Original article

Detection of Enteroviruses from urban sewage in Parma

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Abstract. *Background and aim of the work:* The human enteroviruses (EV) are the most common and widespread human viruses in the world. They have bowel as their natural habitat and they can spread in the environment through the faecal excretion. In the continental climate Regions these viruses may cause epidemic outbreaks in summer and fall, while in the tropical Regions the EV infections present a high incidence during all year. The symptoms can be minor or subclinic, but they can be also associated to rare and serious diseases. The aim of this study was to evaluate the environmental circulation of polioviruses and non-polio enteroviruses (NPEV) using standard methods of urban wastewater surveillance recommended by the WHO. *Methods:* A total of 188 wastewater samples were collected between February 2005 and December 2008 from two sewage treatment plants in Parma. The sampling was carried out twice a month. Environmental variables were collected for each day of sampling. *Results:* Out of the 188 examined wastewater samples, 78,7% were positive to the enterovirus research. One out of the 148 positive samples was identified as poliovirus Sabin-like type 3. The remaining 147 positive samples were enteroviruses non polio: Coxsakieviruses and Echoviruses. All Coxsakieviruses isolated were of type B. *Conclusions:* The proposed method has shown high sensibility, also in presence of very low expected prevalence of vaccine poliovirus. It allows to verify the kind and relative frequency of enteric viruses circulating in the country, whose characteristics (virulence and pathogenicity) may vary with reference to a different epidemiologic and demographic structure of the resident population. (www.actabiomedica.it)

Key words: Enteroviruses, polioviruses, surveillance, environmental circulation, urban wastewater

Introduction

The human enteroviruses (EV) (genus *Enterovirus*, family *Picornaviridae*) are the most common and widespread human viruses in the world (1).

The conventional classification, based on the association with human pathologies and animal models, includes Coxsackieviruses type A and B, Echoviruses, numbered Enteroviruses and Polioviruses type 1-3. They represent a high number of viruses (> 100) that have bowel as their natural habitat and that can spread in the environment through the faecal excretion.

In the continental climate Regions these viruses may cause epidemic outbreaks in summer and fall, while in the tropical Regions the EV infections present a high incidence during all year (2, 3). The symptoms can be minor or subclinic, from a common cold with intestinal symptoms, to Bornholm disease and haemorrhagic conjunctivitis, but they can be also associated to rare and serious diseases such as: aseptic meningitis (4, 5), hepatitis, myocarditis; some authors suggest an association with juvenile type 1 diabetes (6, 7).

In contrast with the wide knowledge on the poliovirus circulation, information on the incidence of

the non polio enterovirus infections (NPEV) and on their diffusion in the population, is incomplete.

The monitoring of sewages , if carried out with a sensible method, may represent a true image of their spreading (8, 9, 10).

Since early 1960, a National Enterovirus Surveillance System (NESS; optional) was established in the USA (11), with the aim to acquire useful information for the public health operators on the different EV serotypes circulation and on their association with clinical expressions (1).

The implementation of the Flaccid Paralysis Surveillance System in the Poliomyelitis Eradication Program with the environmental monitoring offered a great opportunity and it renewed the interest towards a wider knowledge of EV infections epidemiology.

In 1996 in Italy started a pilot study in 4 Regions (Campania, Umbria, Emilia Romagna, Piemonte), that, even if its primary object was the wild poliovirus research, it allowed to acquire data on the EV environmental circulation in a wide area. This active surveillance program, was therefore extended to all national country, following up the Country Polio-free Certification in 2002 (12-14).

This monitoring involved also our area, that was formerly committed in environmental matrix surveys (15-18).

Aim of the project

The aim of this study was to evaluate the environmental circulation of polioviruses and non-polio enteroviruses (NPEV) using standard methods of urban wastewater surveillance recommended by the WHO (19).

