Detection of enteric viruses in Hungarian surface waters: first steps towards environmental surveillance

Anita Kern, Mihaly Kadar, Katalin Szomor, György Berencsi, Beatrix Kapusinszky and Marta Vargha

ABSTRACT

Waterborne viruses infect the human population through the consumption of contaminated drinking water and by direct contact with polluted surface water during recreational activity. Although water related viral outbreaks are a major public health concern, virus detection is not a part of the water quality monitoring scheme, mainly due to the absence of routine analysis methods. In the present study, we implemented various approaches for water concentration and virus detection, and tested on Hungarian surface water samples. Eighty samples were collected from 16 sites in Hungary. Samples were concentrated by glass wool and membrane filtration. Human adenoviruses were detected by conventional and quantitative real-time polymerase chain reaction (PCR) methods in 56% (45/80) of the samples; viral titers ranged from 8.60×10^1 to 3.91×10^4 genome copies per liter. Noroviruses and enteroviruses were detected in 30% (24/80) and 13% (10/80) of samples, respectively, by reverse transcription-PCR assays. Results indicate a high prevalence of viral human pathogens in surface waters, suggesting the necessity of a detailed survey focusing on the quality of natural bathing waters and drinking water sources.

Key words | enteric viruses, method comparison, PCR, surface water, virus concentration method

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INTRODUCTION

Water is the main transport medium for many human pathogens, including bacteria, protozoa and enteric viruses. Infection caused by waterborne pathogenic microorganisms is the most common and widespread health risk associated with water consumption (WHO 2008). Drinking and bathing water related outbreaks, often with unknown etiology, are a serious public health concerns, not only in developing countries but also in countries with a developed infrastructure.

Most of these infections are due to enteric viruses, which spread by the fecal-oral route, such as adenoviruses, noroviruses, enteroviruses, hepatitis A and E viruses, rotaviruses, sapoviruses, Aichi virus, coronaviruses etc. (Wyn-Jones *et al.* 2011). The general symptoms are nausea, diarrhea and vomiting, but some enteric viruses can cause severe diseases, such as hepatitis, myocarditis, aseptic meningitis, conjuctivitis, respiratory diseases and type I diabetes in children, elderly and immunocompromised persons (Bosch 1998; Griffin *et al.* 2003; Kapusinszky *et al.* 2010). Most enteric viruses are excreted into communal sewage, and are only partially eliminated by the current wastewater treatment methods (Fong & Lipp 2005). In Hungary, only about 70% of the wastewater is treated, thus surface water may receive treated and untreated sewage as well.

Despite the well-known risks associated with the presence of human pathogen viruses in surface waters (especially those used for bathing or as a drinking water source), in most countries virus detection is not a part of the monitoring scheme. Until recently, the determination of viruses in natural waters was challenging, mainly due to their low titer. Traditionally, hundreds of liters of water were concentrated by negative or positive charge filtration and/or flocculation (EPA 2001), followed by cell culture assays. The nucleic acid based detection method overcame many of the difficulties associated with cell culture, such as

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low sensitivity (Kopeczka *et al.* 1993), high cost and long analysis time (Tsai *et al.* 1993; Ma *et al.* 1995), and the toxic effects of water constituents that are concentrated in parallel with the viruses (Abbaszadegan *et al.* 1993). Genus specific polymerase chain reaction (PCR) allows detection of non-cultivable viruses and the simultaneous detection of multiple serotypes (Girones *et al.* 2010).

Though PCR-based methods have their limitations in the estimation of viability and infectivity, they are finding their way into natural bathing water quality assessment (e.g. *Enterococcus* enumeration (Wade *et al.* 2006)). There are extensive attempts to quantify sewage impact through novel indicators, such as *Bacteriodales* (Wade *et al.* 2008). Previously reported studies suggest that human adenoviruses and JC polyomaviruses are also suitable and reliable indicators of human fecal pollution of different water bodies (Puig *et al.* 1994; Pina *et al.* 1998; Bofill-Mas *et al.* 2000). Correlation between molecular and culture based detections was confirmed in the case of bacteria (Converse *et al.* 2012) and viruses (Donia *et al.* 2010).

In the present study, we applied various water concentration and virus detection methods and tested their performance and applicability in routine analysis of the surface water in Hungary. To our knowledge, this is the first report on the prevalence of human pathogen viruses in Hungarian surface waters.

MATERIALS AND METHODS

Samples and sampling sites

A total of 80 surface water samples were collected between August 2006 and July 2010 in Hungary (Figure 1 and Table 1). Grab samples were taken at 120 cm water depth, 30 cm below surface in sterile 10 L containers from 16 different sampling sites along Hungarian rivers and streams. Sampling sites were selected to represent rivers of different size and flow rate, and geographically distant areas. The two largest rivers in Hungary, the Danube and the Tisza, were characterized in more detail (four and six sampling sites, respectively). Three sampling points were located on the tributaries of the Tisza (Berettyó, Hármas-Körös, Sebes-Körös). Two streams running into Lake Balaton (Kéki, Koloska), and an oxbow lake of a former Danube branch were also sampled. Seven of the sites were used for recreational water activities ($n = 42$), nine sampling sites had no recreational use ($n = 38$). Recreational water samples were collected during the bathing season (for sampling dates, see Table 1).

