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ORIGINAL PAPER

Molecular Characterization and Phylogenetic Analysis of Enteroviruses and Hepatitis A Viruses in Sewage Samples, Northern Italy, 2016

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Abstract

Enteroviruses (EVs) and Hepatitis A Viruses (HAVs) are human pathogens with a wide spectrum of clinical manifestations. The monitoring of sewage samples enables to monitor the EVs and HAVs in circulation among the general population and recognize possible outbreaks. This study focused on the molecular characterization and phylogenetic analysis of the EVs and HAVs identified in 33 sewage samples collected every 15 days at the influent of a wastewater treatment plant located in Northern Italy from March to October 2016. According to the results of the molecular characterization, the most frequently identified viruses were Echovirus 6 (E-6), E-11 and HAV-IA. The phylogenetic analyses indicated the rapid genetic evolution of E-6 and E-1; noteworthy, most E-11 strains clustered with a strain isolated from a clinical sample collected in the same geographical area over the same period by our laboratory. Most of the HAV strains detected clustered with epidemic HAV-IA strains identified during the European hepatitis A outbreak that occurred in 2016–2017 affecting men who have sex with men (MSM). The detection of environmental HAV strains before and at the beginning of its spread amongst humans demonstrated that this outbreak could have been predicted by monitoring sewage samples. Moreover, conducting a genetic comparison between the HAV and EV strains identified in sewage and clinical samples may improve knowledge of viral epidemiology. EV and HAV molecular environmental surveillance may prove useful for identifying viral circulation and for issuing early warning alerts on possible outbreaks among the human population.

Keywords Enterovirus · Hepatitis A virus · Environmental samples · Virus-concentrate sewage · Molecular characterization · Phylogenetic analysis

Introduction

Enterovirus (EV) and hepatitis A virus (HAV) are small non-enveloped positive-strand RNA viruses belonging to the *Picornaviridae* family. To date, four species of EVs (EV-A to EV-D) comprised of more than 100 types have been identified in humans (Pallansch 2013) and three distinct genotypes of HAVs (I, II and III), further divided into subtypes A and B, have been identified as human pathogens (Vaughan et al. 2014). Mechanisms of EV and HAV genetic variation, such as mutations and genetic recombinations, were characterized by molecular and phylogenetic analyses, demonstrating that new genetic or antigenic variants of these viruses can emerge over time (Pallansch 2013; Vaughan et al. 2014). Worldwide, EV and HAV are responsible for a wide spectrum of clinical manifestations, ranging from asymptomatic presentation to severe and life-threatening diseases (Muehlenbachs et al. 2015; Vaughan et al. 2014). HAV is one of the most common causes of liver disease, which may lead to sporadic acute hepatitis and large outbreaks. EVs can cause from mild to severe disease including systemic and neurological infections (Muehlenbachs et al. 2015). EVs and HAVs are mainly transmitted via the faecal–oral route by direct or indirect contact; while EVs are generally transmitted person-to-person, HAVs are often

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transmitted through the ingestion of contaminated food or water, especially when there is inadequate sanitation and poor hygiene practices (Pallansch 2013; Vaughan et al. 2014). As EV and HAV are non-enveloped viruses, they are resistant to ethers, chloroform and all lipid solvents in general and they can withstand pH and temperature variations; consequently these viruses can survive in the environment and sewage water. It has been established that monitoring sewage samples collected at the influent of waste water treatment plants (WWTPs), it is possible to sample the entire population served by the WWTP, and therefore, track EV and HAV circulation and detect viral outbreaks (Bisseux et al. 2018; Hellmér et al. 2014; Monge et al. 2018).

This study was aimed at determining the prevalence of EVs and HAVs in sewage samples collected at a WWTP in Northern Italy and at describing the molecular characteristics and the phylogenetic analyses of these viruses.

Materials and Methods

From March to October 2016, wastewater samples were collected at the influent of a municipal WWTP in Northern Italy. The WWTP is located in the city of Milan (Northern Italy), and collects and treats wastewater from an urban area with approximately 1,250,000 inhabitants. The WWTP treats wastewater from two different sewers (Nosedo and Ampliamento Est), therefore, the amount of untreated wastewater entering the plant (influent) is 432,000 m^3/day, which is equal to 50% of the sewage flow from the central and eastern areas of the city.

