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Original article

Tracking enterovirus and poliovirus circulation in Parma: Environmental surveillance from 2019 to 2024

Roberta Zoni[1], Lucia Palandri[2,3], Sandra Mezzetta[1], Stefano Fontana[4], Maria Eugenia Colucci[1], Paola Affanni[1], Mariateresa Bracchi[1], Roberto Albertini[1], Cesira Pasquarella[1], Elena Righi[2], Licia Veronesi[1].*

[1]Laboratory of Hygiene and Public Health, Department of Medicine and Surgery, University of Parma, Italy; [2]Public Health Section, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio-Emilia, Modena, Italy; [3]Clinical and Experimental Medicine PhD Program, University of Modena and Reggio Emilia, Modena, Italy; [4]Department of Infectious Disease, Istituto Superiore di Sanità, Roma, Italy

Abstract. *Background and aim:* Environmental Surveillance (ES), which gained renewed interest during the COVID-19 pandemic due to its effectiveness in the early detection of viral spread, has long been recognized as a critical component of the Global Polio Eradication Initiative. This approach has historically played a fundamental role in the early identification of poliovirus transmission and remains an essential tool for monitoring the circulation of enteroviruses, underscoring its enduring significance in public health surveillance. This study aims to present six years ES data on poliovirus and enterovirus in Parma, Italy. *Methods:* The samples were collected every two weeks at the inlet of two sewage treatment plants and underwent a WHO protocol analysis. We detailed the temporal and spatial distribution of non-polio enterovirus (NPEV) and poliovirus based on the virus cultures algorithm from 2019 to 2024. *Results:* A total of 228 sewage samples were collected, a median of 44 samples per year. 68.9% resulted in positive cell cultures, without significant differences among the two treatment plants. The year 2023 had the highest percentage of NPEV isolates (97.7%), followed by 2024 and 2022 (87% and 70.5% respectively). 2021 had the overall lowest positive (35.6%). One Sabin-like strain type 3 was detected in 2022. Seasonal time trends were observed. *Conclusions:* This study contributes to a better understanding of NPEV circulation patterns in a pandemic and post-pandemic period, underlining the importance of ES as a tool in the assessment of the epidemiological spreading of enteroviruses and significantly poliovirus. (www.actabiomedica.it)

Key words: poliovirus, enterovirus, wastewater environmental surveillance, Global Polio Eradication Initiative, COVID-19

Introduction

Environmental Surveillance (ES) consists of systematically testing sewage and wastewater samples and serves as a critical public health tool. It allows pathogen detection, providing a community-representative picture of circulating infectious diseases (1), detection and monitoring of antibiotic resistance (2), and beyond infectious disease tracking, it is used to assess community recreational drug, tobacco, and alcohol use (3). During the COVID-19 pandemic, ES gained renewed interest due to its ability to predict the emergence of pandemic peaks or detect the onset of new SARS-CoV-2 variants (4–6), yet ES is widely used in viral disease epidemiology, encompassing the detections of many viruses as influenza, noroviruses and hepatitis A (7,8). Enteroviruses of the Picornaviridae family include a group of viral species such as polioviruses, coxsackieviruses,

