

Virus Detection in Sewage Samples in Baranya County, Hungary

Ph.D. thesis

Edina Meleg

Program leader: Levente Emőd, M.D., D.Sc.
(Molecular pathogenesis of bacterial infections)

Tutor: Györy Szűcs, M.D., Ph.D.
(Molecular epidemiological investigation of viral gastroenteritis)

Department of Medical Microbiology and Immunology
Faculty of Medicine
University of Pécs, Hungary

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INTRODUCTION

According to the WHO Advisory Committee, the water-demand has increased six-fold between 1900 and 1990 in the world. This growth is two times more than population growth. Although only 0.3% of the water found in the Earth is suitable directly for consumption, water-demand was doubled in every 30 years. The drinking water stock is threatened not only by the extensive use of surface and subsurface waters, but by domestic, industrial and agricultural contamination and wastes too, flowing without any treatment into unprotected waterways. Biological pollution, such as viral contamination of water resources is also considerable.

Large quantities of enteric viruses that cause human diseases (gastroenteritis, ocular, respiratory infections, hepatitis, myocarditis or neurological diseases) can be introduced into the aquatic environment by feces of infected persons (or animals), resulting virus accumulation in community sewage. Many viruses, including enteroviruses, hepatitis A and E viruses, caliciviruses, rotaviruses and enteric adenoviruses were detected in different types of water in the past few decades. Viruses in sewage may contaminate drinking water resources, recreational waters, irrigation waters and natural waters (lakes, rivers), potentially causing a high public health risk. They can be accumulated in bivalve molluscan (shellfish), resulting further infections. Viruses associated with food-borne and waterborne infections are very resistant against different physical and chemical agents. Sewage treatment and disinfection procedures for wastewater have only a limited effect on a number of viruses, and therefore viruses are able to contaminate environmental waters thus preserving a long-term capacity for infection.

Nevertheless, the shortcomings of microorganisms such as fecal coliforms, fecal streptococci, anaerobe bacterial indicators (mainly clostridia and bifidobacteria) and bacteriophages as generally accepted indicators of fecal contamination of water, have been highlighted in several studies, and this has led to call for a reassessment of quality guidelines based on these indicator organisms. Several indicator organisms are more sensitive or more resistant (i.e. bacterial spores) against environmental stress and disinfection procedures than viruses. Hence results gained by traditional routine techniques may lead to false negative results. Therefore the ideal indicator would be the virus itself.

Sewage plants collect and treat community and industrial wastewater, so viral loads in sewage samples may be used as good indicators for viruses present in a community. While large volumes of drinking water, water for bathing or recreational waters are needed to detect viral contaminants, sewage is much better in this purpose because it usually contains much

higher number of viruses. Nevertheless, detection of viruses from any type of water sample without an effective concentration procedure and appropriate virus detection method is difficult and frequently unsuccessful.

Although there are several national and international reports about the presence of viruses in wastewater, surface water and freshwater, still do not exist generally accepted and applicable method for the detection of these viruses from aquatic environment.

Till now five different concentration techniques were described such as the two-phase separation, precipitation, ultrafiltration, ultracentrifugation and the adsorption-elution procedures. Most researchers use a filter adsorption-elution procedure with negatively or positively charged filters. This method is relatively expensive and more appropriate for the detection of viruses in large volumes of water. Another frequently utilized method is the polymer two-phase separation which is applied more widely for concentration of viruses from smaller volumes of different waters, including sewage samples.

Also, many different techniques have been used to recover or detect viruses. The traditional technique for detection of viruses is to isolate them in tissue culture. However, most of the enteric viruses do not readily grow in cell cultures or are even non-cultivable. Bacterial content of sewage also creates difficulties in virus propagation in cell cultures. Therefore at present, molecular techniques offer the best alternative to develop sensitive and specific methods for the detection of enteric viruses from environmental samples.

AIMS

Environmental virology has been a neglected area for the last 20 years in Hungary. In these two decades, however, new enteric viruses were characterized, new methods were developed for concentration of viruses, and the molecular methods were also successfully introduced for viral detection. Furthermore, the outstanding importance of viruses in food-borne and waterborne infections was increasingly recognized. Therefore, our main goal was to restart the water virology in Hungary, first with investigation of sewage, focusing particularly on the detection and characterization of new enteric viruses found in raw and treated sewage samples.

