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ORIGINAL PAPER

Presence of Human and Animal Viruses in Surface Waters in Vojvodina Province of Serbia

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Abstract For the first time in Serbia, a small surveillance study was conducted in order to estimate the presence and frequency of occurrence of selected human [adenoviruses (HAdV), noroviruses, (NoV GI, NoV GII) and hepatitis A virus (HAV)], animal [porcine adenovirus (PAdV) and bovine polyomavirus (BPyV)] and zoonotic [hepatitis E virus (HEV)] viruses in selected surface waters. In total, 60 surface water samples were collected in two sampling occasions at 30 locations, with each sampling time being separated by 1–5 months. In addition, six sewage effluent samples were collected at one sampling site per each of the three tested town sewage systems, in two sampling occasions with 2 months intervals, before their discharge into the surface waters. The most prevalent virus found was HAdV which was detected in 43.33 % samples. NoV GII was found in 40 % samples. NoV GI was found in 10 % samples, and PAdV, BPyV and HEV were detected in 5 (8.33 %), 4 (6.67 %) and 2 (3.33 %) samples, respectively. HAV was not found in any of analysed surface waters or urban sewage samples. The obtained results confirm the presence of pathogenic enteric viruses of both human and animal origin in surface waters in Serbia indicating the existence of diverse contamination sources.

Keywords Surface waters · Untreated sewage · Human and porcine adenovirus · Human norovirus · Hepatitis A and E virus · Bovine polyomavirus · Real time (RT)-PCR · Serbia

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Introduction

The presence of human and animal pathogenic enteric viruses in water environments reflects faecal contamination and indicates a risk to public health. Water is an important vehicle for the transmission of enteric viruses. Rivers, lakes, streams and coastal waters are regularly contaminated by septic tanks, storm water runoff and agricultural run-off or run-off of the animal manure used in agriculture and effluents from inefficiently operated sewage treatment plants. Additionally, water could be also contaminated from overflows of treatment plants impacted by flooding events, or through direct inflow of untreated sewage. However, there is also more direct faecal contamination of the environment from humans and animals, for example, by bathers or by defecation of farm animals and free-range or wild animals onto soil or surface waters (Rodríguez-Lázaro et al. 2012; Cook and Richards 2013; Petrović 2013). In aquatic environments, viruses can survive for prolonged periods of time. Over 100 types of pathogenic viruses are excreted in human and animal wastes (Melnick 1984), which even at low concentrations may cause illness when ingested (Albinana-Gimenez et al. 2006). Virus concentrations in raw water contaminated with faecal wastes such as sewage are often high. Patients suffering from viral gastroenteritis or viral hepatitis may excrete about 10^5–10^{11} virus particles per gram of stool (Bosch 1998), comprising various genera such as adenoviruses (AdV), astroviruses (AstV), noroviruses (NoV), hepatitis E virus (HEV), parvoviruses, enteroviruses (EV) (Coxsackie viruses, echoviruses and polioviruses), hepatitis A virus (HAV) and human rotavirus (HRV) (Carter 2005), so these viruses can be easily present in urban sewage. Above-mentioned viruses are responsible for a large number of epidemics through their presence in the aqueous environment or food, therefore main route of infection could be

mediated by consumption of contaminated water or food (Bofill-Mas et al. 2010; Vantarakis et al. 2010).

Human adenoviruses (HAdV) and human polyomaviruses (HPyV) could be find in all geographical areas throughout the whole year, while EV, NoV, rotaviruses (RVs), AstV, HAV and HEV are detected with variable prevalence in different geographical areas and periods of the year (Gironés and Bofill-Mas 2013). Enteric viruses can survive the water disinfection processes that eliminate bacteria (Carratala et al. 2013). Base on their epidemiologic characteristics, HAdV have been proposed as alternative indicators for human faecal contamination, although the persistence of other enteric viruses over prolonged periods in environmental waters has been also reported (Chigor and Okoh 2012; Carratala et al. 2013).

Since less than ten virus particles can lead to infection and disease, noroviruses (NoV) are very common cause of both endemic and epidemic gastroenteritis (Teunis et al. 2008; Atmar 2010). Transmission of NoV is primarily through faecal–oral route (Atmar 2010), and gastroenteritis outbreaks are result of exposure to contaminated food, water and person-to-person contact (Atmar 2010). Etiological agents of viral hepatitis, like HAV and HEV, can be transmitted through water and food also. HAV represents a threat to human health in several southern Mediterranean countries, and they are responsible for substantial morbidity in both developed and undeveloped countries (Beji-Hamza et al. 2014). It is worth to mention that most of HEV infections are asymptomatic and consequently go unnoticed (Petrović et al. 2014); nevertheless the number of clinical cases is continuously increasing in some countries (Pina et al. 2000).

