



Wastewater-based epidemiology revealed in advance the increase of enterovirus circulation during the Covid-19 pandemic

Laura Pellegrinelli^{a,*}, Cristina Galli^a, Arlinda Seiti^a, Valeria Primache^a, Aurora Hirvonen^a, Silvia Schiarea^b, Giulia Salmoiraghi^b, Sara Castiglioni^b, Emanuela Ammoni^c, Danilo Cereda^c, Sandro Binda^a, Elena Pariani^a

^a Department of Biomedical Sciences for Health, University of Milan, Milan, Italy

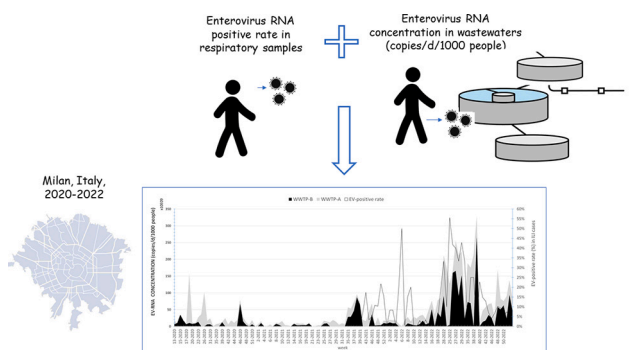
^b Department of Environmental Health Sciences, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy

^c Directorate General for Health, Lombardy Region, Milan, Italy

HIGHLIGHTS

- Enterovirus (EVs) were circulating in Milan metropolitan area between March 2020 and December 2022.
- EV-RNA concentration trend in wastewater samples overlapped with trend in EV-positivity rate in clinical cases
- The epidemiological trends unfolded the accumulation of EV transmission in the population after removal of Covid-19 restrictions.
- The increased of EVs in wastewaters was identified at least 35 days in advance compared to the analysis of clinical data.
- EVs outbreaks can be predicted by using Wastewater-Based Epidemiology

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based epidemiology (WBE) was conducted to track Enteroviruses (EVs) circulation in the Milan metropolitan area (Northern Italy) during Covid-19 pandemic (March 2020–December 2022).

202 composite 24-hour wastewater samples (WWSs) were collected weekly from March 24, 2020, to December 29, 2022 at the inlet of two wastewater treatment plants (WWTP) in Milan (1.5 million inhabitants). EV-RNA was quantified and molecular characterization of non-polio EVs (NPEV) was performed by Sanger sequence analysis. Data from WWS were matched with virological data collected in the framework of Influenza-Like Illness (ILI) surveillance in the same place and time.

EV-RNA was identified in 88.2 % of WWSs. The peak in EVs circulation was observed in late August 2020 (upon conclusion of the first national lockdown), in late August 2021, and in mid-April 2022.

EV-RNA concentration in WWS (normalized as copies/d/1000 people) at peak of circulation presented a yearly increase (2020: 2.47×10^{10} ; 2021: 6.81×10^{10} ; 2022: 2.14×10^{11}). This trend overlapped with trend in EV-positivity rate in ILI cases, expanded from 21.7 % in 2021 to 55.6 % in 2022. EV trends in WWS preceded

* Corresponding author at: Department of Biomedical Sciences for Health, University of Milan, Via C. Pascal 36, 20133 Milan, Italy.

E-mail address: laura.pellegrinelli@unimi.it (L. Pellegrinelli).

clinical sample detections in 2021 and 2022 by eight and five weeks, respectively, acting as an early warning of outbreak.

Although sequencing of EV-positive WWSs revealed the presence of multiple EV strains, typing remained inconclusive. Molecular characterization of EVs in clinical samples revealed the co-circulation of several genotypes: EV-A accounted for 60 % of EVs, EV-B for 16.7 %, EV-D68 for 23.3 %.

EVs were circulating in Milan metropolitan area between March 2020 and December 2022. The epidemiological trends unfolded the progressive accumulation of EV transmission in the population after removal of Covid-19 restrictions. The increased circulation of EVs in 2021–2022 was identified at least 35 days in advance compared to the analysis of clinical data. The inconclusive results of Sanger sequencing lookout for improvement and innovative molecular approaches to deepen track EVs.