Specific aims of the study were to:

- develop and adopt a reliable, effective and reproducible concentration and detection method using direct molecular techniques,
- detect enteric viruses circulating in the Transdanubian region of Hungary (human astroviruses, human rotaviruses, human caliciviruses and human adenoviruses),

- characterize at molecular level of viruses detected in sewage samples,
- determine whether gastroenteritis cases observed among hospitalized children give a good indication on the real impact of the examined viruses in the population, or whether only a small proportion of the true number of infections is being observed by physicians,
- clarify possible correlation between viruses and fecal coliforms as indicators for fecal pollution in wastewater,
- reveal the relative efficiency of treatments in sewage plants for virus elimination.

MATERIALS AND METHODS

Sewage samples: In the first period of investigation, between April, 2004 and November, 2005, 35 raw sewage samples were examined from 22 sewage plants of Baranya County. In the second period, 36 pairs of corresponding raw and treated sewage samples were collected from four sewage treatment plants between November, 2004 and November, 2005. Finally, in the third period, between November, 2005 and August, 2006, 12 influent and effluent sample-pairs were examined from 3 sewage plants.

Concentration, viral nucleic acid extraction: A modified sodium-chloride–polyethylene-glycol method described previously by Minor et al. (1985) was used to concentrate wastewater samples, and the guanidinium thiocyanate-silica procedure published originally by Boom et al. (1990) was applied with modifications for the nucleic acid extraction.

Reverse transcription-polymerase chain reaction (RT-PCR): Viruses were detected by standard RT-PCR, PCR, semi-nested RT-PCR or SYBR Green-based real-time RT-PCR.

Sequencing and phylogenetic analysis: The specificity of amplicons was confirmed by nucleotide sequencing and phylogenetic analysis. Reference sequences downloaded from GenBank data base and environmental sequences were edited using the GeneDoc software. Phylogenetic trees were constructed with the MEGA v 2.1 software using Neighbor-Joining algorithm and Kimura-2 substitution model, with 1000 bootstrapping.

Internal control: To verify the presence/absence of interfering inhibitors, a modified viral nucleic acid was added to a portion of samples.

RESULTS

- Human astrovirus (HAstV) were used to develop a fast, economical and effective concentration and virus detection method. Eighty percentage of spiked HAstV were recovered from water samples with the NaCl-polyethylene glycol concentration method, and after using the viral nucleic acid extraction procedure described by Boom et al. we were able to detect effectively HAstV.
- Astro-, rota-, calici- and enteric adenoviruses were found in sewage samples, possessing public health importance.

Between April, 2004 and November, 2005, 15 out of 35 samples (43%) were positive for HAstV.

In the second and the third investigation periods, a total of 48 raw and treated sewage sample-pairs were collected and transported to the laboratory from four sewage plants of Baranya County. Human astroviruses were detected in 52.5% of the samples, group A and group C rotaviruses (RV-A, RV-C) were found in 54.6% and 97.9%, respectively, human caliciviruses (HuCV) in 34.2%, and adenoviruses were detected in 90.9% of the specimens.

Nine sample-pairs were found, in which only the treated sample contained a certain virus. It could be explained that effluent specimens were not the real pairs of the corresponding raw samples because both samples were collected in the same hour at the influent and effluent points of sewage plants not taking into account the detention time of wastewater.

Seasonal patterns of HAstV, RV-A, HuCV and HAdV were similar to that of variations observed in the clinical investigations.

Human RV-C strains could be detected mainly in one sewage plant, and particularly in the first 6 months in 2005. When the circulation of human RV-C strains decreased in the population, the prevalence of porcine RV-C strains was dominantly seen. Interestingly, human RV-C strains could not be identified in 2006 and the bovine RV-C strains became predominant.

- In the first period of examination, between April, 2004 and November, 2005, and in agreement with the previously published genotyping results of HAstV from clinical specimens in Hungary, HAstV-1 was found as the most abundant genotype in sewage, too. For the first time in Hungary, we could detect HAstV genotype 2 in a single sample.