Materials and methods

A total of 188 wastewater samples were collected between February 2005 and December 2008 from two sewage treatment plants in Parma. The sampling was carried out twice a month. Both plants are located in the northern area of the city; they have a capacity of 168,000 equivalent inhabitants (West plant - WP) and 180.000 equivalent inhabitants (East plant - EP), and serve about 150,000 residents. The first one is a depurative plant of third level using activated sludge technology with biological denitrification, nitrification and defosfatation; the second one is a depurative plant of third level that does not foresee biological defosfatation. The West plant receives raw sewages coming from the west area of Parma included Hospital, Faculty of Veterinary Medicine, Zooprofilactic Institute and General Markets; the East plant receives raw sewages coming from the east area of the city including the industrial area. The treatment plants use automated equipment for collecting samples at regular intervals during a 24-hour period or over the peak hours of household sewage flow. One litre of each sewage sample (24-hour flow-proportional composite sample) was processed in our laboratory no later than 4 hours, according to a WHO method (Guidelines for environmental surveillance of poliovirus circulation). Samples should be kept at +4°C for no more than 24 h, and should be kept cool during their transport to the laboratory. Environmental variables were collected for each day of sampling: flow rate (m^3/h), Chemical Oxigene Demand- COD (mg/l), Ammoniacal nitrogen - $N\text{-}NH_4$ (mg/l), pH, conductivity, mean daily air temperature.

Sample processing, virus isolation and characterization of virus isolates

All samples were concentrated according to the "two-phase separation method" (WHO). A given volume of clarified sewage was mixed with defined amounts of two polymers, dextran (22%) and polyethylene glycol (PEG) (29%) (19). The homogenous mixture obtained by vigorous shaking was left to stand overnight at 4°C in a separation funnel. This allows the polymers to separate and form two distinct layers (phases) in the funnel. Enteroviruses accumulate in the smaller bottom layer and/or at the boundary between the layers (interphase). The bottom layer and the interphase are collected drop-wise. The pellet from the initial centrifugation is suspended in this concentrate, which is then treated with chloroform (40%) and assayed for the presence of virus. The re-

sulting nominal sample concentration is 50-100-fold; 1 ml aliquot of the extracted concentrate was frozen at -20°C for potential future use. The remaining extracted concentrate was inoculated into tissue cultures: BGM (Buffalo green monkey) cells were used for the isolation of enterovirus, while L20B cells were used for selection of poliovirus. Tissue cultures were incubated for 5 days at 37°C. Two blind passages were performed for each cell line. All cultures positive in BGM cells but negative in L20B, were passaged one more time in L20B, since it is known that a small percentage of poliovirus isolates do not grow well in L20B cells during the first passage and may not produce recognizable cytopathic effect.

Typing was performed by microneutralization assay on L20B and BGM isolates, using enterovirus serum pools (anti-Coxsakievirus B, anti-Echovirus) and type specific poliovirus antisera.

Sewage samples were also investigated with molecular biology methods: Reverse Transcription-PCR. Viral RNA was extracted by treatment with phenol and chloroform followed by ethanolic precipitation. The cDNA was synthesized with reverse transcription (RT) and then amplified through nested-polymerase chain reaction (PCR) amplification, using specific primers for enterovirus detection: CX10 5'-att gtg acc ata agc agc ca-3', CX3 5'-cgg tgg ctg cgt tgg cgg cc-3', CX8 5'-aaa cac gga cac cca aag ta-3', CX9 5'-ggc ccc tga atg cgg cta at-3'. The extracts, correspondent to the obtained positive amplification products, underwent again RT and nested-PCR using a different set of specific primers for poliovirus, Coxsakievirus and Echovirus detection (20).

Statistical analysis

Environmental variables were summarized as mean, median, standard deviation (SD).

Temporal trends of measurements were showed in the figures. Differences in the environmental measurement performed during the same day were evaluated through Student's t test for samples on the two sewage treatment plants. Differences in virus isolation frequency were evaluated through X2 test on type of plant, year, month and season of study. Differences between virus isolation frequency and wastewater characteristics were evaluated through Analysis of Variance (F test and Student's t test for 2 independent samples). P value < 0.05 was considered as statistically significant. All statistical analyses were performed by SPSS 16.01.