Porcine adenoviruses (PAdVs) are widely disseminated in the swine population (Gironés et al. 2010; Gironés and Bofill-Mas 2013). Infection with highly prevalent PAdV in pigs is usually asymptomatic and PAdVs have been often isolated from apparently healthy pigs (Fong and Lipp 2005). PAdVs have been proposed as porcine faecal contamination indicators (Maluquer de Motes et al. 2004; Gironés and Bofill-Mas 2013) and were often detected in river water samples with potential porcine faecal contamination (Hundesa et al. 2006). Although bovine polyomaviruses (BPyVs) do not lead to clinically severe diseases in cattle, they have been suggested as potential bovine markers (Hundesa et al. 2006; Gironés et al. 2010; Gironés and Bofill-Mas 2013). In environmental samples with bovine faecal contamination, BPyVs have been found more prevalent than bovine adenoviruses (BAdVs) (Hundesa et al. 2006; Gironés and Bofill-Mas 2013; Petrović 2013).

Although the presence of human and animal viruses, especially zoonotic agents, in water is intensively studied and monitored in countries worldwide (Kokkinos et al. 2012; Hundesa et al. 2006; Kern et al. 2013; Chigor and Okoh 2012; Parasidis et al. 2013a; Rusiñol et al. 2014), so far, there are no similar published available data in Serbia. Furthermore, the treatment of waste water and sewage, especially from small towns and villages, as well as from some large cities in Serbia, is seldom implemented, or works only with partial function. Consequently, for the first time in Serbia, a small surveillance study was conducted in order to estimate the presence and frequency of occurrence of selected human [(HAdV, noroviruses genogroup I and II (NoV GI and NoV GII) and HAV), animal (PAdV and BPyV) and zoonotic (HEV)] viruses in surface waters in the country. Adenoviruses were selected as faecal indicators based on their almost universal shedding and stability in the environment; and NoV and HAV were selected as being the most prevalent gastroenteritis and significant hepatitis agent worldwide and/or because many viral waterborne outbreaks are restricted to those viruses. Animal and zoonotic viruses PAdV, BPyV and HEV were chosen to estimate the possible existence of route of animal faecal contamination, as well as indicate whether the examined surface waters could present a possible hazard for animal and public health.

Materials and Methods

Samples

To evaluate the existence and the level of viral contamination, through human and animal faecal discharge, twelve surface waters (six rivers, one stream, one lake, one special nature reserve wetland and three canals) and three urban sewage systems effluent (untreated sewage from towns Subotica, Sombor and Odzaci) in the territory of Vojvodina Province in Serbia were sampled between July and December 2013 (Fig. 1). In total, 60 surface water samples were collected at 30 locations during two sampling periods: summer (July–October) and autumn (November–December). The sampling locations for surface waters were chosen near all larger towns and as close as possible to a few intensive animal production farms. Among these water samples, ten samples were collected from five urban beaches (sampled twice) with the aim to evaluate the potential risk for swimmers, and four different kind of surface water from five locations in protected natural areas (each location was also sampled twice) were included to distinguish the presence of viruses in surface waters from urban and wild nature areas. In addition, we collected six untreated sewage samples from three urban sewage systems (each of the tested urban sewage was sampled twice). Sewage samples from Subotica and Sombor were collected just before entrance into the sewage treatment plant (TP).