During the study period, two (one for each WWTP sewer) wastewater samples were collected every 15 days. Each sample consisted of 500 ml of wastewater and was collected by an automated sampling system; the wastewater samples were concentrated to a final volume of 10 ml using the two-phase polyethylene-glycol and dextran (PEG-DeX) separation method proposed by the World Health Organization (WHO) guidelines (WHO 2003). The wastewater samples were analysed to detect EVs by virus isolation in L20B [murine transgenic L cells that are susceptible to poliovirus (PV) infection] and RD (human rhabdomyosarcoma, susceptible to EV infection) cell cultures (WHO 2003). Total viral RNA was extracted from 200 µl of both virus-concentrated samples and cell culture supernatants showing cytopathic effect (CPE) using a commercial extraction kit (QIAamp MinElute Virus Spin, Qiagen, Hilden, Germany); the elution volume was 50 µl of RNase-free water. The extracted RNA was tested for the presence of EVs and PVs by RT-nested PCR assays using specific primers targeting a fragment of the 5′ untranslated region (5′UTR) of EVs genome and the VP1 region of PVs, respectively (Delogu et al. 2018). To molecularly characterize EVs and HAVs, specific RT-nested PCR assays were preformed to amplify a fragment of the VP1 gene of EVs (Nix et al. 2006), a portion of the VP1-2A and the VP3-VP1 region in HAVs (Chironna et al. 2003). All amplicons were purified using a commercial kit (NucleoSpin® Gel and PCR Clean-up kit, Macherey–Nagel, Duren, Germany) and sequenced using Sanger sequencing method. EV type identification and HAV genotyping were achieved by nucleotide (nt) sequence similarity searches using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignments were carried out using the ClustalW program implemented in BioEdit software. Nucleotide and amino acid sequence similarity was calculated with the Sequence Identity Matrix tool. Phylogenetic trees were constructed using the Neighbour-Joining method and the Kimura 2-parameter model implemented in MEGA 6.0 software. The reliability of the phylogenetic trees was estimated by performing a bootstrapping analysis with 1.000 replicates; bootstrap values > 70% were considered statistically significant. For the phylogenetic analysis on the space–time distribution, human viral sequences reported in Italy and various European and non-European countries in 2016 (±2 years) were selected as references. The nucleotide reference sequences used for constructing the phylogenetic trees were retrieved from GenBank (Tables S1–S5 of Supplemental material).

Results

During the study period a total of 33 wastewater samples (16 from the Nosedo sewer and 17 from the Ampliamento Est sewer) were collected and analysed for the presence of EVs and HAVs.

Enterovirus

None of the viral concentrates tested positive to PV by specific RT-nested PCR and no CPE was observed in the L20B cell cultures, thus confirming the absence of PV.

Overall, 66.7% (22/33) of the concentrated wastewater samples tested positive to EV by specific RT-nested PCR; all of these samples (100%) only showed CPE in the RD cell line.

20 out of 22 (90%) cultured EV-positive samples were successfully sequenced: 6 different types of EVs were identified: all of the EVs belonged to species B and were classified as Echovirus (E)-3, E-6, E-9, E-11, E-13 and E-14. In particular, the most frequently detected EV type was E-6 (47.6%; 10/21), followed by E-11 (28.6%; 6/21). The other EV types were identified in unitary amounts (4.8%; 1/21). The VP1 sequences of the two untypable EV-positive samples revealed mixed electropherogram peaks, suggesting

the co-presence of more than one EV type in the same supernatant.

A more detailed phylogenetic analysis was performed on the space–time distribution of the most commonly circulating EV types. Taking into account that E-6 can be classified into genogroups defined by letters (from A to C) (Mao et al. 2010) or numbers (from I to V) (Fares et al. 2011), the phylogenetic tree in Fig. 1a suggests that sequences of E-6 identified in this study ($n=10$) segregated into B/III ($n=1$) and C/IV ($n=9$) genogroups. The nucleotide and amino acid sequence similarities ranged from 82.0 to 100.0% and from 96.2 to 100.0%, respectively. More specifically, 8 strains belonging to genogroup C/IV clustered together sharing a very high level of nucleotide and amino acid similarity (range: 98.4–100.0% and 99.0–100.0%, respectively).

