Food and Environmental Virology (2018) 10:333–342
https://doi.org/10.1007/s12560-018-9350-8

ORIGINAL PAPER

Poliovirus and Other Enteroviruses from Environmental Surveillance in Italy, 2009–2015

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Received: 5 February 2018 / Accepted: 8 June 2018 / Published online: 12 June 2018

Abstract

Within the initiatives for poliomyelitis eradication by WHO, Italy activated an environmental surveillance (ES) in 2005. ES complements clinical Acute Flaccid Paralysis (AFP) surveillance for possible polio cases, detects poliovirus circulation in environmental sewage, and is used to monitor transmission in communities. In addition to polioviruses, the analyses comprised: (i) the monitoring of the presence of non-polio enteroviruses in sewage samples and (ii) the temporal and geographical distribution of the detected viruses. From 2009 to 2015, 2880 sewage samples were collected from eight cities participating in the surveillance. Overall, 1479 samples resulted positive for enteroviruses. No wild-type polioviruses were found, although four Sabin-like polioviruses were detected. The low degree of mutation found in the genomes of these four isolates suggests that these viruses have had a limited circulation in the population. All non-polio enteroviruses belonged to species B and the most frequent serotype was CV-B5, followed by CV-B4, E-11, E-6, E-7, CV-B3, and CV-B2. Variations in the frequency of different serotypes were also observed in different seasons and/or Italian areas. Environmental surveillance in Italy, as part of the 'WHO global polio eradication program', is a powerful tool to augment the polio surveillance and to investigate the silent circulation or the re-emergence of enteroviruses in the population.

Keywords Enteroviruses · Poliovirus · Environmental surveillance · Sewage · Italy

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Introduction

Human Enteroviruses belong to the genus Enterovirus (EV), family Picornaviridae, and are small RNA-positive viruses. Currently, they are classified into four species (Enterovirus A, B, C and D), with an increasing number of serotypes for each one (Tapparel et al. 2013; Adams et al. 2016). They include Coxsackieviruses A and B, Echoviruses, a number of enteroviruses and Polioviruses (PVs) 1–3.

Human EVs have a relevant impact on public health, causing severe diseases that can occur as outbreaks. These diseases include meningitis, encephalitis, myocarditis and pancreatitis, foot and mouth disease, febrile illness, and poliomyelitis (also known as polio), one of the most severe human diseases (Mirand et al. 2012).

As EVs are non-enveloped viruses, they are resistant to chloroform and to all other lipid solvents. They persist in the environment also in critical conditions, e.g., extreme changes in pH and temperature (LaBelle and Gerba 1979; de Roda Husman et al. 2009; Petrinca et al. 2009).

In Italy, the last case of poliomyelitis caused by wild-type PV was notified in 1982. In June 2002, Italy and all World Health Organization (WHO) European Region Member States were declared *polio-free* countries by the Regional Commission for the Certification of Poliomyelitis Eradication (RCC). However, Italy, for its particular geographical position and high levels of immigration across the Mediterranean basin, remains at risk of reintroduction of wild-type PV from endemic areas or Sabin-like (SL) and Vaccine-derived polioviruses (VDPV) from countries currently using oral attenuated polio vaccine (OPV, Sabin vaccine). It should be noticed that in Italy since 2002, OPV has been replaced by inactivated polio vaccine (IPV, Salk vaccine) (WHO 2002).

In the global action plan for poliomyelitis eradication by WHO, environmental surveillance complements clinical Acute Flaccid Paralysis (AFP) surveillance for possible polio cases detects poliovirus circulation in environmental sewage and is used to monitor transmission in communities.

Environmental surveillance is based on the observation that infected people, including those who are asymptomatic, shed large amounts of virus into the wastewater system, making effective this type of detection (Hovi et al. 2012; GPEI 2015). In Israel, Egypt, Slovakia, Finland, and Switzerland, where the Salk vaccine has been in use for a long time, wild-type PV and VDPV, with several mutations and regained neurovirulence, were detected in the environment despite the absence of clinical cases reported in the population (Manor et al. 2014; GPEI 2015; Roivainen et al. 2010; Zurbriggen et al. 2008; Blomqvist et al. 2012). In this context, environmental surveillance played a critical role in detecting these viruses. A prompt extensive and coordinated National Public Health response successfully prevented PVs from spreading.