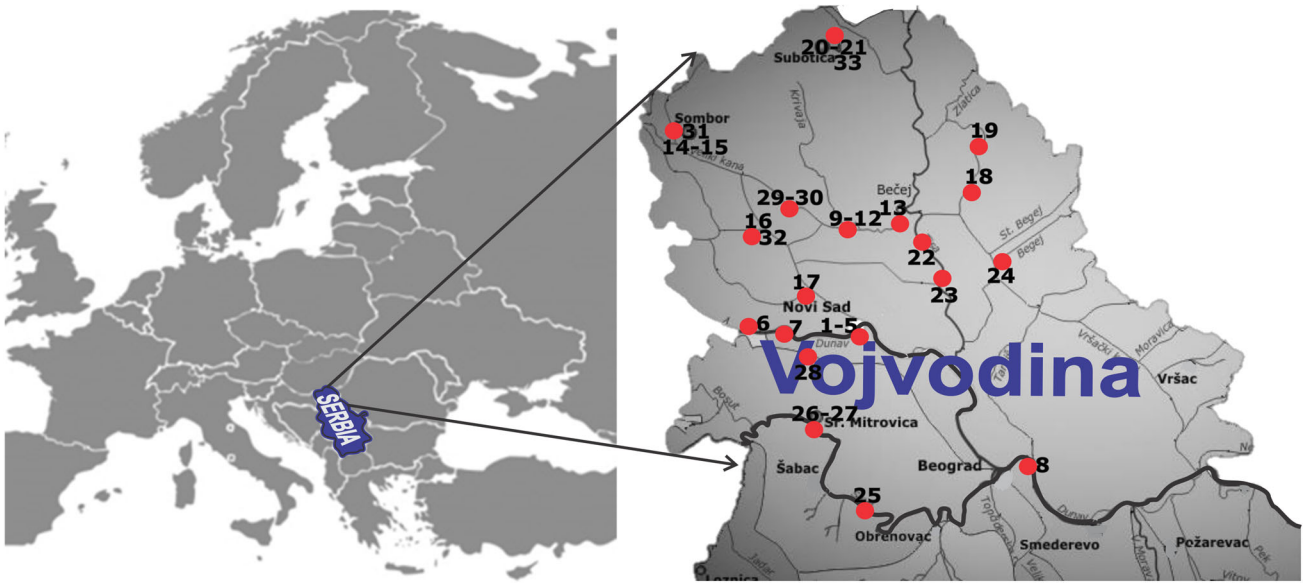

Fig. 1 Sampling locations of surface water and sewage samples (Vojvodina Province—Northern part of Serbia). 1–33 locations of tested samples (see Table 2)

In Odžaci, that does not have sewage TP, we collected untreated sewage samples before the release into the DTD canal. In collected samples, the presence and frequency of occurrence of selected human (HAdV, NoV GI, NoV GII, and HAV), animal (PAdV and BPyV) and zoonotic (HEV) viruses were tested. In order to control the whole process of virus detection in the water samples, the sample process control virus (SPCV) was added to the water samples before samples processing. The virus used as SPCV was murine norovirus 1 (MNV-1) (Diez-Valcarce et al. 2011a), which had been propagated in RAW264.7 cells to a concentration of 10^8 pfu/mL. MNV-1 stocks were kindly provided by the group of Dr. Franco Ruggeri at the Instituto Superiore di Sanità, Rome, Italy, by agreement with Washington University, USA).

Concentration of Water Samples by Glass Wool and Nucleic Acids Extraction

Ten litres of untreated surface water and 5 L of untreated urban sewage samples were collected and concentrated by an adsorption/elution technique with glass wool filtration followed by glycine beef extract buffer elution and centrifugation after low pH flocculation.

The method used was that described by Vilaginès et al. (1993), as modified by Wyn-Jones et al. (2011) and in details described by Kokkinos et al. (2012). Briefly, a glass wool filter was made by compressing 10 g glass wool (type 725; Rantigny, Saint-Gobain, France) into a 30 cm by 3 cm polystyrene column to obtain a filter height of 6–8 cm. The filter was washed by gravity with 50 mL volumes of (in order) 1 M HCl, tap water and 1 M NaOH, followed by tap water until the filtrate pH was neutral. The sample was then passed through the filter using a vacuum pump at a rate not exceeding 1 L/min. When sample had passed through the filter, viruses were eluted from the glass wool by gravity (20–30 min) passage of 200 mL 3 % (w/v) beef extract at pH 9.5 in 0.05 M glycine buffer through the filter. The eluate was flocculated by the addition of 1 M HCl until the pH reaches 3.5–3. The resultant protein floc was deposited by centrifugation at 7000×*g* for 30 min and dissolved in PBS to a final volume of 10 mL. The suspension was stored at −70 °C prior to nucleic acid analysis. Nucleic acids were isolated from 5 mL of water concentrate by NucliSENS miniMAG (bioMerieux) technology with 10 mL of lysis buffer and final repeated elutions resulting in total of 100 µL of nucleic acid extract. The procedure was performed according to the manufacturer's instructions. The samples of isolated nucleic acids were assayed immediately or stored at −70 °C.

Real-Time PCR (qPCR) and Reverse Transcription Real-Time PCR (RT-qPCR)

In this study, the presence of seven viruses in surface waters was tested. In addition, the presence of MNV-1 which is added as SPCV to each of the examined tested water sample before processing was determined, before target virus testing, to evaluate the reliability and success rate of used sample concentration and nucleic acids extraction procedures. The presence of DNA viruses HAdV, PAdV and BPyV were tested by real-time PCR (qPCR) and the presence of RNA viruses NoV GI and GII, HEV, HAV and MNV-1 (SPCV) were tested by reverse transcription

real-time PCR (RT-qPCR) assays. Primers and probes used in reactions are shown in Table 1. Amplification and fluorescence detection was performed on the 7500 Real-Time PCR System (Applied Biosystems) using 96-well PCR plates (Applied Biosystems). Each sample was tested in duplicate with 10 and 5 µL of ten times diluted nucleic acids extracts. Positive controls (DNA and RNA plasmid standards produced by ITACyL, Martinez-Martinez et al. 2011) and negative controls (NTC and negative concentration/extraction control) were included each time in the entire virus detection procedure to guarantee that the procedure was performed correctly. All the samples were first tested for the presence of SPCV and only the samples that tested positive for MNV-1, which guarantee that the entire virus detection procedure was performed correctly, were considered valid for further testing. To control inhibition of the qPCR and RT-qPCR, a target specific internal amplification control (IAC) was included in each reaction. All IACs and their probes were constructed as described by Diez-Valcarce et al. (2011b). The IAC amplicons were detected with the specific VIC-labelled IAC probe IACP (5′-VIC- CCA TAC ACA TAG GTC AGG –MGB-NFQ-3′). The amount of IAC added to the q(RT-)PCR reaction was optimised to determine the lowest consistent limit of detection, which was considered as the optimal working concentration and used in reactions. If inhibition of reaction is observed, one further dilution (10×) of nucleic acids extract was tested. Only the samples with positive signal of IAC were considered valid with the exception of samples that were positive for target virus when IAC could test negative.

