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Detection of Human Enteric Viruses in Freshwater from European Countries

Emilio D'Ugo[1] · Stefania Marcheggiani[1] · Ilaria Fioramonti[1] · Roberto Giuseppetti[1] ·
Roberto Spurio[2] · Karim Helmi[3] · Delphine Guillebault[4] · Linda K. Medlin[4] ·
Ivan Simeonovski[5] · Bas Boots[6] · Ulrich Breitenbach[7] · Latife Koker[8] ·
Meric Albay[8] · Laura Mancini[1]

Abstract The transmission of water-borne pathogens typically occurs by a faecal–oral route, through inhalation of aerosols, or by direct or indirect contact with contaminated water. Previous molecular-based studies have identified viral particles of zoonotic and human nature in surface waters. Contaminated water can lead to human health issues, and the development of rapid methods for the detection of pathogenic microorganisms is a valuable tool for the prevention of their spread. The aims of this work were to determine the presence and identity of representative human pathogenic enteric viruses in water samples from six European countries by quantitative polymerase chain reaction (q-PCR) and to develop two quantitative PCR methods for Adenovirus 41 and Mammalian Orthoreoviruses. A 2-year survey showed that Norovirus, Mammalian Orthoreovirus and Adenoviruses were the most frequently identified enteric viruses in the sampled surface waters. Although it was not possible to establish viability and infectivity of the viruses considered, the detectable presence of pathogenic viruses may represent a potential risk for human health. The methodology developed may aid in rapid detection of these pathogens for monitoring quality of surface waters.

Keywords Enteric viruses · Water-borne pathogens · Real time PCR · Waste water · Faecal pollution · Human health

✉ Emilio D'Ugo
emilio.dugo@iss.it

[1] Quality and Fishfarm Unit, Department Environmental Environment & Primary Prevention, Istituto Superiore di Sanità, Rome, Italy

[2] School of Biosciences and Veterinary Medicine, University of Camerino, Ascoli Piceno, Italy

[3] Veolia Rechercheur & Innovation, Saint-Maurice, France

[4] Microbia Environnement, Observatoire Océanologique, 66650 Banyuls/Mer, France

[5] National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

[6] UCD School of Biosystems Engineering, Agriculture and Food Science Centre, Dublin, Ireland

[7] Marilim Gesellschaft für Gewässeruntersuchung, Schönkirchen, Germany

[8] Fisheries Faculty, Istanbul University, Istanbul, Turkey

Introduction

Water contaminated with pathogenic microbes is a global problem for human health. Although viruses often occur in relatively low concentrations in environmental waters, they remain a potential hazard requiring rapid and sensitive detection methods to help preventing outbreaks.

Indicator organisms of faecal contamination, such as *Escherichia coli*, are commonly used to evaluate the microbiological quality of aquatic ecosystems. Assessing water quality is an essential component of monitoring programmes under the EU water directive and to protect human health (Marcheggiani et al. 2011).

Indicator organisms are relatively easy and inexpensive to monitor; however, their absence does not exclude faecal contamination. Enteric viruses can be excreted in faeces from both ill and healthy individuals, and several studies have reported the presence of enteric viruses in surface waters even when bacterial indicators were not detected.

Viruses may be more resilient to environmental stressors, allowing them to survive for extended periods of time (Carter 2005). For example, Adenovirus F can also be used as an indicator of faecal pollution, but detection requires alternative approaches, mainly based on molecular technologies (Goyal et al. 1984; Jiang and Chu 2004; Fong and Lipp 2005).

Enteric viruses are causative agents of many non-bacterial gastrointestinal and respiratory infections, but also conjunctivitis and hepatitis causing periods of debilitating illness and/or high morbidity and mortality in immunocompromised individuals (Kapikian 2001; Carter 2005; Lenaerts et al. 2008; Okoh et al. 2010). After infection, enteric viruses infect and replicate in the gastrointestinal tract, or in other organs of their hosts, and are released in large quantities; about 10^5–10^{11} virus particles per gram of stool (Bosch 1998). The majority of excreted viruses are non-enveloped, which makes them highly resistant to external stressors in the aquatic environment. They are also resilient to decontamination processes used in drinking and wastewater treatments (Gerba et al. 2002; Meschke and Sobsey 2003; Bofill-Mas et al. 2006), and there is ample evidence for the survival of enteric viruses during wastewater treatment (He and Jiang 2005; Carducci et al. 2008; Fong et al. 2010; Kokkinos et al. 2011; Prado et al. 2011).