To genetically link the E-6 detected in our study to those reported in Italy or other countries over the same period, a phylogenetic tree was constructed with the E-6 prototype strain (D'Amori) as root (Fig. 1b). The phylogenetic tree showed an overall nucleotide and amino acid diversity of 25.5% and 8.9%, respectively (excluding the E-6 prototype strain D'Amori). Our unique E-6 sequence of genogroup B/III clustered with all Italian sequences (isolated from 2012 to 2014) available online, presenting a robust phylogenetic relationship (bootstrap value = 99) and high levels of nucleotide and amino acid similarities (95.5–99.0% and 97.0–100.0%, respectively). Most (8/9) of the E-6 sequences belonging to genogroup C/IV identified in this study, formed a distinct phylogenetic group separated from the other reference sequences (nucleotide diversity ranged from 13.8 to 22.6%; amino acid diversity up to 5.9%); moreover, the remaining sequence clustered with Dutch strains detected in 2014–2015 (nucleotide and amino acid identity range 95.5–100.0%).

As suggested by Oberste et al. (Oberste et al. 2003), E-11 can be subdivided into 4 genogroups (A–D) based on the VP1 region analysis; genogroup D was also divided into at least 5 lineages (D1-D5). The phylogenetic analysis of our E-11 sequences suggested a co-circulation of two genogroups: A ($n=1$) and D ($n=5$), respectively (Fig. 2a). All of the E-11 sequences of genogroup D belonged to the D5 lineage and shared high levels of nucleotide and amino acid similarities (range: 95.3–100.0% and 98.1–100.0%, respectively). Contrastingly, lower similarity values (74.3–75.5% and 89.1%, respectively) were observed within the E-11 sequence of genogroup A. On comparing our E-11 strains sequences with those identified in Italy or other countries in the same study period, the phylogenetic analysis revealed an overall nucleotide and amino acid diversity of 28.5% and 18.7%, respectively (excluding the E-11 prototype strain Gregory which was used as root). Our E-11 genogroup D5 sequences clustered with most of the Italian strains (nucleotide and amino acid similarity range: 92.8–100.0%

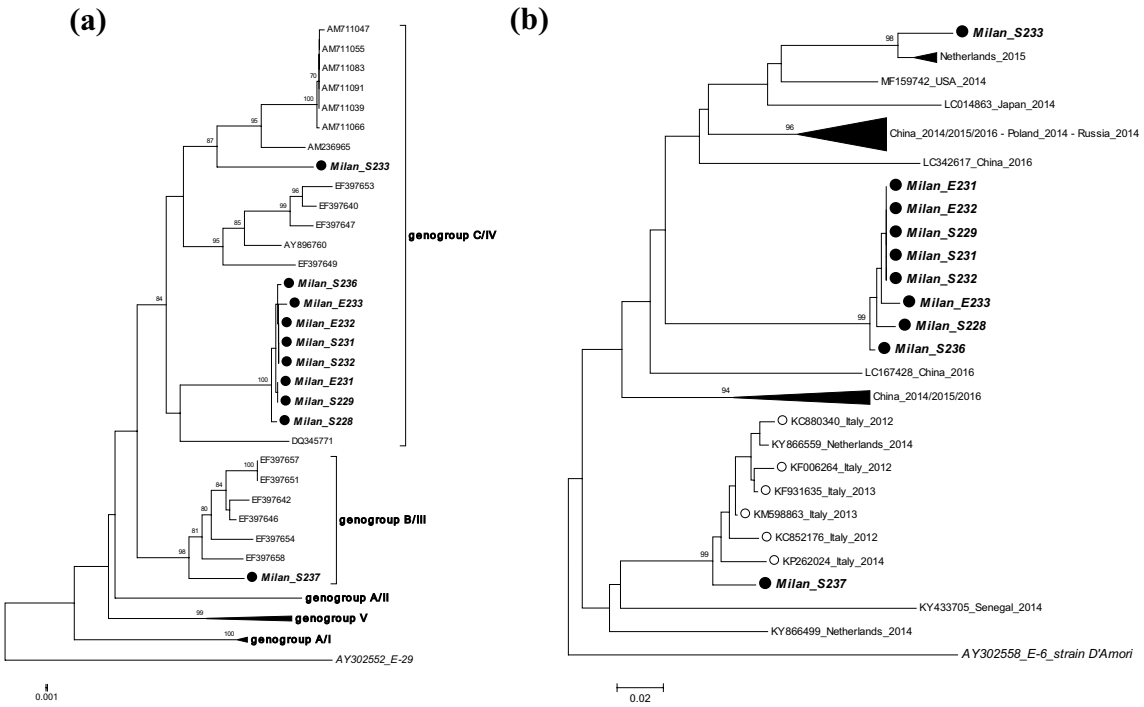

Fig. 1 **a** Rooted phylogenetic tree of E-6 strains identified in this study (filled circle) and genogroup reference strains; **b** rooted phylogenetic tree of E-6 identified in this study (filled circle), strains identified in Italy (open circle) and several European and non-European strains detected in the same study period