Detection of DNA viruses (HAdV, PAdV and BPyV) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in total volume of 25 µL per reaction. The qPCR specific for adenoviruses contained 1× TaqMan Universal PCR Master Mix, 0.9 µM (final concentration) forward and reverse primers and 0.225 µM adenovirus specific probes, 50 nM internal amplification control (IAC) probe, approximately 100 copies of IAC DNA and 10 or 5 µL of template nucleic acid. For BPyV, 25 µL qPCR reaction contains 0.4 µm each primer, 0.12 µM bovine polyomavirus TaqMan probe, 50 nm IAC probe, approximately 300 copies of bovine polyomavirus IAC, and the same amount of template nucleic acids

Table 1 Primers and probes used in the study

Virus	Name	Primer sequence	Reference
HAdV	AdF	5′-CWTACATGCACATCKCSG G-3′	Hernroth et al. 2002
	AdR	5′-CRCGGGCRAAYTGCACCAG-3′	
	AdP1	5′-FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ-3′	
PAdV	PAdVF	5′-AACGGCCGCTACTGCAAG-3′	Hundesa et al. 2009
	PAdVR	5′-AGCAGCAGGCTCTTGAGG-3′	
	PAdVP	5′-FAM-CACATCCAGGTGCCGC-BHQ1-3′	
BPyV	QB-F1-1	5′-CTAGATCCTACCCTCAAGGGAAT-3′	Hundesa et al. 2010
	QB-R1-1	5′-TTACTTGGATCTGGACACCAAC-3′	
	QB-P1-2	5′-FAM-GACAAAGATGGTGTGTATCCTGTTGA-BHQ-3′	
NoV GI	QNIF4	5′-CGCTGGATGCGNTTCCAT-3′	da Silva et al. 2007
	NV1LCR	5′-CCTTAGACGCCATCATCATTTAC-3′	
	NVGG1p	5′-FAM-TGGACAGGAGAYCGCRATCT-BHQ1-3′	
NoV GII	QNIF2	5′-ATGTTCAGRTGGATGAGRTTCTCWGA -3′	Loisy et al. 2005
	CNG2R	5′-TCGACGCCATCTTCATTCACA-3′	
	QNIFS	5′-FAM-AGCACGTGGGAGGGCGATCG-BHQ1-3′	
HAV	HAV68	5′-TCACCGCCGTTTGCC-3′	Costafreda et al. 2006
	HAV240	5′-GGAGAGCCCTGGAAGAAAG-3′	
	HAV150	5′-FAM-CCTGAACCTGCAGGAATTAA-MGB-NFQ-3′	
HEV	HEV-F	5′-GGTGGTTTCTGGGGTGAC-3′	Jothikumar et al. 2006
	HEV-R	5′-AGGGGTTGGTTGGATGAA-3′	
	HEV-P	5′-FAM-TGATTCTCAGCCCTTCGC-BHQ1-3′	
mNoV	FwORF1/2	5′-CACGCCACCGATCTGTTCTG-3′	Baert et al. 2008
	RvORF1/2	5′-GCGCTGCGCCATCACTC-3′	
	ProbORF1/2	5′-FAM-CGCTTTGGAACAATG-MGB-NFQ-3′	

described for adenoviruses. The qPCR protocol, specific for HAdV and BPyV, was initiated by UNG inactivation at 50 °C for 2 min, followed by activation of Ampli*Taq* Gold polymerase (Applied Biosystems) at 95 °C for 10 min and 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The qPCR protocol specific for PAdV is different in annealing and extension conditions, which was 50 cycles of 95 °C for 15 s, 55 °C for 20 s and 60 °C for 40 s.

RNA viruses were detected using RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen) in total volume of 20 μL. The reaction mixture specific for NoV GI, NoV GII, HEV, HAV and MNV-1 (SPCV) contained 1× RNA Ultrasense reaction mix, virus specific primers and probes (0.5 μM forward, 0.9 μM reverse primer and 0.25 μM virus specific probes for NoV GI, NoV GII and HAV; 0.25 μM of both primers and 0.1 μM probe for HEV and 0.2 μM of both primers and probe for MNV-1), 50 nM IAC probe, approximately 300 copies of IAC RNA, 1× ROX reference dye, 1 μL of RNA Ultrasense enzyme mix and 10 or 5 μL of tested nucleic acids. All reactions were performed according to the protocol: reverse transcription at 50 °C for 15 min, preheating at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min, except for HEV where annealing was at 55 °C for 20 s with the addition of extension at 72 °C for 40 s.