Based on the molecular sequence analysis, types 1, 2, 3, 4, 5, and 8 astroviruses were found in the second and third periods indicating concurrent circulation of multiple astrovirus strains and multiple genotypes in Baranya County. Four samples (8.7%) contained HAstV-1, five (10.87%) contained HAstV-2, two (4.35%) contained HAstV-3, 13 (28.26%) contained HAstV-4, two (4.35%) contained HAstV-5 and 11 samples (23.91%) contained HAstV-8.

Of group C rotaviruses, altogether 12 human, 15 bovine and 17 porcine strains were identified in the raw samples by real-time PCR assay.

In the first investigation period phylogenetic analysis included all 11 strains isolated from sewage and reference sequences from all eight genotypes of HAstVs (HAstV 1-8), together with Hungarian strains detected in clinical specimens was carried out. This analysis revealed unambiguous genotype assignments of HAstV strains from sewage. Astrovirus strains detected in sewage samples clustered with the corresponding Hungarian strains found in clinical specimens, but were slightly different from them. Our HAstV-2 virus was most closely related with strains that originated from Mexico and South Africa.

Similar observation was done for HAstV types 1 and 8 in the second and third investigation periods. In this time, type 3 astrovirus sequences were found to be related to that of Egyptian strains. Type 4 astroviruses formed two genetically distinct groups (4a and 4b) and were similar to Dresden strains. HAstV-5 viruses were most closely related with Norwegian sequences.

Twenty group A rotavirus sequences were appropriate for phylogenetic analysis. Three different RV-A genogroups were isolated. The one AU-1 like strain probably originated from feline strains and transmitted to human. Two samples contained porcine strains.

Seven calicivirus sequences were subjected to phylogenetic assay. Three sequences clustered with genogroup I and four with genogroup II viruses, however they were different from the reference sequences.

- The most predominant viruses circulating in Baranya County were adenoviruses. They were found in sewage samples in ~91%, followed by the occurrence of human astroviruses and human rotaviruses (~53% and 54%, respectively) and eventually human caliciviruses (~34%). Taking into account these data and compare them to the detection rate of these viruses from samples of hospitalized persons in the same period in Baranya County, it was established that astroviruses, rotaviruses, caliciviruses and

adenoviruses were more common cause of gastrointestinal infections than it could be expected from the number of clinical cases with viral diagnosis (e.g. HAstV was only detected in 1.6% in the study period), suggesting that most of these infections are probably mild or asymptomatic.

- No correlation between the presence of viruses and indicators of fecal pollution (fecal coliforms) was observed. Bacterial tests were done only in 7% of samples, therefore, our results are not representative for the study period. Also, no differences were seen in the chemical parameters and ions routinely tested.
- Sewage treatment efficiencies could not be unambiguously determined, because in 15 cases only the treated effluent of a sample-pair was virus-positive, while the raw sample (influent) was negative for virus. However, more informative data were obtained when group C rotaviruses were quantified. The treatment efficiencies varied from 60 to 97% for rotavirus while elimination of bacteria was 99.5% in all examined plants.

Adenoviruses were found to be the most resistant viruses against the treatment procedures applied in the sewage plants. Group C rotaviruses, human astroviruses and finally group A rotaviruses, in this order, were less resistant. The most susceptible viruses against wastewater treatments seemed to be human caliciviruses.

Unfortunately, using only molecular techniques for virus detection, we were unable to distinguish between infectious and noninfectious virus particles which is a critical point in the environmental virology and infection control.

SUMMARY, PRESENTATION OF NOVEL FINDINGS

Viral investigations in aquatic samples were carried out again in Hungary after 20 years. Our applied methods were simple, worked well, and we verified that recently recognized enteric viruses could be detected from wastewater samples in the country. Our preliminary studies revealed that these viruses were released into the environment from asymptomatic or mild infections in the most cases. These viruses form genetically heterologous populations, and can be present in effluent sewage samples even after wastewater treatments. We also found that widely used and generally accepted indicators for fecal pollution are not quite suitable for indicating viral contamination. We also suggest, in agreement with others, that adenoviruses as real indicators of viral pollution (detecting by PCR) would be advisable.