1. Introduction

Human enteroviruses (EVs) are common viruses infecting humans and belong to the Enterovirus *genus* within the *Picornaviridae* family (Dunn, 2016). The genetic relationships of individual enteroviruses and rhinoviruses to each other, and to other picornaviruses, form the basis of their current taxonomy. Nowadays, the members of the Enterovirus *genus* are classified based on the molecular sequencing of the variable part of viral protein 1 (VP1) (Oberste et al., 1999) into seven species infecting humans: three rhinovirus (RV) species (RV-A to RV-C) and four EV species (EV-A to EV-D) (Oberste et al., 1999). EV species includes Poliovirus (PV) and Non-Polio Enteroviruses (NPEVs) such as coxsackieviruses (CVs) A and B, echoviruses (Es), and other EV types (i.e. EV-A71, EV-C105, EV-D68) (Kottaridi et al., 2005).

EVs primarily transmit mainly through the fecal-oral route and undergo their replication cycle in the gastrointestinal tract. From here they can spread to all organs of the body, including the central nervous system (CNS) (Baggen et al., 2018). Alternatively, or additionally, EVs such as EV-D68 preferentially reside in the upper respiratory tract and spread principally through respiratory secretions (Gutierrez et al., 2016; Jaramillo-Gutierrez et al., 2013). EV symptoms, if any, normally develop after an incubation period of 3–21 days, while it can be shed from the throat up to 2–3 weeks and in feces up to 4–6 weeks, extending the period of contagion and increasing the likelihood of secondary infections (Baggen et al., 2018). The EVs seasonal pattern varies depending on genotype and the geographical area. In continental climate regions, most impactful epidemic outbreaks occur during summer and fall, although baseline EVs circulation can be observed all year round (Abedi et al., 2015; Khetsuriani et al., 2006).

The main approach used so far for EV surveillance has been the monitoring of infection in symptomatic individuals. However, in Europe, paralytic polio has been the only reportable disease monitored through the acute flaccid paralysis (AFP) surveillance system (Wilkinson et al., 2022). The lack of *ad hoc* EV surveillance makes it difficult to assess the actual disease burden attributable to EV infections, which remain either underdiagnosed or underreported, although EVs can also lead to life-threatening and fatal infection (Fischer et al., 2022; Harvala et al., 2018; Pellegrinelli et al., 2021).

Recently, environmental surveillance (ES) of wastewater samples (WWSs) has added an additional level to research, filling some important epidemiological gaps (Battistone et al., 2014a; Brinkman et al., 2017; Pellegrinelli et al., 2017b; Venkatesan, 2022). Exploration was especially carried out amid the SARS-CoV-2 pandemic, when clinical testing for EVs and other pathogens was not feasible, thus negatively affecting the efficiency of AFP surveillance (Venkatesan, 2022). In fact, over the last three years, ES and wastewater-based epidemiology (WBE) have been recognized as the primary data source to identify EVs circulation (Pellegrinelli et al., 2017b) as well as other pathogens spreading in the population through the fecal-oral route. This approach enables all infected individuals - asymptomatic and symptomatic - to be considered (Pellegrinelli et al., 2019; Pellegrinelli et al., 2022). WBE permits the determination of the EV circulation patterns thanks to the transmission of EVs via human feces. Their strong environmental stability results from being non-enveloped viruses, which allows them to resist extreme

temperatures, pH, disinfectants, chloroform and other lipid solvents (Tambini et al., 1993). The main advantage of wastewater surveillance is that it can serve as an early warning system of viral spread in communities, giving additional, crucial information about virus circulation and prevalence of current infections in the population.

We have previously reported an association between the presence of SARS-CoV-2 and Adenovirus genomes in WWS and positivity rates of clinical samples and related disease and hospitalization within the general population (Castiglioni et al., 2022; Nattino et al., 2022; Pellegrinelli et al., 2022). Here, we conducted a molecular monitoring of EVs circulation in the general population by the application of WBE methodology and we compared EV genome concentration in WWSs to rate of EV-positive cases in the community during the SARS-CoV-2 pandemic (from March 24, 2020 to December 29, 2022).