Because of their potential low, but infective doses, large volumes of environmental water are required (up to 100 L) in order to obtain representative samples for the detection of viruses. Viral particles can be concentrated from the water samples using several approaches, including ultrafiltration, ultracentrifugation, and adsorption–elution (Hata et al. 2014; Ikner et al. 2012). Ultrafiltration and ultracentrifugation have been successfully used to capture and concentrate viruses from large sample volumes of environmental water (Smith and Hill 2009; Smith et al. 2008). Standardisation of the sampling methodology is essential to obtain reliable and comparable samples for spatial and temporal monitoring of water quality. The FP7-EU funded project μAQUA developed a standard operating procedure allowing for the parallel sampling of different water types in a pan-European monitoring campaign. The goal of this project was (1) to obtain environmental water samples from six European countries as part of a monitoring campaign, concentrate each sample and apply q-PCR technology for the detection and quantification of water-borne enteric viruses; and (2) to design new primers and probes to efficiently detect, by q-PCR, two enteric viruses, including Adenovirus F (ADV41) and the Mammalian Orthoreovirus belonging to the family of Reoviridae.

The species targeted are commonly found in surface waters and included Norovirus (NoV: genotype II: NoGGII), Human Enterovirus (HE), Hepatitis A Virus (HAV) and Adenovirus (ADV41).

Furthermore, Hepatitis E virus (HEV) and a member of the Reoviridae, the Mammalian Orthoreovirus (MRV), were included, because little information is available on these viruses from an environmental and epidemiological perspective. In particular, the recently characterisation of MRV isolates having high pathogenic potential by Wang (2015) and evidence that MRV are found in many wild and domesticated animals (Tyler 2001), implies that monitoring freshwater for MRV could be a useful method to assess the spread of this virus in the environment. There is not much information on the current distribution of these viruses in European freshwaters.

Experimental Section

Viruses

Standards for the target viruses were obtained from the following sources: the HAV was provided by National Institute for Biological Standards and Control (NIBSC): WHO International Standard, WHO First International Standard For HAV RNA Nucleic Acid Amplification Techniques (NAT) assays NIBSC code: 00/560. HEV was provided by Paul Erlich Institut (PEI): World Health Organization International Standard for HEV RNA NAT-Based assays PEI code 6329/10. Norovirus GII (NoGGII) RNA (was gently provided by Dip. SPVSA—Ademipimenti comunitari e sanità pubblica, National Institute of Health of Rome. Human Adenovirus 41 (ADV41) and Human Enteroviruses (HE, Poliovirus 1), was gently provided by CRIVIB—Viral Vaccines, National Institute of Health of Rome. Mammalian Orthoreovirus type 3 (MRV3) was provided by Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna Department of Virology (IZSLER, Italy). Mammalian Orthoreovirus types 1 and 2 (MRV1–MRV2) were provided by General and Applied Hygiene University of Tor Vergata of Rome.

Sampling of Water and Ultrafiltration

Representative samples (Table 1) were taken from six countries for different types of water, totalling 50 L of environmental water per sample. Upon arriving in the laboratory, samples were immediately processed from each partner following ultrafiltration protocol adopted in μAQUA project. Initial concentration was performed using a polysulfone hollow fibre module (HF-80S, Fresenius Medical Care, Bad Homburg, Germany) with a filtration surface of 1.8 m^2 and a cut-off size of 20 kDa. Using a peristaltic pump, water was concentrated at an average flow rate of 3.5 L min^{-1}, and the average filtrate flow rate was 1.7 L min^{-1}. Because of the 200 μm internal diameter of

Table 1 Details on water samples taken sample code: S000: (number of site, i.e. S1A; S1B for the second sample of the same site)

