PRESENCE OF HUMAN PATHOGEN VIRUSES IN HUNGARIAN SURFACE AND RECREATIONAL WATERS

PhD thesis

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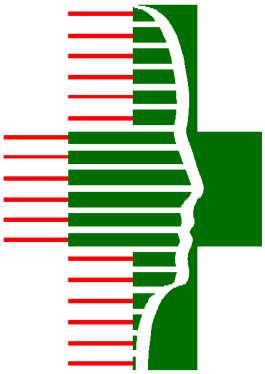

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I. Introduction

Viruses persist widely in the environment, thus they may be present in different water bodies. Raw sewage is the most contaminated, it contains enteric viruses in high concentrations. Enteric viruses are usually transmitted via the fecal-oral route and primarily infect and replicate in the gastrointestinal tract of the host. Although a significant part of the viruses may be eliminated during waste water treatment, a small proportion of viruses may get into the treated waste water owing to the small size and high resistance of viruses. A significant risk should be taken into account if the treated waste water is shed into surface water which are used as drinking water or for recreational use. The contamination of recreational waters may originate from the bathers, which directly increases the risk of viral infection.

The contamination of surface waters with communal and agricultural waste water is a worldwide public health concern. In developing countries the enteric viral epidemics occur frequently due to the lack of water treatment or insufficient water management. Children under the age of 5, elderly population and immunosuppressive persons are the most susceptible for enteric viral infections. This problem also shows up in developed countries, but only occasionally, mainly in connection with extreme weather events. Extreme weather events may occur more frequently in the future owing to the climate change, which supports the spread of waterborne viruses. The more frequent heavy rainfalls, the increasing average temperature and the frequent worldwide floods promote the spread and survival of pathogen microorganisms.

Water has been identified more and more often as the source of viral epidemics in Hungary for the last few decades. The spread of pathogens have been identified indirectly – except for the drinking water epidemics in Miskolc in June 2006 –, drinking or recreational water has been proven to be the medium which spreads the contamination on the basis of human samples, fecal indicator bacteria detected from water samples and epidemiological data. In the present study methods for virus detection have been implemented, which render the detection of the potential pathogen viruses from large amounts – 10 liters – of water samples possible. The use of virus concentration and virus detection techniques the analyses of water as the potential medium which spreads the infection may be a part of routine virus diagnostics in Hungary, as well.

The detection of viruses from water presents a great challenge for several reasons due to the high variety and the complexity of the different water types and the low titer of viruses in

water. There are no standard procedures either for the whole virus concentration and detection process, or for each step of the process. Contrary to the clinical samples, the virus titers are low in water samples, hence, the first step is to concentrate the viruses from the water. Concentration methods can be divided into four main categories: adsorption-elution, ultrafiltration, ultracentrifugation and direct flocculation. Two adsorption-elution techniques and one direct flocculation concentration method were compared. Several methods are available for the detection of viruses from concentrated samples: they range from the direct electronmicroscopic observation through the detection of cytopathogen effects on different cell lines to the indirect diagnosis with immunological or molecular techniques. In the present study molecular methods (PCR and qPCR) were applied after nucleic acid isolation and purification for the detection and quantification of viruses in raw sewages and secondary treated waste waters, natural and artificial bathing waters and other surface waters. In addition, the effects of different physico-chemical, hydrological and meteorological parameters were studied on the fate, survival and transport of viruses in aquatic environments during a one and a half year sampling period within the confines of Viroclime project. In the course of the project the relationship of pathogen viruses and indicator bacteria was examined.

II. Objectives

- ✓ Implementation and comparison of virus concentration methods, virus nucleic acid isolation and purification techniques and molecular virus detection methods.
- ✓ Detection and quantification of enteric viruses from different aquatic environments in Hungary.
- ✓ Examination of the effect of physico-chemical, hydrological and meteorological parameters on fate, survival and transport of viruses in aquatic environments.
- ✓ Evaluation of the efficacy of secondary (biological) waste water treatment on the removal of pathogen viruses and indicator bacteria.
- ✓ Analysis of the relationship between human enteric viruses, animal enteric viruses and indicator bacteria in surface waters and waste waters.
- ✓ Assessment of the source of contamination with microbial source tracking.

III. Test methods

Ten liters of surface and recreational water samples and 100 - 1000 mL waste water samples were collected for detection of human and animal pathogen viruses. For indicator bacteria count analysis 250 mL water was sampled. The tested samples originated from 41 different sites: 13 artificial bathing waters, 6 natural bathing waters, 2 raw sewages, 1 secondary treated waste water and 19 other surface water sampling sites.

