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Monitoring of enterovirus diversity in wastewater by ultra-deep sequencing: An effective complementary tool for clinical enterovirus surveillance

Maxime Bisseux [a, b, *], Debroas Didier [a], Mirand Audrey [a, b], Archimbaud Christine [a, b], Peigue-Lafeuille Hélène [a, b], Bailly Jean-Luc [a, b], Henquell Cécile [a, b]

[a] Université Clermont Auvergne, CNRS, Laboratoire Microorganismes: Genome et Environnement, F-63000, Clermont-Ferrand, France
[b] CHU Clermont-Ferrand, 3 IHP, Centre National de Référence des entérovirus et parechovirus - Laboratoire Associé, Laboratoire de Virologie, F-63000, Clermont-Ferrand, France

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ABSTRACT

In a one-year (October 2014–October 2015) pilot study, we assessed wastewater monitoring with sustained sampling for analysis of global enterovirus (EV) infections in an urban community. Wastewater was analysed by ultra-deep sequencing (UDS) after PCR amplification of the partial VP1 capsid protein gene. The nucleotide sequence analysis showed an unprecedented diversity of 48 EV types within the community, which were assigned to the taxonomic species A (n = 13), B (n = 23), and C (n = 12). During the same period, 26 EV types, of which 22 were detected in wastewater, were identified in patients referred to the teaching hospital serving the same urban population. Wastewater surveillance detected a silent circulation of 26 EV types including viruses reported in clinically rare respiratory diseases. Wastewater monitoring as a supplementary procedure can complement clinical surveillance of severe diseases related to non-polio EVs and contribute to the final stages of poliomyelitis eradication.

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1. Introduction

Enteroviruses (EVs) are small, non-enveloped RNA viruses within the *Picornaviridae* family. EVs involved in human infections include polioviruses (PVs, n = 3), coxsackieviruses (CVs) A (n = 21) and B (n = 6), echoviruses (Es, n = 29) and 49 other types numbered 68–121 which are grouped among four taxonomic species EV-A to EV-D. EV-B types are the most frequent causes of aseptic meningitis outbreaks and have been occasionally reported in other central nervous system syndromes (Mirand et al., 2008). Several EV types have been observed in sporadic encephalitis cases, in particular, infections caused by EV-A71, that are marked by utmost severity (Xing et al., 2014).

The analysis of environmental and wastewater samples collected from defined communities has been used to estimate the prevalence of specific enteric pathogens in populations for which no clinical data are available, including EVs (Berchenko et al., 2017). The World Health Organization has included environmental PV surveillance in the new Strategic Plan of the Global Polio Eradication Initiative as a supplement to the surveillance of acute flaccid paralysis (AFP) (Polio Eradication & Endgame Strategic Plan, 2013–2018, 2013). Environmental surveillance has detected infections with wild or vaccine-derived recombinant PVs without observation of paralysis cases and the early introduction of virulent strains within communities (Cowger et al., 2017; Manor et al., 2014). As circulation of wild PVs has been virtually interrupted, poliomyelitis is becoming a rare cause of AFP worldwide (Tangermann et al., 2017). However, non-polio EVs are increasingly reported in diseases with similar manifestations. In this respect, the EV types EV-A71 and EV-D68 have emerged as a growing cause of concern in children (Casas-Alba et al., 2017; Holm-Hansen et al., 2016). Most EVs replicate in the small intestine mucosa and are excreted in the faeces for 3–4 weeks, regardless of whether infections are symptomatic or not. Health risks to susceptible individuals can stem from poor sanitation and the release of untreated or inadequately treated wastewater effluents, which

* Corresponding author. CHU Clermont-Ferrand, Laboratoire de Virologie, Centre National de Référence des Entérovirus-Parechovirus – Laboratoire associé, Clermont-Ferrand, France.
E-mail address: mbisseux@chu-clermontferrand.fr (M. Bisseux).

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increase the risk of exposure to disease-causing EVs and virus transmission in the community. Accordingly, systematic environmental sampling could provide important supplementary surveillance data for non-polio EVs (Adeniji and Faleye, 2014).

We previously performed studies combining surveillance of clinical EV manifestations and virological data to investigate the epidemiologic patterns of EV diseases in France (Antona et al., 2016; Lang et al., 2014; Mirand et al., 2016, 2010, 2008). In other studies, we recorded changing patterns of several EV types each year among patients finding a wide genetic diversity of virus lineages within a same epidemic EV type and described the circulation of EV strains between countries (Hassel et al., 2017, 2015; Henquell et al., 2013; Othman et al., 2015). The findings suggested that susceptible individuals are exposed to a wide array of EV strains and that the epidemiological patterns of clinical manifestations are driven by constant flows of virus strains from different countries, regardless of whether they are neighbouring or distant countries. We hypothesized that yearly outbreaks caused by a given EV type result not only from the transmission of one epidemic virus strain within the community but also from the exposure of individuals to multiple viruses of distinct lineages and geographic origins.

To investigate how environmental surveillance could be implemented to complement clinical surveillance and improve public health monitoring and evaluation, we performed a one-year pilot study of municipal wastewater effluents of the city of Clermont-Ferrand, France, to analyse enteric infections (Bisseux et al., 2018). In the present study, we analysed wastewater samples by ultra-deep sequencing (UDS) to determine the pattern of EV infections of the whole community and compared the data with those derived from clinical surveillance of the same population and the entire French population. We discuss the relevance and limitations of the present pilot study and the lessons learnt in terms of epidemiologic surveillance and future applications.

