# LETTER TO THE EDITOR
# SHOULD ENTEROVIRUSES BE MONITORED IN NATURAL RECREATIONAL WATERS?

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**SUMMARY**

Enteroviruses (EVs) infections occur worldwide. Although, infections by these viruses are often asymptomatic and go unnoticed, they can be shed in stool for several weeks. The EVs are associated with sporadic outbreaks and a wide range of clinical symptoms, occasionally accompanied with fatal consequences. Presently in the Slovak Republic (SR) recreational waters are tested only for bacterial indicators. Our aim was to monitor EVs in recreational waters. Water samples were collected during the years 2012–2014 from different recreational natural lakes in Central and West regions of SR. The samples were concentrated by centrifugation using the two-phase separation method recommended by the World Health Organization (WHO) used for EVs surveillance in the treated sewage waste water. Each of the two phases collected from the samples was analysed by polymerase chain reaction for detection of EVs and primary sequencing was done. Our study demonstrated presence of EVs in three localities consecutively for three years, indicating a probability of constant local source of faecal contamination. This is the first monitoring report on the occurrence of EVs in the natural recreational waters in SR.

*Key words:* enteroviruses, natural recreational waters, monitoring

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## INTRODUCTION

Human and animal pathogens survive in the environment and may spread via infection of other hosts, making these pathogens important for public health. Contaminated drinking, recreational or surface waters which contain enteric viruses such as adenoviruses, rotaviruses, noroviruses, astroviruses, and picornaviruses remain a serious public health problem (1). Recreational-epidemiological studies show that diarrhoea and respiratory infections are commonly reported, and it is believed that these may be associated with a variety of enteric viruses of which some remain unidentified (2).

The proposal for a directive of the European Union Parliament and the Council concerning the quality of bathing waters (3) and World Health Organization (WHO) guidelines for safe recreational waters (4) include only bacterial indicators as microbiological quality parameters. However, the bacteria alone may not always be sufficient as indicator. The European directive 76/160/ECC contemplated on monitoring for viruses where an agreement was made considering the presence of a standard of 0 plaque forming units of enteroviruses (EVs) per 10 litres (5). Yet, the present European directive 2006/7/EC and the legislation of the Slovak Republic (SR) for testing of recreational waters does not include viral analysis in the quality parameters of the recreational waters (6).

Presently in the Slovak Republic the recreational waters are tested only for bacterial indicators. EVs are not included in this monitoring program. The EV-surveillance in SR includes sampling of waters from the sewage treatment plants as a part of the screening of acute flaccid paralysis (AFP) cases for poliovirus (PV), and a part of the PV-surveillance according to the new strategic plan of the Global Polio Eradication Initiative (GPEI) supplementing surveillance of AFP (7). The EVs cause a wide spectrum of diseases ranging from sub-clinical mild flu-like illness to serious manifestations such as meningo-encephalitis, paralysis, myocarditis, and in neonates a fulminant sepsis-like syndrome.

The recreational lakes are very often surrounded by summer cottages, which may lack a municipal sewer system, and the sewage dump is collected by the municipality on payment basis at defined time intervals. Long intervals between collections of the sewage dumps may allow the seeping of the ground waters

into the lakes. Furthermore, a high number of summer tourists and a high population density increase the chances and spread of infections. The deterioration in bathing water quality is also affected by gradual urbanization and tourism.

Aim of the present work was to monitor the presence of enteroviruses in recreational waters by polymerase chain reaction (PCR) analysis.

## MATERIALS AND METHODS

Bathing water quality and sanitary conditions of natural recreation sites (which include some of the natural lakes in SR) are monitored by the Public Health Authority of the Slovak Republic, Bratislava and 36 different Regional Authorities of Public Health in accordance with the European Parliament and the Council 2006/7/EC (6) and the applicable national legislation related to the management of the quality of bathing waters. The 'summer tourist season' lasts from 15th June to 15th September. Monitoring of the water quality in natural bathing waters (natural lakes) begins generally before the start of the season. The samples were collected for the present study accordingly.

For the monitoring of natural bathing waters in Western and Central regions of Slovakia in the years 2012–2014, samples were collected approximately during the period from 15th June to 15th September each year. The sampling size and frequency were as follows: 24 to 38 water samples were collected yearly, of which 22 to 31 water samples were from natural bathing sites. One water sample was collected from one permanent monitoring point per year. Only under rare conditions were 2 samples collected from the same point per year. One litre of each water sample was concentrated by centrifugation using the two-phase method of separation with 29% polyethylene-glycol 6000 (Serva) and 22% dextran (Serva) both prepared in 5M Sodium chloride, as recommended by the WHO (4) and previously reported by Klement et al. (8). EVs are present in the smaller bottom layer (bottom phase) and/or at the boundary between the layers (interphase) (4).