Following the order of our aims, the next conclusions and achievements were made:

- We were able to restart the detection of viruses from aquatic samples in Hungary.
- We optimized and adopted a modified rapid, effective and reliable method for concentration and direct molecular detection of viruses from sewage samples.
- Molecular methods for detection of viruses from wastewater samples were applied first in Hungary.
- Raw and treated sewage sample-pairs were examined first for viruses in Hungary.
- We detected astroviruses, rotaviruses, adenoviruses and caliciviruses from wastewater samples also first in the country.
- It was established that high rates of astro-, rota-, calici- and adenovirus-positive samples provide convincing evidence that these viruses are more common cause of gastrointestinal infections than it could be expected from the number of patients required hospital treatment, and most of these infections are probably mild or asymptomatic.
- An internal viral nucleic acid control was created for the detection of PCR inhibitors.
- We proved that our method optimized for virus detection effectively removed inhibitors from samples.
- No correlation between the presence of viruses and number of indicators for fecal pollution (fecal coliforms) was confirmed. It was proved that viruses show increased resistance against water treatment. Therefore, waters found to be excellent by routine water qualification procedures may contain enteric viruses.
- Treatment efficiency could not be clearly determined. Nevertheless, in agreement with studies done abroad, wastewater treatment and disinfection procedures used in Hungary are not efficient enough to eliminate viruses from sewage.
- Quantitative methods were used with molecular procedures first in Hungary. Results of these experiments suggest that wastewaters in Hungary can be a source of enteric viral infections.
- We observed that genotype of virus strains in sewage differed from genotype of clinical strains.
- Different HAstV, RV-A, RV-C and HuCV strains and multiple genotypes were found concurrently within a given geographical area in the examined period.
- HAstV-2 was detected first in Hungary.

- Seasonal patterns of examined viruses were similar to seasonal occurrence of viruses obtained from clinical samples.
- Animal rotavirus strains were identified, and as a possible route at least for group C rotaviruses, zoonotic transmission was suggested.
- Viruses detected in sewage samples seem to be endemic in Baranya County.

LIST OF PUBLICATIONS

Publications served as basis for the thesis

➤ *Oral presentations, posters*

- **Meleg, E.**, Jakab, F., Kocsis, B., Bányai, K., Melegh, B., Szűcs, Gy.: Humán astrovírusok első kimutatása szennyvízmintákból Magyarországon. (*Oral presentation, Semmelweis Egyetem Doktori Iskola PhD Tudományos Napok 2005, April; Budapest; Hungarian*)
- **Meleg, E.**, Jakab, F., Kocsis, B., Bányai, K., Melegh, B., Szűcs, Gy.: Humán astrovírusok első kimutatása nyers szennyvízből Magyarországon. (*Oral presentation, Népegészségügyi Tudományos Társaság XV. Nagygyűlése 2006; Siófok; Hungarian*)
- **Meleg, E.**, Jakab, F., Kocsis, B., Bányai, K., Bogdán, Á., Szűcs, Gy.: Baranya megyei szennyvizek virológiai vizsgálata. (*Oral presentation, Magyar Mikrobiológiai Társaság 2006. évi Nagygyűlése, October, 2006.; Keszthely; Hungarian*)
- **Meleg, E.**, Jakab, F., Kocsis, B., Bányai, K., Melegh, B., Szűcs, Gy.: First detection of human astroviruses in raw sewage samples in Baranya County, Hungary. (*Poster, Ist Central European Forum for Microbiology, 2005, October; Keszthely*)
- **Meleg, E.**, Bányai, K., Jakab, F., Kocsis, B., Bogdán, Á., Melegh, B., Szűcs, Gy.: C csoportú rotavírusok kimutatása szennyvízből real-time PCR módszerrel Baranya megyében. (*Poster, Magyar Mikrobiológiai Társaság 2006. évi Nagygyűlése, October, 2006.; Keszthely; Hungarian*)
- **Meleg, E.**, Bányai, K., Szűcs, Gy.: Detection of human, porcine and bovine group C rotaviruses in the communal sewage: zoonosis or contamination? (*Poster, II. DIVINE EVENT Meeting, September, 2006.; Rome*)