2. Material and methods

2.1. Wastewater sampling and virus concentration

Sampling procedure and virus concentration steps have been previously described (Bisseux et al., 2018). Briefly, from October 2014 to October 2015, we sampled 20 L of raw wastewater every 2 weeks at the wastewater treatment plant (WWTP) of the urban area of Clermont-Ferrand that currently treats the waste of 250,000 people. A total of 27 samples were collected from the influent of WWTP. They were immediately filtered under gravity through a 50 μm filter and transferred to the laboratory within 1 h after collection. After a second gravity filtration stage (20 μm), samples were concentrated by tangential flow ultrafiltration using a hallow fibre filter (molecular cut-off 30 kDa, inner diameter 200 μm) and a transmembrane pressure of 0.07–0.1 bar. After the addition of 10 mM sodium pyrophosphate decahydrate and sonication, samples were centrifuged at 8,000 x g for 20 min. The pH of supernatants was neutralised to pH 7. Virus precipitation was further performed with polyethylene glycol (PEG). PEG 8000 and sodium chloride were added to the supernatant at a final concentration of, respectively, 10% and 0.6% (w/v) and incubated at 4 °C for 24–48 h. The white phase containing viruses was centrifuged at 8,000 x g at 4 °C for 20 min. Pellets were suspended in buffer (0.1 M NaCl, 8 mM MsSO-7H_2O, 50 mM Tris-HCl and 0.005% (w/v) glycerol, pH 7). After addition of 1 M KCl, the mixture was incubated on ice for 40 min to precipitate PEG, leaving purified virus particles in suspension. After centrifugation (12,000 x g, 10 min at 4 °C), the supernatant was stored at −20 °C. The raw wastewater samples were concentrated to an average of 60 mL (concentration factor: 333). Total nucleic acids were extracted from 1 mL of viral concentrates with the NucliSENS® EasyMAG® platform (bioMérieux, Marcy l'Etoile, France), and eluates were immediately aliquoted and stored at −80 °C until analysis.

2.2. Target gene amplification and deep-sequencing

All the 27 samples tested positive for EV ARN (Enterovirus@ceeram, bioMérieux, France) and quantified as previously reported (Bisseux et al., 2018) with an in-house assay (Volle et al., 2012), and were subjected to deep sequencing. Two manipulators in two distant laboratories performed all reactions in duplicate independently following standard precautions to prevent PCR contamination. The partial gene (360–390 nucleotides) encoding the major VP1 capsid protein was amplified with pan-EV RT-nested PCR (Nix et al., 2006). After the first amplification (40 cycles, first set of Nix primers), the second round (40 cycles) used the second set of Nix primers tagged with a single sequence of eight nucleotides for each replicate of each sample.

The positive control was a stool collected from a healthy child containing a mixture of echovirus 13 (E−13) and coxsackievirus A2 (CV-A2), identified in a specific Illumina sequencing run. The negative control was DNA- and RNA-free sterile water.

PCR products and controls were checked on standard 1% agarose gel and purified with the MinElute® gel extraction kit (Qiagen, Paris, France). All purified DNA samples were quantified with Agilent D1000 Screen Tape system and mixed in the same quantity. Sequencing was performed with the Illumina MiSeq sequencing platform, which uses 2 x 300 bp paired-end chemistry at the GATC platform, Germany. The sequencing run contained 58 tagged amplicons from 27 WWTP samples, the positive and negative controls, all in duplicate.

2.3. Reference databases for EV identification from partial $1D^{VP1}$

Two different databases were built for the bio-informatics analysis. The first database, named database I, was built by aligning sequences from all human and non-human EV reference strains downloaded from the NCBI GenBank database. Sequences of Sapelovirus were added as an outgroup. The second database (database II) was built to span intratypic EV diversity. The partial VP1 gene sequences targeted by the PCR primers used in this study were recovered from all nucleotide sequences for each human EV type available in GenBank (as of March 2015). These published sequences were compiled with those determined in our laboratory. All the sequence data sets were constructed with the BioEdit software version 7.1.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Assignment and sequence quality were checked with phylogenetic trees reconstructed by the neighbour joining method implemented in MEGA software v5.01 (Tamura et al., 2011). The sequences with assignment error or with suspected sequencing error were discarded. For each type, we selected sequences representing each cluster (defined as sequences with less than 1% of nucleic acid differences). The final database contained 3,256.

2.4. Bioinformatic analysis and quality control of sequence data

The VSEARCH open source tool for metagenomics (https://github.com/torognes/vsearch) was used for processing and preparing sequence data. After assembly of paired-end reads, sequences were aligned and those containing ambiguous bases (N) or mismatches in primers were removed. Putative chimaeras were identified and removed with the VSEARCH tool. The remaining sequences that shared >98% nucleotide sequence identity were assigned to a single operational taxonomic unit (OTU). The taxonomic assignment of the sequence representing each OTU was

performed by similarity and phylogeny using the two reference databases described above. For each OTU, the closest sequences in database II were selected by similarity for a first assignment. These sequences were then added to the phylogenetic tree reconstructed with FastTree (Price et al., 2010) from sequences of database I for a second phylogenetic assignment. The consistency of the results provided by the two methods was verified. This process was performed with a modified version of the pipeline PANAM (Phylogenetic Analysis of Next-generation AMplicons https://github.com/panammeb/) (Taib et al., 2013). We checked the data for detecting eventual mistagging effect (Esling et al., 2015; Schnell et al., 2015). By removing sequences representing less 0.5% of the total abundance, we eliminated the spurious ones (Technical appendix A, supplementary data). Duplicates were then pooled for the final analysis. We normalised the WWTP samples using the vegan package of R software, which consists in a random selection of the same number of sequences in each sample. This number was defined as the number of sequences present in the sample containing the lowest sequence number (16,250 sequences in our run).