echoviruses, and novel enteroviruses commonly associated with asymptomatic to mild infections. However, under specific circumstances, they can cause a wide range of severe syndromes as aseptic meningitis and encephalitis, paralysis, hand-foot-and-mouth disease, myocarditis, severe neonatal enterovirus infections, and others. Viral detection in wastewater started over 75 years ago to detect poliovirus and other enterovirus in sewage specimens (9) and has been subsequently integrated into the WHO's Global Polio Eradication Initiative (GPEI) to evaluate the effectiveness of these programs alongside the clinical surveillance for acute flaccid paralysis (AFP) (10–12). ES allows for the detection of a wider range of viruses than clinical surveillance on patients, as it covers viruses shed by asymptomatic and pauci-symptomatic individuals thereby enabling the detection of polioviruses and enteroviruses transmission before clinical cases occur. In the context of the GPEI, ES can detect both wild-type poliovirus (WPV) and circulating vaccine-derived poliovirus (cVDPV), as well Sabin-like virus, the live attenuated vaccine virus used for the oral polio vaccine (OPV). In polio-free countries, ES aims to detect the potential reintroduction of WPV or the emergence of cVDPV, particularly in settings with non-existent or suboptimal AFP surveillance and/or use of inactivated polio vaccine (IPV). Additionally, it seeks to document the release of poliovirus from manufacturing facilities or laboratories and to monitor the disappearance of Sabin strains following the withdrawal of oral polio vaccine (13). The GPEI was launched in 1988 with a global effort to immunize children worldwide against polio. The effort of public health campaigns worldwide has brought the elimination of polio in the Americas (1994), in the Western Pacific Region (1997), in the European Region (2002), in the South-East Asia region (2014), and in the African region (2020). Today the WPV1 continues to circulate only in two countries (Afghanistan and Pakistan). Due to low immunization rates, the circulation of cVDPV has increased in recent years. Through ES, public health workers can detect a surge in the circulation of cVDPV and address this with targeted supplementary immunization campaigns (14). Furthermore, samples collected through ES may be sequenced helping researchers trace the transmission path and where the circulating virus originated from. During the last quarter of 2024, ES detected cVDPV type 2 in 14 European cities and genetic sequencing of European viral isolates indicated that the virus had been circulating undetected for about one year (15). Even though the importance of ES is accepted, 11 Member states of the European Union do not have an active ES for polioviruses. In January 2025, the European Centre for Disease Prevention and Control (ECDC) encouraged Member States to implement ES, enhancing geographical coverage and sampling frequency to improve overall surveillance sensitivity (16). The Laboratory of Public Health of the Department of Medicine and Surgery of the University of Parma has been part of the network of sub-national reference laboratories involved in poliovirus ES since 2005. This network operates within the national framework coordinated Istituto Superiore di Sanità (ISS) as National Reference Laboratory, and globally, by the WHO (17–19). This study aimed to detail six years of environmental surveillance, focusing on the temporal and spatial distribution of enterovirus and poliovirus based on virus cultures from 2019 to 2024.

Materials and methods

Wastewater sampling

Samples of wastewater (1 L of 24-hour composite raw sewage) were collected from two treatment plants, the East Treatment Plant (ETP) and the West Treatment Plant (WTP), located in Parma, a major city in the Emilia-Romagna region. These plants serve approximately 198,000 inhabitants, including about 30,000 individuals under the age of 17.

Samples were collected twice a month, from January 1, 2019, to December 31, 2024, at the inlet of the primary sedimentation tanks. For all sites, the collection time was between 8:30 and 10:30 a.m. They were immediately transported to the laboratory using a reverse cold chain (4–8°C) and processed within 4 hours of collection.

Samples concentration by two-phase separation method

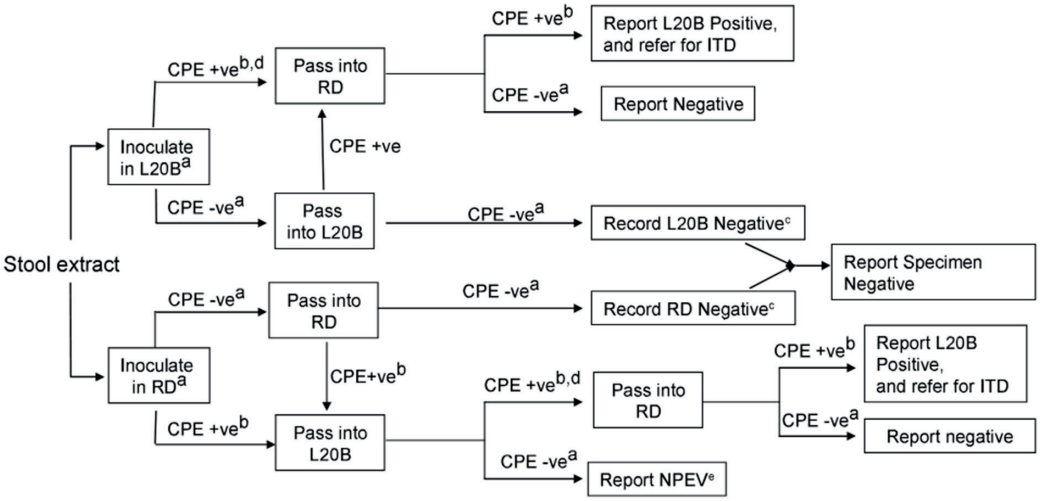
The quality (colour, smell, and turbidity) of each sewage specimen was checked before analysis.