➤ **Abstracts of the oral presentations**

- **Meleg, E.,** Jakab, F., Kocsis, B., Bányai, K., Melegh, B., Szűcs, Gy.: First Detection of Human Astroviruses in Raw Sewage Samples in Baranya County, Hungary. *Acta Microbiologica et Immunologica Hungarica* 2005, **52(Suppl)**, 95. (Abstract)
- **Meleg, E.,** Jakab, F., Kocsis, B., Bányai, K., Melegh, B., Szűcs, Gy.: First Detection of Human Astroviruses in Raw Sewage Samples in Baranya County, Hungary. *Clinical Microbiology and Infection* 2006, **12 (Suppl 4): R2209**, 112. (Abstract)
- **Meleg, E.,** Bányai, K., Jakab, F., Kocsis, B., Bogdán, Á., Szűcs, Gy.: Detection of group C rotaviruses by real-time PCR in sewage samples in Baranya County, Hungary. *Acta Microbiologica et Immunologica Hungarica* 2006, **53(3)**, 316-317. (Abstract)
- **Meleg, E.,** Jakab, F., Kocsis, B., Bányai, K., Bogdán, Á., Melegh, B., Szűcs, Gy.: Viral examination of sewage samples in Baranya County, Hungary. *Acta Microbiologica et Immunologica Hungarica* 2006, **53(3)**, 315-316. (Abstract)

➤ **Articles**

- **Meleg, E.,** Jakab, F., Kocsis, B., Bányai, K., Melegh, B., Szűcs, Gy. (2005) Humán astrovírusok első kimutatása nyers szennyvízből Magyarországon. *Egészségtudomány* **49**, 318-327. (IF: -); *Hungarian*
- **Meleg, E.,** Jakab, F., Kocsis, B., Bányai, K., Melegh, B., Szűcs, Gy. (2006) Human astroviruses in raw sewage samples in Hungary. *Journal of Applied Microbiology* **101**, 1123-1129. (IF: 2,127)
- **Meleg, E.,** Bányai, K., Martella, V., Jiang, B., Kocsis, B., Kisfali, P., Melegh, B., Szűcs, Gy. Detection and quantification of group C rotaviruses in the communal sewage. (submitted to *Applied and Environmental Virology*)

Other publications

➤ **Oral presentations, posters**

- Bogdán, Á., Bányai, K., Jakab, F., **Meleg, E.,** Martella, V., Melegh, B., Szűcs, Gy.: Humán C csoportú rotavírusok kimutatása és molekuláris jellemzése Baranya megyében (Poster, *Népegészségügyi Tudományos Társaság XV. Nagygyűlése 2006; Siófok*)

➤ **Articles**

- Jakab, F., **Meleg, E.**, Bányai, K., Melegh, B., Tímár, L., Péterfai, J., Szűcs, Gy. (2004) One year survey of astrovirus infection in children with gastroenteritis in a large hospital in Hungary - Occurrence and genetic analysis of astroviruses. *Journal of Medical Virology* **74**, 71-77. (IF: 2,52)
- Bányai, K., Gentsch, J. R., Schipp, R., Jakab, F., **Meleg, E.**, Mihály I., Szűcs, Gy. (2005) Dominating prevalence of P[8],G1 and P[8],G9 rotavirus strains among children admitted to hospital between 2000 and 2003 in Budapest, Hungary. *Journal of Medical Virology* **76**, 414-423. (IF: 2,52)
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- Jakab, F., Péterfai, J., Verebély, T., **Meleg, E.**, Bányai, K., Mitchell, D. K., Szűcs, Gy. (2007) Human astrovirus infection associated with childhood intussusception. *Pediatrics International* **49**, 103-105. (IF: 0,666)
- Bányai, K., Jiang, B., Bogdán, Á., Horváth, B., Jakab, F., **Meleg, E.**, Martella, V., Magyar, L., Melegh, B., Szűcs, Gy. (2006) Prevalence and molecular characterization of human group C rotaviruses in Hungary. *Journal of Clinical Virology*, **37**, 317-322. (IF: 2,623)

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