2.5. EV sequences from clinical infections over the same period

Between October 2014 and October 2015, 3191 EV infections were reported to the French national laboratory network of enterovirus surveillance (RSE), coordinated by the Agency Santé publique France and the National Reference Centre for enteroviruses and parechoviruses. EVs were detected by molecular typing in 2124 patients, of whom 122 were seen at the teaching hospital of Clermont-Ferrand. Typing was performed prospectively in all clinical samples (cerebrospinal fluid [CSF], blood, respiratory, stool and throat). Nucleotide sequences were obtained after complete VP1 gene amplification with species specific primers (Mirand et al., 2016, 2006; Schuffenecker et al., 2016) or with pan-EV primers (Nix et al., 2006) targeting a partial region of the VP1 gene. The amplicons were sequenced by the Sanger technique using the BigDye Terminator kit (Applied Biosystems).

2.6. Phylogenetic analysis

Phylogenetic analysis based on the partial VP1 gene sequences was performed with the maximum parsimony method implemented in the Bionumerics software (bioMérieux, Marcy l'Etoile, France). The sequences that shared $\geq$98% nucleotide identity were clustered in a single sphere.

2.7. Accession numbers

The partial VP1 gene sequences have been deposited in the GenBank sequence database under accession numbers MK086267 to MK086510 and MK086163 to MK086266 for wastewater (OTUs) and clinical sequences, respectively.

3. Results

3.1. Sequencing data

Of the merged sequences (assembly of forward and reverse reads), 33% were discarded by the quality control analyses. Of the remaining sequences assigned to an EV type, 0.4% were removed because of incorrect or inaccurate assignment. The sequences assigned to rhinovirus types (0.7%) were also excluded, leaving a set of 2,001,179 sequences for further analyses. The cleaning analyses applied to the entire dataset left 1,546,641 sequences in the wastewater samples and 438,750 sequences after the normalisation of data (Table 1).

3.2. Diversity of EV types in the WWTP samples

OTUs were assigned to 3 taxonomic species EV-A, B and C (Table 1). No sequences were allocated to the EV-D species. A total of 48 EV types were detected in the wastewater samples. Among them, 15 types (CV-A1, A11, A13, A19, A20, and A22, EV-A76, A89, A90, A119, EV-B78, EV-C99, C105, C109, and C116) are rarely reported in the clinical setting at a national level. Some, such as EV-A119 and EV-A76, were detected sporadically in the wastewater samples and others, such as CV-A11 and CV-A22, were present over time in several consecutive samples. A set of 16 sequences clustered in a single OTU and shared 99.4% nucleotide identity with the Sabin PV-1 vaccine sequence (accession number V01150.1) and accordingly were classified as Sabin-like PV-1.

After cleaning and normalisation, sequences were clustered into 180 OTUs, which were assigned to 33 EV types (Table 1). The sequences were distributed among EV-B (60.1%), EV-A (26%) and EV-C (13.9%) species. Four EV types accounted for more than 70% of all normalised sequences (Table 1): CV-B5 (43.9% of sequences), CV-A16 (13.3%), CV-A5 (7.4%), and CV-A11 (6.2%). A set of 10 minority EV types accounted for 1.5% of all sequences. Fifteen EV types represented by a handful of sequences (less than 0.5% of the sequences from a respective sample; 2 to 703 sequences by type in the whole sequence set) were removed during mistagging cleaning and were excluded from the temporal analysis because their allocation to a given sampling date was unreliable. However, they were included in the analysis of the global diversity of EVs within the community during the study period (Table 1).

3.3. Distribution of EV types in the clinical samples tested over the study period

From October 2014 to October 2015, 126 patients (median age = 3.07 years [range 4 days–53.8 years]) referred to the teaching hospital of Clermont-Ferrand had an EV infection, mainly meningitis (Table 2). The 26 EV types identified in these patients were distributed among EV-A (n = 7), EV-B (n = 15), EV-C (n = 3) and EV-D (n = 1) species (Table 3). The two most frequent EV types were CV-B5 and EV-D68. CV-B5 was detected in 30 patients and was responsible for 15/52 (28.5%) meningitis cases observed during the summer of 2015. EV-D68 (n = 17 patients), a cause of severe respiratory syndromes, was recorded only in October and November 2014. E–25, the third EV type, was reported in eight patients. The EV types identified in stools during systematic examination for adoption were EV-A120, CV-B5, CV-A13, and EV-C99.

During the same period, members of the EV surveillance national network (excluding the Clermont-Ferrand reporting site) reported 45 types, which were assigned to EV-A (n = 8), EV-B (n = 28), EV-C (n = 8) and EV-D (n = 1) species (unpublished data). The overall EV-B species accounted for 1643 (82%) infections reported in France, followed by EV-A (n = 172 infections, 8.6%), EV-D (n = 165, 8.2%) and EV-C (n = 22, 1%) species.The three most frequent EV types reported in France during 2014–2015 were E6 (18.6%), CV-B5 (14.5%), and EV-D68 (8.2%).

3.4. Comparison of EV types detected by clinical and environmental surveillance

Over the study period, 48 EV types were detected in wastewater and 26 in patients referred to the teaching hospital of Clermont-Ferrand (Fig. 1). Of the 26 types identified in the patients, 22 (85%) were detected in wastewater. More than half (13/22, 60%) were assigned to species B. The EV types EV-A120, EV-D68, E–7 and E–13 were detected only by clinical surveillance and 26 types only by environmental surveillance.