The collected specimens were analysed using a two-phase separation method, following the World Health Organization (WHO) guidelines on environmental surveillance for Poliovirus detection (20).

Briefly, five hundred millilitres of the specimen were centrifuged to settle the suspended matter (1500 g/20 min at 4°C). The supernatant was collected, and the pellet was stored at 4°C. After adjusting pH to neutral, 287 ml of 29% PEG 6000, 39.5 ml of 22% Dextran, and 35 ml of NaCl 5N were added to the supernatant, and the mix was stirred for one hour. Then the samples were poured into the separation funnel and kept at 4°C overnight. The lower and middle layers were collected carefully, mixed with the sediment previously stored, and then processed with chloroform for decontamination (20% v/v). After stirring for 20 minutes at room temperature, the specimens were centrifuged at 1500g for 20 minutes at 4°C, and the supernatant was collected. Penicillin and streptomycin were added to a final concentration of 100 µg/ml and 100 IU/ml, respectively. The sewage concentrate obtained was utilized for cell culture inoculation.

Cell culture

According to the Global Polio Laboratory Network guidelines, two cell lines have been routinely used for the isolation of Poliovirus and non-polio Enteroviruses (NPEV): mouse genetically engineered cell line (L20B) expressing the human poliovirus receptor was used for Poliovirus isolation and human Rhabdomyosarcoma cell line (RD) for NPEV isolation (21). The concentrate of each specimen was inoculated (0.5 ml) into five 25-cm^2 flasks of L20B fresh monolayer cultures and one flask of RD cells and kept in a 36°C incubator. To observe cytopathic effects (CPE), inoculated cell culture flasks were checked by an inverted microscope for at least five consecutive days. According



a Observed for a minimum of 5 days
b Observe until ≥ 3+ CPE obtained (usually 1-2 days, 5 days maximum; re-inoculate when toxicity or contamination observed)
c Total minimum observation time of 10 days (2 x 5 days)
d Pool positive tubes (if both tubes show > 3+ CPE on the same day) before final RD passage
e Isolates can be serotyped by laboratories with an interest in NPEV diagnosis or to confirm proficiency

Figure 1. Algorithm for Poliovirus Isolation modified by "World Health Organization. Department of Immunization, Vaccines and Biologicals. Supplement 1 to the WHO Polio Laboratory Manual. An alternative test algorithm for poliovirus isolation and characterization. Geneva: World Health Organization; 2017" (22).

to the flowchart in Figure 1, each positive culture from both arms (RD and L20B) underwent cross-passaging in the other culture. For intratypic characterization and sequencing to differentiate Wild-Type Poliovirus (WPV), Sabin-like Poliovirus (SL), and Vaccine-Derived Poliovirus (VDPV), Poliovirus isolates were sent to the National WHO Reference Laboratory at ISS.

Following the new algorithm for poliovirus isolation, RNA from the "L20B positive isolate" was extracted and analysed by Intratypic Differentiation (ITD) rRT-PCR with specific primers and probes, to determine the serotype and intratype of poliovirus isolates. The Sabin-like isolates were also analysed by rRT-PCR VDPV assay to identify VDPV. Finally, the VP1 region of RNA extracted from poliovirus isolates was amplified by RT-PCR and sequenced. Nucleotide sequences were aligned using Sequencer software and compared with the reference type sequences to identify nucleotide substitutions and percentage of identity (23).

