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Seasonal prevalence of potentially infectious enteric viruses in surface waters below treated wastewater discharge

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Abstract

Introduction and Objective. Enteric viruses are widely distributed in the natural water environment. The aim of the study was to assess the prevalence of potentially infectious adenoviruses (AdV) and rotaviruses (RoV) in surface water near treated wastewater discharge.

Materials and method. Water samples were collected from surface water below the treated wastewater effluent discharge located near a wastewater treatment plant receiving sewage from an urban area. Water samples were concentrated by ultrafiltration and treated with propidium monoazide dye, followed with v-qPCR/v-RT-qPCR analysis. Simultaneously, the temperature and pH of the collected samples were measured to check the influence of these parameters on the concentrations of potentially infectious viruses.

Results. The average concentrations of potentially infectious AdV and RoV particles in collected samples ranged between $\log_{10}$ 1.86 ÷ 3.94 gc/L and $\log_{10}$ 2.39 ÷ 3.82 gc/L in the winter season, and between $\log_{10}$ 2.18 ÷ 3.59 gc/L and $\log_{10}$ 1.85 ÷ 2.10 gc/L in the summer season, respectively. In general, AdVs were detected more often than RoVs, while RoV-positive samples were more frequent in the winter than in the summer season (Chi^2: $p = 0.028$; Fisher's Exact test $p = 0.033$). Negative correlations between log10 concentration of viral particles and temperature and pH for both viruses were observed.

Conclusions. The presence of potentially infectious AdVs and RoVs in the surface waters may constitute a health risk for the local population. Application of v-PCR-based methods and considering AdV as a viral contamination indicator should be introduced into virological water quality monitoring for estimations of public health risks.

Key words

enteric viruses, surface waters, viability PCR, wastewater discharge

INTRODUCTION

The term surface waters means any body of water above ground, including streams, rivers, lakes, reservoirs, and creeks. Most of the surface waters are considered suitable for recreational use and have areas dedicated for swimming, kayaking, scuba diving, wading and boating. These waters, after proper purification, are sometimes also a source of drinking water. Waterborne diseases, caused by enteric viruses, may result from the recreational use of contaminated water reservoirs, especially when they are near the discharge of a wastewater treatment plant [1]. Although sewage treatment processes should remove all pathogens, the technical difficulties associated with proper water sanitation for viral agents remain a significant problem [2]. Enteric viruses, which are responsible for many cases of non-bacterial gastroenteritis, respiratory infection, conjunctivitis and hepatitis, are known to be more resistant to common wastewater treatments than bacterial pathogens [3–8]. Moreover, unexpected failures in wastewater treatment plants and sewage systems often necessitate the emergency discharge of untreated wastewater directly to receiving water courses [9].

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Rotaviruses (RoVs) are the most common cause of acute gastroenteritis in children under two years of age, but infections and diseases also occur in older children and adults [5, 10]. These viruses are widely distributed in the natural environment and are excreted in large numbers in the faeces of infected individuals [11]. Rotaviruses have been isolated from various types of waterborne samples, i.e. from sewage [12], river [13], ground [14] and drinking waters [15]. In turn, adenoviruses (AdVs) have been reported to be the second most important viral pathogens of gastroenteritis after rotaviruses; however, depending on the species, they can be also responsible for different infections, including respiratory and ocular, as well as meningitis, encephalitis and hepatitis [16, 17]. AdVs have been found to be prevalent worldwide in rivers, coastal waters, swimming pool waters, and drinking water supplies [18, 19]. The characteristics of AdVs and RoVs, their transmission routes, seasonality and related disease are listed in Table 1.