The water samples were concentrated with two adsorption-elution methods on two different adsorbents: membrane filter and glasswool. The water pH was adjusted to 3.5 and then the water was filtered through the membrane filter or the glasswool. The virus particles were eluted with 3 % pH 9.5 glycine beef extract solution and flocculated with 1 N HCl. The flocculated virus particles were sedimented with centrifuging. The supernatant was discarded and the virus particles were resuspended in pH 7.2 phosphate buffer to a final volume of 10 mL. In case of direct flocculation concentration the conductivity of the surface water sample was adjusted above 1600 µS, while the 50 mL of waste water samples were mixed with glycine buffer 0.25 N, pH 9.5 (1:2 v/v) to elute viruses form organic matter. The samples were centrifuged after 30 minutes shaking on ice. The resulting supernatant of waste water and the surface water samples were acidified, the pre-flocculated skimmed milk was added (1:100 v/v) and were stirred for a minimum of 8 hours to allow the viruses present to adsorb to the flocculates. The samples were allowed to sediment for a minimum of 8 hours. The waste water samples were then centrifuged and the pellet re-suspended in phosphate buffer to a 2 mL final volume. The supernatant of the surface waters were discarded leaving a maximum of 500 mL sediment. The sediment was centrifuged, the supernatant was discarded and the pellet was re-suspended in pH 7.2 phosphate buffer to a 10 mL final volume.

Magnetic silica bead – NucliSens® miniMAG^{TM} – nucleic acid isolation and purification method was applied during the initial tests, which render the handling of a larger volume of virus concentrate possible. After the comparison of the two nucleic acid isolation and purification techniques, we switched to a silica spin column method – QIAGEN QIAamp Viral RNA Mini kit – which has a higher sensitivity and results in a purer nucleic acid solution. One hundred µL purified nucleic acid solution was isolated from 5 mL of virus concentrate using the magnetic silica bead method. The silica spin column method required 140 µL virus concentrate and resulted in 80 µL purified nucleic acid solution.

Human adenovirus, enterovirus, norovirus genogroup I and II, JC polyomavirus, porcine adenovirus and bovine polyomavirus genome were detected with polymerase chain reaction

(PCR) using virus specific primers. The verification of the presence of human adenovirus, porcine adenovirus and bovine polyomavirus was performed with nested PCR. The enterovirus RNA was detected with reverse transcription nested PCR, the norovirus genogroup I and II RNA was detected with reverse transcription seminested PCR. The quantification of human and porcine adenovirus, JC and bovine polyomavirus, norovirus genogroup I and II was performed with quantitative PCR (qPCR). For qPCR the determined region of the target genes were cloned into plasmids transformed into competent cells. The target sequences were obtained after plasmid linearization and digestion with restriction endonucleases. For each virus a stock solution was made according to the size of the insert. Ten µL of the stock solution contained 10^6 genome copies (GC) of the target sequence. Dilutions from 10^0 to 10^6 /10µl were used for standard curves of the qPCRs and for absolute quantification.

Besides the virus detection, the bacterial counts (*Escherichia coli*, intestinal *Enterococcus*, in case of artificial bathing waters *Staphylococcus aureus*, *Pseudomonas aeruginosa*, total cocci, too) were also determined in waters. The microtiter method was applied in case of surface waters and waste waters, and membrane filtration in case of artificial bathing waters. The somatic coliphage titer was determined using the standardized pour plate method.

IV. New scientific results

1. The adsorption-elution virus concentration technique was tested on two – negatively charged membrane filter and glasswool – adsorbents with 37 paired surface water samples. In case of human adenovirus and enterovirus more positive samples resulted after glasswool filtration, whilst 15-15 samples were positive for norovirus genogroup II with both adsorbents. The detected human adenovirus copy numbers were higher after glasswool concentration. The comparison of glasswool filtration and direct flocculation concentration methods in case of surface waters and waste waters did not show significant differences either with respect to virus recovery, or with respect to virus detection. Since the efficacy of both virus concentration methods is rather similar, and the direct flocculation method is more simple, cost and time effective, more samples can be handled at the same time, and the chance for cross-contamination is lower, too. After performing the comparison of the virus concentration methods, the samples were concentrated only with direct flocculation.