Statistical analysis

We performed descriptive statistics, calculating the number and percentage for categorical variables and time trends of sample isolates. A heatmap of frequencies of enterovirus detection in wastewater against monthly distribution was created to assess seasonal trend. All statistical analyses and visualizations were performed using Microsoft Excel version 2502.

Results

A total of 228 sewage samples were collected, 114 collected from ETP and 114 from WTP between January 2019 and December 2024. A median of 44 samples were analysed each year, except for 2020 when we analysed 6 samples. In 2020, Northern Italy, including Parma, was among the areas most heavily impacted by the COVID-19 pandemic (24–28). Consequently, samples were collected and sent directly to the ISS. Table 1 shows a summary of the results of the enterovirus environmental surveillance in Parma between January 2019 and December 2024.

Positive proportion of viruses isolated in sewage samples

On the totality of samples, 157/228 (68.9%) resulted cell culture positive (RD or L20B), without significant differences among the two treatment plants (68.4% ETP vs 69.3% WTP). The year 2023 had the highest percentage of isolates (97.7%), followed by 2024 and 2022 (87.0% and 72.7%, respectively). The year 2021 had the overall lowest positive rate with 35.6%, higher in WTP than ETP, 45.5% vs 26.1%.

NPEV

Out of 157 samples that tested positive in cell culture, 156 were identified as NPEV, representing 99.4% of the isolates. The positivity proportion was lowest in 2021 (35.6%), with a proportion higher in WTP than ETP (45.5% vs 26.1%), and highest in 2023 followed by 2024 and 2022 (97.7%, 87.0% and 70.5%, respectively) (Figure 2).

Poliovirus

Out of 157 positive samples, one sample in March 2022 (0.6%) exhibited a polio-like behaviour with a cytopathic effect on L20B cells. It was sent to the ISS for ITD typing and genotypic sequencing. A poliovirus was detected and identified as a Sabin-like strain type 3. Sequence analysis revealed a single nucleotide substitution in the poliovirus isolate over the 900-base span of the PV3 VP1 reference sequence. During the study period, no wild polioviruses or cVDPVs were detected.

Temporal distribution

Figure 3 shows the monthly distribution for NPEV isolates in Parma. Overall, the highest percentage of NPEV isolates was observed at the end of the summer and autumn, peaking in the fourth trimester (October to December). In this study, we observed two distribution patterns. From 2019 to 2021, low levels of detection were observed during the winter months (January to March), followed by a steady increase leading up to a peak in October–November. Years 2022 to 2024 showed an overall high detection rate

Table 1. Parma (Northern Italy) poliovirus and NPEV environmental surveillance summary results of isolation between January 2019 and December 2024.

	Year	Samples	NPEV	L20B CPE positive sample	Total Isolates
ETP	**2019**	21	10	0	10
	2020	3	2	0	2
	2021	23	6	0	6
	2022	22	16	0	16
	2023	22	21	0	21
	2024	23	23	0	23
	Total	114	78	0	78
WTP	**2019**	22	12	0	12
	2020	3	2	0	2
	2021	22	10	0	10
	2022	22	15	**1**[a]	16
	2023	22	22	0	22
	2024	23	17	0	17
	Total	114	78	**1**[a]	79
Total	**2019**	43	22	0	22
	2020	6	4	0	4
	2021	45	16	0	16
	2022	44	31	**1**[a]	32
	2023	44	43	0	43
	2024	46	40	0	40
	Total	228	156	**1**[a]	157

Abbreviations: ETP: East Treatment Plant, WTP: West Treatment Plant, NPEV: Non-polio enterovirus, CPE: cytopathic effect. [a]L20B positive sample sent to the ISS and confirmed a Sabin-like virus type 3.