In addition to PV, Coxsackievirus (CV), Echovirus (E), and other EVs represent a public health issue in industrialized countries, causing severe diseases particularly among young people (Battistone et al. 2014a).

EVs were detected in wastewaters, rivers, seas, and recreational waters (Pianetti et al. 2000; Hughes et al. 1992; Lee et al. 2005), which can all represent efficient virus transmission routes to humans (Rajtar et al. 2008; Amvrosieva et al. 2001; Scarcella et al. 2009; Chikhi-Brachet et al. 2002). Since crop irrigation with treated wastewaters is frequently used (Steele and Odumeru 2004; Munoz et al. 2010; Viau et al. 2011; Moazeni et al. 2017), vegetables or fruits contaminated with viruses contained in sewage can be a threat for the consumer. Data from environmental monitoring and the increasing attention paid to water-transmitted diseases have prompted technological improvement in wastewater-treatment protocols (Petrinca et al. 2009; Simmons and Xagoraraki 2011; Francy et al. 2012; Battistone et al. 2014b).

A National Plan for environmental surveillance of human EVs has been implemented by the Italian Ministry of Health since 2005 in collaboration with the Istituto Superiore di Sanità (ISS) and several universities in different Italian Regions. As data on the distribution of specific human EV serotypes in sewage in Italy are currently limited (Battistone et al. 2014a; Pellegrinelli et al. 2017; Pennino et al. 2018), we decided to carry out the present study with the following aims: (i) to rule out the reintroduction of PV in Italy in 7 years of environmental surveillance (2009–2015); (ii) to monitor the presence of non-polio enteroviruses (NPEVs) in sewage specimens; and (iii) to evaluate temporal and geographical distribution of the identified viruses.

Materials and Methods

Wastewater Sampling

Sewage samples, collected from 2009 to 2015 at the inlet Wastewater-Treatment Plants (WWTPs) from the collector sewers serving Bolzano, Venice, Sassari, Naples, Bari and Palermo, were sent and analyzed at the ISS, the WHO Regional Reference Laboratory (RRL). In the case of the samples from Parma and Milan, these were locally analyzed at the accredited subnational polio reference laboratories (University of Parma and University of Milan, respectively). The analysis carried out at these laboratories consisted in discriminating the presence of either PVs or NPEVs in the samples by means of cell cultures and virus isolation. At the ISS, this analysis was performed on the samples from all the

other Italian cities. In addition, the ISS carried out on all samples the characterization of PVs and NPEVs by means of real-time PCR and RT-nested-PCR. In accordance with the WHO guidelines (WHO 2003), the number and volume of samples for each collector were defined. In fact, samples were collected every month and the number of samples per site was calculated under the catchment area: from a minimum of 1–2 for smaller collectors to a maximum of 4 for the larger ones. Usually, only one sample per month was taken from WWTPs serving less than 100,000 inhabitants and two or more per month from WWTPs serving more than 100,000 inhabitants. One-liter samples of raw sewage were collected during the peak hours of household sewage flow (Table 1).

Virus Concentration

Sewage samples were collected in appropriate sterile polypropylene bottles and stored at –20 °C until transported to the ISS under refrigerated conditions. Five hundred ml of each samples were then concentrated by the two-phase separation method [Polyethylene Glycol (PEG)-dextran] and decontaminated by chloroform extraction to kill bacteria and to eliminate mildew, as recommended by the WHO guidelines for environmental surveillance (WHO 2003). After treatment, 10 ml of concentrated sample were obtained, corresponding to an approximately 50-fold volume reduction.