Sample code	Land	Water type	Expected faecal sources of contamination	Country
S.01.	Open spaces	River	NPSP	Italy (IT)
S.02.	Grazing land	River	PSP; NPSP	Italy (IT)
S.03.	Artificial area	Lake	NPSP	Italy (IT)
S.04.	Grazing land	Sea	NPSP	France (FR)
S.05.	Grazing land	River	NPSP	France (FR)
S.06A.	Grazing land	Lake	NPSP	Italy (IT)
S.06B.	Grazing land	Lake	NPSP	Italy (IT)
S.07.	Urban	River	PSP; NPSP	Italy (IT)
S.08.	Urban	River	NPSP	Ireland (IE)
S.09A.	Grazing land	Reservoir	NPSP	Bulgaria (BG)
S.09B.	Grazing land	Reservoir	NPSP	Bulgaria (BG)
S.10A.	Grazing land	Black sea	PSP; NPSP	Bulgaria (BG)
S.10B.	Grazing land	Black sea	PSP; NPSP	Bulgaria (BG)
S.11.	Urban	River	PSP	Germany (DE)
S.12.	Grazing land Urban	Lake	PSP; NPSP	Turkey (TR)

Land: dominant land use of the catchments near the considered site. water type: body of water. Expected faecal sources of contamination: point source pollution

PSP water treatment plants; factories, confined animal feedlots, or combined sewers, *NPSP* non-point source pollution; pollution that results from water runoff from urban areas, construction sites, agricultural and silvicultural operations; National water quality monitoring council http://acwi.gov/monitoring/glossary.html)

the hollow fibres, the system blocks and removes any larger particles and macroorganisms. Microorganisms are captured by adhering to the filter, which are eluted once 50 L of environmental water has passed through. Elution was done by "backflushing" the filter in reversed order so that all captured particles, including microorganisms, are released. A backflush buffer was made with 0.01 % sodium hexametaphosphate (Sigma, St. Louis, USA), 0.5 % Tween 80 (Sigma, St. Louis, USA), 0.001 % of antifoam B emulsion (Sigma) in 1 L of deionized H_2O. This concentrated the sample 50-fold. The eluent (50 mL) of backflush solution was sent (on dry ice) from each partner to our laboratory for viral detection.

The efficiency of capturing viral particles using this method was evaluated on Adenovirus 5 and bacteriophage MS2. The percentages of recovery of viral particles evaluated with q-PCR for ADV5 were 46 and 76 % of bacteriophage MS2 respectively (see Electronic Supplementary Material, ESM 1).

Viral Nucleic Acid Extraction

From each eluted (ultrafiltered) sample (1 L), 20 mL was filtered on a 0.025-µm filter (Merck Millipore) under continuous vacuum. From these filters, viral DNA and RNA were simultaneously extracted using NucliSens magnetic extraction reagents (Rutjes et al. 2005) according

to the manufacturer's instructions (Biomerieux, Florence, Italy). Samples were stored at −80 °C until further processing. Briefly, 2 mL of the kit's Lysis Buffer (Biomerieux) was used to resuspend particles captured on the filter. Each resuspended samples was divided into two parts: (1) aliquot A (1.8 mL) and (2) Aliquot B (200 µL). In order to evaluate the efficiency of viral genomes and the presence of inhibitors, a known amount of a virus not existing in the environmental samples (Poliovirus 1, see below) was added to aliquot B. Both aliquots were incubated for 10 min at room temperature. Magnetic silica beads (50 µL) were added to the mixture for 10 min and centrifuged $1500 \times g$ for 2 min at room temperature, followed by washing with buffer (WB1), and the beads were transferred to a 1.5-mL microcentrifuge tube. Using a magnetic rack (DynaMag™-2 Magnet, Thermo Fisher Scientific, Milan, Italy), the sample was washed three times (WB1, WB2, WB3) and the final pellet resuspended in 100 µL of elution buffer (Biomerieux, Florence, Italy). To separate the beads from the elution buffer containing nucleic acids, the mixture was incubated in a thermomixer (Eppendorf Thermomixer 5436) at $1400 \times g$ at 60 °C for 5 min and the eluate (100 µL) collected using a magnetic rack. Nucleic acids from the reference material, including HAV, HEV, HE (Human Enteroviruses: Poliovirus 1, Echovirus 7, Coxsackievirus) and ADV 41, were extracted in a similar fashion after dissolving 200 µL of the organic fluid (sera or

cellular fluid) in 2-mL tubes containing the Lysis Buffer (Biomerieux).