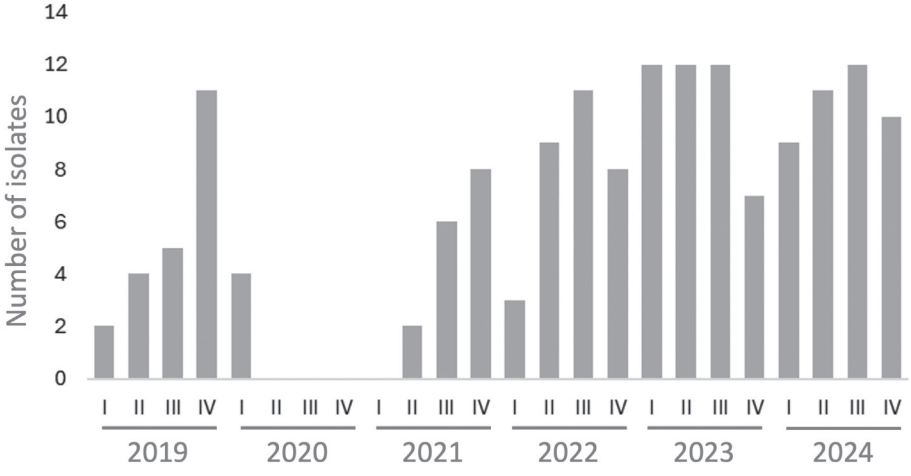


Figure 2. Temporal distribution for NPEV isolated from environmental samples in Parma from 2019 and 2024, divided by trimesters.

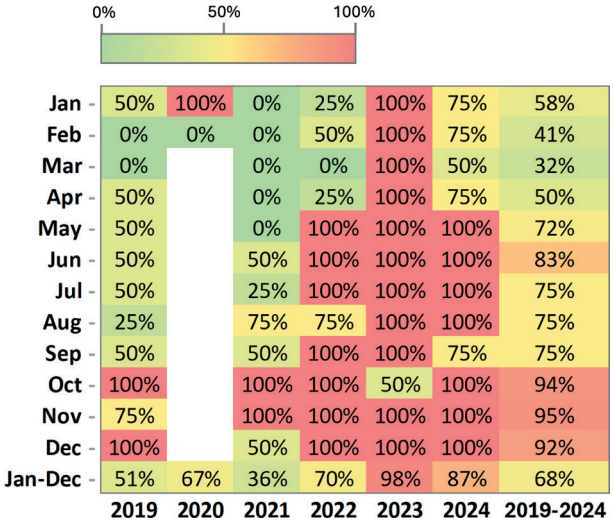


Figure 3. Monthly distribution for NPEV isolates in Parma from 2019 and 2024. Each cell represents the percentage of environmental NPEV isolates detected by month.

throughout all seasons, with a 100% positive rate from May to December.

Discussion

This study describes the results of 6 years of Environmental Surveillance in Parma, documenting the temporal-spatial distribution of viral isolates from 2019 to 2024.

NPEV

NPEV circulation showed a seasonal pattern, with higher prevalence in summer and fall months, data that has been reported in previous environmental studies. Interestingly, in 2021, during the earlier phases of the COVID-19 pandemic, we detected a lower prevalence of NPEV in sewage samples, followed by a significant increase in detection in the following years. As Italy was one of the countries that was most heavily hit by the COVID-19 pandemic, stringent public health measures aimed at controlling the pandemic diminished person-to-person contact, and social restrictions were adopted and took place since the end of February 2020 and were progressively lifted during the summer of 2021 (26). At the same time, as soon as these measures were eased, NPEV reappeared at higher than pre-pandemic percentages. Similar studies conducted worldwide described similar patterns. A study conducted in the Netherlands showed changes in NPEV epidemiology with a reduction of enterovirus transmission during the pandemic, followed by a rebound peak (starting from May, with a smaller peak in June and higher in October), higher than pre-pandemic levels, in 2022 (29). Gad et al. detected a similar pattern of NPEV in Poland, counting the highest peak in 2023 as well (30). Enterovirus detection in wastewater represents a quality indicator for ES. It is considered a highly sensitive technique, complementary to clinical surveillance methods, especially in those countries where polio has been eradicated. The decrease in NPEV detection during 2021 can be attributed to social distancing measures; however, the authors also hypothesize a direct contribution from the excessive use of disinfectants during this period, indeed, regular disinfection of public places, indoor areas, and hospitals with aid of chlorine-based disinfectants (CBDs) was the most widely practiced approach (31). Sewage systems function as diffuse collectors of human behaviours, and through wastewater analysis, we can study these patterns. In the initial phase of the pandemic, when excessive use of hand and surface disinfectants was predominant, we cannot exclude the possibility that sewage contained increased concentrations of disinfecting agents, which may have subsequently impacted viruses circulating in the wastewater system.