Determination of Virus Recovery Efficiency

To evaluate the virus detection capability of the WHO two-phase concentration method, six raw sewage samples (500 ml each) were autoclaved at 121 °C for 30 min to inactivate possible live viruses. Five positive controls were obtained by spiking sewage samples with 2×10^5, 2×10^3, 20, 2, and 0.2 50% cell culture infective doses ($CCID_{50}$) of PV type 1 Sabin, while a sixth non-spiked sample was added as negative control. Both types of controls were concentrated up to 10 ml. A rate of 0.5 ml volume of chloroform-extracted sewage concentrate was inoculated in duplicate on human rhabdomyosarcoma (RD) cell monolayers in 50-ml cell culture flasks. Two serial blind passages were carried out.

Cell Cultures and Virus Isolation

Virus isolation was performed inoculating 0.5 ml of the 10-ml chloroform-extracted sewage concentrate on two RD cell monolayers (permissive to EVs) and two L20B cell monolayers (murine cell line L-series, genetically modified, selective for PVs, transfected with the receptor for human

Table 1 Sampling per year for each collector and city during environmental surveillance in Italy, 2009–2015

Cities	Collector	Catchment area	No, Samples							Total
		(inhabitants)	2009	2010	2011	2012	2013	2014	2015	
Bolzano	Bolzano	374,000	18	23	24	24	24	23	23	159
Milan	Nosedo	300,000	24	29	23	18	25	24	24	167
	Nosedo Est	300,000	24	30	14	18	25	23	24	158
	Peschiera	300,000	21	30	23	27	24	24	24	173
Venice	Cavarzere	17,500	27	22	25	18	–	–	–	92
	Ceggia	5000	24	21	22	15	–	–	–	82
	Musile	10,000	25	21	23	16	–	–	–	85
	Campalto	110,000	27	22	24	17	-	–	–	90
	Fusina	330,000	29	24	26	19	–	–	–	98
Parma	Est	130,000	–	32	23	22	24	23	23	147
	Ovest	160,000	–	32	23	22	24	23	24	148
Sassari	Caniggia	120,000	24	24	24	24	21	24	18	159
Bari	Fesca	300,000	25	26	24	24	–	–	7	106
	Mola	300,000	24	25	24	24	–	–	8	105
	Japigia	300,000	24	25	24	24	–	–	8	105
Naples	Cuma	1,000,000	19	26	47	49	46	50	49	286
	S.G. Teduccio	700,000	10	12	24	25	23	24	23	141
	Est	500,000	10	12	24	25	23	24	23	141
Palermo	Acqua dei Corsari	130,000	26	23	20	28	30	31	26	184
	Fondo Verde	70,000	12	11	10	12	14	14	12	85
	Via Diaz	70,000	12	11	10	12	14	14	12	85
	Jolly Hotel	70,000	11	11	10	12	14	14	12	84
Total		7,806,500	416	492	491	475	331	335	340	2880

PV) in 50-ml cell culture flasks in accordance with the WHO algorithm (WHO 2004, 2014).

Two serial blind passages were carried out with both cell lines and samples with cytopathic effect (CPE) on RD cells were passaged on L20B to amplify PV, if present. Samples showing CPE in both cell lines were classified as "suspected PVs", whereas those showing CPE only in RD cells were classified as "NPEVs".

Identification and Characterization of Viruses

For rapid virus identification, the CPE-positive cell culture lysates were tested by reverse transcription and nested PCR, using specific primers for the VP1 region (nt. 2602–2977) common to all EVs (Battistone et al. 2014a; Nix et al. 2006).

For molecular characterization, viral RNA was extracted from 200 µl of lysate using Viral Nucleic Acid Extraction Kit II (Geneaid, New Taipei, Taiwan) in accordance with the manufacture's instruction. RNA, eluted in 50 µl of RNase-free water, was stored at –30 °C. Reverse transcription and amplification were performed as previously described (Battistone et al. 2014a; Nix et al. 2006). Briefly, RT-nested PCR was carried out with an initial reverse transcription step at 42 °C for 60 min, followed by two sequential PCRs with an activation step at 95 °C for 2 min, and 40 cycles of PCR in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Inc., Foster City, CA) at the following conditions: 30 s at 95 °C, 30 s at 42 °C (60 °C for the second PCR), 45 s at 60 °C, and a final extension for 5 min at 72 °C.