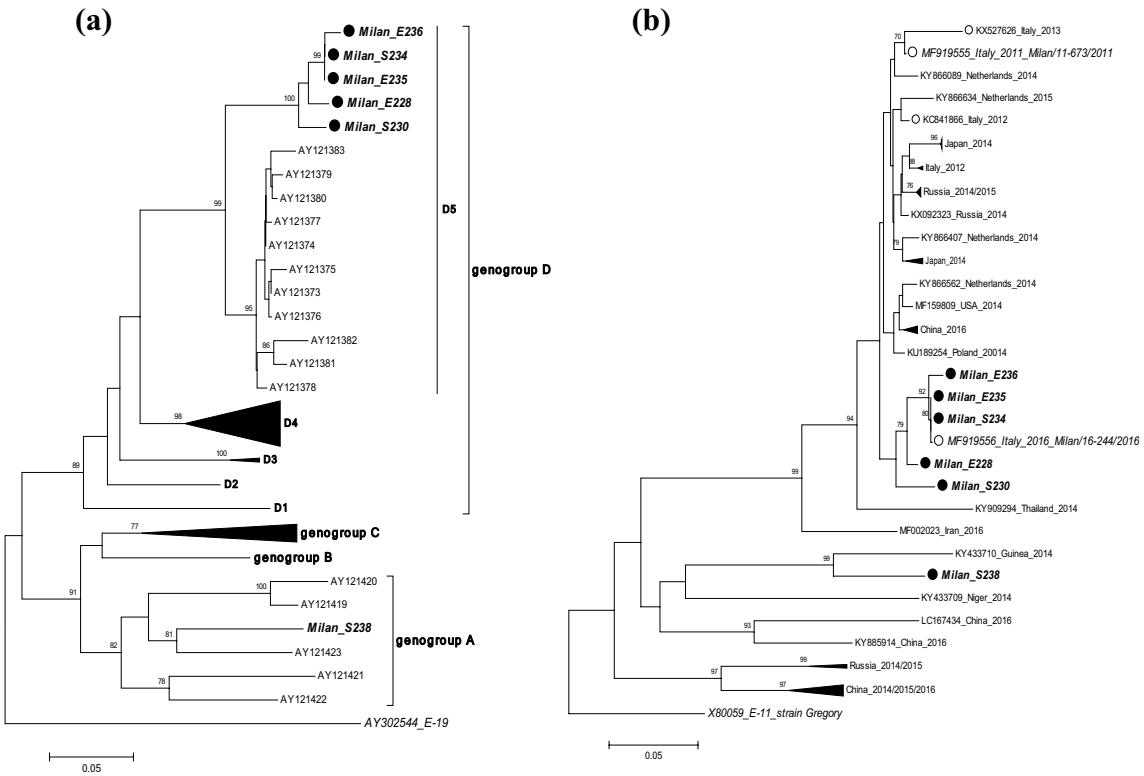

Fig. 2 **a** Rooted phylogenetic tree of E-11 identified in this study (filled circle) and genogroup reference strains; **b** rooted phylogenetic tree of E-11 identified in this study (filled circle), strains identified in Italy (open circle) and several European and non-European strains detected in the same study period

and 94.6–100.0%, respectively) and with other European, Asian and American sequences (nucleotide and amino acid similarity range: 88.4–100.0% and 89.3–100.0%, respectively). All of the E-11 sequences of genogroup D5 clustered with an E-11 strain previously identified from a clinical sample collected and analysed in our laboratory in 2016 (nucleotide and amino acid similarity range: 96.0–97.7% and 98.6–100.0%, respectively) (Fig. 2b) (Pellegrinelli et al. 2017). The only E-11 sequence belonging to genogroup A showed the highest percentage of nucleotide and amino acid similarity (89.3% and 97.3%, respectively) with an African strain isolated in 2014.

Hepatitis A Virus

Overall, HAV-RNA was detected in 15.1% (5/33) of the wastewater samples. The HAV-positive samples showed positivity for one or both of the amplified regions: more specifically, 40% (2/5) of samples tested positive for VP3-VP1 region and 80% (4/5) for VP1-2A region. All HAV positive strains belonged to genotype IA and were all collected in April ($n=1$) or between September and October ($n=4$) 2016.