Wastewater treatment plants, releasing secondary (activated sludge) or tertiary (phosphorus removal or chlorination) treated effluent, were located close to several sampling points, but there was generally no direct contact with the effluent, as the point of inflow was downstream

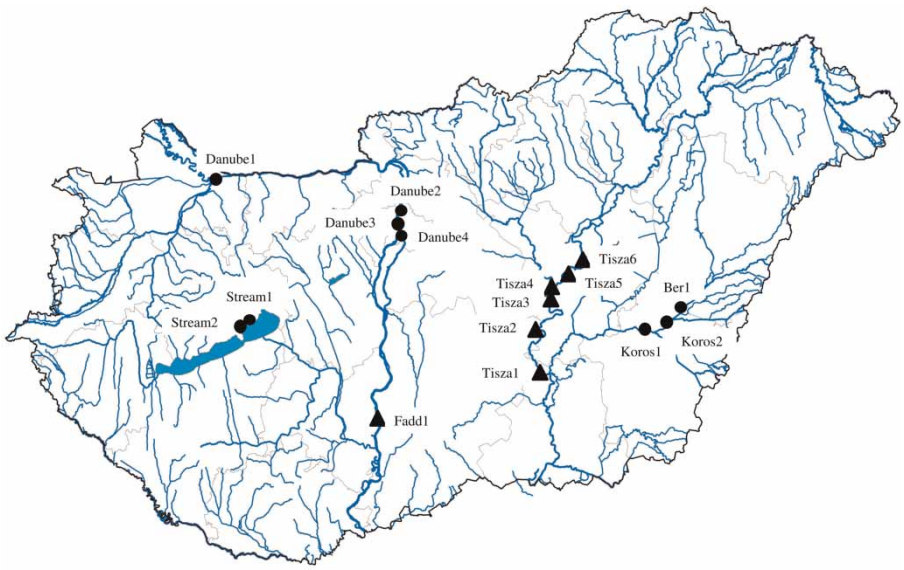

Figure 1 | Location of the sampling sites in Hungary. Black squares are surface natural bathing water sites ($n = 7$), black circles are surface water without recreational use ($n = 9$).

Table 1 | Sampling sites and sampling events

Sampling site ID	Sampling site	Wastewater impact	Collection date (dd/mm/yyyy)	Number of samples	Sample type
Ber1	Berettyó River, Szeghalom	Diffuse[a]	18.08.2008	2	Surface water
Danube1	Danube River, Györ	Diffuse[a]	09.09.2008	1	Surface water
Danube2	Danube River, Budapest	Tertiary treated effluent[b]	19.03.2008	2	Surface water
Danube2	Danube River, Budapest	Tertiary treated effluent[b]	19.11.2008	2	Surface water
Danube2	Danube River, Budapest	Tertiary treated effluent[b]	16.02.2009	2	Surface water
Danube2	Danube River, Budapest	Tertiary treated effluent[b]	13.05.2009	2	Surface water
Danube2	Danube River, Budapest	Tertiary treated effluent[b]	10.08.2009	2	Surface water
Danube3	Danube River, Budapest	Primary treated effluent[c]	20.11.2006	1	Surface water
Danube3	Danube River, Budapest	Primary treated effluent[c]	19.11.2008	2	Surface water
Danube3	Danube River, Budapest	Primary treated effluent[c]	16.02.2009	2	Surface water
Danube3	Danube River, Budapest	Primary treated effluent[c]	13.05.2009	2	Surface water
Danube3	Danube River, Budapest	Primary treated effluent[c]	10.08.2009	2	Surface water
Danube4	Danube River, Budapest	Primary treated effluent[c]	19.11.2008	2	Surface water
Danube4	Danube River, Budapest	Primary treated effluent[c]	16.02.2009	2	Surface water
Danube4	Danube River, Budapest	Primary treated effluent[c]	13.05.2009	2	Surface water
Danube4	Danube River, Budapest	Primary treated effluent[c]	10.08.2009	2	Surface water
Fadd1	Danube River oxbow, Fadd	Na	13.08.2006	4	Natural bathing water
Koros1	Hármas Körös River, Gyomaendröd	Diffuse[a]	18.08.2008	2	Surface water
Koros2	Sebes Körös River, Körösladány	Diffuse[a]	18.08.2008	2	Surface water
Stream1	Koloska Rivulet, Balatonfüred	Diffuse[a]	11.09.2008	2	Surface water
Stream2	Kéki Rivulet, Balatonfüred	Diffuse[a]	11.09.2008	2	Surface water
Tisza1	Csongrád, Tisza River	Na	05.08.2007	12	Natural bathing water
Tisza1	Csongrád, Tisza River	Na	06.08.2008	3	Natural bathing water
Tisza2	Tiszakécske, Tisza River	Na	01.07.2007	12	Natural bathing water
Tisza2	Tiszakécske, Tisza River	Na	06.08.2008	3	Natural bathing water
Tisza3	Rákócziújfalu, Tisza River	Na	20.07.2009	2	Natural bathing water
Tisza4	Rákóczifalva, Tisza River	Secondary treated effluent[d]	20.07.2009	2	Natural bathing water
Tisza5	Tiszapüspöki, Tisza River	Na	27.07.2010	2	Natural bathing water
Tisza6	Nagykörű, Tisza River	Na	15.07.2009	2	Natural bathing water

Na, not applicable.
[a]Illicit discharge from septic tanks or holiday homes.
[b]$FeCl_3$ treatment for phosphorus removal.
[c]Mechanical filtration.
[d]Activated sludge treatment.
Samples were collected from eight riverine locations between 2006 and 2010, five sites were sampled on multiple occasions. Sites are specified by the investigated river and the nearest municipality.

to all sampling sites except Danube2–4. Danube2 is close to the efflux of a tertiary treated wastewater, Danube3 is near a discharge point of primary treated (mechanically filtered) wastewater and Danube4 is located 2 river km downstream from Danube3. Tisza4 is the only natural bathing water sampling site directly affected by secondary treated communal sewage. There was no drinking water abstraction from surface water at any of the sampling sites, though bank filtered wells are located along the Danube. Drinking water in the sampled areas is mainly from groundwater.