Quantification of the positive reactions was performed by the most probable number approach as it was described by Kokkinos et al. (2012) and Maunula et al. (2013). The nucleic acid extract was tested in 10^{-1} dilution in 10 and 5 μL amounts, and two replicate assays were performed for each amount. If both 5 μL × 10^{-1} replicates produced a positive signal, subsequent dilutions were assayed until both replicates of a dilution were negative.

Results

In total, 60 samples of untreated surface water and six samples of untreated urban sewage samples were tested by qPCR and RT-qPCR. PCR inhibition was observed in 8 out of 66 tested samples (12.12 %), and nucleic acids extracts of those samples were further diluted 10× and retested. The most prevalent virus found was HAdV which was detected in 43.33 % (26/60) samples from 60 % (18/30) tested locations and in 66.67 % (8/12) examined surface waters (Danube, Sava, Begej and Krivaja rivers, and DTD, Great Backa, KCIII canals and Palic Lake). NoV GII was found in 40 % (24/60) samples from 56.57 % (16/30) locations and in 75 % (9/12) surface waters (Danube, Sava, Begej and Krivaja rivers, and DTD, Great Backa, KCIII canals, Palic Lake, and Rakovac stream). NoV GI was found in 10 % (6/60) samples from 16.17 % (5/30) locations and in 25 % (3/12) surface waters (Danube and Sava rivers, and Great Backa canal), and PAdV, BPyV and HEV were detected in 5 (8.33 %), 4 (6.67 %) and 2 (3.33 %) samples from 5 (16.67 %), 4 (13.33 %) and 2 (6.67 %) tested locations and in 4 (33.33 %; Krivaja, Sava and Begej rivers and DTD canal), 3 (25 %; Danube, Sava and Krivaja rivers) and 2 (16.67 %; Sava river and KCIII canal) examined surface waters. HAV was not found in any of analysed surface waters or urban sewage samples. Viruses were not detected in 25 % (3/12) of the examined surface waters (Tisa and Jegricka rivers, and the Special Nature Reserve Obedska bara—wetland) (Table 2).

Among six analysed sewage samples, 5 (83.33 %) were found positive for the target viruses. On both sampling occasions in two urban sewage systems HAdV and NoV GII and in one occasion NoV GI were detected, and in the third urban sewage system, only NoV GII was found in one of two sampling occasions (Tables 2, 3).

The simultaneous presence of three to four viruses was detected in samples of four tested surface waters (Danube and Sava rivers, Great Backa and KCIII canals) (Table 2). The Sava river was found to be the most contaminated surface water by viruses (presence of six out of seven tested viruses was found—all except HAV). In Danube and Krivaja rivers the presence of four (HAdV, NoV GII, BPyV and NoV GI or PAdV), and in Begej river and in all three examined canals the presence of three (HAdV, NoV GII and PAdV or NoV GI or HEV) out of seven tested viruses were found. The presence of two viruses (HAdV and NoV GII) was detected in Palic Lake and just one virus (NoV GII) was observed in Rakovac stream. Virus presence was not found in Tisa and Jegricka rivers and Obedska bara wetland (Table 3). The values of quantitative data for detected viruses presented as estimated number of PCR detectable units (PDU) for each of the analysed surface water and untreated urban sewage are shown in Table 3.

Discussion

The presence of human and animal viruses in faecal contaminated recreational waters is a great public health and economic problem (Bofill-Mas et al. 2010). The European Bathing Water Directive (2006/7/EC) came into force in March 2006 to protect the health of European bathers and this Directive does not include the analysis of viruses as one of the microbiological parameters listed (Bofill-Mas et al. 2010). In Serbia, drinking, surface, ground and recreational water sanitary condition is controlled mainly and more often only, on the basis of bacteriological indicators and on bacteriological quality.

In Serbia, untreated urban sewage and wastewater inflow directly into the different surface waters. The sewage

Table 2 Virus presence detected per each sampling locations in two sampling occasions

