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. The European directive 2006/7/EC 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. 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) by using a single tube method with the SuperScript III One-Step 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 H2O. 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 from 19–25% in the bottom phase samples. Our results show that three localities (Ivanka pri Dunaji, Zlate piesky and Ruzina-Ruzina) and 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.

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>p41–1</td>
<td>5'-CAAGCACTTCTGTGTTCCCCGG - 3'</td>
<td>165–185*</td>
</tr>
<tr>
<td>p41–2</td>
<td>5'-CACCAGATGGCCACATCCA - 3'</td>
<td>625–642</td>
</tr>
<tr>
<td>p17–1</td>
<td>5'-GCTAGAATTCCAGTCCTCCGGCCCCTGAATG - 3'</td>
<td>433–462</td>
</tr>
<tr>
<td>p17–2</td>
<td>5'-AACAATGGATCCATTGTCACCATAAGCAGCCA - 3'</td>
<td>580–611</td>
</tr>
<tr>
<td>ENTNES-F</td>
<td>5'-GAYACWATGCARACVMGRCAYGT - 3'</td>
<td>2595–2617**</td>
</tr>
<tr>
<td>ENTNES-R</td>
<td>5'-GRGCAVTWCYCYCTGTCACA - 3'</td>
<td>2994–2976</td>
</tr>
<tr>
<td>β1</td>
<td>5'-ATCATGGTGGACCTTCACAA - 3'</td>
<td>424–443</td>
</tr>
<tr>
<td>β2</td>
<td>5'-CATCTGGTCGAAATCCAA - 3'</td>
<td>723–742</td>
</tr>
</tbody>
</table>

*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
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., the limits of enterococci within the limit value for the indicators: E. coli to 500 CFU/100 ml and enterococci to 200 CFU/100 ml (15). Although

<table>
<thead>
<tr>
<th>Region</th>
<th>Samples tested in 2012</th>
<th>Samples tested in 2013</th>
<th>Samples tested in 2014</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Interphase</td>
<td>Bottom phase</td>
<td>Interphase</td>
</tr>
<tr>
<td>West</td>
<td>2/13</td>
<td>2/13</td>
<td>2/10</td>
</tr>
<tr>
<td>Central</td>
<td>3/10</td>
<td>3/10</td>
<td>1/10</td>
</tr>
<tr>
<td>Percent positives</td>
<td>22%</td>
<td>22%</td>
<td>15%</td>
</tr>
</tbody>
</table>

Acknowledgements

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Conflict of Interests

None declared
REFERENCES


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