2. The detection and recovery of human adenovirus nucleic acid were more efficient and reliable from the concentrate of large volume surface samples and lower volume waste water using the silica spin column technique compared to the magnetic silica bead method. In addition, given quantities of human adenovirus were added to the water samples and these titers were closer to the initial stock solution titer determined with qPCR after silica spin column nucleic acid isolation and purification.
3. More than the two thirds of the samples (53/74) showed human adenovirus positivity using nested PCR during the method implementation period. Norovirus genogroup II RNA was detected from 31 samples, enterovirus RNA was detected from 13 samples. Fifty-six samples contained at least one of the tested viruses. The tested viruses were generally present in other surface waters. The potential pathogen virus genomes were detected on a large scale both in natural and artificial bathing waters. At least one of the tested virus nucleic acids was detected at each natural bathing water sampling point and from each tested pool water. In contrast, the microbial parameters applied for water qualification showed good quality in more cases.
4. All 129 surface water samples were tested for viruses and indicator bacteria during the systematic analysis. Almost every water sample (127) contained human adenovirus. The other tested viruses were detected from a large number of the tested water samples: porcine adenovirus from 106, bovine polyomavirus from 101, JC polyomavirus from 83 samples. Less than half of the samples showed norovirus genogroup I positivity (51 samples) and norovirus genogroup II positivity (52 samples). With respect to the virus concentration the highest genome copy numbers were detected in case of DNA viruses. On average 10^3-10^4 GC/L DNA virus copy numbers were detected, whilst RNA titer varied between 10^2-10^3 GC/L. There was no significant difference between the virus titers detected on the tested four sampling sites.
5. The effects of the physico-chemical, hydrological and meteorological parameters on viruses were also tested as a part of the project. The highest (negative) correlation was observed between every described hydrological variable and animal virus titers. The difference between average DNA virus titers detected at low and high flow rates reached two magnitudes, which difference was also observed in case of river discharge. Seasonality was observed in case of human and animal viruses, as well. Human adenovirus was continuously present in river Tisza water in the tested region. Spectacular seasonality was shown in case of JC polyomavirus: the difference between the spring lowest and the autumn highest titers reached two logs. Norovirus genogroup I titers were the highest in

winter and spring 2011, but this peak did not occur again during samplings in 2012. However, the winter-spring peak of norovirus genogroup II titers was detected in 2011 and 2012, too. The most conspicuous differences were observed in case of animal viruses. The minimum concentrations were showed in the winter and early spring months in both years. Two maximum peaks were observed in case of both animal viruses: in June and in autumn months. Calculations confirm that the animal virus concentrations are higher in warm seasons, and the JC polyomavirus titer is lower in winter months. The amount of the precipitation 24, 48 and 72 hours before the sampling had no effect on the microorganism concentrations, if all surface samples were handled together. However, if the sampling points are analyzed separately, the animal virus concentrations were one log higher in case of samples taken after a 24-hour precipitation than samples taken in a dry period at Tisza4 sampling point. A negative trend was observed in case of the amount of the solar radiation 24, 48 and 72 hours before the sampling and the JC polyomavirus titers if the surface samples were handled together and separately, too. The pH of the water samples did not have a significant effect on virus titers. The water samples with higher conductivity contained higher DNA virus titers in case of every tested DNA viruses. Lower DNA virus titers were detected in case of high suspended material concentration. It was observed in more cases during comparison of the environmental parameters and the microbial results that the animal viruses generally behaved similarly in given environmental conditions, which was also confirmed with calculations. On the other hand, the indicator bacteria typically showed opposite trends.

6. During the 18-month monitoring period altogether 33 raw sewage samples and 32 secondary treated effluent samples were tested from a waste water treatment plant, from which the effluent is discharged into river Tisza. All of the tested raw sewage samples contained human and porcine adenovirus, JC polyomavirus and norovirus genogroup II. Norovirus genogroup I was detected from 27, bovine polyomavirus from 29 raw sewage samples. The ratio of the virus positive samples decreased after mechanical and activated sludge treatment. From the 32 effluent samples 29 showed porcine adenovirus positivity, 27-27 were above the detection limit for JC and bovine polyomavirus, 25 samples showed norovirus genogroup II positivity, and almost the half of the effluent samples (18/32) were positive for norovirus genogroup I. On the other hand, human adenovirus presence was observed in every tested effluent samples. The average virus titer of the activated sludge treated effluents varied between 10^4-10^6 GC/L, this is the virus burden for the recipient water body. The average virus titer was higher in case of DNA viruses (10^5-10^6 GC/L), the

RNA virus concentration ranged from 10^4 to 10^5 GC/L. The genome copy numbers of the enteric viruses decreased 0.5-1.5 log in average during the primary and secondary waste water treatment. RNA viruses were eliminated with the highest efficiency, whereas the animal (DNA) viruses gave evidence of the highest resistance to secondary waste water treatment. The elimination of indicator bacteria was more effective than the virus elimination.