Table 1
Detection of EV types in wastewater and evolution of their abundance in the successive steps of the bio-informatics analysis.

species	types	before mistagging detection			after mistagging cleaning			after data normalisation		
		sequences (n)	sequences (%)	OTUs (n)	sequences (n)	sequences (%)	OTUs (n)	sequences (n)	sequences (%)	OTUs (n)
EV-A	CV-A16	365 880	18,3	199	291 124	18,8	19	58 344	13,3	19
	CV-A5	163 623	8,2	333	103 424	6,7	14	32 404	7,4	14
	EV-A119	72 236	3,6	138	41 054	2,7	6	11 098	2,5	6
	CV-A2	30 844	1,5	93	16 641	1,1	4	5 222	1,2	4
	CV-A4	25 517	1,3	145	14 866	1,0	4	2 474	0,6	4
	CV-A12	18 355	0,9	14	7 576	0,5	3	1 980	0,5	3
	EV-A76	9 516	0,5	8	8 215	0,5	1	2 217	0,5	1
	CV-A6	703	0,0	14	-	-	-	-	-	-
	EV-A89	676	0,0	4	529	0,0	1	276	0,1	1
	CV-A14	490	0,0	11	-	-	-	-	-	-
	CV-A8	252	0,0	4	-	-	-	-	-	-
	EV-A71	62	0,0	5	-	-	-	-	-	-
	EV-A90	22	0,0	2	-	-	-	-	-	-
total EV-A		***688 176***	***34,4***	***970***	***483 429***	***31,3***	***52***	***114 015***	***26,0***	***52***
EV-B	CV-B5	705 235	35,2	868	606 460	39,2	43	192 616	43,9	43
	E−9	72 306	3,6	89	56 796	3,7	7	17 021	3,9	7
	E−33	46 597	2,3	24	45 525	2,9	4	13 081	3,0	4
	CV-B2	36 093	1,8	43	26 971	1,7	5	8 582	2,0	5
	E−11	33 361	1,7	72	23 125	1,5	3	5 475	1,2	3
	E−6	22 469	1,1	17	19 269	1,2	1	3 875	0,9	1
	E−14	21 476	1,1	23	20 180	1,3	4	8 843	2,0	4
	CV-A9	20 377	1,0	77	4 774	0,3	6	1 831	0,4	6
	CV-B4	14 630	0,7	145	8 055	0,5	2	2 950	0,7	2
	CV-B1	12 691	0,6	30	4 403	0,3	4	889	0,2	4
	E−16	9 015	0,5	18	2 839	0,2	2	799	0,2	2
	CV-B3	6 161	0,3	9	4 937	0,3	1	1 631	0,4	1
	E−18	5 277	0,3	25	2 777	0,2	3	623	0,1	3
	E−5	5 011	0,3	15	2 170	0,1	1	631	0,1	1
	CV-B6	4 771	0,2	6	4 680	0,3	1	4 275	1,0	1
	E−1	1 708	0,1	4	1 383	0,1	1	300	0,1	1
	E−20	729	0,0	3	705	0,0	1	170	0,0	1
	E−25	477	0,0	6	-	-	-	-	-	-
	E−21	80	0,0	3	-	-	-	-	-	-
	E−30	50	0,0	3	-	-	-	-	-	-
	EV-B78	45	0,0	1	-	-	-	-	-	-
	E−3	38	0,0	2	-	-	-	-	-	-
	E−27	2	0,0	1	-	-	-	-	-	-
total EV-B		***1 018 599***	***50,9***	***1484***	***835 049***	***54,0***	***89***	***263 592***	***60,1***	***89***
EV-C	CV-A11	136 481	6,8	186	116 318	7,5	13	27 133	6,2	13
	CV-A22	79 259	4,0	72	56 409	3,6	5	15 980	3,6	5
	EV-C109	20 189	1,0	65	9 992	0,6	9	2 929	0,7	9
	EV-C99	20 085	1,0	52	11 907	0,8	3	8 543	1,9	3
	CV-A1	15 606	0,8	32	14 636	0,9	5	3 140	0,7	5
	CV-A13	10 597	0,5	15	7 089	0,5	2	1 891	0,4	2
	CV-A20	10 133	0,5	5	10 036	0,6	1	931	0,2	1
	CV-A24	1 909	0,1	7	1 776	0,1	1	596	0,1	1
	EV-C116	122	0,0	6	-	-	-	-	-	-
	Sabin-like-PV-1	16	0,0	1	-	-	-	-	-	-
	EV-C105	4	0,0	1	-	-	-	-	-	-
	CV-A19	3	0,0	1	-	-	-	-	-	-
total EV-C		***294 404***	***14,7***	***443***	***228 163***	***14,8***	***39***	***61 143***	***13,9***	***39***
total EV		**2 001 179**	**100,0**	**2897**	**1 546 641**	**100,0**	**180**	**438 750**	**100,0**	**180**

Table 2
Clinical manifestations in the 130 hospitalized patients from October 2014 to October 2015.