Nucleic acids were quantified using a spectrophotometer (SPECTROSTAR nano, BMG LABTECH, Germany) by assessing the 260/230 and 260/280 ratios of 2 μL of the extracted material. Nucleic acids were further purified by overnight precipitation (in absolute ethanol, with 1/10 of volume of 3 M sodium acetate) at −20 °C followed by two washes with 70 % ethanol where necessary.

All nucleic acids obtained from environmental samples were subjected to a q-PCR inhibition and extraction efficiency analysis. For extraction efficiency analysis, 10 μL of aliquot B was measured in terms of the presence of spiked virus (see above). In addition, q-PCR inhibition was measured by diluting 10 μL of aliquot B 10- and 100-fold. The q-PCR values (genomes equivalent and ΔCq variation between dilutions) were compared with the standard curve of Poliovirus 1.

Primers and Probes Used

The HE, HAV, HEV and NoGGII were targeted using primers and probes published elsewhere (Oberste et al. 2010; Costafreda et al. 2006; Jothikumar et al. 2006; Loisy et al. 2005). Primers and probes were obtained from Eurofins, Germany. The probes used were Light Cycler type probes or dual hybridisation probes, except those targeting HEV, which were a modified TaqMan MGB probe (Life Technologies). Probes and primers targeting ADV41 and MRVs were designed using Bioedit software (Tom Hall Ibis Biosciences), by searching for oligonucleotide sequences in the conserved regions in the genomes of the two viruses. Exon gene and L1 segment were selected for ADV41 and MRVs, respectively. The primers and probes used are detailed in Table 2.

The design of five primers for the detection of the virus ADV41 (see Table 2) has provided a number of oligonucleotides that can be combined (using GenBank no. AB610527 as a reference) to produce the following fragments in base pairs (bp): P1-P2ADV41: 204 bp, P1–P4 ADV41:168 bp, P2–P3 ADV41:145 bp, P3–P4 ADV41: 90 bp. To confirm specificity of the designed primers, a PCR was performed as follows: first round PCR, 15 min at 95 °C to activate the Taq (Master Mix Qt, QIAGEN) followed by 40 cycles of 30 s at 94 °C, 40 s at 45 °C, 1 min at 72 °C, with a final extension of 72 °C for 7 min. The 204 bp PCR product was subsequently evaluated by electrophoresis on agarose gel using 100 bp ladder as reference (Promega, Madison, USA) and purified using spin columns (Millipore, Darmstadt, Germany), following the manufacturer's instructions. Sequencing was carried out with the ABI prism Dye-Terminator Sequencing Kit (Applied Biosystems by Life Technologies, version 1.1) in an ABI

PRISM 310 Genetic Analyzer (Applied Biosystems by Life Technologies), using the same primers used for amplification P1 and P2 and two internal primers P3 and P4. The obtained sequence was compared to the GeneBank database using the BLAST programmes available on National Center for Biotechnology Information (NCBI).

The design of five primers (P1-P5MOV) for the detection of three serotypes of the virus MRV1-2-3 (see Table 2) has yielded a number of oligonucleotides that can be combined (using Gene Bank n KF154724 as a reference) to produce the following fragments in base pairs: P1–P2 MOV: −336 bp, P1–P4 MOV: 212 bp, P2–P3 MOV: 232 bp, P3–P4 MOV: 108 bp. To confirm primers specificity PCR cycling was performed as follows: first round PCR, 15 min at 95 °C to activate the Taq (Master Mix Qt, QIAGEN) followed by 45 cycles of 30 s at 94 °C, 40 s at 45 °C, 1 min at 72 °C, and a final extension of 72 °C for 7 min and evaluated as detailed above using the same primers used for amplification (ESM 2–4).