Nowadays, molecular methods, such as polymerase chain reaction (PCR) and quantitative PCR (qPCR), are the 'gold standard' in the detection and identification of viruses. PCR-based methods, however, are not able to discriminate between capsid integrated, potentially infectious and damaged non-infectious viral particles [20]. Propidium monoazide (PMA) is a DNA/RNA intercalating dye with a photo-inducible azide group that binds and covalently cross-links to nucleic acids upon exposure to bright light [21]. As PMA crosses damaged

Table 1. Characteristics of adenoviruses and rotaviruses, their transmission routes, seasonality, and related diseases (after Rusiñol and Girones, 2017)

Genus (Family)	Genome	Size [nm]	Most important human pathogens	Related diseases	Transmission routes	Seasonality
Adenoviruses (*Adenoviridae*)	dsDNA	70–90	Human adenovirus A–G (HAdV)	Gastroenteritis, respiratory disease, conjunctivitis, cystitis	Faecal–oral: contaminated food, person-to-person, drinking water; airborne: respiratory secretions; bathing water	Without clear seasonality
Rotaviruses (*Reoviridae*)	dsRNA	70–75	Rotavirus A–G (RoV)	Gastroenteritis	Faecal–oral: contaminated food, person-to-person, drinking water	Winter peaks

dsDNA/RNA – double stranded DNA/RNA

membrane barriers only, the coupling of PMA with qPCR or RT-qPCR, also called viability-PCR (v-qPCR/v-RT-qPCR), is a promising solution to distinguish between potentially infectious and non-infectious viral particles [20].

Enteric viruses are resistant to disinfectants, heat, proteolytic enzymes and environmental pH changes between 3 and 10. Their capacity to survive for long periods and in various conditions contributes to their broad prevalence in the environment [22]. In general, their survival time in the environment is longer than that recorded for traditional bacterial indicators of faecal contamination [18]. Hence, the presence or absence of these bacteria does not indicate, in absolute terms, the quality of the water or the level of health risk for the organisms that use 'such water'. This also suggests that viral pathogens should be independently analyzed.

Considering the above, the purpose of this study was to detect and quantitatively assess the potentially infectious AdVs and RoVs in surface waters sampled below the treated wastewater discharge.

MATERIALS AND METHOD

Water samples. Thirty surface water samples (1 L each) for viral analysis were collected below the treated wastewater discharge before entering watercourse in the Mazovian Province and kept in 4 °C (not longer than 24h) until further analysis (see below). The samples were collected during winter (WS; a period of 6 months from October – March, when the average outdoor air temperature was below 10 °C for at least 7 consecutive days) and summer (SS; a period of 6 months from April – September, when the average outdoor air temperature was above 10 °C for at least 7 consecutive days).

Sample concentration method. Water samples were concentrated by ultrafiltration using an Amicon® Ultra-15 (molecular weight cut-off 30 kDa) centrifugal filter device (Merck Millipore Ltd., Livingston, UK) at 3,200 × g for 20 min in 4 °C. Centrifugal concentration step was repeated until the entire volume of the sample passed through the filter. The concentrated samples (200 µL) were intended for further analysis.

PMA dye pre-treatment. Concentrated samples were treated with PMAxx™ Dye (20 mM in H_2O; Biotium, Inc., Hayward, USA) for a final concentration of 60 µM. Tubes were gently mixed by inverting several times and then incubated in the dark for 15 min at room temperature, with rotation at 200 rpm. The treated samples were exposed to 40 W LED light with a wavelength of 460 nm for 15 min using a photo-activation system (PMA-Lite™ LED Photolysis Device; Biotum Inc., Fremont (CA) USA?).

Viral DNA/RNA extraction. The extraction of viral RNA from all samples was carried out with Kogene Power Prep Viral DNA/RNA Extraction Kit CE-IVD (Kogene Biotech, Seoul, South Korea) according to the manufacturer's instructions to produce a final volume of 30 µL. Obtained RNA samples were stored in –20 °C until further analysis.

Viability quantitative Reverse-Transcription quantitative PCR (v-RT-qPCR) assay. The v-RT-qPCR assays was performed using CFX96 real-time PCR thermocycler (Bio-Rad, Hercules (CA), USA). The detection of AdVs and RoVs was carried out with Adenovirus and Rotavirus VIASURE Real Time PCR Detection Kits (all: CerTest Biotec S.L., Zaragoza, Spain), respectively, according to procedures recommended by the manufacturer. The applied PCR kits have a detection limit of ≥ 10 RNA/DNA copies per reaction. The target genes employed for PCR-based detection and identification of viruses represent conserved regions with the hexon gene for AdVs and the NSP3 gene for RoVs.