2. Materials and methods

2.1. Wastewater sampling

Consecutive 24-h composite weekly WWSs (1 L) were collected at the inlet of two urban wastewater treatment plants (WWTPs), Nosedo and San Rocco, hereafter referred as WWTP-A and WWTP-B, respectively, in high-density urban settings in the Milan metropolitan area. Sampling was performed with a 24-h-automatic sampler, and samples were collected in polypropylene bottles and shipped frozen (-20°C) to the Sub-National Reference Laboratory (SNRL) at the Department of Biomedical Sciences for Health, University of Milan (Lombardy region, Italy) for PVs and NPEVs identification and molecular characterization. WWSs were kept frozen (-80°C) until virological assessment. Sampling and storage of WWSs were carried out according to WHO method (WHO/V&B/03.03, 2003). Consecutive wastewater sampling was completed from March 24, 2020 (week [w]13–2020) to December 29, 2022 (w52–2022) resulting in a total of 202 samples: 107 samples were collected from WWTP-A, and 95 from WWTP-B. These WWTPs allowed the investigation of about 90 % of the Milan metropolitan area, which serves around 1.5 million inhabitants.

2.2. Virus concentration of wastewater samples and molecular quantification of EV genome

Samples were concentrated following the method proposed by Wu et al. (2020) and adapted from Castiglioni et al. (2022). Briefly, 45 mL of WWS were concentrated by using a polyethylene glycol 8000-based (PEG-8000) concentration method. The pellet resulted from the centrifugation resuspended in 600 μL of phosphate-buffered saline (PBS) (Lonza, Belgium) and centrifuged once more at $2000 \times g$ for 30 s, at 4°C without break (Sigma 3-16PK, Sigma, Germany). The resuspended pellet was transferred to 2 mL clean microcentrifuge tubes and stored at 4°C if used immediately or at -80°C if used later.

To assess process efficiency exogenous virus controls (Mengovirus) was seeded into WWS before the concentration.

RNA extraction from concentrated WWSs was performed by a commercial method (QIAamp MinElute Virus Spin Kit, Qiagen, Germany) according to a modified protocol (Castiglioni et al., 2022).

EV-RNA identification was performed by using a one-step real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay, amplifying the 5' non-coding region (5'NCR, nucleotide [nt.] 415–536) common to all EVs, as previously described (Bubba et al., 2015). A standard curve was established for semi-quantification of the EV-RNA. Real-Time PCRs were run with QuantStudio5 Real-Time PCR system (Thermo Fisher Scientific, USA). Positive and negative controls were included in each real-time RT-PCR run. Samples were considered EV-positive if cycle threshold (Ct) value was below 40.

All samples were subjected to molecular detection of PV (VP1 fragment; nt. 3204–4852) by a RT-nested-PCR (RT-nPCR) previously described (Pellegrinelli et al., 2017b). The three-step method included a cDNA synthesis, a first PCR step (nPCR1) and a second PCR step (nPCR2). All RT-nPCR reactions were run on GeneAmp PCR System 9700 (Applied Biosystem, USA) (Battistone et al., 2014a; Delogu et al., 2018; Pellegrinelli et al., 2017b).

To minimize potential PCR contamination, RNA extraction, molecular assays set-up and PCR runs were all performed in separate rooms.

2.3. Genotype identification of EVs by Sanger sequencing

Molecular characterization of EVs was performed by sequence analysis of a fragment of the VP1/VP3 gene (nt. 2602–2965), as described by WHO/CDC Enterovirus surveillance guidelines (WHO. Enterovirus surveillance guidelines).

Sequencing was performed by the Sanger method by means of an automated sequencer with capillary electrophoresis technology (ABI PRISM®3100 GeneticAnalyser, Applied Biosystems, USA). Electropherograms were edited with the bioinformatics software BioEdit (version 7.2).

2.4. Data source for epidemiological data

As EV infections (excluding poliomyelitis) are not notifiable conditions in Italy, epidemiological data of clinically confirmed EV infections were retrieved from the database of virological influenza and Covid-19 surveillance of the regional reference laboratory for Lombardy within the Italian influenza surveillance network (Istituto Superiore di Sanità, Sorveglianza virologica dell'influenza; rete Influnet). Influnet relies on the voluntary participation of sentinel physicians who survey nearly 4 % of the general population, ensuring the representativeness of all age groups. Respiratory samples are collected from outpatients with influenza-like illness (ILI) seeking care in ambulatory setting (Ministero della Salute).