Reverse Transcription of Viral RNA and q-PCR Standard Curves

cDNA was constructed by reverse transcription of viral RNA for each type of virus on all environmental samples using the reverse primer P4 in Table 2. Briefly, 5 μL of the extracted nucleic acids was added to 6.1 μL RNAse free water, 0.9 μL of 100 μM virus-specific primer and 1 μL of dNTP mix at 10 mM (Life Technologies). The mixture was heated at 65 °C for 5 min in a Thermomixer and kept on ice thereafter. To this, 4 μL of 5× first-strand buffer (Invitrogen, Carlsbad, USA), 1 μL of 200 U μL^{-1} Super-Script II reverse transcriptase (Invitrogen), 1 μL of 100 mM dithiothreitol (Invitrogen) and 1 μL of 20 U μL^{-1} RNase inhibitor (Invitrogen) were added, and the mixture was incubated at 50 °C for 60 min. Enzymes were deactivated by heating at 70 °C for 15 min.

The cDNAs obtained from the reference RNA viruses were subsequently purified using an Amicon Ultra-0.5 clean-up kit (Millipore) and quantified using a spectrophotometer (SPECTROSTAR nano, BMG LABTECH, Ortenberg, Germany). The molecular weight for each cDNA was estimated based on the length of the amplified fragment (Haramoto et al. 2008; Brooks et al. 2005).

The limit of detection (LOD) of the q-PCR was determined on tenfold serial dilutions of cDNA (for each virus), prepared with quantities ranging from 10^6 at 0.1 genomic equivalents. All q-PCR reactions were done using 5 μL of cDNA/DNA to which were added: 0.90 μL of 100 mM of sense primer (P3; see Table 2), 0.45 μL of 100 mM of antisense primer (P4; see Table 2), 0.125 μL of 100 mM of probe (P5; see Table 2), 25 μL of Mastermix QT (Qiagen, Florence, Italy) and 18.55 μL of nuclease free water.

Table 2 Sequences of primers used to detect viruses in freshwater by q-PCR assays

Virus	Primer/probe	Sequence	Number position GB
HE	P3Pan	GGC CCCTGAATGCGGCTAATCC	JQ806378: 451–472; 5′-untranslated region (UTR)
	P4Pan	GCGATTGTCACTWAGCAGYCA	JQ806378: 599–580; 5-untranslated region (UTR)
	P5Pan	CCGACTACTTTGGGWGTCCGTGT.FAM-BHQ1	JQ806378: 539–561; 5′-untranslated region (UTR)
HAV	P3HAV	TCACCGCCGTTTGCCTAG	AB279735: 67–84; 5′-untranslated region (UTR)
	P4HAV	GGAGAGCCCTGGAAGAAAG	AB279735: 223–206; 5′-untranslated region (UTR)
	P5HAV	CCTGAACCTGCAGGAATTAA.FAM-BHQ1	AB279735: 152–133; 5′-untranslated region (UTR)
NoGGII	P3GII	TCWGAGAAYCTCATCCAYCTGAACAT	JX023286: 5012–5037; RNA-directed RNA polymerase
	P4GII	TCGACGCCATCT TCATTCACA	JX023286: 5100–5080; RNA-directed RNA polymerase
HEV	P3HEV	GGTGGTTTCTGGGGTGACAGGGT	JQ655736: 5300–5322; capsid protein
	P4HEV	AGGGGTTGGTTGGATGAA	JQ655736: 5369–5352; capsid protein
ADV41	P1ADV41	CAGGACGCCTCGGAGTATCT	AB610527: 52–71; hexon protein
	P2ADV41	GCGTAAAGCGCACTTTGTAA	AB610527: 256–237; hexon protein
	P3ADV41	GTACTTCAGCCTGGGGAACA	AB610527: 111–130; hexon protein
	P4ADV41	GGTCGACTGGCACGAATC	AB610527: 217–220; hexon protein
	P5ADV41	AGACAGGTCACAGCGACTGA.FAM-BHQ1	AB610527: 174–193; hexon protein
MRV	P1MOV	TATCRGGRATGCAGAACATGATYC AG	KF154724: 1941–1965 L1 segment
	P2MOV	AATRGTTTCRCTDTYYACCTTACC	KF154724: 2277–2254 L1 segment
	P3MOV	TGACYACCACTTTYCCRTCAGGYT	KF154724: 2045–2069 L1 segment
	P4MOV	TCA GTR TGT TCD GGT CCC CAY ACY GTC	KF154724: 2153–2127 L1-L1 Segment
	P5MOV	ACYGCYAAYAAYAGYACGATGATGG.FAM-BHQ1	KF154724: 2095–2116 L1 Segment

HE Human Enterovirus, *HAV* Hepatitis A Virus, *NoGGII* Norovirus GGII, *HEV* Hepatitis E Virus, *ADV41* Adenovirus 41, *MRV* Mammalian Orthoreovirus

GenBank (GB) reference number and selected target region for each virus are also reported

Reactions were carried out in duplicate to account for procedural errors on a LightCycler (Roche Diagnostic) thermocycler using the following settings.