Results

Between 1th February 2005 and 16th December 2008 a total of 188 wastewater samples were collected from two sewage treatment plants in Parma, 94 samples for each plant. Descriptive statistics of the observed environmental variables are reported in Table 1. The east plant showed a high mean flow (45586 m^3/h) and low variability (p<0,001). The west plant showed a high variability in organic content and in N-NH_4, expressed as percent and absolute value (p<0,001). Wastewater samples reported different ph values: 7,53 (0,30) in the east plant, 7,31 (0,70) more acid and variable in the west plant. Out of the 188 examined wastewater samples, 148 (78,7%) were positive to the enterovirus research: 80/94 (85,1%) in west plant and 68/94 (72,3%) in east plant (table 2, figure 1). The virus isolation percentage was significantly lower during the first year of sampling in both plants (2005: 20/44, 45,5%); it reached in the following 3 years of study the mean value of 93% in west plant and of 84,7% in east plant. The flow rate stratified in quartile was not associated to a different viral isolation frequency. East plant showed high frequency of positivity compared to high COD values; this result was not observed in west plant. If pH was stratified in quartile, in east plant 95,7% of positive samples appeared distributed in the third quartile (between 7,56 and 7,73) (table 3). The frequency of positive samples on month sampling was reported in figure 1. During the 4 years of study (except January) the sampling was carried out twice a month, with a total of 4 sampling/month. Differences (not statistically significant) in the percentage of positive samples were found in summer and fall months (82%) compared to winter months (70,5%) (table 4).

One out of the 148 positive samples was identified as poliovirus Sabin-like type 3. This poliovirus was isolated from a wastewater sample collected from

Table 1. Wastewater characteristics

Parameter	West Plant		East Plant	
	Mean (SD)	Median (25-75 percentile)	Mean (SD)	Median (25-75 percentile)
Flow rate (m^3/h)	28398 (13876)	23639 (21655 - 29250)	45586 (9070)	45399 (38127 - 51748)
COD (mg/l)	340 (124)	319 (264 - 387)	201 (91)	176 (135 -245)
Organic content (kg/h)	9143 (4051)	8348 (6457 - 10440)	8828 (3755)	7820 (6547 - 10410)
NH_4^+	22 (6)	22 (18,35 - 26,1)	15 (5,8)	14,2 (11,2 -17,73)
pH	7,31 (0,70)	7,45 (6,98 - 7,8)	7,53 (0,30)	7,56 (7,3 - 7,73)
Conductivity	1,41 (0,32)	1,4 (1,3 - 1,6)	1,31 (0,25)	1,3 (1,18 - 1,46)
Air temperature	14,72 (7,98)	13,8 (8,15 - 21,82)	14,72 (7,98)	13,8 (8,15 - 21,82)

Table 2. Number of positive samples/year

Year	West Plant Positive samples		East Plant Positive samples		Total Positive samples	
	N	%	N	%	N	%
2005	13/22	59,1	7/22	31,8	20/44	45,5
2006	23/24	95,8	20/24	83,3	43/48	89,6
2007	21/24	87,5	23/24	95,8	44/48	91,7
2008	23/24	95,8	18/24	75,0	41/48	85,4
Total	80/94	85,1	68/94	72,3	148/188	78,7

west plant during 2005. The remaining 147 positive samples were enteroviruses non polio; out of them, 140 samples (94,6%) were identified as Coxsakieviruses, 2 samples (1,3%) as Echoviruses (type 4, 30) and 5 samples (3,4%) were not characterized. All Coxsakieviruses isolated were of type B, in particular 20,7% type B5, 19,3% type B4, 17,2% type B3 and 11,4% type B2 (table 5, figure 2).

Discussion

Enterovirus contamination of raw sewage is due to high faecal excretion and its environmental resistance. Large numbers of enterovirus particles are excreted in human faecal matter by infected subjects and remain infectious in the environment for different periods of time depending on the immediate conditions: an infected individual can eliminate 10^{10} viral particles every day through faeces.