The reaction products, separated on 1.5% agarose gel with ethidium bromide, were detected with Molecular Imager Gel Doc XR with the Quantity-One software (BioRad, Segrate, Italy).

Sanger nucleotide sequencing of partial VP1 gene was also performed using the primers of the nested PCR (Macrogen Inc Seoul, South Korea). Sequence analysis and comparison were carried out with software Sequencer 5.2 (Gene Codes Corporation, Ann Arbor Michigan, USA) and NCBI GenBank (http://www.ncbi.nlm.nih.gov).

Poliovirus identification, typing, and intratypic differentiation were performed at the ISS in accordance with the WHO guidelines (CDC 2015). Two assays were successively performed: Real-time ITD Assay to determine the serotype and the intra-type of isolated PV and Real-time VDPV Assay to identify VDPV.

Sequencing of the 5′NCR (nt 179–575) and VP1 (nt 2480–3382) genomic regions was also performed for all isolated polioviruses, in accordance with WHO guidelines (CDC 2015), and their genomes were compared with the sequences of PV reference strains AY184219.1, AY184220.1, and AY184221.1 for PV1, PV2, and PV3, respectively.

Table 2 Polioviruses isolated in sewage samples during the surveillance in Italy, 2009–2015

City	Date	PV serotype	ITD / VDPV	Sequence	
				5′ NCR	VP1
Venice	May 2010	PV 2	SL	481 A > G	–
Parma	June 2011	PV 1	SL	–	2765 G > A 2795 A > G
Parma	April 2015	PV 3	SL	–	2493 C > T
Parma	May 2015	PV 3	SL	–	2493 C > T

SL Sabin-like

The nucleotide sequences of the 5′NCR and VP1 regions of PVs (Table 2) were deposited in the GenBank database (AC#MG808047, MG808054).

Results

Virus Recovery and Detection Limit

Both the positive controls spiked with 2×10^5 and those spiked with 2×10^3 $CCID_{50}$ of Sabin type 1, poliovirus suspension produced a CPE on the first RD cells passage, while CPE was only observed on the second passage using 20 $CCID_{50}$ of virus. The positive sample spiked with 2 $CCID_{50}$ of Sabin type 1 poliovirus was CPE negative. The resulting detection limit of 20 $CCID_{50}$/sample is in line with the WHO requirements for a satisfactory environmental surveillance of poliovirus and other enteroviruses (WHO 2003).

Enterovirus Typing

During the 7-year study, 2880 sewage samples were collected from 22 WWTPs in eight cities involved in the environmental surveillance (Table 1). Overall, 1497 sewage samples were CPE positive on cell culture (52.0%), ranging from 40.2% in 2013 to 56.8% in 2011 (Fig. 1) with an average of 50.9% throughout the investigated period (2009–2015).

Of the CPE-positive samples, 1479 (98.8.%) were confirmed by RT-nested-PCR typing (Table 3), whereas 18 samples (1.2%) were untypable with specific primers for enteroviruses and were classified as "non-enteroviruses" (NEVs). These samples were no further investigated and the presence of other viruses was not ruled out.

Among all EV-positive samples, 1475 (99.7%) showed CPE only in RD cells (NPEVs), and were typed as Coxsackievirus (815 samples, 55.1%) and Echovirus (660, 44.6%), whereas four samples (0.3%), collected in Venice in 2010 (one sample) and in Parma in 2011 and 2015 (one and two