To obtain quantitative data on the titre of viral cDNA in each well, the cDNA samples and standards were then subjected to q-PCR simultaneously, followed by analysis using LightCycler Nano Software 1.1 (Roche).

For quantification of ADV41, the only DNA virus, dilutions of P1–P2 PCR fragments (contained within the target sequence for the q-PCR, see Table 2) were prepared, ranging from 10^6 at 0.1 genomic equivalents. q-PCR was carried out with the same conditions with 5 μL out of 100 μL of sample DNA extracted.

Results

Specificities of ADV 41 and MRV Primers

Amplification using the designed primers in Table 2 produced the predicted 232, 212 and 108 bp fragments with MRV1, MRV2, MRV3 serotypes and 204 and 90 bp for ADV41. BLAST searches of the 204 bp ADV41 and

212 bp MRV3 fragments using the GenBank database confirmed that the amplified sequences corresponded to the exon region of ADV41 and L1 segment for MRV respectively (see ESM 5 and 6). Tables 3 and 4 show the obtained standard curves of the q-PCR for ADV41 and MRVs and q-PCRs thermocycler conditions (the other viral standard curves and q-PCR conditions are also showed), using primers P3–P4 and probes P5. The LOD of the q-PCR assays was ten genome equivalents with a *Cq* value ≥40 considered negative.

PCR Detection of Viruses in the Environmental Samples

Table 5 shows the viruses detected using q-PCR. None of the water samples contained detectable amounts of HE, but MRV and ADV41 were frequently detected. The absence of HE in the aliquots from same samples was independently confirmed by the Veolia Rechercheur & Innovation, Saint-Maurice, France using q-PCR.

Only a sample (S.05) taken from a rural river in France was negative for all enteric viruses tested, whereas all other samples showed the presence of at least one viral species.

Table 3 Viral standard curve equations and LOD

Virus	Cq	A	E (%)	R^2	LOD
NoGGII	$= -3.39 \log 10 \cdot (q) + 40.00$	1.971	97.24	0.997	10
HE	$= -3.34 \log 10 \cdot (q) + 38.44$	1.991	99.25	0.992	10
MRV	$= -3.63 \log 10 \cdot (q) + 40.99$	1.890	88.57	0.995	10
HAV	$= -3.30 \log 10 \cdot (q) + 40.81$	2.010	100.00	0.997	10
HEV	$= -3.59 \log 10 \cdot (q) + 41.10$	1.900	89.91	0.993	10
ADV41	$= -3.31 \log 10 \cdot (q) + 39.54$	2.007	100.00	0.997	10

$Cq = K \cdot \log 10 \cdot (q) + I$; (Cq quantitative cycle, K curve slope, $\log 10 \cdot (q)$ logarithm of quantity, I axial intercept, A amplification factor, E efficiency, R^2 coefficient of determination)

Table 4 q-PCR conditions for the different viruses targeted

Virus	Denaturation	Annealing	Extension
HE	95 °C, 15 s	58 °C, 60 s	72 °C, 20 s
HAV	95 °C, 15 s	60 °C, 60 s	70 °C, 60 s
NoGGII	95 °C, 15 s	56 °C, 60 s	72 °C, 15 s
HEV	95 °C, 15 s	55 °C, 20 s	72 °C, 15 s
MRV	95 °C, 20 s	55 °C, 60 s	72 °C, 30 s
ADV41	95 °C, 30 s	50 °C, 60 s	72 °C, 30 s

Each reaction started with a Taq polymerase activation of 15 min at 95 °C, followed by 45 cycles of above conditions