Poliovirus

The detection of a Sabin-like virus in Parma in spring 2022 indicates the presence of immigration from populations in regions where the OPV vaccination campaign was conducted. Following the outbreak of war in Ukraine, in the months of March and April, Parma received numerous immigrants, young women and children especially. In December 2021, in Ukraine, the local Ministry of Health, in collaboration with WHO and GPEI, promoted a comprehensive polio outbreak response plan, following the identification of polio cases in two children. This plan included an initial phase of vaccination with IPV and a second phase

with OPV for all Ukrainian children under 6 years old. The hypothesis is that the Sabin-like virus found in Parma, differing by only a single nucleotide substitution in the VP1 reference sequence, may have originated from this migratory flow (32). The absence of WPV and VDPV in sewage samples is consistent with the European Region having been declared polio-free in 2002. Effective environmental surveillance of poliovirus and enterovirus requires ongoing professional development in both infectious disease methodologies and public health communication (33,34). Recent literature is focusing on the development of early warning systems in conjunction with wastewater-based epidemiology (35,36). Technical training ensures accurate detection and interpretation of emerging threats, while communication skills enable translation of findings into timely interventions. Together, these competencies transform surveillance data into meaningful public health action. There are limitations to this report. The current study was limited to EVs found in Parma in sewage. The lack of clinical data does not allow the authors to further assess the public health impact of the increased detection of NPEVs. However, studies have reported similar patterns between clinical and environmental isolates (37), suggesting that in areas where no clinical surveillance systems are in place, ES might be a suitable alternative to detect temporal variability and inform programs for prevention and control (38). Furthermore, this study covers 6 years of ES, of which 3 were in the pandemic era. This might prove insufficient to conclusively confirm a change in NPEV epidemiology. Finally, the lack of next-generation sequencing (NGS) techniques is a limitation. NGS methods have certainly increased sampling specificity and given important insights in terms of molecular epidemiology. However, these techniques only inform about viral presence but provide no information on whether the viruses in wastewater are live and infectious. For the latter purpose, viral isolation through cell culture remains the gold standard. Clinical and environmental surveillance is crucial for preventing the spread of infectious diseases, and the literature agrees that efforts by the medical and organizational sectors are essential for this purpose. Improving training in the control and prevention of infectious diseases is fundamental in this regard (33,39). Environmental surveillance has proven to be highly sensitive in detecting social and behavioural changes, while also offering valuable insights into the presence or absence of poliovirus circulation within specific geographical areas. In conclusion, this study contributes to a better understanding of NPEV circulation patterns in a pandemic and post-pandemic period, underlining the importance of ES as a tool in the assessment of the epidemiological spreading of enteroviruses.

Ethic Approval: This study did not require ethical approval, as no data or samples from human subjects or animals were used.

Conflict of Interest: The author declares that he has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Authors Contribution: LV, RZ and LP conceptualized and designed the study. RZ, MTB, SM, MEC, PA and SF conducted the sample collection and laboratory analyses. RZ, LP, SF and LV, performed the data analysis and interpretation. LP, ER, CP, RA and LV contributed to the literature review and manuscript drafting. LV, RZ supervised the research and provided critical revisions. All authors reviewed and approved the final version of the manuscript.

Declaration on the Use of AI: During the preparation of this work the authors used Claude AI and Microsoft Copilot to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Correspondence:
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Lucia Palandri, MD, DrPH, PhD Candidate
Public Health Section, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia
E-mail: lucia.palandri@unimore.it
ORCID: 0000-0001-9787-7965