RNA for the reverse transcription PCR (RT-PCR) was isolated from each of the two separated phases. RNA was extracted from prepared suspensions by using Mammalian Total RNA Kit (Invitrogen). cDNA synthesis and amplification were performed by using a single tube method with the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen) and nested reaction with Platinum PCR SuperMix (Invitrogen). Primers and conditions used for identifying the enteroviral RNA have been described by de Leeuw et al. (9). PCR reactions were performed in C1000 Touch Thermal Cycler (BioRad). Nested-PCR products from the gels were purified using QIAquick Gel Extraction Kit (Qiagen). The primers used for the PCR analysis and sequencing are described in Table 1.

The sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Mastermix contained 1.5 µl sequencing buffer, 1 µl BigDye Terminator v3.1 Ready Reaction Mix, 0.5 µl of the primer p17.1 (1 pmol/ µl) and 6 µl distilled water. To this mixture 1 µl of purified DNA from agarose gel was added. The same mastermix was prepared with primer p17.2. Sequencing program consisted of 1st cycle at 105°C, 2nd cycle of initial denaturation at 96°C for 3 min., 25 cycles of denaturation at 96°C for 15 sec., annealing at 50°C for 7 sec. and extension at 60°C for 4 min., followed by 1 cycle of final hold at 4°C. The products were purified by precipitation solution which was prepared as 40 µl of mix contained 1.5 µl 3M sodium acetate (pH 4.6), 31.25 µl 96% ethyl alcohol and 7.25 µl $H_2O$. Sequencing denaturation reaction was performed with hot start as 1st cycle at 100°C, 2nd cycle of initial denaturation at 94°C for 5 min., and final extension was stopped by a final hold at 4°C. Analysis of the sequencing product was carried out as capillary electrophoresis in the Applied Biosystems 3130 Genetic Analyzer. Sequences were analyzed using software SeqMan, EditSeq, and MegAlign (DNA STAR).

Virus isolations were attempted on tissue cultures Hep-2 (Human epidermoid carcinoma cells) and Vero (African Green Monkey kidney cells) cell lines. Both cell lines were received from the Public Health Authority, Bratislava.

## RESULTS

As seen in Table 2 EVs were detected in both the 'interphase' and in the 'bottom phase'. The table shows detection of enteroviruses ranging from 12–22% in the interphase samples and from19–25% in the bottom phase samples. Our results show that three localities (Ivanka pri Dunaji, Zlate piesky and Ruzina-Ruzina) were consecutively positive in the studied years. In the primary sequence analysis using the non-coding 5' end nested-primers and VP 1 primers the positive samples were identified as coxsackievirus B4-Tuscany strain, accession number DQ480420.1. Attempts to isolate the viruses from the concentrated samples (collected phases) on cell lines Hep-2 and Vero failed.

***Table 1.*** *The primers for the polymerase chain reaction and sequencing*

| Primer | Sequence | Localization |
|---|---|---|
| p41–1 | 5' -CAAGCACTTCTGTTTCCCCGG - 3' | 165–185* |
| p41–2 | 5' -CACCGGATGGCCAATCCA - 3' | 625–642 |
| p17–1 | 5' -GCTAGAATTCCAGTCCTCCGGCCCCTGAATG - 3' | 433–462 |
| p17–2 | 5' -AACAATGGATCCATTGTCACCATAAGCAGCCA - 3' | 580–611 |
| ENTNES-F | 5' -GAYACWATGCARACVMGRCAYGT - 3' | 2595–2617** |
| ENTNES-R | 5' -GRGCAYTVCCYTCTGTCCA - 3' | 2994–2976 |
| ß1 | 5' -ATCATGTTTGAGACCTCCAA - 3' | 424–443 |
| ß2 | 5' -CATCTCTTGCTCGAAGTCCA - 3' | 723–742 |

*Positions refer to the CVB3 sequence of Lindberg et al. (10)
**Y is C or T; D is A, T or G; R is A or G; N is A, C, T or G; V is A, C or G; M is A or C; H is A, T or C; B is T, C or G; W is A or T

***Table 2.*** *Enteroviral RNA in recreational water samples from West and Central regions of the Slovak Republic*

| Region | Samples tested in 2012 | | Samples tested in 2013 | | Samples tested in 2014 | |
|---|---|---|---|---|---|---|
| | Interphase | Bottom phase | Interphase | Bottom phase | Interphase | Bottom phase |
| West | 2/13 | 2/13 | 2/10 | 2/10 | 2/14 | 2/14 |
| Central | 3/10 | 3/10 | 1/10 | 3/10 | 1/12 | 3/12 |
| Total | 5/23 | 5/23 | 3/20 | 5/20 | 3/26 | 5/26 |
| Percent positives | 22% | 22% | 15% | 25% | 12% | 19% |