All samples were analysed for efficiency of extraction and inhibition as described above (see "Experimental Section" section), using enteric viruses (HE) that resulted negative in q-PCR analysis on all environmental samples. Poliovirus 1 (HE) was chosen as reference virus and a q-PCR, previously developed, as method of detection (Oberste et al. 2010). 10 μL of aliquot B containing 10^5 genomic equivalents (see "Experimental Section" section) was used to test extraction efficiency and the other 10 μL containing 10^5 genomic equivalents, diluted 10- and 100-fold to evaluate the inhibition. The values obtained were compared to tenfold serial dilutions of Poliovirus 1

Table 5 Number of genome copies as a result of nucleic acids amplification using q-PCR

Sample	NoGGII Viral copies/(L)	HE Viral copies/(L)	MRV Viral copies/(L)	HAV Viral copies/(L)	HEV Viral copies/(L)	ADV41 Viral copies/(L)	Land	Water type/expected faecal sources of contamination
S.01	–	–	10^3	–	–	10^3	Open spaces	River surface water/NPSP
S.02	–	–	10^3	–	–	10^3	Grazing land	River surface water/PSP; NPSP
S.03	–	–	–	–	10^4	–	Artificial area	Lake/NPSP
S.04	10^3	–	10^3	–	–	10^4	Grazing land	Sea surface water/NPSP
S.05	–	–	–	–	–	–	Grazing land	River surface water/NPSP
S.06A	10^4	–	10^5	–	–	10^5	Grazing land	Lake/NPSP
S.06B	–	–	–	–	–	10^6	Grazing land	Lake/NPSP
S.07	–	–	10^4	–	10^4	10^6	Urban	River Surface water/PSP; NPSP
S.08	10^3	–	10^3	10^3	–	–	Urban	River Surface water/NPSP
S.09A	10^4	–	10^4	–	–	10^4	Grazing land	Dam Surface water/NPSP
S.09B	–	–	10^4	–	–	10^6	Grazing land	Dam Surface water/NPSP
S.10A	10^3	–	10^3	–	–	10^4	Grazing land	Black Sea/PSP; NPSP
S.10B	10^3	–	10^3	–	–	10^3	Grazing land	Black Sea/PSP; NPSP
S.11	10^3	–	–	–	–	–	Urban	River Surface water/PSP
S.12	10^3	–	10^3	–	–	–	Grazing land urban	Lake/PSP; NPSP

HE Human Enterovirus, HAV Hepatitis A Virus, NoGGII Norovirus GGII, HEV Hepatitis E Virus, ADV41 Adenovirus 41, MRV Mammalian Orthoreovirus:

In the respective columns are reported the converted copies to 1 L of raw water

cDNA as described in the Experimental Section. No significant reduction in extraction efficiency or evidence of inhibition was observed in the samples analysed.

Discussion

This study focused on using q-PCR as a detection tool for pathogenic viruses, in particular HAV, HE, NoGGII and HEV (Svraka et al. 2007; Wilhelmi et al. 2003) in environmental waters. For this work, primers targeting MRV and ADV 41 viruses were developed and tested. We successfully designed and carried out q-PCRs to detect ADV 41 and MRV, which may assist in the monitoring of water quality by detecting the most prevalent circulating serotypes (Leary et al. 2002).

None of the waters sampled contained detectable amounts of HE but MRV and ADV41 were frequently detected. The negative sample obtained from a rural river in France (S.05) was from a catchment with few animal and human activities present in the area at the time of sampling.

MRV is found in many wild and domesticated mammals, which are the sources for viral and zoonotic infections (Tyler 2001; Ya et al. 2015; Steyer et al. 2013). Human and animal sewage, runoff and leaching of contaminated soil are dissemination pathways of viruses into watersheds. Indeed, several (11 out of 15 = 73 %) of the European waters sampled contained MRV, including water bodies intended for direct use.

Although human MRV infections are often asymptomatic, the characterisation of isolates with high pathogenic potential suggests that there is a significant public health risk (Tyler et al. 2004). Moreover genetic shift and drift, which is common for segmented RNA genomes, can increase the pathogenic properties of viruses. In fact, a recent isolation and characterization of an Orthoreovirus type 2 was described as the result of genetic shift between bat, human and pig (Wang et al. 2015). Symptoms included diarrhoea, acute gastroenteritis and necrotising encephalopathy in animals and humans. It is therefore necessary to monitor spatial and temporal distributions of such viruses in the environment, and to identify sources of contamination (Shoeib et al. 2009).