Historically, virological surveillance within Influnet has had an observation period of 28 consecutive weeks. The timeframe during which clinical samples for influenza viruses are tested normally extends from week 46 to week 17 of the following year. Given the increased frequency in non-specific clinical manifestations of viral respiratory infections and the recent emergence of SARS-CoV-2, virological surveillance of ILI was implemented. Therefore, since 2021, the period under surveillance has been extended such that samples were continuously collected from w39–2021 to w52–2022.

The queries used to extract epidemiological data from this database were: i) samples collected from w13–2020 to w52–2022, AND ii) samples collected from outpatients with ILI symptoms attending the ambulatory of sentinel general practitioners in Milan metropolitan area, AND iii) samples tested for EV-RNA detection. The following epidemiological and virological surveillance data were collected: number of respiratory samples collected per week; number of respiratory samples tested positive for EVs per week and, if available, data on molecular characterization of EV cases (Pellegrinelli et al., 2017a).

2.5. Data analysis

EV-RNA concentrations were expressed as genome copies per liter (copies/L) of WWS by application of the virtual standard curve equation

These concentrations were multiplied by the daily flow rate of each WWTP (m^3/d) to obtain the viral loads entering the WWTPs daily. Viral loads were normalized to the population, reported as copies/d/1000 people, to compare results from the different communities. The residential population was available for both the WWTPs and was used for normalization (Castiglioni et al., 2013). The fraction of EV-positive WWSs and the mean value of copies/d/1000 people of EV-RNA were evaluated through a two-tailed Student's *t*-test assuming different variances. A *p*-value below 0.05 was considered statistically significant. Seasonal characteristics of EVs, including season onset (or start), duration, peak and offset (or end) were estimated from temporal data of WWSs.

EV-positivity rate among ILI cases was calculated by week and it was expressed as a crude proportion (numerator: number of EV-positive samples; denominator: number of EV-positive samples plus EV-negative samples). Seasonal EV characteristics among ILI cases were estimated by applying the RS10 method, which defines the start of an epidemic season as the first two consecutive weeks when virus detection exceeds 10 % of virus positivity (Midgley et al., 2017).

3. Results

3.1. Detection and quantification of EV-RNA in wastewater samples

In this study we considered WWSs collected for consecutive weeks from March 24, 2020 (w13–2020) to December, 29, 2022 (52–2022), during Covid-19 pandemic in Italy. EV-RNA was identified in 88.1 % (178/202) of WWSs collected in the area of Milan. In particular, the EV-positivity rate in WWSs resulted statistically higher ($p < 0.05$) during 2022 (94 %; 79/84) compared to that observed in 2020 (86.7 %; 39/45) and 2021 (82.2 %; 60/73). Overall, the percentage of positive samples was similar ($p > 0.05$) in the two WWTPs under investigation; 87.8 % (94/107) in WWTP-A and 88.4 % (84/95) in WWTP-B.

The temporal and quantified data of EV circulation formed the epidemic curve of EVs across the Milan metropolitan area. These findings uncovered at least three significant and consecutive epidemic peaks (Fig. 1). Table 1 summarizes the characteristics of EV epidemic identified by WBE. The onset of the EV epidemic waves occurred at the end of August 2020, the end of August 2021 and in mid-April 2022 (w35–2020, w35–2021 and w14–2022, respectively). The length of EV epidemics increased each year: 8 weeks in 2020, 14 weeks in 2021 and 39 weeks in 2022.

The concentration of EV genome in WWSs collected during the peak of the three epidemic waves was highest ($p < 0.005$) in 2022 (2.14×10^{11} copies/d/1000 people) followed by 2021 (2.47×10^{10} copies/d/1000 people) and 2020 (6.81×10^{10} copies/d/1000 people) (Table 1). A similar trend was revealed by statistical analysis where mean viral loads of WWSs collected during the 2022 epidemic (6.55×10^{10} copies/d/1000 people) were significantly higher ($p < 0.005$) than those in 2020 (9.74×10^9 copies/d/1000 people) and in 2021 (3.12×10^{10} copies/d/1000 people) (Table 1). The EV epidemics observed in 2020 and 2021 were followed by a steep decline (Fig. 1).