Samples were stored at +2–8 °C until processing (<24 h).

Sample processing

Virus concentration was performed by the methods developed in the European Union funded project Virobathe (Wyn-Jones *et al.* 2011). Ten liters of water samples were concentrated using two different adsorbents: glass wool, type 725 (Rantigny, Saint-Gobain, France) and mixed cellulose ester membrane with 0.45 mm pore size, and 142 mm diameter (Whatman International Ltd) with a glass wool pre-filter, diameter 125 mm (Sartorius, Germany). Briefly, pH of the samples was reduced to 3.5 by adding 1 N HCl (Molar Chemicals, Hungary), and filtered. The positively charged viral particles were adsorbed to the negatively charged filters. Viruses were eluted from the filter with 200 mL of 3% glycine beef extract solution, pH 9.5 (Biolab, Hungary). The pH of the beef extracted elute was adjusted to 3.5 by 1 N HCl, thus flocking the protein fraction including the viral particles. The flocked elute was centrifuged at 8000 × g for 30 min and the pellet was resuspended in phosphate buffer to 10 mL final volume (Figure 2).

Twenty-four samples (six recreational and 18 other surface water) were processed by both membrane- and by glass wool concentration methods in order to compare the virus binding efficiency.

Escherichia coli and intestinal enterococci counts were determined from all surface water samples by the microtiter most probable number (MPN) method (ISO 9308-3:1998 and ISO 7899-1:1998, respectively) on MUG/EC and MUD/SF microplates (Bio-Rad, France).

Virus detection

Viral nucleic acids were extracted from 5 mL sample concentrates using the magnetic silica bead kit NucliSens® miniMAG™ (Biomérieux, France) according to manufacturer's instructions. The final volume of the purified nucleic acid was 100 μL. Nucleic acid extract was stored at −20 °C until further analysis.

Human adenoviruses (HAdV) were detected by nested PCR as previously reported (Allard *et al.* 2001). The reaction parameters were based on the protocol of Wyn-Jones *et al.* (2011) with modifications. The PCR reactions included 1× Platinum® Taq buffer, 1.5 mM $MgCl_2$ (Applied Biosystems), 250 μM of each dNTP (Thermo Scientific (Fermentas), Lithuania), 500–500 nM forward and reverse primer (Table 2) (Integrated DNA Technologies Inc.), 0.4 M bovine serum albumin (Thermo Scientific (Fermentas), Lithuania), 1 U/μL Platinum® *Taq* (Applied Biosystems), 10 or 2 μL nucleic acid (first and second round, respectively) in 50 μL total volume. The quantity of HAdV genome copies were determined by quantitative real-time PCR (qPCR) (Hernroth *et al.* 2002). The qPCR was performed in a total volume of 25 μL using the corresponding primers at 0.9 M and TaqMan® FAM-TAMRA-labeled probe at 0.225 M and 1× TaqMan® Environmental Master Mix (Applied Biosystems) and 10 μL DNA. The reactions were performed with an ABI7300 sequence detector system (Applied Biosystems). Linearized pBR322 plasmid containing the adenovirus hexon region was used as a standard (10^6–10^0 GC/10 μL range). The detection limit of the assay was 1 genome copy per 10 μL of nucleic acid. Each dilution of the standard curve, undiluted DNA and 10-fold dilutions of the extracted DNA were run in duplicate for each reaction. Human enteroviruses were detected by RT-nested PCR described by Puig *et al.* (1994) (Table 2). Human noroviruses were detected by RT-seminested PCR with oligonucleotide primers by Vennema *et al.* 2002 (Table 2). The parameters of the reaction were previously reported by Wyn-Jones *et al.* (2011). Bovine serum albumin (Thermo Scientific (Fermentas), Lithuania) was added to every reaction mixture in a final concentration of 0.4 M. Conventional PCR products were detected by electrophoresis in a 2% agarose – 1× TAE gel stained with ethidium bromide.

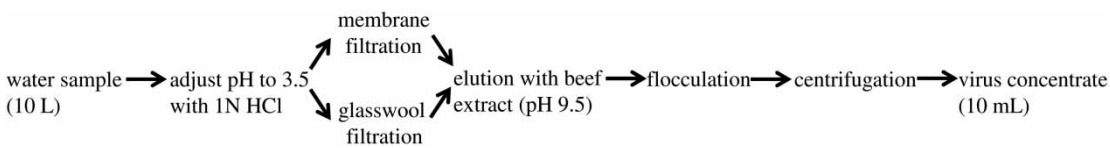

Figure 2 | Summary of the virus concentration procedure.

