] strain BA46 detected in Bari 1 year later and to other G6P[14] RVA strains identified in Italy during 1988 to 2005. The P[14] genotype found in Bari sewage sample strain M11-07 showed 93.5% nucleotide identity with G6P[14] clinical strain BA46 (Fig. 2e).

Five of the common P[8] sequences from Naples, Bari, and Sassari WWTPs exhibited high nucleotide identity to two clinical P[8] sequences identified in Naples and to other strains from GenBank. The only sewage P[4] strain sequenced presented 98% identity to Italian strains PA84/2008 and PA3/2004.

A rare P[19] genotype was detected in sewage sample strain NA11-144 (Fig. 2e), which also yielded the uncommon G26 genotype (Fig. 2c). This P[19] sequence was 97 to 98% identical to two human G1P[19] and G9P[19] strains previously identified in India.

The P[6] genotype from sewage sample strain NA11-148 (also

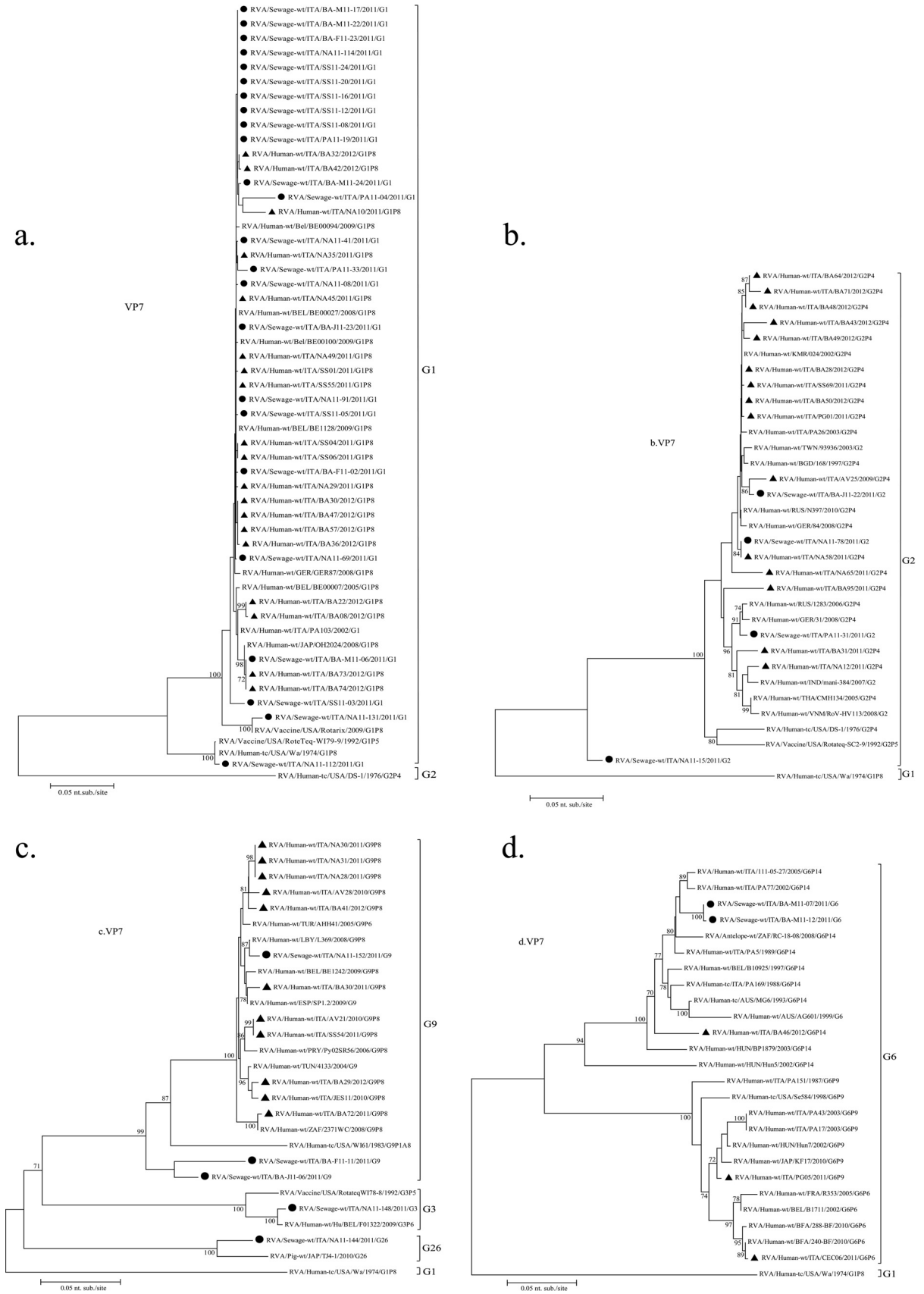

FIG 2 Phylogenetic dendrograms based on partial VP7 sequences of genotypes G1 (a), G2 (b), G3 and G9 (c), and G6 (d) and on partial VP4 nucleotide sequences of genotypes P[8], P[4], P[14], P[19], P[6], and P[9] (e). RVA strains from Italy were detected in sewage and clinical samples. All sequences obtained from GenBank are named as described previously by Matthijnssens et al. (7), and G and P genotypes are indicated on the right. Environmental samples are marked with filled circles; AGE samples are marked with filled triangles. The scale bar at the bottom of the tree indicates the number nucleotide substitutions/site. Bootstrap values (2,000 replicates) are shown at the branch nodes; values of <70 are not shown.