A detailed phylogenetic analysis on the time spatial distributions of the HAV-IA sequences was performed on the strains sequenced in the VP1-2A region ($n=4$). HAV-IA sequences isolated in our study showed overall nucleotide and amino acid sequence similarities with other human HAV-IA sequences isolated in Italy in 2016 (± 2 years) or in other countries, which ranged from 91.5–100% to 94.3–100%, respectively. Nucleotide and amino acid similarities were higher (range 92.4–100% and 95.7–100%, respectively) when the analysis was only performed with the Italian HAV-IA strains. Three out of 4 HAV-IA sequences identified from samples collected in September–October 2016 clustered with the 3 HAV-IA strains involved in the European HAV outbreak affecting men who have sex with men (MSM), identified in June 2016 which has circulated Italy since August 2016 (nucleotide similarity ranged between 99.5 and 100% with a maximum of 1 different nucleotide; amino acid similarity 100%) (Fig. 3) (Ndumbi et al. 2018). The other HAV-IA sequences showed the highest nucleotide similarity (99.0%) with a VP1-2A sequence of an HAV strain identified in a human faecal sample collected in Southern Italy in 2008.

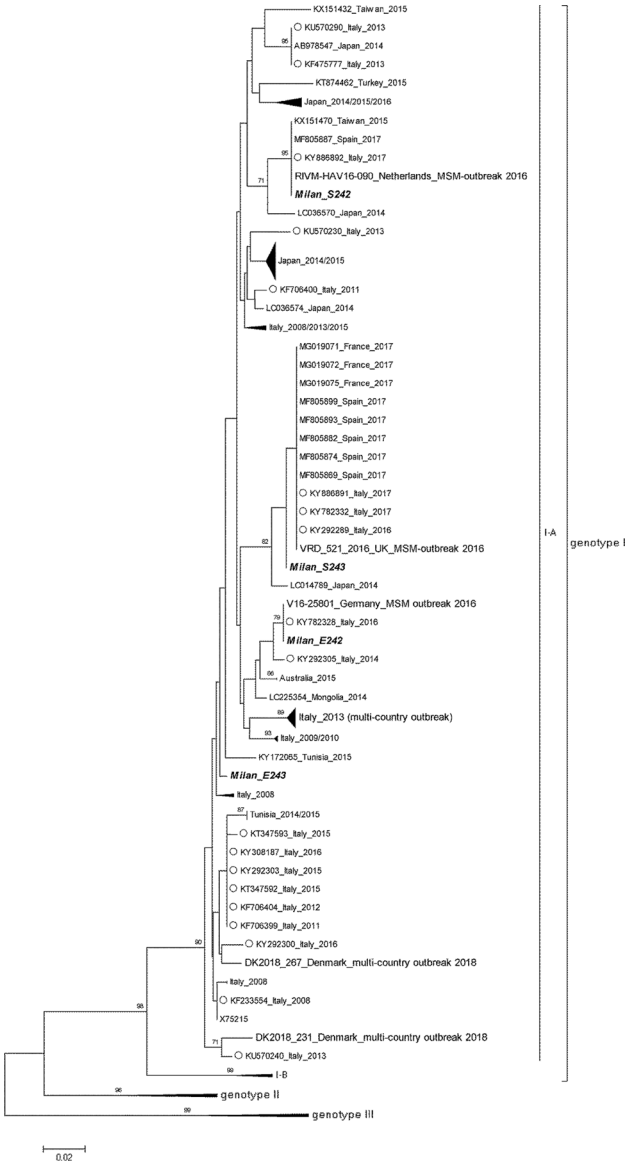

Fig. 3 Phylogenetic tree of HAV identified in this study (filled circle), HAV genotype reference sequences (italics), a number of HAV-IA strains identified in Italy (open circle) and several HAV-IA European and non-European strains detected in the same study period, including reference sequences of recent HAV outbreaks (filled diamond)

Discussion

In this study, the molecular characterization and the phylogenetic analysis of EVs and HAVs detected in sewage samples collected at a WWTP in Milan were carried out to gain valuable insights into their distribution and circulation.

EVs are widespread viruses that can cause asymptomatic infections or more serious illnesses with a wide range of symptoms, from the common cold to severe and life-threatening diseases (Muehlenbachs et al. 2015). Our study confirmed the epidemiological data on the absence of PV circulation in Italy. In this study, 66.7% of viral concentrate wastewater samples proved to be EV-positive and all (100%) were grown in RD cell cultures, as described by Battistone et al. (Battistone et al. 2014). The prevalence of EV in wastewater samples was higher than that reported in other Italian studies (Iaconelli et al. 2017a; Pennino et al. 2018), suggesting a wide circulation of EVs among the study population. According to the results of the molecular characterisation, six different types of EV species B were identified in this study; all of them were Echovirus, and E-6 and E-11 were the most frequently detected EV types, which were similar to the results obtained in another study carried out in Italy and other European countries from 2009 to 2015 (Delogu et al. 2018; Monge et al. 2018).