Table 2 | Oligonucleotide primers and probe used for virus detection

Virus detection assay	Region	Primer	Sequence	PCR product size (bp)	Reference
HAdV/nested PCR	hexon	Forward	5′-GCCSCARTGGKCWTACATGCACATC-3′	301	Allard *et al.* (2001)
		Reverse	5′-CAGCACSCCICGRATGTCAAA-3′		
		Nested forward	5′-GCCCGYGCMACIGAIACSTACTTC-3′	171	
		Nested reverse	5′-CCYACRGCCAGIGTRWAICGMRCYTTGTA-3′		
HAdV/qPCR	hexon	Forward	5′-CWTACATGCACATCKCSGG-3′	na	Hernroth *et al.* (2002)
		Reverse	5′-CRCGGGCRAAYTGCACCAG-3′		
		Probe	5′-FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-TAMRA-3′		
HEV/RT-nested PCR	5′NTR	Forward	5′-CGGTACCTGTACGCCTGT-3′	534	Puig *et al.* (1994)
		Reverse	5′-ATTGTCACCATAAGCAGCCA-3′		
		Nested forward	5′-TCCGGCCCCTGAATGCGGCTA-3′	138	
		Nested reverse	5′-GAAACACGGACACCCAAAGTA-3′		
HNoV GII/RT-seminested PCR	RdRp	Forward	5′-ATACCACTATGATGCAGAYTA-3′	326	Vennema *et al.* (2002)
		Reverse	5′-TCATCATCACCATAGAAIGAG-3′		
		Nested forward	5′-ATACCACTATGATGCAGAYTA-3′	236	
		Nested reverse	5′-AGCCAGTGGGCGATGGAATTC-3′		

HAdV, human adenovirus; HEV, human enterovirus; HNoV GII, human norovirus genogroup II; 5′NTR, 5′non-translated region; RdRp, RNA-dependent RNA polymerase.
Na, not applicable; RT, reverse transcription; FAM, 6-carboxyfluorescein; TAMRA, tetramethylrhodamine.

The sequencing reaction was done by the DYEnamic ET Dye Terminator Cycle kit (Amersham Biosciences Inc.) following the manufacturer's instructions. Sequencing was performed in MegaBACE^{TM} 1000 DNS sequencer (Amersham Biosciences Inc.). Acquired sequences were analyzed for nucleotide homology by BLAST program in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Quality control

For quality control purposes, 10 L of dechlorinated, sterile tap water was spiked with echovirus serotype 11 (ECHO-11) or human adenovirus type 2 (HAdV-2) and processed in parallel with the surface water samples (positive process control). Dechlorinated, sterile tap water was used as a negative process control with every batch. In addition, reaction control was included in every PCR run; a negative (reagent) control and purified viral nucleic acid (HAdV-2, ECHO-11 and human norovirus genogroup II genotype 13) as positive control. Internal amplification control was omitted to increase sensitivity, but all PCRs were run using three dilutions (using undiluted, 10-fold and 100-fold diluted nucleic acids) to overcome inhibition effects.

RESULTS

Virus detection and quantification

Over 56% (45/80) of the samples were positive for adenovirus by nested PCR. Norovirus and enterovirus RNA were

Table 3 | Presence of human adenovirus, enterovirus and norovirus (genogroup II) in surface water samples by taxon specific (RT)-PCR given as positive samples/all samples

Sampling site ID	Adenovirus	Enterovirus	Norovirus
Ber1	1/2 (na[a]) 0/2 0/2		
Danube1	0/1	0/1 0/1	
Danube2	8/10 (1.91×10^2–3.72×10^3 GC/L)	0/10	5/10
Danube3	9/9 (3.73×10^3–2.98×10^4 GC/L)	3/9	8/9
Danube4	8/8 (2.24×10^2–9.46×10^3 GC/L)	2/8	5/8
Koros1	0/2	0/2	0/2
Koros2	0/2	0/2	0/2
Fadd1	2/4 (na)	0/4	4/4
Stream1	0/2	0/2	0/2
Stream2	0/2	0/2	0/2
Tisza1	7/10 (8.60×10^1–3.09×10^2 GC/L)	0/15	0/15
Tisza2	7/15 (1.02×10^3 GC/L)	0/15	0/15
Tisza3	0/2	1/2	0/2
Tisza4	1/2 (1.33×10^2 GC/L)	2/2	1/2
Tisza5	0/2	0/2	1/2
Tisza6	2/2 (4.26×10^2–8.90×10^2 GC/L)	2/2	0/2

[a]Na, not analysed; GC, genome copies.
Quantitative information (range genome copy numbers determined by quantitative PCR) is listed in brackets after the prevalence data where available.

detected in 24 (30.0%) and 10 samples (12.5%), respectively (Table 3). Approximately one-third of the samples contained at least two of the targeted viral groups; four samples were positive for both HAdV and human enteroviruses (5.0%), 17 samples contained HAdV and human noroviruses (21.3%), and all three virus groups were detected in five samples (6.3%). A total of 62.5% (50/80) of the samples contained at least one of the examined viruses.

The HAdV quantity was determined in 30 out of 45 (66.7%) PCR positive samples, viral load varied from 8.60×10^1 to 3.91×10^4 genome copies per liter (GC/L) with a geometric mean value of 1.84×10^3 GC/L. Nineteen of the 45 samples that contained HAdV DNA originated from natural bathing waters (8.60×10^1–1.02×10^3 GC/L, with a mean value of 3.33×10^2 GC/L), the other 26 HAdV-positive samples were collected from rivers, where bathing is forbidden or not possible (1.91×10^2–3.91×10^4 GC/L, with a mean value of 2.88×10^3 GC/L).

The detection rate of human enteroviruses in surface water for recreational and non-recreational use was 11.9% (5/42) and 13.2% (5/38), respectively. Noroviruses were detected in six out of 42 (14.3%) natural bathing water samples and in 18 out of 38 (47.4%) water samples with no recreational use.

Nine of the 45 HAdV positive samples were further analyzed to determine virus serotype. The detected HAdV serotypes were as follows: type 41 ($n=5$), type 40 ($n=2$), type 12 ($n=1$) and type 2 ($n=1$). Human norovirus genogroup II was confirmed from four samples and human enteroviruses from two samples by sequence analysis.