No.	Location	HAdV		NoV GII		NoV GI		PAdV		BPyV		HEV		HAV	
		I	II	I	II	I	II	I	II	I	II	I	II	I	II
1.	Danube river—GC-1 Novi Sad	+	+	+	+	+	−	−	−	−	−	−	−	−	−
2.	Danube river—GC-2 Novi Sad	+	+	+	+	+	+	−	−	−	−	−	−	−	−
3.	Danube river—Oficirac beach, Novi Sad	+	+	+	+	−	−	−	−	−	−	−	−	−	−
4.	Danube river—Štrand 1 beach, Novi Sad	−	+	−	+	−	−	−	−	−	+	−	−	−	−
5.	Danube river—Štrand 2 beach, Novi Sad	−	+	−	−	+	−	−	−	−	+	−	−	−	−
6.	Danube river—Plavna	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7.	Danube river—Karadjordjevo	−	+	−	−	−	−	−	−	−	−	−	−	−	−
8.	Danube river—Pancevo	+	−	+	+	−	−	−	−	−	−	−	−	−	−
9.	Krivaja river-Srbobran—near cattle farm	−	+	−	+	−	−	+	−	−	−	−	−	−	−
10.	Krivaja river-Srbobran—between 2 farms	−	+	−	+	−	−	−	−	−	−	−	−	−	−
11.	Krivaja river—Srbobran-Farm Matić	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12.	Krivaja river—Radičevic	−	−	−	−	−	−	−	−	−	+	−	−	−	−
13.	Krivaja river—Bečej	−	+	−	−	−	−	−	−	−	−	−	−	−	−
14.	Sombor—after TP in Mostonga DTD canal	+	+	+	−	−	−	−	−	−	−	−	−	−	−
15.	Sombor—DTD canal	−	−	−	−	−	−	−	−	−	−	−	−	−	−
16.	Odžaci—DTD canal	+	+	−	−	−	−	+	−	−	−	−	−	−	−
17.	Bački Petrovac—DTD canal	−	−	−	−	−	−	−	−	−	−	−	−	−	−
18.	Melenci-Bašaid—DTD canal	−	−	−	−	−	−	−	−	−	−	−	−	−	−
19.	Kikinda—DTD canal	−	−	+	−	−	−	+	−	−	−	−	−	−	−
20.	Palic Lake—after TP	+	+	+	+	−	−	−	−	−	−	−	−	−	−
21.	Palić Lake—beach	−	−	−	−	−	−	−	−	−	−	−	−	−	−
22.	Tisa—Čurug—beach	−	−	−	−	−	−	−	−	−	−	−	−	−	−
23.	Jegrička–Žabalj	−	−	−	−	−	−	−	−	−	−	−	−	−	−
24.	Begej—Zrenjanin	+	−	+	−	−	−	−	+	−	−	−	−	−	−
25.	Obedska bara—wetland—Obrež	−	−	−	−	−	−	−	−	−	−	−	−	−	−
26.	Sava river-Sr. Mitrovica-beach	+	+	+	−	+	−	−	+	+	−	−	−	−	−
27.	Sava river-Sr. Mitrovic	+	+	+	+	−	−	−	−	−	−	+	−	−	−
28.	Rakovac stream-Rakovac	−	−	+	−	−	−	−	−	−	−	−	−	−	−
29.	Great Bačka canal—Vrbas	+	−	+	+	+	−	−	−	−	−	−	−	−	−
30.	KCIII canal—Vrbas	+	−	+	+	−	−	−	−	−	−	+	−	−	−
31.	Urban sewage Sombor	−	−	+	−	−	−	−	−	−	−	−	−	−	−
32.	Urban sewage Odzaci	+	−	+	+	−	+	−	−	−	−	−	−	−	−
33.	Urban sewage Subotica	+	+	+	+	−	−	−	−	−	−	−	−	−	−

1–30, locations of surface water samples; 31–33, locations of sewage samples; I, first samplings (July–October); II, second samplings (November–December); +, positive samples; −, negative samples; TP, treatment plant

treatment plants usually do not exist, or there are only mechanical and partial-treatment sewage systems on place. This situation is common for small towns, and also for urban sewage from big towns and cities in Serbia. Only a few sewage treatment plants have been built in the last few years. Due to this situation, the main aim of the present study was to determine to which extent the surface waters in Serbia are contaminated with human and animal viruses. For this purpose, 30 sampling locations were chosen from most of the existing surface waters in Vojvodina Province. Vojvodina is situated in the northern part of Serbia (total surface area of 21.500 km^2; 25 % of total Serbia territory) and represents area with intensive agriculture and approximately 2 million inhabitants (26.88 % of Serbia's total population) (http://www.britannica.com/EBchecked/topic/631952/Vojvodina). The sampling locations for surface waters were chosen near all larger towns and as close as possible to a few intensive animal production farms, but also a few surface waters from protected natural areas were included. Water samples were also collected from five

Table 3 Viruses detected per examined surface water with estimated number of PCR detectable units (PDU) per litter