Our results show that two out of the fifteen environmental samples were positive for the detection of HEV (S.03 and S.07). S.03 was obtained from an artificial lake in an urbanised area, which may indicate contamination by faecal matter of either animal or human origin. Another positive sample (S.07) was obtained from a site in the vicinity of an urban area already with a history of enteric viral contamination (Marcheggiani et al. 2015). Although HEV infections do not occur frequently in European countries, its environmental distribution and zoonotic potential could play a role in the epidemiology of future human HEV infections.

In addition, human NoV are the main viral agents responsible, together with Rotavirus, for infections of the gastrointestinal tract in children and in adults worldwide (Carter 2005). Compared to all other enteric viruses, numerous reinfections by NoV are possible, which makes it a highly prevalent cause of epidemics of viral gastroenteritis (Dolin 2007). NoV were detected in more than half of the samples, mostly from urbanised or agriculturally intense catchments. Our findings are consistent with a recent study where 30–80 % of water samples from two geographical regions show traces of these enteric viruses (Calgua et al. 2013). A monitoring study over a 1-year period revealed a NoV contamination of about 90 % of Norwegian surface waters which were used as drinking water reservoirs (Grøndahl-Rosado et al. 2014).

Although the presence of NoV is mostly associated with pollution by waste water from urban areas, also waste water from rural areas can contribute to the presence of NoV in surface waters. The sources of human faecal contamination could come from direct activities on site as well as from agriculture activities including animal husbandry or spread of manure on cultivated fields.

The development of ADV41 q-PCR was successful, and eleven of fifteen environmental samples were positive for this virus. Although ADV41 is only one of the two genotypes (ADV40 and 41) belonging to F Adenovirus, these data suggest that ADV41 is prevalent in surface water along with other Adenoviruses.

The viral load of ADV41 varied from 10^3 copies per litre to 10^6 per litre, with the highest numbers found in water from a site downstream of an urban area in proximity of a waste water treatment plant (sample S.07). Furthermore, Human HAV was recorded in only one sample (S.0.8), which was collected in river water from an urbanised area. Finally, HE was not detected in any of the water samples, whereas this group of enteric viruses has been frequently found in surface and waste water (Noble et al. 2003).

It is likely that our method of ultrafiltration, which permitted a cut-off of 25–30 nm, is not able to retain HE. This, however, contrasts with the results of HAV, a virus of similar size (25–27 nm) and with the methodology used to develop the ultrafiltration. Spiking water samples with known concentrations of HE, which are subjected to the entire processing will help elucidate this. Nevertheless, the extraction efficiency of viral nucleic acids involves the use of phage MS2 whose dimensions are comparable to those of HE (25 n–30 nm) and was efficiently found in the eluate solution after ultrafiltration (see ESM 1). It is also possible the HE was not present in the water, or at levels below the detection limit.

The results on HE were confirmed by a second, independent laboratory (Département Environnement & Santé Rechercheur & Innovation, Saint-Maurice, France) who performed q-PCR tests.

Conclusions

Overall, these data show human faecal contamination in various water bodies within Europe, including those used recreationally and as a source for human consumption. The enteric viruses detected in this study can be considered of human origin (HAV, NoGGII and ADV41) and may potentially be associated with zoonotic transmission (HEV and MRV). Our findings also suggest that monitoring of environmental waters for the presence of these viruses may provide an additional tool to determine potential dissemination of enteric viruses in a given community, which helps protecting human health.

Author Contributions Emilio D'Ugo proposed experimental design, searched articles and written the work. Laura Mancini and Stefania Marcheggiani implemented the study and contributed to writing the manuscript. Ilaria Fioramonti, collected data and performed q-PCR experiments. Roberto Giuseppetti carried out sampling and water concentration. Roberto Spurio, Karim Helmi, Delphine Guillebault, Linda K. Medlin, Ivan Simeonovski, Bas Boots, Ulrich Breitenbach˙ Stefania Marcheggiani, Latife Koker and Meric Albayhave have participated actively in the sampling campaign and carefully read the work. All the authors read, approved and significantly contributed to the final version of the manuscript.

Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflict of interest.

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