All negative process controls reagent controls (15/15) and PCR reagent controls were negative. Spiked virus was successfully recovered from positive process controls in every case (15/15).

Comparison of membrane and glass wool filtration methods

In order to compare the efficiency of two ubiquitously applied adsorbents, 24 samples were concentrated by both membrane filtration and glass wool adsorbent (Figure 3). Eighteen of the 24 samples (75.0%) were positive for at least one of the examined viruses after glass wool filtration and 15 samples (62.5%) after membrane filtration. Adenoviruses were detected in 16 (66.7%) and 14 samples (58.3%) by glass wool and membrane filtration, respectively. A similar trend was observed for the concentration of

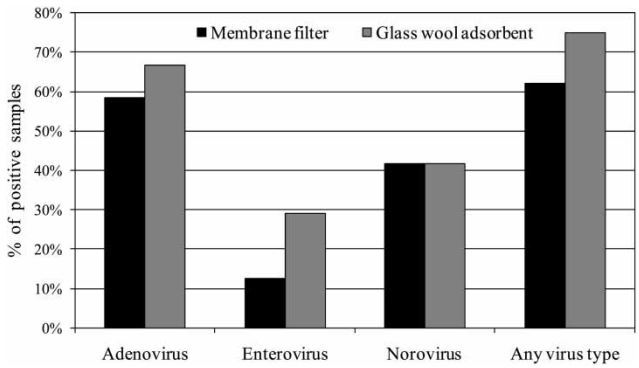

Figure 3 | Comparison of the rate of positive samples using membrane and glass wool filtration. The difference in virus binding efficiency varied with the virus type.

enteroviruses: seven (29.2%) and three positive samples (12.5%), respectively after glass wool and membrane filtration. The same number (though not identical set) of the samples (10/24; 41.7%) was positive for norovirus by both concentration methods.

The average titer of HAdV was higher after the glass wool concentration method than after the membrane filtration (geometric mean titer 1.97×10^3 GC/L and 1.58×10^3 GC/L, respectively).

Fecal indicator counts

In the investigated natural bathing waters *E. coli* counts ranged from 3.0×10^1 to 7.3×10^2 MPN/100 mL, with a mean value of 2.0×10^2 MPN/100 mL, and intestinal enterococci counts from 1.5×10^1 to 1.0×10^3 MPN/100 mL, with a mean value of 1.0×10^2 MPN/100 mL. In other surface water samples *E. coli* were detected between 6.1×10^1 MPN/100 mL and 9.5×10^4 MPN/100 mL with a mean value of 8.9×10^2 MPN/100 mL and intestinal enterococci between 1.5×10^1 MPN/100 mL and 2.0×10^4 MPN/100 mL with a mean value of 5.7×10^2 MPN/100.

DISCUSSION

A high prevalence of enteric viruses was detected in the eight investigated Hungarian rivers and streams (Table 3). Smaller streams and rivers were usually less contaminated (though the average sample number was also lower), while the samples from large rivers (Tisza and Danube) were almost 70% positive for at least one of the targeted viruses.

There is no previous systematic prevalence information available on enteric viruses in Hungarian surface waters. Recent information on viruses in Hungarian water environments is limited to reports on raw and treated sewage (Daniel & Dömök 1962; Meleg *et al.* 2006, 2008). The presence of rotavirus (group C) and human astrovirus (genotype I) was confirmed in the majority of raw sewage samples, and secondary treatment only reduced the rate of positive samples by 50%. Other studies also indicate that secondary wastewater is a main source of enteric virus load in surface water (Bosch 1998; Pina *et al.* 1998; Okoh *et al.* 2010).

Wastewater impact was clearly reflected both by the fecal indicator counts and the viral prevalence in the Danube2, 3 and 4 sampling sites (all located in Budapest). Danube2 was near a tertiary treated (phosphorus removal by $FeCl_3$) effluent inflow, while Danube3 was receiving practically untreated sewage (mechanical filtration only). Danube4 is located 2 km downstream from Danube3, without further sewage impact in between. Accordingly, bacterial counts, HAdV titers and the rate of positivity for the other viruses were lowest at Danube2, highest at Danube3 and showed the effect of dilution (a decrease of at least an order of magnitude in bacterial counts and HAdV titers) at Danube4 (Table 2, Figure 4).

The Tisza river is also known to receive (mostly biologically treated) effluent from wastewater treatment plants. However, only one of the sampling points (Tisza4) was directly impacted by effluent (within 5 river km), the others were at least 5 river km downstream from the nearest sewage inlet. Secondary treated wastewater in the area is not disinfected continuously, though there is usually a possibility to perform emergency disinfection by chlorine-based disinfectants in the case of outbreaks or sewage overflow. In the case of the Tisza river, the effect of sewage impact was less obvious. Tisza4 samples were positive for all viruses, but other Tisza sites also showed high prevalence, and adenovirus titers were even higher at some points. The bacterial water quality was similar for all sites. The results suggest other sources of contamination, such as diffuse discharge from septic tanks, surface run-off and streams with wastewater impact, or holiday houses near the river illicitly releasing untreated sewage to the river. Though there is a known input from industrial wastewater near some Tisza sampling sites, from chemical industries, food and energy industry, these are not considered significant sources of human viruses. The natural bathing water sites were sampled during the bathing season, often when bathers were also present, thus the viruses might have originated directly from the bathers.

Sites used for bathing or other recreational activities, such as kayaking or canoeing, were a special focus of the study, as the presence of enteric viruses only poses a risk if there are also potential exposure routes. Of the seven natural bathing sites, adenoviruses were detected at five locations, while norovirus and enterovirus were detected at three locations.