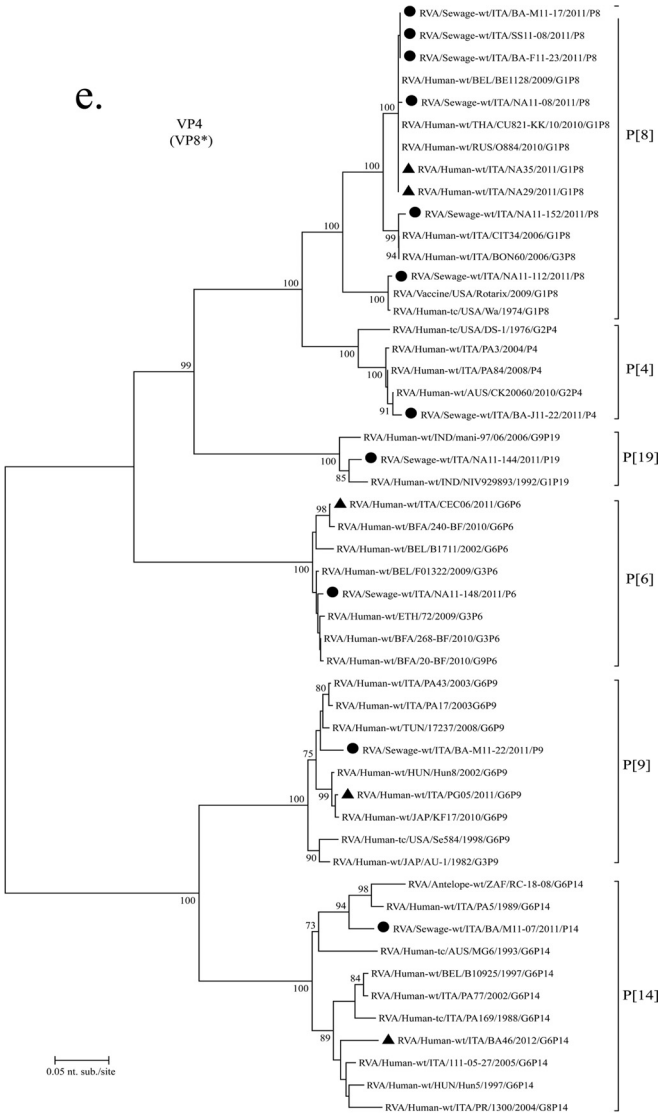

FIG 2 continued

containing G3) presented 98% identity with pediatric strain RVA/Human-wt/ITA/CEC06/2011/G6P[6], reported in central Italy in 2011. The P[9] genotype from sewage sample strain BA-M11-22 (also containing genotype G1) showed only 95% nucleotide identity to the contemporary strain RVA/Human-wt/ITA/PG05/2011/G6P[9] but coclustered with older G6P[9] Italian strains.

DISCUSSION

We investigated the correlation between rotavirus genotypes G and P in strains from inflowing water in the WWTPs of four Italian cities and the RVA strains isolated from children with severe gastroenteritis, identified by the RotaNet-Italy surveillance network in the same cities, in 2011.

Combined ultracentrifugation and molecular methods permitted efficient rotavirus recovery and RNA detection in sewage samples, altogether yielding a 20-fold rotavirus concentration or higher with removal of PCR inhibitors. This method of viral concentration in sewage samples was described previously by Fumian and coworkers (30) and was demonstrated to work better than adsorption-elution protocols. In our hands, this method allowed a rate of recovery of the original virus in spiked samples of at least 30% to between 50 and 67%, which is in the same order as that reported by Fumian and coworkers (45%), with some differences between samples.

Besides allowing rapid and sensitive rotavirus identification, the molecular methods applied offer the additional advantage of being easily extended to the simultaneous detection of other enteric viruses.

Although molecular detection may not assess infectious RVA in sewage, the detection of viral RNA is meaningful, since wild RVA strains are normally resistant in the environment (39).

The finding of RVA in a large fraction of the sewage samples examined indicates both that considerable and continuous virus circulation occurs in the populations of the cities investigated and that RVA is shed with feces in large amounts, permitting its detection despite extensive dilution in sewage.