The cycling conditions for AdVs were as follows: polymerase activation at 95 °C for 2 min, then 45 cycles of denaturation at 95 °C for 10 s, and annealing at 60 °C for 50 s. In the case of RoVs, the reverse transcription at 45 °C for 15 min was followed by initial denaturation at 95 °C for 2 min, then 45 cycles of denaturation at 95 °C for 10 s, and annealing at 60 °C for 50 s. In accordance with the manufacturer's procedure, the fluorogenic data were collected through the FAM, ROX and HEX channels. Both negative and positive controls (CerTest Biotec, Zaragoza, Spain), were included in each run. All samples were tested in duplicate.

All v-qPCR/v-RT-qPCR data were collected and quantification cycles (Cq) calculated using CFX96 manager software (Bio-Rad). According to the manufacturer's instructions, the samples with Cq ≤ 40 for AdVs and RoVs were considered as positive. The negative samples and the samples with Cq > 40 were re-analysed after 10-fold dilution to evaluate the possible presence of inhibitors. Quantification analyses were performed based on standard curves, obtained by amplification of positive control 10-fold dilutions (standard from 1×10^1 to 1×10^6 gene copies/reaction), and log RNA/DNA copies were plotted against Cq value. All standard curves had efficiencies between 90% and 110% and r^2 above 0.98.

To minimize potential contamination, all analytical steps were performed in separate rooms, including RNA/DNA isolation, preparation of reagents, sample preparation, and amplification. All analyses were carried out using the sterile RNase/DNase-free filter pipette tips only. The obtained results were expressed as the number of viral genome copies per 1 L of water (gc/L).

Temperature and pH of water samples. Temperature and pH value of the water samples were determined immediate after

samples collection with thermometer T-11 (Termoprodukt, Bielawa, Poland) and pH meter Five go F2 (Mettler Toledo, Greifensee, Switzerland) [24].

Statistical analysis. Statistical analyses were carried out with Mann Whitney U, Spearman correlation, Chi-squared, and Fisher Exact tests using STATISTICA, version 7.1 (StatSoft, USA). P values <0.05 were considered statistically significant.

RESULTS

The v-RT-qPCR-based studies revealed the presence of potentially infectious AdVs and RoVs in the examined water samples. In general, 73.3% of samples were AdV-positive and 50% of samples were RoV-postive, with average concentrations equal to $\log_{10}$ 2.57 gc/L and $\log_{10}$ 2.80 gc/L, respectively (Fig. 1). Taking into account the seasonality, in winter season 80.0% samples were AdV-positive and 73.3% samples were RoV-positive, while in the summer season AdVs and RoVs were detected in 66.7% and 26.7% of water samples, respectively (Fig. 2). In general, AdVs were detected more often than RoVs; this difference, however, was not statistically significant. On the other hand, when taking into account the seasonality, in the summer season AdVs were significantly more often detected than RoVs (Chi^2: p = 0.011; Fisher's Exact test: p = 0.013). In turn, RoV-positive samples showed higher prevalence in winter than in summer season (Chi^2: p = 0.028; Fisher's Exact test: p = 0.033).

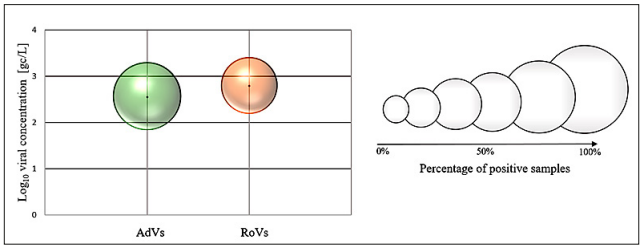

Figure 1. The average log10 concentration of potentially infectious AdV and RoV in positive water samples (the size of the bubbles indicate the percentage of positive samples within all tested samples); AdV, RoV