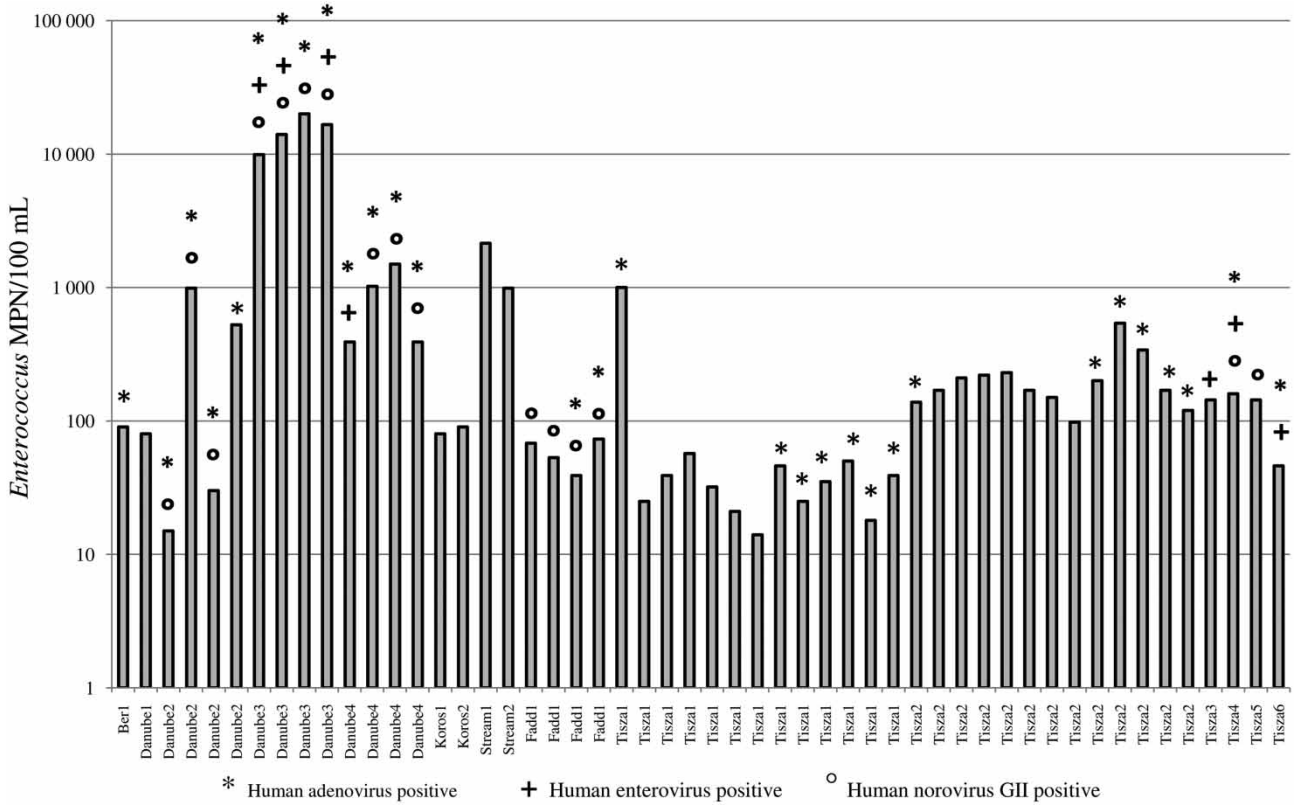

Figure 4 | *Enterococcus* counts and presence of human pathogen viruses.

Water quality assessment of natural bathing waters is based on the fecal indicator bacteria, usually determined by culture, though some regulations recently incorporated real-time PCR method for *Enterococcus* (EPA 2012). The currently most widely accepted regulation for bathing water quality assessment is the European Union Bathing Water Directive 2006/7/EC and the US EPA 2012. However, both are using a risk based approach, rather than a single quality threshold, and thus cannot be used in the context of the current study to evaluate water quality based on the observed fecal indicator counts. The former EU Bathing Water Directive, 76/160/EEC (CEU 2006) defined limit values of 'good' quality for *E. coli* and *Enterococcus* (1000 CFU/100 mL and 200 CFU/100 mL, respectively). All natural bathing water samples complied with the *E. coli* criteria and only four single samples (in the case of Tisza1 and Tisza2) exceeded the limit value for *Enterococcus*, which were positive for HAdV. All other virus positive samples were compliant with good quality limit values. Most of the investigated natural bathing sites also qualify as good according to the current EU Bathing Water Directive, with the exception of Tisza 1 and Tisza2.

The results show that the good quality of the natural bathing water does not mean the absence of viruses and potentially human pathogen viruses were detected in recreational waters of excellent quality according to the bacterial hygiene parameters. Though the disease burden of waterborne viruses in association with bathing is still being assessed, this is a further implication for introducing enteric viruses as a water quality parameter.

In the present study, human adenoviruses had the highest prevalence of the three examined virus groups in accordance with previous observations (Puig *et al.* 1994; Pina *et al.* 1998; Wyn-Jones *et al.* 2011). It was detected in the majority of the Danube and Tisza sites (3/4 and 4/6, respectively), and two additional points (Fadd Oxbow and Berettyó). Norovirus was present at three Danube, two Tisza sampling points and in the Fadd oxbow, while human enterovirus was only detected from the two major rivers. The higher stability of adenovirus particles and its

genetic material might have also contributed to its higher detection rate (Enriquez *et al.* 1995; Pina *et al.* 1998). Both the stability and its ubiquitous presence in communal sewage were arguments for proposing human adenoviruses as indicators of fecal contamination (Pina *et al.* 1998); and the results of the present study also support their applicability.