As a consequence their monitoring as environmental indicators is very important from an epidemiologic point of view because they represent a reflec-

Table 3. Flow rate, COD, pH: positive samples/quartile

	West Plant						East Plant					
	Flow rate (m3/h)		COD (kg//h)		pH		Flow rate (m3/h)		COD (kg//h)		pH	
	N	%	N	%	N	%	N	%	N	%	N	%
I quartile	18/23	78,3	21/23	91,3	20/23	87	19/23	82,6	14/23	60,9	12/22	54,5
II quartile	21/24	87,5	18/24	75	21/24	87,5	15/24	62,5	17/24	70,8	15/24	62,5
III quartile	20/24	83,3	21/24	87,5	18/21	85,7	15/24	62,5	18/24	75	22/23	95,7
IV quartile	21/23	91,3	20/23	87	21/26	80,8	19/23	82,6	19/23	82,6	19/25	76

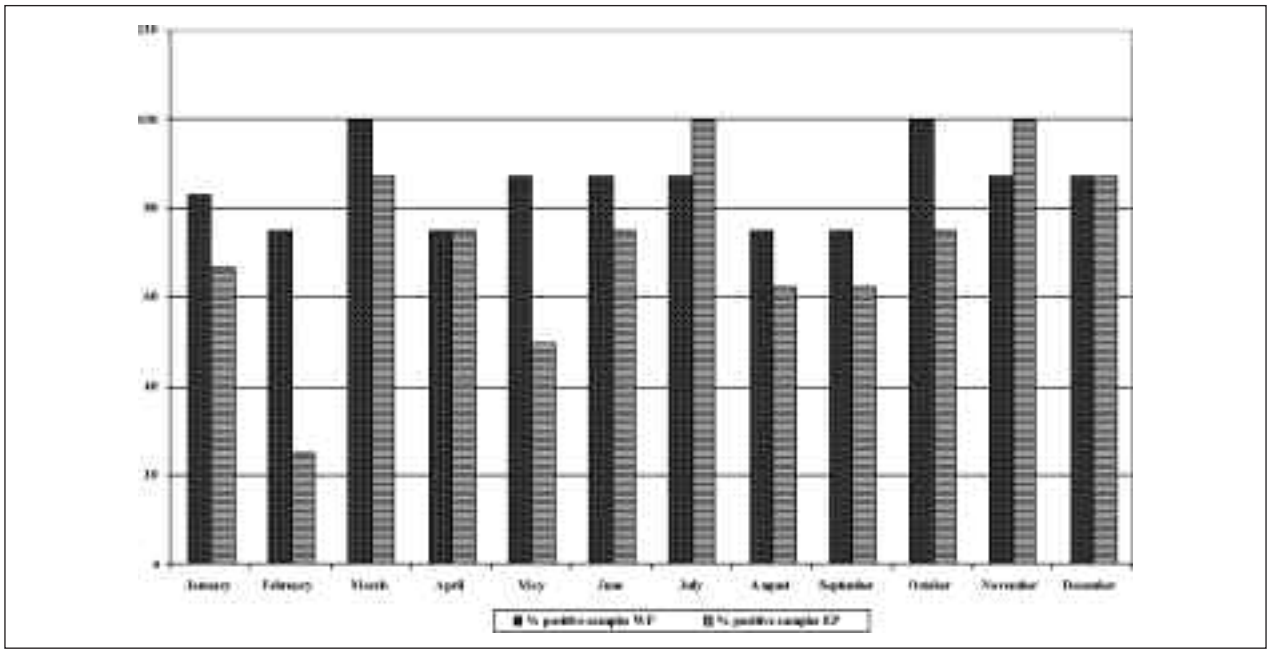

Figure 1. Percentage of positive samples /month

Table 4. Number of positive samples/season

Year	West Plant Positive samples		East Plant Positive samples		Total Positive samples	
	N	%	N	%	N	%
Winter	18/22	81,8	13/22	59,1	31/44	70,5
Spring	21/24	87,5	17/24	70,8	38/48	79,2
Summer	20/24	83,3	19/24	79,2	39/48	81,3
Autumn	21/24	87,5	19/24	79,2	40/48	83,3