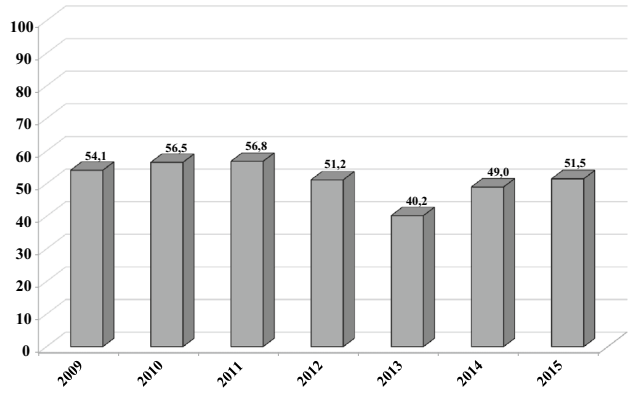

Fig. 1 Prevalence of positive samples from all waste water treatment plants in Italy, 2009–2015

samples, respectively), showed CPE in both cell lines (RD and L20B) and were characterized as Sabin-like PVs by Real-time ITD and VDPV Assay. In particular, PV type 2 was detected in the Venice sample while PV type 1 in the 2011 Parma sample and PV-type 3 in the two 2015 Parma samples (Table 2). No wild type or VDPV were found during the 7 years of the study.

Nucleotide sequencing of the 5′NCR, a common region to all enteroviruses, showed a single point mutation at nucleotide position 481 (A > G), associated with neurovirulent reversion in the PV-type 2 isolated in Venice in 2010. No mutations in the same sequenced region were identified in the other three PV-positive samples isolated in Parma in 2011 and 2015. Conversely, some mutations in the VP1 coding region at positions 2765 (G > A) and 2795 (A > G) were detected in the PV-type 1 isolated in Parma in 2011 as well as in the PV-type 3 strains isolated in Parma in 2015 at position 2493 (C > T). No mutation in the VP1 coding region (Table 2) was detected in the Venice strain.

All NPEVs belonged to species B and the most frequently isolated serotype was CV-B5 (366 samples, 24.4%), followed by CV-B4 (207, 13.8%), E-11 (203, 13.6%), E-6 (155, 10.4%), E-7 (126, 8.4%), CV-B3 (118, 7.9%), and CV-B2 (101, 6.7%), whereas the proportions of the other serotypes ranged from 0.1 to 3.7% (Table 3). The trend of serotypes changed over the years (Table 3), with an endemic circulation of NPEVs peaking between March and August (Fig. 2), and a prevalence of Coxsackievirus vs Echovirus. Conversely, PVs were exclusively detected between April and June.

Table 3 Temporal distribution of enteroviruses serotypes identified in sewage water samples (%)