Symptoms	Patients (n)
meningitis/neurological disease	52
infection in pregnant women and neonates	27
respiratory infection	23
febrile syndrome	12
myocarditis/pericarditis	4
examination for international adoption dossier	4
hand foot and mouth disease	2
diarrhea	2
not specified	4

Phylogenetic analysis showed high intra-type genetic diversity, a pattern indicating that multiple viral lineages co-circulated within the community (Fig. 2). High genetic diversity was observed for the CV-B5 and CV-A6 types, both of which were detected in wastewater and patients, and for other types detected only in wastewater (e.g. CV-A11). Most viral sequences (54%) recorded in patients clustered with wastewater OTUs, evidence of close genetic relationships between viruses within the community and those detected in patients. Marked differences were observed among EV types in the genetic clustering rate of clinical sequences with wastewater OTUs. This rate ranged from 0% (CV-A2, E−6, and E−25) to 75% (CV-A6) and 100% (E−11). The CV-B5 sequences

Table 3
EV types identified in patients.

Species	Types	Patients (n)
EV-A	CV-A4	4
	CV-A6	4
	CV-A2	3
	CV-A5	1
	CV-A8	1
	EV-A71	1
	EV-A120	1
EV-B	CV-B5	30
	E−25	8
	E−18	7
	E−5	6
	E−6	6
	E−7	6
	E−11	6
	E−16	4
	CV-B3	3
	E−9	3
	E−13	2
	E−21	2
	CV-A9	1
	CV-B1	1
	CV-B4	1
EV-C	EV-C109	2
	CV-A13	1
	EV-C99	1
EV-D	EV-D68	17
NT		8

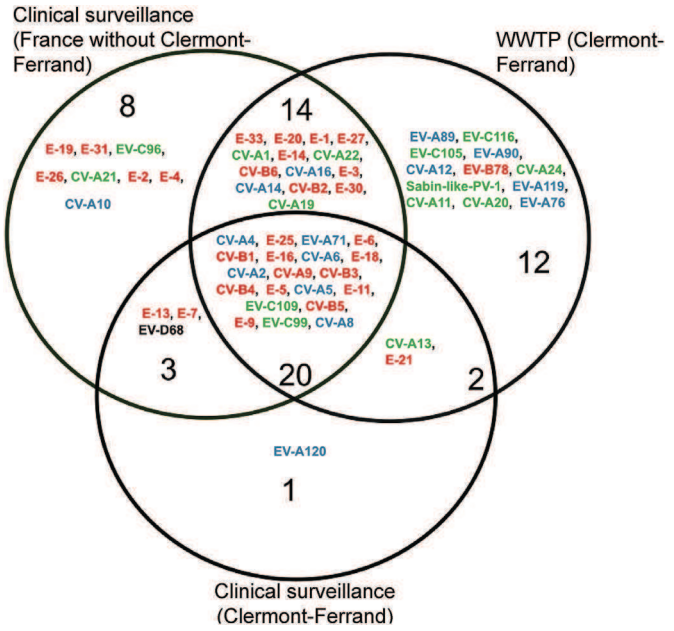


Fig. 1. Comparison of EV types detected by environmental surveillance in Clermont-Ferrand (France) and by clinical surveillance in Clermont-Ferrand and nationwide during the period 2014-2015.
The 48 EV types detected in the WWTP and types associated with patient hospitalization in Clermont-Ferrand (n = 26) and France (n = 45) were compared and represented in a Venn diagram. Types in the intersection were detected by both surveillance systems. EV-A species is shown in blue, EV-B species in red, EV-C species in green and EV-D species in black.

detected in patients clustered with OTUs (29/30, 97%) and one major genetic cluster included OTUs collected in March−October 2015 and clinical sequences detected in April−September 2015. One OTU detected in December 2014 only in wastewater was genetically distant from all other sequences and was identified as CV-B5 genogroup A by phylogenetic analysis (data not shown).

3.5. Temporal dynamics of viral diversity

The temporal distribution was performed for wastewater samples after normalisation of data and for clinical data using only local infection in Clermont-Ferrand city. On the basis of the viral diversity observed, distinct patterns defining four successive periods were distinguished (Fig. 3). Between 28 October, 2014 and 3 March 2015, half of the viral sequences (50.5%) in wastewater samples were assigned to types of the EV-A species. The samples contained a range of 6−12 EV types, of which CV-A16 was predominant (35.2% of the total sequences collected over the period). During the same period, the viral sequences assigned to the EV-C species represented 27.5% of the total sequences. CV-A11 (15.6%) and CV-A22 (7.3%) were, respectively, the second and third most frequent types. The EV-B species accounted for 22% of sequences, and the most frequent types were E−33 (6.3%), CV-B2 (5.2%), and CV-B5 (3.3%). The first CV-B5 detected in December 2014 was identified as belonging to genogroup A while the following sequences detected during this period and after were identified as belonging to genogroup B.

The total viral load determined in the wastewater samples during the four months was the lowest of the whole study period with a mean 1,057 (range 102−1,678) viral genome copies/mL. Clinical surveillance detected few EV infection cases except in October and November 2014, two months marked by 17 respiratory infections associated with EV-D68. CV-A16, the predominant EV type in wastewater, was associated with no clinical cases. EV-C109 was associated with two respiratory infections and was detected in wastewater.

During the second period, 16 March 2015 to 18 August 2015, the CV-B5 sequences accounted for 77.1% of the total sequences and ranged between 40% in a sample collected in May to 100% in a sample collected in June. With 9.1% of the total viral sequences, CV-A5 was the second most frequent type. The total viral load in wastewater was 4,072 (range 290−4072) genome copies/mL. The seasonal peak of clinical EV infections occurred during this second period, in July 2015 (n = 56/130 cases, 43%). CV-B5 was the most frequent type identified in patients (n = 24/56). Environmental surveillance detected CV-B5 in March 2015 before the first clinical case in May 2015 and the summer meningitis outbreak in July. During this period, 16 other EV types were also associated with clinical cases and assigned to three EV species (B, n = 11, A, n = 4 and C, n = 1), but only half were detected in wastewater.