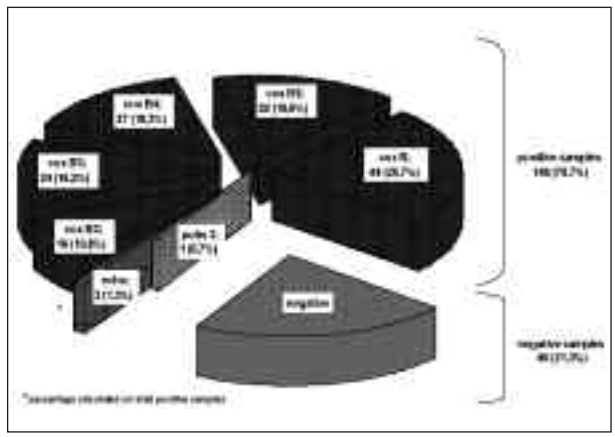

Figure 2. Percent distribution of isolated Enteroviruses

ted image of the viral circulation in the population (15, 21).

In this study wastewater samples collected from two sewage treatment plants in Parma between February 2005 and December 2008 were investigated in order to monitor the environmental circulation of polioviruses and non-polio enteroviruses.

As part of the poliomyelitis eradication program supported by the World Health Organization, the epidemiologic and virologic surveillance in environmental matrix (even in countries declared polio-free), allows an early detection of any (re)emergence of wild or vaccine strains, with different meaning in terms of public health. The isolation of wild strains would indicate a re-introduction of poliovirus in a country already declared polio-free, while the isolation of vaccine strains is explained by the presence of individuals coming from countries where it is still performed vaccination with OPV (22).

In our study, only one wastewater sample, during the first year of the study, was identified as poliovirus (poliovirus type 3 Sabine-like) (23). Among the isolated NPEV, the most frequent ones (94,6% of isolates) were Coxsackieviruses type B. The viral isolations took place during the summer months, according to

Table 5. Enteroviruses characterization/year

Year	EV positive samples	Poliovirus	Echovirus	Coxsackievirus					NT
				B2	B3	B4	B5	B	
2005	20/44 (45%)	1/20 (5%)	2/20 (10%)	0 0	8/20 (40%)	1/20 (5%)	1/20 (5%)	7/20 (35%)	0
2006	43/48 (89,6%)	0	0	7/43 (16,4%)	4/43 (9,3%)	10/43 (23,3%)	10/43 (23,3%)	10/43 (23,3%)	2/43 (4,7%)
2007	44/48 (91,7%)	0	0	6/44 (13,6%)	0	14/44 (31,8%)	11/44 (25%)	12/44 (27,3%)	1/44 (2,3%)
2008	41/48 (85,4%)	0	0	3/41 (7,3%)	12/41 (29,3%)	2/41 (4,9%)	7/41 (17%)	15/41 (36,6%)	2/41 (4,9%)
Totale	148/188 (78,7%)	1/148 (0,7%)	2/148 (1,3%)	16/148 (10,8%)	24/148 (16,2%)	27/148 (18,3%)	29/148 (19,6%)	44/148 (29,7%)	5/148 (3,4%)

the greater incidence of gastro-enteric diseases in this period.

Comparing the current results with those obtained in a similar investigation carried out from 1987 to 1989 in the same urban area (14), a drastic reduction of poliovirus isolates was shown (0,7% vs 31,5%) due to the introduction, in 2002, of the new vaccination card with 4 doses of IPV, On the contrary a very high-frequency of Coxsackievirus isolates was confirmed. Many similar studies, performed in different geographic areas, showed similar frequency of isolation (2, 24). The most frequently isolated sub-types were B5 followed by B4 and B3.

Although the two plants present different characteristics with reference to the kind of collected sewage (COD, pH, NH4+, etc), significant differences in viral isolation percentage was not shown

The proposed method, studied by the WHO for the endemic Countries, has shown high sensibility, also in presence of very low expected prevalence of vaccine poliovirus.

In addition, it allows to verify the kind and relative frequency of enteric viruses circulating in the country, whose characteristics (virulence and pathogenicity) may vary with reference to a different epidemiologic and demographic structure of the resident population. In such perspective, this method will be adapted and tested for searching other enteric viruses, that in the last years have become important gastroenteritis agents.

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Accepted: March 17th 2010
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