7. According to the composition of the potential contamination sources along river Tisza between Szolnok and Tiszakécske, human and animal (porcine and bovine) source of contamination was confirmed. For the sake of the determination of reason for the high virus concentrations detected in river Tisza further points were sampled and tested for presence of enteric viruses. The concentration of the two human viruses varied the same way during the additional sampling; human viruses were present in every sample. One of the canals did not contain porcine adenovirus (above detection limit) during the two sampling, whilst bovine polyomavirus was detected from both samples. No significant differences could be observed in the ratios of human and animal virus content of river Tisza and of the tested influents of river Tisza.

V. Conclusions

The first part of the thesis is about the implementation and comparison of virus detection methods: concentration and molecular detection techniques. In case of surface waters and artificial bathing waters, glasswool binds viruses more effectively than the membrane filter adsorbent. The direct flocculation method is more time and cost effective than the adsorption-elution method containing two concentration steps. Our studies confirmed that the direct flocculation method is easier, consequently, the use of this method is suggested for detection of human adenoviruses from surface and waste waters. Furthermore, the human adenovirus recovery is more reliable. According to the comparison of the magnetic silica bead and the silica spin column nucleic acid isolation and purification methods, it can be concluded that the PCR inhibitors are removed more effectively by the silica spin column method. The removal of the inhibitors has no impact on the nucleic acid concentration and the pureness of the nucleic acid. It was confirmed in the course of a ring trial with participation of five laboratories that the applied virus specific qPCRs are sensitive, reproducible and repeatable.

During waste water treatment, the amount of viruses decreases but cannot be eliminated totally with the applied treatment method. The treated effluent is discharged into surface

water, which explains the general human adenovirus presence in other surface waters and natural bathing waters and the high ratio of RNA virus samples. The highest health risk belongs to those surface waters, which are used for recreational activities or as drinking water source. Viruses can be shed into the waters by the infected bathers, thereby artificial bathing waters may be contaminated, as well. Pool waters with water circulation are continuously treated with disinfectants, however, approximately the same proportion of circulated pool water contained pathogen virus nucleic acid as non-treated pools. This may be explained with the inadequate water treatment. Each pool waters contained at least one of the tested virus genomes, although some of them had good quality according to the bacteriological parameter test results. This confirms the previous observation that the indicator bacteria do not predict the presence of human pathogen viruses appropriately. These results imply that the infections in connection with recreational activities may be more frequent, but the connection may not be recognized in every case.

Human and animal enteric viruses persisted not only in raw sewage, but in secondary (biologically) treated effluent and in surface water in river Tisza between Szolnok and Tiszakécske during the test period. There was no significant difference between the detected virus titers on the four sampling sites. The change of hydrological characteristics and virus titers showed reverse trend. Some of the tested viruses showed seasonal periodicity, while others were present continuously. The virus elimination effect of the UV radiation of the sun was observed to a small extent in case of JC polyomavirus. There was a small amount of precipitation during the test period, and it was found that precipitation had no effect on the distribution of viruses. Virus titers changed in parallel with conductivity of the water samples of river Tisza The water turbidity had a negative effect on the virus titer as well as on the virus detection. The two tested animal viruses responded in the same way on the change of meteorological, hydrological and physico-chemical characteristics of river Tisza.

The regional epidemiological data showed permanent presence of enteric viruses in the human population, thus, the high load of, human adenovirus, JC polyomavirus and norovirus of waste waters and surface waters is not surprising. The virus titers were more balanced in raw sewage, but there was a slight seasonality in the titer variability and in the efficacy of waste water treatment. The primary and the secondary (biological) waste water treatment decreased the virus titers in the effluent generally with 1-2 logs, maximally with 3 logs. RNA viruses were removed with higher efficiency than the DNA viruses. The viral load was 10^4-10^5 GC/L in the effluent discharged into the streamline of river Tisza, which was diluted in river Tisza water body.

Animal viruses were detected both in surface waters and waste waters. Human and animal viruses were equally present in the influent of river Tisza and in canals running into river Tisza. This finding confirms that the pollutions in the region have animal and human sources, as well. The lack of relationship between indicator bacteria and enteric viruses was experimentally confirmed. This confirmation is essential because human pathogen viruses were detected in high concentration on sampling points, which is used for drinking water supply and/or for recreational purposes. The present tests broadened greatly our knowledge about the presence of pathogen viruses in Hungarian aquatic environments, provided remarkable results in international aspect about the effects of environmental factors on the presence of viruses in the environment. The implemented methods give opportunity to the detection of human pathogen viruses from waters with high health risk, even during routine water qualification, even during epidemiological inspections.

VI. Publication list in connection with the thesis

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