The phylogenetic analyses of the E-6 and E-11 identified revealed that E-6 segregated into genogroups B/III and C/IV while E-11 segregated into genogroups A and D5, as reported by several previous studies (Fares et al. 2011; Mao et al. 2010; Oberste et al. 2003; Savolainen-Kopra et al. 2009). In this study, E-6 and E-11 were characterised by nucleotide diversity levels of 22.6% and 18%, respectively, when compared to the genotypes that circulated in Italy or in other parts of the world in 2016 (± 2 years). This variability suggests that the EVs have undergone a genetic evolution as already underlined by other authors (Fares et al. 2011; Oberste et al. 2003). More specifically, most of E-6 genogroup C/IV formed one distinct phylogenetic group clearly separated from the other reference sequences. Despite the high-genetic variability, the unique E-6 strain identified in this study within genogroup B/III had high similarities with all of the sequences identified in Italy from 2012 to 2014, suggesting that this strain has been circulating in Italy for many years. Similarly, the E-11 sequences of genogroup D segregated with the majority of the Italian strains and with other European and non-European sequences, suggesting a wide spread circulation of these EV genogroups. Noteworthy, the E-11 strains of genogroup D clustered with an E-11 strain previously identified by our laboratory from a clinical sample collected in the same geographical area and in the same year of the current study (Pellegrinelli et al. 2017).

HAV is an ongoing challenge of public health, as it is one of the most common causes of acute liver disease (Vaughan et al. 2014). In Europe HAV is associated with the ingestion of food and water that has been contaminated by feces resulting in sporadic cases or large outbreaks, as demonstrated by the recent HAV outbreak caused by the consumption of contaminated frozen fruit (Enkirch et al. 2018). During the study period, HAV-RNA was identified in approximately 15% of the wastewater samples; this low prevalence in of HAV-RNA in wastewater seems to be consistent with national epidemiological data showing that the HAV infection rate is less than 1 case per 100,000 inhabitants (Istituto Superiore di Sanità—ISS)

in absence of outbreak. All of the characterised HAV strains belonged to genotype IA, which is one of the main genotypes circulating in Italy and Europe (Vaughan et al. 2014). A high degree of nucleotide and amino acid similarity was observed between our HAV-IA sequences and other sequences isolated in Italy or in other countries in the same period. The phylogenetic analysis revealed that the HAV strains identified from the wastewater samples collected from September to October 2016 clustered with HAV-IA outbreak strains, identified during a large European outbreak affecting MSM, that have been reported in Italy since August 2016 (Ndumbi et al. 2018). The detection of environmental HAV strains before and at the beginning of its spread amongst humans demonstrated that this outbreak could have been predicted by monitoring sewage samples, as previously reported by other authors (Hellmér et al. 2014). Moreover, the comparison of environmental data and human surveillance could deepen our understanding of the epidemiology of enteric viruses (Bisseux et al. 2018; Hellmér et al. 2014; Monge et al. 2018), as observed for both HAV and E-11.

A limitation of this study is that using only the conventional Sanger sequencing method on EV-positive cell supernatants and HAV-positive wastewater samples, it was not possible to detect multiple virus genotypes in the same sample. The fact that VP1 sequencing of the two cultured EV-positive samples that resulted untypable showed mixed peaks within the electropherogram may suggest the co-presence of more than one EV type in the same supernatant. As recently demonstrated, next generation sequencing (NGS) can enable to better estimate viral genetic diversity in the environmental samples (Iaconelli et al. 2017b).

In conclusion, molecular characterization and phylogenetic analyses of EVs and HAVs in environmental samples demonstrated a widespread circulation of EVs among the population and showed that HAV-IA outbreak strains had already been circulating in Italy before the HAV outbreak. A routine sentinel surveillance system for viruses transmitted via the oral–faecal route could prove useful for characterizing the strains circulating among the human population. It could also strengthen disease surveillance at community level and provide early warning of disease outbreaks among the general population.

Accession Numbers for All Newly Published Sequences

All of the sequences of E-6, E-11 and HAV analysed in this study were deposited in the GenBank database under the accession numbers: MK737874–MK737893 (Table S6 of Supplemental material).

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