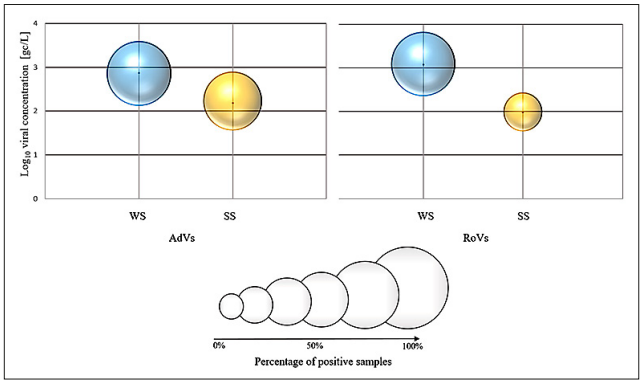

Figure 2. The average log10 concentration of potentially infectious viruses in water samples collected during water and summer season (the size of the bubbles indicate the pecentage of positive samples); WS – winter season, SS – summer season .

The concentrations of potentially infectious AdVs and RoVs in the winter season were $\log_{10}$ 2.86 gc/L (range: $\log_{10}$ 1.86 ÷ 3.94 gc/L) and $\log_{10}$ 3.09 gc/L (range: $\log_{10}$ 2.39 ÷ 3.82 gc/L), respectively (Tab. 2). In the summer season, the observed average concentrations were lower, i.e. $\log_{10}$ 2.23 gc/L (range: $\log_{10}$ 2.18 ÷ 3.59 gc/L) for AdVs and $\log_{10}$ 1.99 gc/L (range: $\log_{10}$ 1.85 ÷ 2.10 gc/L) for RoVs. However, statistically significant difference in concentrations between seasons was confirmed only in the case of AdVs (p = 0.038).

The average temperature of water samples collected in the winter season was equal – 6.8 °C (range: 1.5 ÷ 12.4 °C), while in summer season was equal to 17.2 °C (range: 12.3 ÷ 20.7 °C) (Tab. 2). In turn, pH values were observed at the level of 7.7 (range: 7.2 ÷ 8.2) in the winter and at the level of 8.4 (range: 8.0 ÷ 8.7) in the summer season. Statistical analysis showed a significant negative correlation between $\log_{10}$ concentration of viral particles and temperature, and between $\log_{10}$ concentration of viral particles and pH. In the case of AdV, these correlations were moderate (Spearman correlations: r = -0.616; p = 0.002 and r = -0.580; p = 0.005, respectively), whereas for RoVs, the observed correlations were high (Spearman correlations: r = -0.739; p = 0.002 and r = -0.724; p = 0.003) (Fig. 3). The relationships between concentrations of potentially infectious RoV particles and physical parameters (temperature, pH) of water samples are illustrated on Fig. 4.

DISCUSSION

The study revealed that both potentially infectious AdVs and RoVs were present in analysed surface water samples collected below treated wastewater discharge. According to available data, the presence of enteric viruses has been reported in various types of waters, including treated sewage, ground, marine, fresh (rivers, streams), recreational and drinking waters [4, 25, 26, 27]. The presence of human enteric viruses, including AdVs and RoVs, in post-treatment wastewater, has been reported in several studies [8, 28]. The main source of enteric viruses in surface water is faecal matter, excreted in large numbers with the faeces of infected individuals (up to 10^{11} viral particles per gram of stool), and introduced into the environment mainly through the discharge of treated and untreated sewage [29, 30]. The oral infectious dose of enteric viruses is very low, generally 1–10 viral units, and thus their presence in surface water, even in low concentrations, may represent a serious risk to human health [31]. Enteric viruses are mostly non-enveloped and, as such, in humid conditions demonstrate a high environmental persistence, being resistant to inactivation by temperature, pH changes and exposure to ultraviolet light. For these reasons, they show high biological stability in a water environment and can be transported over long distances, up to several kilometres, from their discharge site [32, 33].