The sequence analyses confirmed that potentially human pathogen viruses were detected during this study. Predominantly enteric adenoviruses were present as it was previously reported (Hamza *et al.* 2009). In the case of enterovirus typing, the molecular analysis of the virus protein 1 (VP1) region would be more appropriate instead of the highly conserved 5′NTR region and needs further analysis (Oberste *et al.* 1999).

Comparison of the concentration methods on the same samples set demonstrated a slightly better efficiency of glass wool filtration, in accordance with previous results of method characterization for freshwater (Albinana-Gimenez *et al.* 2009; Maunula *et al.* 2012). The ratio of positive samples was generally higher for glass wool filtered samples, and adenovirus titers, where determined, were also higher, though the difference was not statistically significant (data not shown). In freshwater, suspended and dissolved solids are the main limiting factors of the adsorption-elution methods. Suspended solids clog the pores of the filter material, while dissolved or colloidal organic material competes with viral particles for the active binding sites (Sobsey 1976). Glass wool was less sensitive to clogging, and its larger active surface may explain the better concentration efficiency. It was also easier to process multiple samples by glass wool filtration in parallel, thus the overall time-demand of the process was considerably lower than that of membrane filtration.

Not only the choice of the concentration method, but other steps of the analysis steps may influence the detection results. Suspended solids sediment during centrifugation and may hinder nucleic acid extraction. Certain materials in the water, such as metal ions or organic acids, can be concentrated during samples processing, and interfere with the molecular detection through the inhibition of the polymerase enzyme (Tsai & Olson 1991; Abbaszadegan *et al.* 1993). In the present study, inhibition was not investigated in detail, but samples were analyzed in three dilutions to overcome the effect. Around 16% of the positive samples (10 adenovirus and three norovirus detections) only gave a positive reaction in dilution.

CONCLUSIONS

A high prevalence of human enteric viruses in Hungarian surface waters was demonstrated using a combination of negatively charged filtration and flocculation with molecular detection methods. The results demonstrate that even natural bathing waters, which comply with the quality criteria based on bacterial fecal indicators, are often contaminated with potentially pathogenic viruses. This indicates the necessity of a more detailed survey extended to other types of surface waters, such as lakes or reservoirs, and targeting issues of seasonality and viability. The ubiquitous presence of adenoviruses in sewage impacted surface waters and the relation of its titer to the sewage load were observed even in this preliminary study. The findings support the proposals of adenovirus as an indicator of fecal contamination from human sources.

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REFERENCES

Abbaszadegan, M., Huber, M. S., Gerba, C. P. & Pepper, I. L. 1993 Detection of enteroviruses in groundwater with the

polymerase chain reaction. *Appl. Environ. Microbiol.* **59** (5), 1318–1324.

Albinana-Gimenez, N., Clemente-Casares, P., Calgua, B., Huguet, J. M., Courtois, S. & Girones, R. 2009 Comparison of methods for concentrating human adenoviruses, polyomavirus JC and noroviruses in source waters and drinking water using quantitative PCR. *J. Virol. Methods* **158**, 104–109.

Allard, A., Albinson, B. & Wadell, G. 2001 Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. *J. Clin. Microbiol.* **39** (2), 498–505.

Bofill-Mas, S., Pina, S. & Girones, R. 2000 Documenting the epidemiologic patterns of polyomaviruses in human populations studying their presence in urban sewage. *Appl. Environ. Microbiol.* **66** (1), 238–245.

Bosch, A. 1998 Human enteric viruses in the environment: a minireview. *Int. Microbiol.* **1** (1), 191–196.

CEU 2006 Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC.

Converse, R. R., Griffith, J. F., Noble, R. T., Haugland, R. A., Schiff, K. C. & Weisbergb, S. B. 2012 Correlation between quantitative PCR and culture-based methods for measuring *Enterococcus* spp. over various temporal scales at three California marine beaches. *Appl. Environ. Microbiol.* **78** (4), 1237–1242.

Daniel, M. & Dömök, I. 1962 Virological examination of urban sewage in periods of mass immunization with live attenuated polioviruses. *Acta Microbiol. Acad. Sci. Hung.* **9**, 251–259.

Donia, D., Bonanni, E., Diaco, L. & Divizia, M. 2010 Statistical correlation between enterovirus genome copy numbers and infectious viral particles in wastewater samples. *Lett. Appl. Microbiol.* **50** (2), 237–240.

Enriquez, C. E., Hurst, C. J. & Gerba, C. P. 1995 Survival of the enteric adenoviruses 40 and 41 in tap, sea, and wastewater. *Water Res.* **29** (11), 2548–2553.

EPA 2001 *US EPA Manual of Methods for Virology*. EPA 600/4-84/013 (N14), Environmental Protection Agency.

EPA 2012 *US EPA Recreational Water Quality Criteria*. EPA Office of Water 820-F-12-058, Environmental Protection Agency.

Fong, T.-T. & Lipp, E. K. 2005 Enteric viruses of humans and animals in the aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiol. Mol. Biol. Rev.* **69** (2), 357–371.

Girones, R., Ferrus, M. A., Alonso, J. L., Rodriguez-Manzano, J., Calgua, B., de Abreu Correa, A., Hundesa, A., Carratala, A. & Bofill-Mas, S. 2010 Molecular detection of pathogens in water: The pros and cons of molecular techniques. *Water Res.* **44** (15), 4325–4339.

Griffin, D. W., Donaldson, K. A., Paul, J. H. & Rose, J. B. 2003 Pathogenic human viruses in coastal waters. *Clin. Microbiol. Rev.* **16** (1), 129–143.