EV Serotype	2009	2010	2011	2012	2013	2014	2015	2009–2015
CV-B1	7 (3.1)	5 (1.8)	–	–	8 (6.0)	1 (0.6)	2 (1.1)	23 (1.5)
CV-B2	9 (4.0)	9 (3.2)	22 (7.9)	22 (9.1)	16 (12.0)	17 (10.4)	6 (3.4)	101 (6.7)
CV-B3	10 (4.4)	11 (4.0)	10 (3.6)	36 (14.8)	14 (10.5)	37 (22.6)	–	118 (7.9)
CV-B4	7 (3.1)	116 (41.7)	12 (4.3)	11 (4.5)	16 (12.0)	13 (7.9)	32 (18.3)	207 (13.8)
CV-B5	101 (44.9)	78 (28.1)	60 (21.5)	29 (11.9)	26 (19.5)	36 (22.0)	36 (20.6)	366 (24.4)
E-1	3 (1.3)	–	–	–	1 (0.8)	1 (0.6)	7 (4.0)	12 (0.8)
E-3	1 (0.4)	4 (1.4)	20 (7.2)	–	3 (2.3)	10 (6.1)	2 (1.1)	40 (2.7)
E-4	37 (16.4)	14 (5.0)	2 (0.7)	3 (1.2)	–	–	–	56 (3.7)
E-6	17 (7.6)	17 (6.1)	50 (17.9)	26 (10.7)	9 (6.8)	24 (14.6)	12 (6.9)	155 (10.4)
E-7	11 (4.9)	3 (1.1)	43 (15.4)	10 (4.1)	3 (2.3)	2 (1.2)	54 (30.9)	126 (8.4)
E-9	–	–	–	–	–	1 (0.6)	–	1 (0.1)
E-11	11 (4.9)	11 (4.0)	41 (14.7)	98 (4.03)	21 (15.8)	12 (7.3)	9 (5.1)	203 (13.6)
E-12	–	2 (0.7)	2 (0.7)	–	–	–	1 (0.6)	5 (0.3)
E-13	1 (0.4)	–	3 (1.1)	–	2 (1.5)	–	1 (0.6)	7 (0.5)
E-14	–	–	–	–	2 (1.5)	–	3 (1.7)	5 (0.3)
E-19	–	–	4 (1.4)	1 (0.4)	–	1 (0.6)	1 (0.6)	7 (0.5)
E-20	–	–	1 (0.4)	1 (0.4)	1 (0.8)	–	–	3 (0.2)
E-24	–	–	1 (0.4)	1 (0.4)	–	–	–	2 (0.1)
E-25	4 (1.8)	1 (0.4)	4 (1.4)	–	2 (1.5)	3 (1.8)	2 (1.1)	16 (1.1)
E-30	3 (1.3)	4 (1.4)	1 (0.4)	1 (0.4)	8 (6.0)	3 (1.8)	1 (0.6)	21 (1.4)
E-33	–	–	–	1 (0.4)	–	–	–	1 (0.1)
PV	–	1 (0.4)	1 (0.4)	–	–	–	2 (1.1)	4 (0.3)
NEV	3 (1.3)	2 (0.7)	2 (0.7)	3 (1.2)	1 (0.8)	3 (1.8)	4 (2.3)	18 (1.2)
Total	225 (100)	278 (100)	279 (100)	243 (100)	133 (100)	164 (100)	175 (100)	1497 (100)

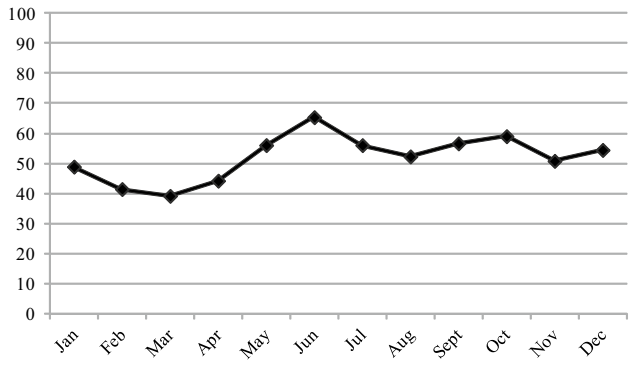

Fig. 2 Percentage of seasonal distribution of enteroviruses by month, Italy 2009–2015

Table 4 shows the EV serotypes distribution by geographical areas, with data clustered according to the Italian Regions (Northern, Central and Southern Italy). Among the most frequent isolated serotypes, E-6 and E-11 were mainly detected in Northern Italy, whereas CV-B2 and CV-B4 showed a higher prevalence in Central Italy. Finally, CV-B3 and E-7 were more prevalent in Southern Italy than in the remaining part of Italy. CV-B5 was the most frequent serotype identified in this study, recovered throughout Italy every year during the investigated period, except in 2010 and 2012. Three out of the four PV-positive samples were from Central Italy (Parma), whereas only one was from Northern Italy (Venice).

Discussion

Environmental surveillance provides both a system for routine surveillance of PVs circulation independent of AFP cases, particularly in urban populations, where a silent virus transmission might occur, and valuable supplementary information about the circulation of NPEVs in the environment. Even in *polio-free* countries as Italy, the risk of PVs reintroduction (wild or Sabin) remains high due to the population movements from countries, where either the OPV vaccine is still routinely used (Zurbriggen et al. 2008; Ruggeri and Fiore 2012; Battistone et al. 2014a; Foiadelli et al. 2016) or the polio is still endemic (Lopalco 2017).