During the study period, the percentage of respiratory samples collected from ILI outpatients that resulted EV-positive was 10.3 %. No EVs were identified in samples collected until October 2021 (w40–2021). With the exclusion of the season preceding October 2021 when no EVs were detected, the mean weekly EV-positivity rate was 12.7 %, ranging from 3 % (w12–2022) to 55.6 % (w25–2022) (Fig. 1).

The temporal analysis of EV-positivity rate in clinical samples by week (Fig. 1) shows two EV epidemics, of which the characteristics are detailed in Table 1. The onset of EV epidemics identified by WBE preceded the upsurge in ILI cases observed in 2021 and 2022 by 8 and 5 weeks, respectively (Fig. 1, Table 1). In fact, the onset of the EV epidemic identified in 2021 was in the end of October 2021 (w43–2021) and peaked in December 2021 (w51–2021), whereas the upsurge of EV cases in 2022 was identified in mid-May 2022 (w19–2022) and peaked at the end of June 2022 (w25–2022). The length of the EV epidemics was

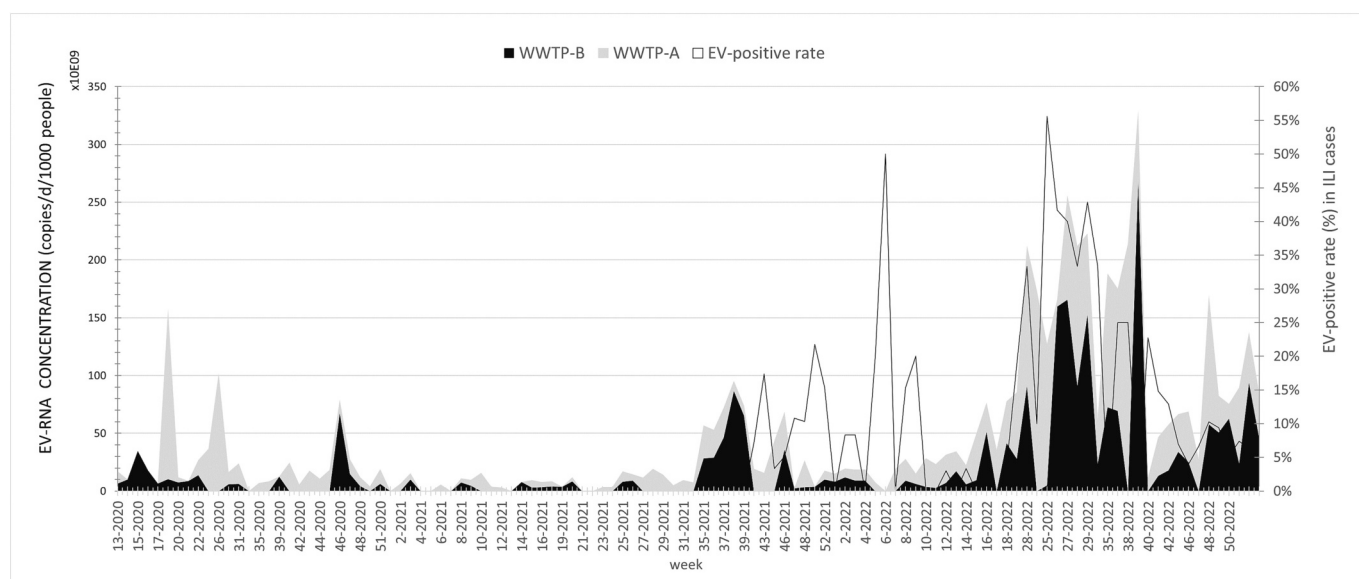


Fig. 1. Quantification of EV-RNA (copies/d/1000 people) in wastewater samples (WWS) (blue) and EV-positivity rate by week in clinical samples (gray) collected from ILIs in the Milan metropolitan area from March 24, 2020 (w13–2020) to December 29, 2022 (w52–2022).