	HAdV Mean PDU (95 % CI)	NoV GII Mean PDU (95 % CI)	NoV GI Mean PDU (95 % CI)	PAdV Mean PDU (95 % CI)	BPyV Mean PDU (95 % CI)	HEV Mean PDU (95 % CI)	HAV Mean PDU (95 % CI)	No. of viruses detected
Danube river	48 (4–369)–1300 (172–4800)	132 (17–486)–960 (81–3500)	132 (17–486)	–	480 (40–960)	–	–	4/7
Sava river	48 (4–369)–264 (34–730)	96 (8–477)–264 (34–730)	96 (8–477)	264 (34–730)	96 (8–477)	96 (8–477)	–	6/7
Begej river	264 (34–730)	96 (8–477)	–	132 (17–486)	–	–	–	3/7
Krivaja river	132 (17–486)	96 (8–477)	–	132 (17–486)	132 (17–486)	–	–	4/7
Tisa river	–	–	–	–	–	–	–	0/7
Jegricka river	–	–	–	–	–	–	–	0/7
Rakovac stream	–	26 (2–161)	–	–	–	–	–	1/7
Palic Lake	1300 (172–4800)	96 (8–477)	–	–	–	–	–	2/7
Obedska bara-wetland	–	–	–	–	–	–	–	0/7
DTD canal	480 (40–960)	264 (34–730)	–	264 (34–730)	–	–	–	3/7
Great Bačka canal	480 (40–960)	960 (81–3.5 × 10^3)	96 (8–477)	–	–	–	–	3/7
KCIII canal	480 (40–960)	264 (34–730)	–	–	–	96 (8–477)	–	3/7
USW Subotica	1.3 × 10^3 (172–4.8 × 10^3)	96 (8–477)	–	–	–	–	–	2/7
USW Sombor	–	96 (8–477)	–	–	–	–	–	1/7
USW Odzaci	6.1 × 10^3 (406–70.9 × 10^3)	264 (34–730)–2.6 × 10^3 (344–14 × 10^3)	96 (8–477)	–	–	–	–	3/7

–, negative samples; USW, urban sewage

urban beaches (two beaches on Danube river, one beach on Tisa and Sava rivers, and Palic Lake) near towns Novi Sad, Čurug, Sremska Mitrovica and Subotica), to evaluate the potential risk for swimmers. In addition, in order to evaluate the level of virus contamination that originating from urban areas, untreated sewage samples from 3 urban sewage systems were tested.

For surface waters, the main source of contamination is sewage discharge from urban areas (Maalouf et al. 2010). In addition, rural run-off and run-off caused by heavy rains or melting snow can also contribute to contamination of surface water by viruses. Among all virus types, HAdVs are most commonly found in surface waters such as lakes and rivers, sewage systems, treated waters and swimming pools (Wyn-Jones et al. 2011; Silva et al. 2011). The most prevalent virus found in the present study was HAdV detected in 43.33 % (26/60) samples from 60 % (18/30) tested locations and in 66.67 % (8/12) examined surface waters. Danube, Sava, Krivaja and Begej rivers, and DTD, KCIII and Great Backa canals have a direct inflow of untreated sewage or leaking sewage and septic systems, urban run-off, agricultural run-off or run-off of animal manure used in agriculture. In addition, HAdV was detected in samples from two untreated urban sewage. Two out of three tested samples from urban sewage systems were positive for HAdV in the first samplings during summer (July–October), and 1/3 samples were positive for HAdV in the second sampling in autumn (November–December). HAdV has been proposed as an indicator of the presence of human viral pathogens in the environment and is used as virus marker of human faecal contamination because of their almost universal shedding and stability in the environment (Bofill-Mas et al. 2006; Silva et al. 2011). Results of our study confirm this observation and are in line with the results of many other studies conducted around the world (Rusiñol et al. 2014; Kern et al. 2013; Silva et al. 2011; van Heerden et al. 2005; Pina et al. 1998). We found almost the same prevalence of HAdV in tested surface waters in both sampling seasons. During our first sampling period (summer) from July to October, HAdV was detected in 40 % (12/30) samples, and during the second sampling time from November to December (autumn), HAdV was detected in 46.67 % (14/30) samples. Similar results were reported by testing of Wieprz river from Poland during 2007, where HAdV were detected in 28.3 % out of 60 examined water samples and were present throughout the whole year (Kozyra et al. 2011).

Low infectious dose, resistant to disinfection, multiple routes of transmission and strain diversity give reasons for the high prevalence and persistence of Noroviruses in the human population and in environmental samples. NoV has been often detected in raw urban sewage and surface waters in many studies around the world (Parasidis et al.