As reported in Israel, Egypt and some European countries (Manor et al. 2014; GPEI 2015; Roivainen et al. 2010;

Table 4 Distribution by city of enteroviruses isolated from sewage samples in Italy, 2009–2015 (%)

EV Serotype	BZ	VE	MI	Tot (%)	PR	SS	Tot (%)	NA	PA	BA	Tot (%)
CV-B1	1	–	–	1 (0.1)	13	5	18 (6.6)	2	–	2	4 (0.8)
CV-B2	2	5	10	17 (2.4)	38	7	45 (16.5)	11	17	11	39 (7.7)
CV-B3	6	13	11	30 (4.3)	25	–	25 (9.2)	32	22	9	63 (12.4)
CV-B4	18	31	28	77 (11.0)	59	4	63 (23.2)	40	7	20	67 (13.2)
CV-B5	33	79	51	163 (23.4)	51	25	76 (27.9)	44	33	50	127 (25.0)
E-1	–	1	8	9 (1.3)	–	1	1 (0.4)	–	2	–	2 (0.4)
E-3	–	21	14	35 (5.0)	–	–	–	–	5	–	5 (1.0)
E-4	3	13	15	31 (4.4)	–	1	1 (0.4)	–	7	17	24 (4.7)
E-6	8	42	64	114 (16.3)	4	2	6 (2.2)	18	14	3	35 (6.9)
E-7	10	18	14	42 (6.0)	11	1	12 (4.4)	14	27	31	72 (14.1)
E-9	–	–	–	–	–	–	–	1	–	–	1 (0.2)
E-11	11	38	80	129 (18.5)	2	12	14 (5.1)	18	33	9	60 (11.8)
E-12	–	4	1	5 (0.7)	–	–	–	–	–	–	–
E-13	–	3	3	6 (0.9)	1	–	1 (0.4)	–	–	–	–
E-14	–	–	5	5 (0.7)	–	–	–	–	–	–	–
E-19	–	–	6	6 (0.9)	–	–	–	1	–	–	1 (0.2)
E-20	1	–	2	3 (0.4)	–	–	–	–	–	–	–
E-24	1	–	1	2 (0.3)	–	–	–	–	–	–	–
E-25	–	5	4	9 (1.3)	–	2	2 (0.7)	2	3	–	5 (1.0)
E-30	–	3	9	12 (1.7)	–	5	5 (1.8)	3	–	1	4 (0.8)
E-33	–	1	–	1 (0.1)	–	–	–	–	–	–	–
PV	–	1	–	1 (0.1)	3	–	3 (1.1)	–	–	–	–
Total	94	278	326		207	65		186	170	153	
	North				Centre				South		
	698				272				509		

BZ Bolzano, *VE* Venice, *MI* Milan, *PR* Parma, *SS* Sassari, *NA* Naples, *PA* Palermo, *BA* Bari

Zurbriggen et al. 2008; Blomqvist et al. 2012), human EVs, able to survive in a variety of environmental conditions, are excreted in high numbers by infected, symptomatic or asymptomatic people, and might spread among a highly immune population.

In Italy, since 2002, after the replacement of OPV and the introduction of IPV, no PVs have been isolated from suspected AFP cases (Battistone et al. 2014a; Pellegrinelli et al. 2017; Pennino et al. 2018).

In this study, four Sabin-like PVs were isolated in the environment: one, PV-type 2, in Northern Italy (Venice) in 2010 and three, 1 PV-type 1 and 2 PV-type 3, in Central Italy (Parma) in 2011 and 2015, respectively. These Sabin-like PVs were probably excreted by individuals immunized with OPV abroad, suggesting poliovirus importation. The low degree of mutation found in the four Sabin-like genomes suggests a limited circulation of the viruses in the population.