Table 1

Temporal data for EV identification in wastewater samples (WWS), clinical samples collected from ILIs and EV-RNA concentration (copies/d/1000 people) in the Milan metropolitan area from March 24, 2020 (w13–2020) to December 29, 2022 (w52–2022). n.a.: samples not available.

Wastewater samples	Year of study					
	2020		2021		2022	
	WWS	ILIs	WWS	ILIs	WWS	ILIs
Week of onset	w35–2020	n.a.	w35–2021	w43–2021	w14–2022	w19–2022
Week of peak	w41–2020	n.a.	w40–2021	w51–2021	w35–2022	w25–2022
Week of offset	w42–2020	n.a.	w48–2021	w52–2022	w52–2022	w47–2022
Epidemic length (N. of weeks)	8	n.a.	14	10	39	30
EV-RNA concentration in WWS and EV-positivity rate in ILIs at peak	2.47×10^{10}	n.a.	6.81×10^{10}	21.7 %	2.14×10^{11}	55.6 %
Mean value of EV-RNA concentration and EV-positivity rate in ILIs during the epidemic	9.74×10^9	n.a.	3.12×10^{10}	11.9 %	6.55×10^{10}	19 %

of 10 weeks in 2021 and 30 weeks in 2022.

EV-positivity rate during the epidemic peak was statistically higher during the upsurge in 2022 than that observed in 2021 (55.6 % vs. 21.7 %, $p < 0.005$). In the same manner, the mean value of EV-positivity during the 2022 epidemic was higher with respect to that observed in 2021 (19 % vs. 11.9 %, $p < 0.005$).

3.2. Molecular characterization of EV in wastewater samples and clinical samples

A total of 56 out of 178 (31.5 %) EV-positive samples were randomly selected and successfully sequenced. Unfortunately, interpretation of EV genotypes resulted inconclusive. In fact, the electropherograms of the sequences revealed multiple overlapping peaks, making molecular characterization of EVs impossible, but uncovering the probable co-presence of more than one EV genotype in the same sample.

According to the molecular characterization of EV genotypes identified in respiratory samples collected from ILIs (Fig. 2), the circulation of different EV genotypes was observed. Of these, group A (CV-A2, CV-A4, CV-A5, CV-A6, CV-A9, CV-A16) accounted for 60 % of EVs, group B (E-3, E-11, E-25, CV-B5, CV-B2) accounted for 16.7 % of EVs, and group D - namely EV-D68 - accounted for 23.3 % of EVs. To specify, in ILI series, two epidemics of EV-D68 were observed, the first one between the end of October and the end of December 2021 (w43/52–2021; epidemic length 9 weeks) and the second between the end of August and the end of October 2022 (w35/44–2022; epidemic length 10 weeks).

4. Discussion

The experience from the Covid-19 pandemic as well as the ever-increasing scientific evidence on WBE have demonstrated the potential for scanning pathogens from sewage samples (Karthikeyan et al., 2021; La Rosa et al., 2022), although wastewater EVs surveillance is not a new concept. In fact, it has been applied for decades to track polio, and it is playing an important role in the WHO polio-eradication campaign (Battistone et al., 2014a; Delogu et al., 2018; Pellegrinelli et al., 2013).

In the present study, wastewater samples collected from the inlet of two WWTPs in the Milan metropolitan area (Lombardy, Northern Italy) for nearly three consecutive years amid the SARS-CoV-2 pandemic, (March 24, 2020–December 29, 2022) were analysed to molecularly quantify the circulation of EVs in its 1.5 million inhabitants. Data from WBE were compared to those collected in the framework of influenza and Covid-19 virological syndromic surveillance as data source for EV prevalence in the population.

Here, no PV-RNA was identified, excluding any PV circulation in the Milan metropolitan area and confirming the epidemiological situation in Italy, which has been polio-free since replacement of OPV with IPV in 2002 (Smith et al., 2004). In the context of the “Polio Endgame Strategy 2019–2023”, coordinated by the WHO (WHO/V&B/03.03. 200), the decline in AFP surveillance system performance was contrasted by the implementation of WBE, recognized as the primary data source to identify PV circulation in New York (USA) and in the UK during the Covid-19 pandemic (Link-Gelles et al., 2022; Uwishema et al., 2022; Wilkinson et al., 2022; Zomahoun et al., 2021).