During the third period (September 2015), the mean viral load was the highest determined over the year: 17,046 (range 2769−17046) copies/mL. The proportion of all the EV-B types, including CV-B5, dropped to 64% of the total number of sequences. Two other EV types, EV-C99 and CV-A22, accounted for 21.4% of the total number of sequences. The number of clinical cases dropped to 10, 9 of which were associated with 5 types of EV-B species and 1 with CV-A6 (A species).

The total viral load (mean 2,107 [range 138−4075] genome copies/mL) decreased over the fourth period, from 14 October−26 October 2015, and the distribution of EV types shifted again with 89.7% and 10.3% of sequences being assigned to EV species B and A, respectively. CV-B5 was once again predominant in wastewater (74.8% of viral sequences) while the number of clinical cases remained low.

4. Discussion

This one-year pilot study to analyse wastewater sampled from a

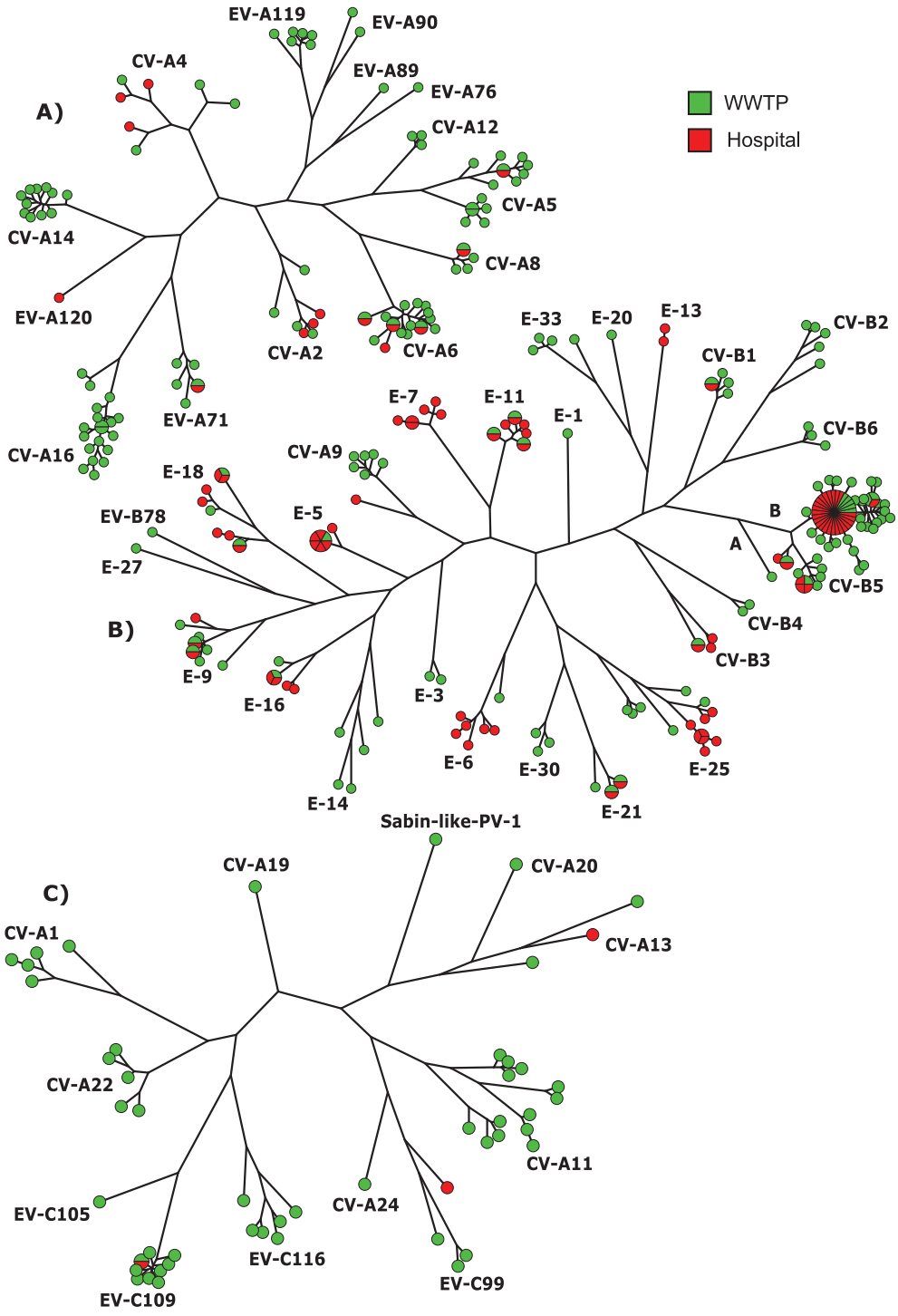


Fig. 2. Phylogenetic network of EV.
All EV types detected during the 2014–2015 study period are represented in phylogenetic networks for species A (A), B (B) and C (C). Strains detected in wastewater are in green and clinical strains in red. Networks were built with Bionumerics software and maximum parsimony methods. Strains with less than 2% nucleotide differences were grouped into a single sphere with a diameter proportional to the number of strains inside. For CV-B5, the two genogroups A and B, as defined in a previous study (Henquell et al., 2013), are indicated.