Moreover, detection of rotavirus in sewage also points out possible risks for human health, particularly in the case of wastewater treatment failures, as may occur during heavy raining and flooding.

Only a few other studies compared rotavirus strains in samples from pediatric AGE patients and sewage in the same location and year (40–43). In this study, RVA genotyping and phylogenetic analysis of common VP7 genotype G1, G2, G3, and G9 sequences showed high similarity between clinical and environmental strains. These findings indicate that rotaviruses released into urban sewage largely match the RVA strains that cause severe disease in children, implying that common RVA genotypes are also involved in asymptomatic or mild infection of adults and children not requiring hospitalization.

It should be considered that coverage with rotavirus vaccine in Italy, particularly in the period considered in this study, did not exceed 5% of the pediatric population, including the cities monitored. Therefore, the genotypes found to be circulating were not receiving any selective pressure, which Matthijnssens et al. (44) hypothesized was induced by mass vaccination, favoring specific viral strains. However, other studies disagree with any occurrence of genotype replacement in largely vaccinated populations (5, 45), rather highlighting that common genotypes, particularly G1P[8], remain predominant despite the overall decrease in the number of cases.

A high prevalence of genotypes G2 and G9 was observed in sewage samples from Palermo and Bari, while these genotypes were infrequently associated with cases of disease in children in this study. The prevalence of genotype G2 in sewage was confirmed by the correspondingly high rate of detection of genotype P[4], normally associated with G2P[4] strains. It is possible that the higher rate of detection of G2 and P[4] merely reflects temporal fluctuations in the circulation of different genotypes in the population. Nevertheless, this finding might otherwise indicate a different persistence of distinct viruses in sewage or higher rates of intestinal replication of individual strains, which would result in different viral concentrations in wastewater. In fact, fewer sewage samples were found to be positive for rotavirus in Naples, where a higher dilution of fecal viruses in the city WWTPs is very likely, since these WWTPs also collect industrial wastewaters in addition to urban waste.

Uncommon G6 and P[14] sequences were detected in sewage twice in 2011 in Bari. Interestingly, these RVA strains were related

phylogenetically to a genotype G6P[14] strain detected in the stool samples of a patient in the same city in 2012. This findings may suggest that a rare G6P[14] RVA strain circulated in the city population for at least a year, being shed into the sewer system at high concentrations. It is tempting to believe that environmental surveillance may help predict the circulation of emerging RVA strains before symptomatic cases are detected, as observed with other enteric viruses (46).

Although related, the G6 strains from sewage samples in 2011 and the patient in 2012 in Bari were not identical, suggesting that the same autochthonous strain evolved obviously between 2011 and 2012. All other G6 sequences from other cities belonged to completely separate clusters.

The rare G26 strain NA11-144 detected in sewage samples from Naples showed high sequence similarity to a Japanese strain of apparent swine origin. The sewage sample also contained a rare P[19] genotype, previously detected in a G1P[19] strain from a patient in India, which was proposed to represent a human/swine reassortant (47). The simultaneous presence of typically swine genotypes G26 and P[19] in the same sewage sample suggests that animal feces may have been disposed of into the urban wastewater sewer system of Naples. Although animal waste should not merge with human drainage, we cannot exclude that unauthorized dumping from a nearby swine farm or slaughterhouse may have occurred.

The initial erroneous identification of G26 RVA as G3 was likely due to sequence conservation of the G3 primer-binding region in the G26 strain, generating a typical G3-like amplicon by nested PCR. Because this may in principle lead to wrong genotyping and a lack of detection of other uncommon emerging RVAs, the G3-specific primer may need to be redesigned. At present, whenever an uncommon G or P genotype is identified in association with an apparently common P or G type, sequencing of both genes may be recommended to avoid mistyping.

Interestingly, whereas most hospitalized rotavirus cases clustered between January and April 2011 and cases were virtually absent in July and August, RVA was constantly found in sewage samples in all months investigated. Similar observations of the persistence of RVA in sewage have been reported previously, mostly independent from the seasonal pattern of hospitalized RVA cases (40, 48, 49), and indicate that the population maintains a high level of virus shedding throughout the year, independent of pediatric clinical disease. The lack of seasonality in virus discharge in sewage suggests that rotaviruses might circulate through asymptomatic or subclinical reinfections of children and adults, before a new epidemic starts among susceptible young children.

In conclusion, this paper suggests that environmental monitoring of sewage provides a good assessment of RVA genotypes circulating in the local human population, with minor differences with respect to the strains causing severe infantile diarrhea. Also, uncommon RVA strains might be detected in sewage earlier than in patients, which might help in preparation for the future spread of emerging strains. Environmental monitoring of WWTPs might be complementary to molecular RVA surveillance of clinical cases within vaccine monitoring programs.

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