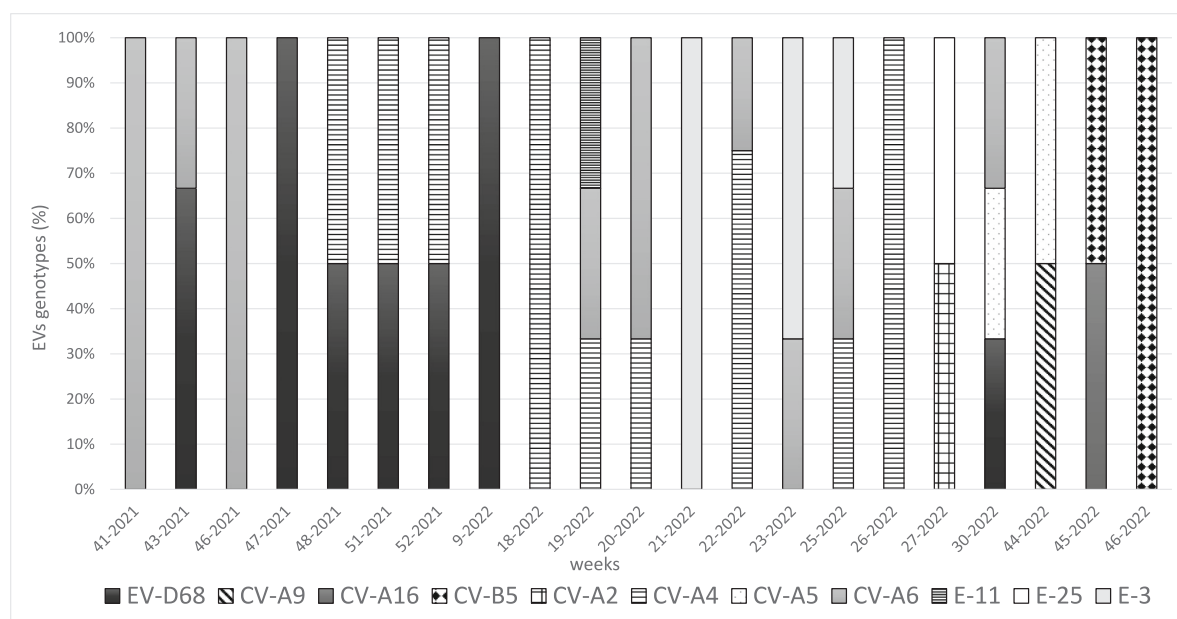


Fig. 2. Prevalence (%) of EV genotypes by week in clinical samples collected from ILIs in the Milan metropolitan area from March 24, 2020 (w13-2020) to December 29, 2022 (w52-2022).

A sustained presence of NPEVs was observed in WWSs collected from the WWTPs under investigation. In fact, nearly 88 % of WWSs tested positive for EV-RNA and the quantification of the EV genome allowed us to identify consecutive upsurges of EV circulation in Milan. The peaks of EV concentration were observed in WWSs collected in late August 2020 and 2021, aligning with the historical greater incidence of EVs circulation and EV-related diseases during the months at the turn of summer and fall (Abedi et al., 2015; Brinkman et al., 2017; Pellegrinelli et al., 2017b). Furthermore, another surge of EVs with an onset in mid-April 2022 was also detected.

It is of great interest that an EV epidemic was also identified during 2020. Concurrently, Italy was under national lockdown until June 2020, followed by other intermittent lockdowns until December 2020. This latter year also saw the massive implementation of non-pharmaceutical interventions (NPI) to limit the spread of Covid-19. The increase of EVs in 2020 came upon the conclusion of the first national lockdown - declared on March 22, 2020 (w12-2020) and that ended on May 3, 2020 (w18-2020) - thus allowing some EVs circulation. In this light, mitigation measures put in place during the Covid-19 pandemic, such as face masking and social distancing, did not fully silence EV circulation. A probable reason is that EVs are non-enveloped viruses: this makes them more resistant to disinfectants and their transmission might not be prevented enough by facemasks, as pointed out by others (Eldesouki et al., 2022; Piret and Boivin, 2022).