WWTP serving an urban community showed a highly dynamic co-circulation of 48 EV types assigned to the taxonomic species A, B, and C. Our results were consistent with those of a previous American study (Brinkman et al., 2017) that described a similar seasonal distribution of EV species with an alternative predominance of EV-A and EV-B, a major peak of EV-B during summer and fall and the circulation of EV-C throughout the year. Unlike in our study, the highest viral concentration in the sewage from the two WWTP analysed was observed in July. To our knowledge, our study yields the first environmental and clinical data on the surveillance of non-polio EV infections without cell culture bias concomitantly obtained in a same urban population. The results showed the consistency between the two surveillance systems: 22/26 EV types identified in patients referred to the teaching hospital serving the

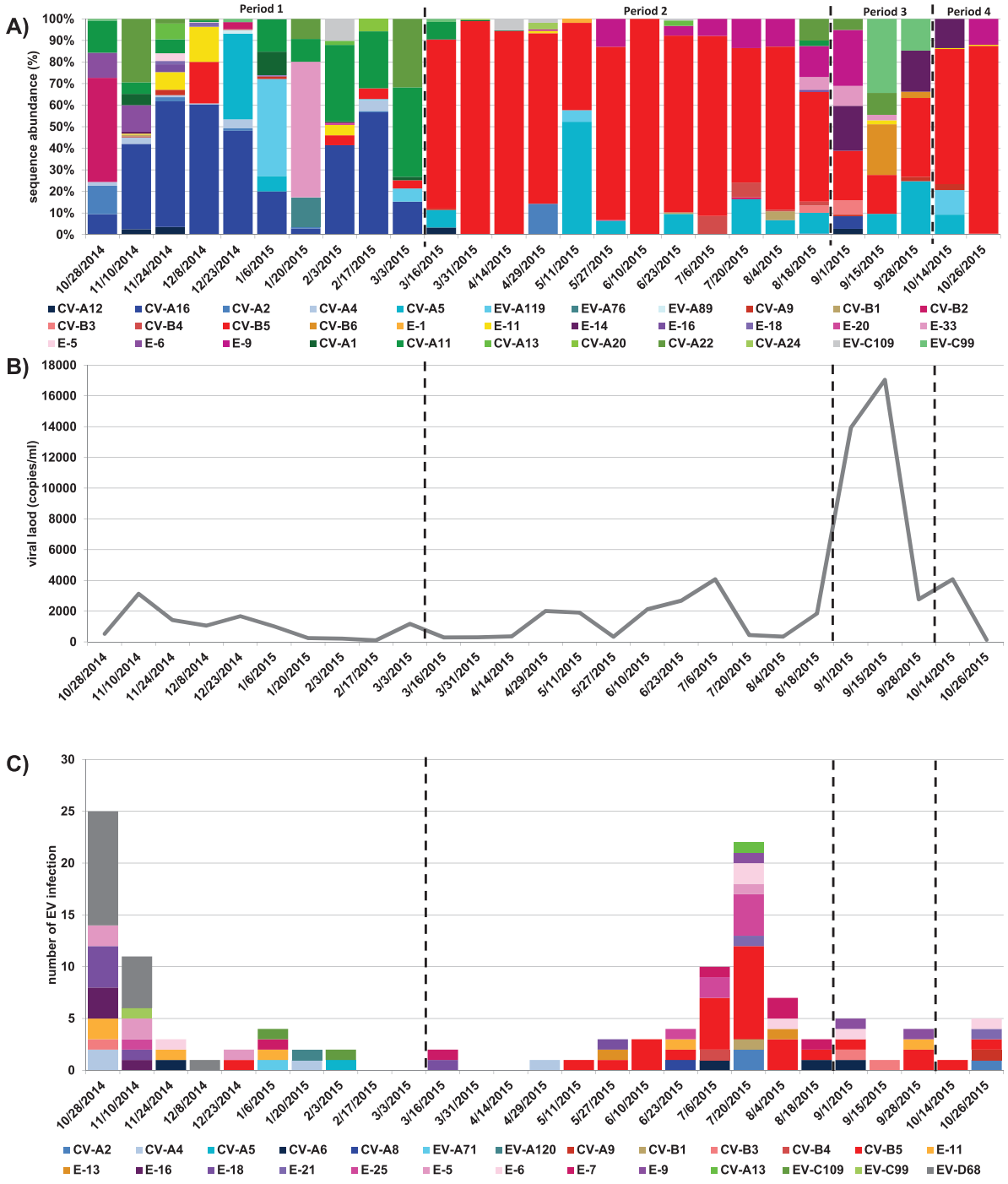


Fig. 3. Quantity and diversity of EVs detected in the Clermont-Ferrand area over one year by the two surveillance systems.
(A) Relative abundance of the 33 conserved EV types detected in the WWTP of Clermont-Ferrand during 2014–2015 are given for each sample.(B) The quantity of EV expressed as genome copies/mL. of WWTP water was determined in a previous study (Bisseux et al., 2018).(C) The diversity of EV types associated with patient hospitalization occurring in the same city in the same period is represented for every two weeks preceding the mentioned date.

population under study were also detected in wastewater monitoring. There were close phylogenetic relationships between virus sequences detected in patients and wastewater. Yet, analysis of the wastewater showed that EVs associated with community-acquired infections in the urban population were much more diverse than those associated with infections reported in the clinical setting during the same period. The net number of EV types (intertype diversity) was higher in the whole urban population than in patients. Notably, the intertype diversity within the urban population was actually similar to that reported in patients admitted to hospitals nationwide during the same period. These findings support the conclusion that UDS with a multiplexed amplicon approach can be used in raw urban wastewater to investigate the ecology and transmission of EVs and assess the burden of these enteric viruses in the general population.