Hamza, I. A., Jurzik, L., Stang, A., Sure, K., Überla, K. & Wilhelm, M. 2009 Detection of human viruses in rivers of a densely-populated area in Germany using a virus adsorption elution method optimized for PCR analyses. *Water Res.* **43** (10), 2657–2668.

Hernroth, B. E., Conden-Hansson, A.-C., Rehnstam-Holm, A.-S., Girones, R. & Allard, A. 2002 Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. *Appl. Environ. Microbiol.* **68** (9), 4523–4533.

International Organization for Standardization (ISO) 7899-1 1998 *Water quality – Detection and enumeration of intestinal enterococci – Part 1: Miniaturized method (Most Probable Number) for surface and waste water*. International Organization for Standardization, Geneva, Switzerland.

International Organization for Standardization (ISO) 9308-3 1998 *Water quality – Detection and enumeration of Escherichia coli and coliform bacteria – Part 3: Miniaturized method (Most Probable Number) for the detection and enumeration of E. coli in surface and waste water*. International Organization for Standardization, Geneva, Switzerland.

Kapusinszky, B., Szomor, K. N., Farkas, A., Takács, M. & Berencsi, G. 2010 Detection of non-polio enteroviruses in Hungary 2000–2008 and molecular epidemiology of enterovirus 71, coxsackievirus A16, and echovirus 30. *Virus Genes* **40** (2), 163–173.

Kopeczka, H., Dubrou, S., Prevot, J., Marechal, J. & López-Pila, J. M. 1993 Detection of naturally occurring enteroviruses in water by reverse transcription, polymerase chain reaction, and hybridization. *Appl. Environ. Microbiol.* **59** (4), 1213–1219.

Ma, J.-F., Gerba, C. P. & Pepper, I. L. 1995 Increased sensitivity of poliovirus detection in tap water concentrates by reverse-transcriptase polimerase chain reaction. *J. Virol. Methods* **55** (3), 295–302.

Maunula, L., Söderberg, K., Vahtera, H., Vuorilehto, V. P., von Bonsdorff, C. H., Valtari, M., Laakso, T. & Lahti, K. 2012 Presence of human noro- and adenoviruses in river and treated wastewater, a longitudinal study and method comparison. *J. Water Health* **10** (1), 87–99.

Meleg, E., Bányai, K., Martella, V., Baoming, J., Kocsis, B., Kisfali, P., Melegh, B. & Szűcs, Gy. 2008 Detection and quantification of group C rotaviruses in the communal sewage. *Appl. Environ. Microbiol.* **74** (11), 3394–3399.

Meleg, E., Jakab, F., Kocsis, B., Bányai, K., Melegh, B. & Szűcs, Gy. 2006 Human astroviruses in raw sewage samples in Hungary. *J. Appl. Microbiol.* **101** (5), 1123–1129.

Oberste, M. S., Maher, K., Kilpatrick, D. R., Flemister, M. R., Brown, B. A. & Pallansch, M. A. 1999 Typing of human enteroviruses by partial sequencing of VP1. *J. Clin. Microbiol.* **37** (5), 1288–1293.

Okoh, A. I., Sibanda, T. & Gusha, S. S. 2010 Inadequately treated wastewater as a source of human enteric viruses in the environment. *Int. J. Environ. Res. Public Health* **7**, 2620–2637.

Pina, S., Puig, M., Lucena, F., Jofre, J. & Girones, R. 1998 Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. *Appl. Environ. Microbiol.* **64** (9), 3376–3382.

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, F. & Girones, R. 1994 Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Appl. Environ. Microbiol.* **60** (8), 2963–2970.

Sobsey, M. D. 1976 Methods for detecting enteric viruses in water and wastewater. In: *Viruses in Water* (G. Berg, H. L. Bodily, E. H. Lennette, J. L. Melnick & T. G. Metcalf, eds). American Public Health Association, Inc., Washington, pp. 88–127.

Tsai, Y.-L. & Olson, B. H. 1991 Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Appl. Environ. Microbiol.* **58** (2), 754–757.

Tsai, Y.-L., Sobsey, M. D., Sangermano, L. R. & Palmer, C. J. 1993 Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse-transcriptase polymerase chain reaction. *Appl. Environ. Microbiol.* **59** (10), 3488–3491.

Vennema, H., de Bruin, E. & Koopmans, M. 2002 Rational optimization of generic primers used for Norwalk-like virus detection by reverse transcriptase polymerase chain reaction. *J. Clin. Virol.* **25** (2), 233–235.

Wade, T. J., Calderon, R. L., Sams, E., Beach, M., Brenner, K. P., Williams, A. H. & Dufour, A. P. 2006 Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. *Environ. Health Perspect.* **114** (1), 24–28.

Wade, T. J., Calderon, R. L., Brenner, K. P., Sams, E., Haughland, R., Wymer, L. & Dufour, A. P. 2008 High sensitivity of children to swimming-associated gastrointestinal illness. *Epidemiology* **19** (3), 375–383.

World Health Organization (WHO) 2008 *Guidelines for Drinking-Water Quality*. Recommendations, Vol 1, 3rd edn. WHO, Geneva, Schwitzerland.

Wyn-Jones, A. P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleisher, J., Gantzer, C., Gawler, A., Girones, R., Höller, C., de Roda Husman, A. M., Kay, D., Kozyra, I., López-Pila, J. M., Muscillo, M., Nascimento, M. S. J., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R. & Wyer, M. 2011 Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Res.* **45** (3), 1025–1038.

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