Another interesting insight of our data series is that the EV epidemics identified by WWSs analysis were observed weeks before the increase in ILI cases. The increase of EV infections among ILIs in 2021 and 2022, confirmed and underlined that wastewater trends often precede the corresponding clinical detections, acting as an epidemiological alert and helm (Karthikeyan et al., 2021; Levy et al., 2023). The progressive reduction of NPIs, from reopening of public places and the coincidental rise in the displacement of the population, helped the increase of EVs spread in 2021, culminating to the removal of all restrictions from May 2022 onwards. The progressive accumulation in EV transmission explains the statistically significant highest activity in EV onset in May 2022.

Our data from WWSs matched with data of EV circulation in clinical samples coincides with that of previous research. They have indicated an increase in EV infections in the European and US populations since June/July 2021, particularly of EV-D68 (Benschop et al., 2021; Erster

et al., 2022; Fall et al., 2022; Tedcastle et al., 2022). The increase of EV-D68 cases is not only highlighted by our data but also mirrored by recent next generation sequencing (NGS)-based studies conducted on WWSs in the UK, Israel and the USA between July and November 2021 (Benschop et al., 2021; Erster et al., 2022; Fall et al., 2022; Tedcastle et al., 2022).

Our unfeasible genotype identification by Sanger sequencing did not permit to uncover the circulation of EV-D68 in WWSs. This is a known limitation of WBE. In fact, genomic surveillance of wastewater is technically challenging because of low viral loads, eventual PCR inhibitors and fragmented RNA. However, it is important to note that while Sanger sequencing might not have resolved EV mixtures in our sample, it was able to signal the possible presence of EVs, and more importantly, the possibility that multiple EV types might be present in a single sample, as reported also by Faleye et al. (2021). This evidence is supported by the distribution of multiple EV genotypes - group A, B and D - that were observed in the clinical samples collected among the same population served by the WWTPs.

The implementation of whole-genome sequencing by NGS may uncover the circulation of multiple EV types, groups or lineages. Furthermore, it must be noted that investigation on environmental circulation, in particular that of EV-D68, reveals to be more challenging than other EV types, as the virus is predominantly found in the respiratory tract, and less in human feces, and infected prevalently children still in diapers (Larsson et al., 2022).

As the Covid-19 pandemic has demonstrated, combining NGS and the quantification of EV by RT-PCR assays should be the best approach for WBE in the future. Ideally, this detection process should be implemented at different stages, with RT-PCR being conducted for routine assessment and early warning whereas NGS testing being used at sentinel sites to uncover genotypes and emerging EVs that may pose a risk to human health.

5. Conclusion

Despite the SARS-CoV-2 pandemic and implementation of NPIs to contrast the pandemic, WBE confirmed the circulation of EVs in the Milan metropolitan area between March 2020 and December 2022. Viral concentration increased as measures to contrast Covid-19 were relaxed. The two upsurges of EV circulation in 2021 and 2022 were identified weeks in advance and later confirmed by the analysis of

clinical epidemiological data.

On one hand, these findings urge to identify a threshold value for EV concentration in wastewater to scan and recognize epidemic events more rapidly. On the other hand, the approach still longs for the implementation of innovative molecular methods, such as NGS, to identify specific genotype circulation and to uncover the emergence of viruses of concern to public health, such as EV-D68 and EV-A71.

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Ethical approval statement

Not required.

CRediT authorship contribution statement

Laura Pellegrinelli: Conceptualization, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Cristina Galli:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Arlinda Seiti:** Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing. **Valeria Primache:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Aurora Hirvonen:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Silvia Schiarea:** Investigation, Writing – review & editing. **Giulia Salmoiraghi:** Investigation, Writing – review & editing. **Sara Castiglioni:** Methodology, Writing – review & editing. **Emanuela Ammoni:** Supervision, Writing – review & editing. **Danilo Cereda:** Supervision, Writing – review & editing. **Sandro Binda:** Conceptualization, Supervision, Writing – review & editing. **Elena Pariani:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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