The study revealed that the average concentrations of potentially infectious viral particles in collected surface water samples were equal to $\log_{10}$ 2.57 gc/L for AdVs and $\log_{10}$ 2.80 gc/L for RoVs. These levels are lower than those reported by other authors who found concentrations of AdVs and RoVs of $\log_{10}$ 4.55 gc/L and $\log_{10}$ 5.35 gc/L, respectively [30]. It should be mentioned here that most of available data regarding viral water contamination were obtained by the 'classic' PCR methods, which are not able to discriminate between potentially infectious and non-infectious viral particles. This may lead to an overestimation of the concentrations of these viruses [34] and thereby distort the real picture of

Table 2. Average log_{10} viral concentrations, temperature and pH in studied seasons

Viruses	Season					
	Winter			Summer		
	Log_{10} concentration [gc/L]	Temperature [°C]	pH	Log_{10} concentration [gc/L]	Temperature [°C]	pH
AdVs	2.86 (range: 1.86 ÷ 3.94)	6.8 (range: 1.5 ÷ 12.4)	7.7 (range: 7.2 ÷ 8.2)	2.23 (range: 2.18 ÷ 3.59)	17.2 (range: 12.3 ÷ 20.7)	8.4 (range: 8.0 ÷ 8.7)
RoVs	3.09 (range: 2.39 ÷ 3.82)			1.99 (range: 1.85 ÷ 2.10)		

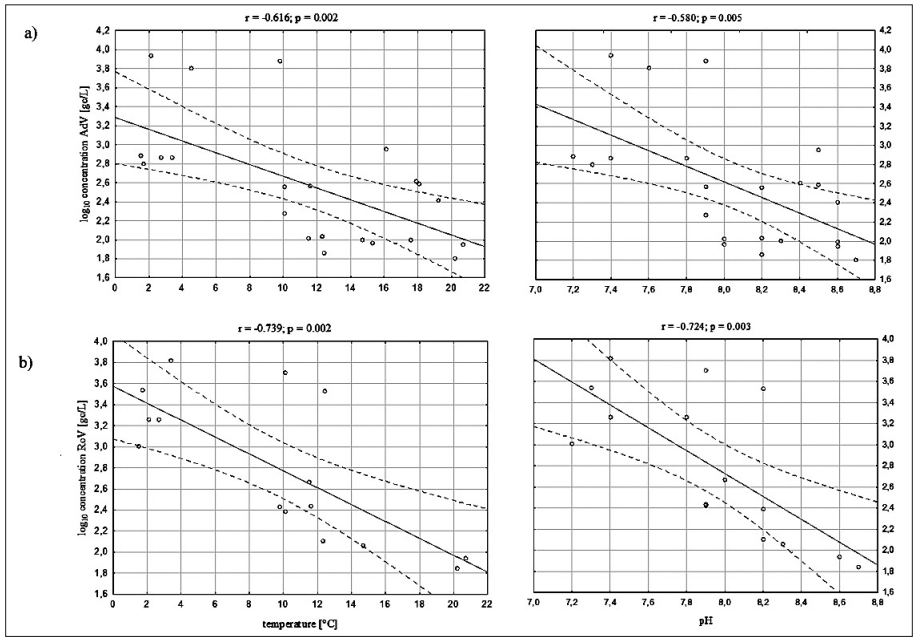

Figure 3. Correlation scatter plots of the log_{10} concentration of viral particles [gc/L], temperature and pH for AdVs (a) and RoVs (b)

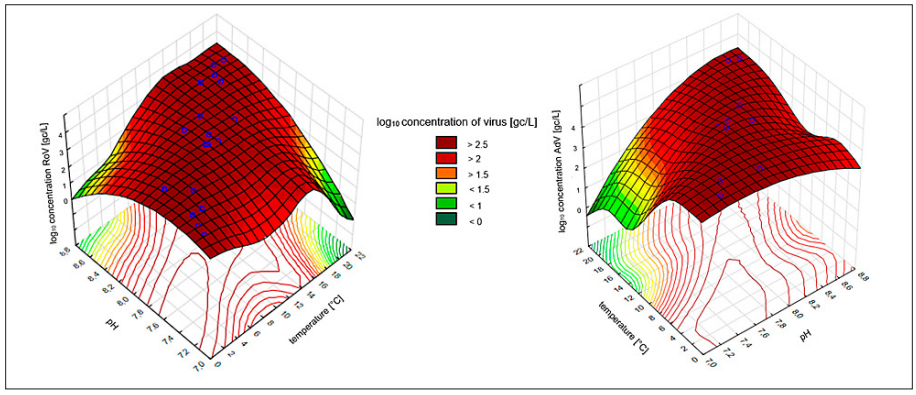

Figure 4. A 3D surface plots of relationships between concentration of potentially infectious RoV and AdV viral particles and physical parametres of water samples. The graph is a 3D projection of log10 concentration of viruses, temperature and pH of the samples. The blue dots represent values on which basis the surface were plottes