## DISCUSSION AND CONCLUSION

In the period 1986–1996, 710 outbreaks of waterborne diseases were reported in nineteen European countries; 65 of these were of viral aetiology (1, 11). The largest enteroviral European outbreak occurred in Minsk, Belarus, in the year 2003. The sources of infection were mainly echovirus 30, 6, and coxsackievirus B5 (CVB5) contaminated waters (12). In recreational waters of Northern Ireland, EVs were present in 4/46 (8–7%) water samples during the year 1986 (13). In Slovakia, EVs in the Danube River waters were monitored by using tissue culture method (14). In the year 1981 this study pointed out the necessity of monitoring of viruses in natural waters.

In the eleven year sewage monitoring reported by Klement et al. (8), the most frequently identified non-polio viruses in Slovakia were the coxsackieviruses. Among the CVB were CVB5 (15%) and CVB2 (11%), to a lesser degree CVB3 (8%) and CVB4 (6%).

The present results show repeated detection of enteroviruses in three lakes. Our sequence analysis identified the viruses as coxsackievirus B4. Repeated finding of EV RNA in the localities Ivanka pri Dunaji, Zlate piesky and Ruzina-Ruzina lakes implicates presence of a constant source of contamination. These results and the recurrent presence of the same virus suggest a constant local source of contaminated faeces and circulation of this CVB4 strain in the population. We have focussed our study on West and Central regions of the Slovak Republic as systematic sample collection was not done in East Slovakia lakes.

Failure to isolate EVs on tissue cultures Hep-2 and Vero cell lines could be related to a low concentration of viruses or the small range of tissue culture cell lines used by us. In the period from 2012 to 2014, the recreational waters from the site Ivanka pri Dunaji, where enteroviruses were monitored, the concentrations of *E. coli* ranged from 0–72 colony forming units (CFU)/100 ml and intestinal enterococci at a concentration of 4–150 CFU/100 ml. Furthermore, *E. coli* were in the range of 8–10 CFU/100 ml and intestinal enterococci from 15–45 CFU/100 ml in the locality Zlate piesky.

In the recreational waters from the site Ruzina-Ruzina – *E. coli* ranged from a concentration of 4–74 CFU/100 ml and the intestinal enterococci from 37–80 CFU/100 ml. These results show low levels of *E. coli* and intestinal enterococci as an indicator of faecal contamination. According to the Decree of the Ministry of Health of the Slovak Republic No. 309/2012 Coll. on Requirements for recreational bathing water as amended by the Decree of the Ministry of Health of the Slovak Republic No. 397/2013 Coll., they can be referred to as "excellent water quality" within the limit value for the indicators: *E. coli* to 500 CFU/100 ml and enterococci to 200 CFU/100 ml (15). Although the analyzed samples of natural bathing waters from a microbiological point of view were classified as 'satisfactory' in the localities of Ivanka pri Dunaji, Zlate piesky, and Ruzina-Ruzina, presence of enteroviruses in the period 2012–2014 was repeatedly demonstrated by us.

We point out that the EVs studies were only qualitative (presence/absence) and we do not know if the contamination can be classified as serious or only mild. Among the illnesses and their aetiological agents which are suggested to be significant risk factors by the WHO report (16) enteroviruses, namely the coxsackieviruses and echoviruses, are listed. The review also brings attention to the fact that recreational activities involving contact with water have increased and easy means of travel has altered the public use of water for recreational purposes, increasing the potential risk factors.

In conclusion we suggest that detection of enteroviral RNA by PCR analysis, supported by the tissue culture method and/or sequencing/molecular typing will not only substantiate the faecal-pollution in recreational waters along with bacterial indicators but will be useful information for the public health authorities as well. EVs could be valuable as a viral-faecal contamination indicator. The route of infection of these viruses is the faecal-oral route, and EVs are shed in the stool over a long period after the infection. Presence of viral RNA may not always indicate the viability of the virus, but the detection of viruses by this method is relatively fast, sensitive, and devoid of false positives or false negatives when good laboratory practice is followed and the necessary controls are included.

The results of monitoring and state regulation of bathing waters are the basis for preparation of legislation and addressing specific situations in practice. The present study is the first monitoring report on EVs in the recreational waters of the Slovak Republic using the PCR analysis. Within the public health sector in SR currently EVs are being monitored only in treated sewage water surveillance included by the WHO in the new strategic plan of GPEI. We suggest that the screening of EVs in the recreational waters should be included in the monitoring program of recreational water quality.

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**Conflict of Interests**

None declared

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