The epidemiology of EV infections in 2014–2015 was marked by

the epidemic circulation of CV-B5, which accounted for 44% of the sequences in wastewater and 28.5% (15/52) of meningitis cases. The abrupt upsurge of CV-B5 was clearly identified in March 2015 by wastewater analysis. The large circulation of CV-B5 was also observed by the national network for EV surveillance, which recorded this virus type as the second most frequent in France in 2015. Ten other EV-B types were co-detected with CV-B5 during the winter 2014-2015 by environmental surveillance, notably E−11 (3% of all sequences over the winter) and E−6 (2.4%). These viruses were also detected during the meningitis outbreak in the summer. In contrast, EV types E−33 (6.3%) and CV-B2 (5.2%) were only detected in wastewater. Accordingly, the winter pattern of EV diversity in wastewater was not completely predictive of the pattern during the following seasonal outbreak. Longer follow-up studies including WWTPs serving distinct urban communities are required to provide a complete description of viral circulation dynamics. Striking differences in the distribution pattern of EV-A and EV-C types were also observed. Few infections associated with EV-A types required hospitalization but the viruses circulated widely in the community (26% of reads in wastewater), indicating that most infected individuals had subclinical infections or self-limited illnesses managed in ambulatory settings. CV-A16 is an example in this respect. Conversely, while CV-A6 accounted for 0.03% reads in wastewater, the virus was detected in four patients reported with meningitis, myocarditis, and HFMD (including a case of atypical HFMD clinical presentation). Our data are consistent with the emergence of CV-A6 as a cause of worldwide disease concern (Bian et al., 2015). Environmental and clinical surveillance revealed the unexpected epidemiologic pattern of the EV-C types: large circulation in the urban community and few disease cases (Apostol et al., 2012; Benschop et al., 2017; Harvala et al., 2014). All the three EV-C types identified in patients were traced back to wastewater. EV-C109 was detected in two patients referred for respiratory diseases and clustered close to viruses in wastewater. In contrast, CV-A13 and EV-C99 detected in the stools of two adopted children and in wastewater did not cluster.

Based on the structure of the sequence database and phylogenetic techniques used in our study, the sequence assignment rate was high (99.6%) and enabled us to detect less abundant viruses. Two EV types with direct public health relevance (EV-A71 and PV-1 Sabin) were detected in wastewater and identified down to the strain level by analysis of sequence data. The low nucleotide variation in the wastewater PV-1 sequence compared with that of the vaccine strain suggested no or limited circulation in the population. Our French data are consistent with earlier results from the United States and other European countries showing the importation of vaccine PV strains into polio-free countries (Benschop et al., 2017; Delogu et al., 2018). The findings clearly show the need to maintain a high immunization coverage to avoid the occurrence of an outbreak associated with the circulation of vaccine-derived polioviruses (VDPV), as reported in 2015 in Ukraine (Khetsuriani et al., 2017). CV-A11 and CV-A22 were among the five most abundant types in this study. These unexpectedly prevalent non-polio EV-C types could be a gene reservoir for the emergence of recombinant VDPV (Bessaud et al., 2011). We also evidenced the circulation of rare EV-A (*eg* -A76, -A89), EV-B (*eg* -B78) and EV-C (*eg* -C105, -C116) types previously unreported in the French population and for which few epidemiological and genomic data are available. Notably, our environmental surveillance identified 'new' EV-C types associated with severe respiratory diseases and neurological disease conditions, whose detection in the faeces was considered to be difficult (Holm-Hansen et al., 2016). Our findings suggest that the circulation of these viruses in the community could be higher than anticipated from the number of reported clinical infections and are consistent with suggestions to enhance the surveillance of EV-C species in respiratory specimens (Barnadas et al., 2017; Van Leer-Buter et al., 2016).

Our study has two limitations. First, although the pan-EV primers used included a high number of degenerate positions, variations in the levels of PCR amplification exist among EV types and could explain why we did not detect EV-D68. This virus was previously detected, however, by type-specific amplification, confirmed by direct Sanger sequencing in 7/27 samples (Bisseux et al., 2018). Second, we showed that incorrect assignment of reads caused the random spread of signals within multiplexed samples and artificially inflated the diversity detected in each sample. This bias, referred to as mistagging (Esling et al., 2015) or tag jump (Schnell et al., 2015), has been rarely documented and taken into account in UDS analyses despite its critical impact on the quality of data collected for ecological and epidemiological studies. Minimising incorrect assignments and obtaining reliable results is a real challenge for metabarcoding studies on Illumina sequencing platforms. Strategies including controls must be incorporated into the experimental design to perform sequence data filtering (Esling et al., 2015; Galan et al., 2016). Despite these limits, amplicon deep-sequencing in wastewater for surveillance and epidemiological studies clearly surpasses earlier techniques used in this field. With the implementation of robust quality controls, monitoring EVs in wastewater can be implemented as a supplementary surveillance system of the community at large to complement clinical surveillance.

5. Conclusion

The combination of temporal wastewater sampling, efficient virus concentration from samples, analysis with amplicon deep-sequencing and robust sequence assignment allow the detection of a large array of EV types while avoiding the bias caused by cell culture. Sustained environmental surveillance is an advanced approach for a better understanding of the diversity of the EV landscape within an urban community. The environmental findings of this study show the circulation of clinically uncommon EVs, including respiratory EV-C species strains and Sabin-like PV-1, and provide a powerful complement to the clinical data.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.115246.

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