2013a; Lodder and de Roda Husman 2005). In the present study, we found NoV GII in 40 % and NoV GI in 10 % of tested surface water samples. Our results are in accordance with the results of the studies conducted in Hungary, where noroviruses were detected in 30 % examined surface water samples (Kern et al. 2013) and in the region of North-Eastern Greece where NoV GI strains were found in 17.3 % and NoV GII strains in 34 % samples (Parasidis et al. 2013b). During our first sampling period (summer) from July to October, NoV GII and NoV GI were detected in 43.33 % (13/30) and in 16.67 % (5/30) samples. On the second sampling occasion (autumn) from November to December, NoV GII and NoV GI were detected at similar 36.37 % (11/30) and much lower 3.33 % (1/30) prevalence than during summer time. Similar results were reported by Kozyra et al. (2011) in the study of Wieprz river during 2007, when NoVs were detected in 11.6 % out of the 60 examined samples, but unusually NoV was detected only during the summer period. These results are possibly the consequence of an occasional epidemic of NoV in human populations in Serbia and Poland during the summer time and just before the sampling was conducted. This observation is also confirmed with the results of untreated urban sewage testing. Samples from all (3/3) untreated urban sewage tested during summer were positive for NoV GII, and two out of three and one out of three samples from untreated urban sewage were tested positive for NoV GII and NoV GI in the second sampling period from November to December. Noroviruses are generally more prevalent in winter (Allen et al. 2013), but detection of noroviruses in water samples taken in warmer periods of the year is relevant when surface waters are used for recreational purposes (Lodder and de Roda Husman 2005). We found NoV GI in samples from 2 out of 5 examined beaches during the summer time (Danube river beach (Novi Sad) and Sava river beach (Sremska Mitrovica)). NoV GII was detected in the water samples on another Danube river beach (also in Novi Sad) and on the same beach on Sava river (Sremska Mitrovica). As very low number of NoV particles is enough for infection to occur, this result suggests that the risk for health exist on those beaches at the time when they were occupied with swimmers (approx. more than 15,000 per day in city Novi Sad). According to our results, NoV infection, mostly caused by NoV GII, existed in the human population in Vojvodina from July to December 2013.

Hepatitis A and E viruses are both transmitted via the faecal–oral route, most often through contaminated water and from person to person (Cook and Rzezutka 2006). The presence of HAV varies in different geographical areas, but in endemic areas, it is frequently detected in urban sewage throughout the year. HEV is more common in developing countries where sanitation is poor. Autochthonous strains of HEV have been reported in urban sewage in several highly developed countries, as well as related cases of sporadic acute hepatitis caused by these non-imported strains (Pina et al. 2000). In our study HEV is detected in 16.67 % of examined surface waters and just during the first (summer) sampling occasion. As HEV is highly prevalent in pig population in Serbia (Lupulović et al. 2010; Petrović et al. 2014), the source of the HEV detected in the samples could be infected humans and/or pigs. HAV is the primary agent for acute hepatitis with a distribution around the world. Hepatitis A viruses present in the stool of infected patients are discharged into sewage which ultimately may contaminate surface waters and seawater (Pinto et al. 2010). In developed countries, the incidence of hepatitis A illness is low; while in developing countries is significantly higher (Parasidis et al. 2013a). For example in the Klip river and Vaal Dam in Gauteng, South Africa HAV was detected in 18 (35.3 %) river and 19 (37.3 %) dam water samples (Taylor et al. 2001). In our study HAV was not detected in any of tested water samples. This result could be the consequence of the fact that the prevalence of HAV is low and it is not recognised as an endemic infection in Serbia. According to the "Health statistical yearbook of Republic of Serbia 2013", in total 243 HAV infection cases (3.38 cases per population of 100.000) was reported in Serbia in 2013, and from these number 94 cases (4.87 cases per population of 100.000) are reported on the territory of Vojvodina Province (http://www.batut.org.rs/download/publikacije/pub2013.pdf).

For source-tracking of animal faecal contamination in the environment, PAdV and BPyV were used as marker viruses. We found PAdV and BPyV presence in 8.33 % (5/60) and in 6.67 % (4/60) samples. Both viruses were detected in a similar lower level during both sampling occasions. During the summer sampling, PAdV and BPyV were detected in 3/30 (10 %) and 1/30 (3.33 %) samples, and during autumn in 2/30 (6.67 %) and 3/30 (10 %) samples. In our study, we found PAdV and BPyV presence in tested surface waters in Serbia in much lower extent than it is published in some other studies conducted in Hungary and Spain (Rusiñol et al. 2014; Hundesa et al. 2006, Hundesa et al. 2009). One of the possible reasons why we detected a very low prevalence of animal viruses in the analysed surface waters is because the animal production in Serbia has been quite low in recent years due to the economic crisis. A lot of big industrial pig and cattle farms in the past are now closed or raise a limited number of animals. Due to this situation, there is a very low production of animal manure and wastewaters from farms and consequently a low contamination level of animal viruses in the environment could be observed.

Conclusions

The results confirm the presence of pathogenic enteric viruses of both human and animal origin in surface waters in Serbia, indicating the existence of diverse contamination sources. Furthermore, the results show that the potential risk for public and animal health exists if the examined surface waters are used in agricultural and recreational purposes, and suggest the necessity for further and more extensive studies. These studies should include testing of human and animal pathogenic virus presence and prevalence, but also molecular typing of detected virus isolates as bases for molecular source-tracking. As far as we know, this is the first study on human and animal enteric virus presence in surface water environments in Serbia. Our results present the need for assessing water sources for viral contamination to help protect public health.

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