environmental contaminations. In this study, PMA dye pre-treatment was coupled with qPCR/RT-qPCR, and the application of v-qPCR/v-RT-qPCR eliminated the number of damaged viral particles from the results providing information only about potentially infectious intact viruses. This analytical approach is highly useful for estimating the public health risks posed by the presence of potentially infectious viruses in the environment [35, 36]. On the other hand, not all intact virions retain aninfectious capacity in the environment. Due to that fact, even the concentrations of potentially infectious viruses detected with v-qPCR/v-RT-qPCR may still be overestimated, and further *in vitro* investigations may be needed to confirm their real infectivity and subsequent actual impact on human's health.

This study has shown that RoVs were significantly more often detected in samples collected during the winter season which is consistent with the data gathered by Pang et al. [30]. Higher prevalence of RoVs in population, in treated sewage and in surface waters during cold season, was also found by Silva-Sales et al. [37]. RoVs stability and infectious ability decrease with an increase in temperature and UV radiation [36]. RoV decay rates positively associated with temperature were also confirmed by Kraay et al. [38]. Similarly, in the current study, significantly high negative correlations were

between observed concentrations of potentially infectious RoVs and the temperature of water samples (r = -0.739; p = 0.002). In the case of pH, rotaviral virions seems to be stable at any pH from 3 – 7 [39]. In the current study, pH of water samples ranged from 7.2 – 8.7, and statistical analysis showed significantly high negative correlations between the concentrations of potentially infectious RoVs and pH (r = -0.724; p = 0.003). In turn, for AdVs, the correlations between the concentration of viral particles and the values of the physical parameters were moderate, which is probably due to the high resistance of AdVs to these factors. According to Rexroad et al. [40], AdVs are resistant to a wide range of temperatures and pH, maintaining stability at 10 ÷ 85 °C and in the pH range 4–8.

In general, enteric viruses are considered to be more stable in the environment and more resistant to wastewater treatment methods, compared to enteric bacteria. Stable viral particles have been reported of up to 130 days in seawater, up to 120 days in freshwater and sewage, and up to 100 days in soil at 20 °C – 30 °C [41]. A previous study by the authors of the current study demonstrated that potentially infectious enteric viruses were present in the post-treatment wastewater effluents, which suggest that hitherto applied water purification technologies are, at present, insufficient for viral particles inactivation [42]. All the above-mentioned examples clearly show that the presence of pathogenic viruses should be monitored in water reservoirs into which sewage is discharged.

The contamination of surface waters with potentially infectious viruses creates a need for the development of a comprehensive approach to monitor such environmental health risks and to identify sewage sources, in order to facilitate both the remedial actions for surface water and assessment of its quality. In this context, some authors have proposed AdVs as a viral indicator of faecal water pollution due to their high prevalence in the population and high stability in the environment [43, 44]. The year-round frequency of AdVs in water samples suggests that these viruses can be considered an an indicator of contamination in monitoring the quality of surface water.

CONCLUSIONS

Both potentially infectious AdVs and RoVs were detected in surface water below the treated wastewater discharge. These viruses were more often detected in the winter season with average $\log_{10}$ concentration equal to 2.86 and 3.09 gc/L for AdVs and RoVs, respectively. It was observed that the concentrations of potentially infectious viruses were significantly negatively correlated with temperature and pH of water samples; however, in the case of AdVs, this correlation was moderate. AdVs were also detected in over 70% of samples, regardless of the season. This suggests that AdVs may be considered a viral indicator of faecal water pollution. Detection and quantification of potentially infectious enteric viruses in surface water based on v-PCR methods should be introduced as an inherent part of the standard monitoring of surface water quality, as their presence may pose a real health threat to the local population. The results of this study also confirmed the importance of year-round environmental surveillance, which can be a key tool for both pollution control and assessment of population exposure.

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