It is known that OPV, containing live attenuated viruses, may revert to the neurovirulent phenotype as circulating vaccine-derived poliovirus (cVDPV) with a risk for unvaccinated population (Fontana et al. 2017). Moreover, the risk of circulation of cVDPV strains is higher when the vaccination coverage for poliovirus is low, as recently reported in Ukraine (Khetsuriani et al. 2017).

No wild type or cVDPV were found in sewage samples during the 7-year surveillance, confirming the *polio-free* status of Italy.

In addition to PVs, several NPEVs were also detected in Italy. Overall, the findings of NPEVs in 52% of samples demonstrated the wide spread of these viruses in the population during all seasons, peaking between March and August (Fig. 2), differently from other countries with temperate climate, with incidence peaks in summer and autumn (Khetsuriani et al. 2006a; van der Sanden et al. 2013).

EV serotypes distribution showed a different prevalence per geographical areas and years. Overall, Coxsackievirus B strains were isolated more often than Echovirus strains (55.1% *vs* 44.6%). These findings are in line with data reported from environmental surveillances in Europe (Costan-Longares et al. 2008; Antona et al. 2007; Richter et al. 2011; Battistone et al. 2014a; Pennino et al. 2018). In particular, CV-B5 (24.7%), CV-B4 (14.0%), E-11 (13.7%) and E-6 (10.5%) were the main serotypes isolated in the present study. CV-B5, constantly circulating throughout Italy, is correlated with outbreaks of meningitis and other diseases. The most isolated Echoviruses, E-11 and E-6, circulating in Northern Italy, are associated with childhood diseases, specifically meningitis, respiratory and gastrointestinal illnesses for E-11, and outbreaks of respiratory diseases for E-6 (Ramelli et al. 2004; Groneck et al. 2011; Cao et al. 2014; Chen et al. 2015). E-30, identified in the present study, is nowadays considered an important emerging serotype and is commonly isolated in meningitis outbreaks in several countries (Milia et al. 2013; Chomel et al. 2003; Rudolph et al. 2017; Holmes et al. 2016; Leveque et al. 2010). Since no systematic NPEV hospital- and community-based surveillance systems are in place in Italy, and an increasing number of epidemic outbreaks associated with NPEVs have been described worldwide (Wikswo et al. 2009; Khetsuriani et al. 2006b; Richter et al. 2006; Mirand et al. 2016; Molet et al. 2016), environmental surveillance may play a pivotal role in monitoring the circulation of human EVs and other enteric agents in the population (Ruggeri et al. 2015; Di Bartolo et al. 2015; Vieira et al. 2012; Aw and Gin 2010; Werber et al. 2009; Scarcella et al. 2009; Chikhi-Brachet et al. 2002). Their presence in rivers, seas, recreational waters and treated wastewaters used for crop irrigation (Steele and Odumeru 2004; Munoz et al. 2010; Viau et al. 2011; Lodder et al. 2010; Moazeni et al. 2017) highlights the need for an efficient monitoring system. Although most infections are asymptomatic, NPEVs are responsible for a high number of neurological and other diseases. More rarely, EVs may recombine with PVs favoring the emergence of new serotypes (Combelas et al. 2011; Jiang et al. 2007).

Overall, the use of RD cells may have led to an underestimation of some EV serotypes, since not all EVs are able to grow on these cell lines (Tsao et al. 2010; Prim et al. 2013). The use of conventional cell culture isolation methods together with RT-PCR permits to rapidly detect EVs in sewage samples also in the presence of a low amount of viral concentrations and/or of RT-PCR inhibitors in the sample, even though mixed infections with different serotypes are not always detected. This method was standardized to monitor PV circulation in the context of the 'WHO global polio eradication program' (WHO 2003). In fact, both environmental surveillance and AFP surveillance provide additional insight in the dynamic of PVs and other enteroviruses.

Acknowledgements The authors wish to thank the workers of the wastewater-treatment plants in charge of collecting samples for the environmental surveillance.

Funding This work was partially supported by grants from the Italian Ministry of Health, CCM, "AFP surveillance in Italy. Research of Poliovirus and other enteroviruses in immunodeficient patients and in the environment".

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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