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Hepatitis E virus genotype 3 strains and a plethora of other viruses detected in raw and still in tap water

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ABSTRACT

In this study, next generation sequencing was used to explore the virome in 20L up to 10,000L water from different purification steps at two Swedish drinking water treatment plants (DWTPs), and in tap water. One DWTP used ultrafiltration (UF) with 20 nm pores, the other UV light treatment after conventional treatment of the water. Viruses belonging to 26 different families were detected in raw water, in which 6–9 times more sequence reads were found for phages than for known environmental, plant or vertebrate viruses. The total number of viral reads was reduced more than 4-log10 after UF and 3-log10 over UV treatment. However, for some viruses the reduction was 3.5-log10 after UF, as for hepatitis E virus (HEV), which was also detected in tap water, with sequences similar to those in raw water and after treatment. This indicates that HEV had passed through the treatment and entered into the supply network. However, the viability of the viruses is unknown. In tap water 10–130 International Units of HEV RNA/mL were identified, which is a comparable low amount of virus. The risk of getting infected through consumption of tap water is probably negligible, but needs to be investigated. The HEV strains in the waters belonged to subtypes HEV3a and HEV3c/i, which is associated with unknown source of infection in humans infected in Sweden. None of these subtypes are common among pigs or wild boar, the major reservoirs for HEV, indicating that water may play a role in transmitting this virus. The results indicate that monitoring small fecal/oral transmitted viruses in DWTPs may be considered, especially during community outbreaks, to prevent potential transmission by tap water.

1. Introduction

Human pathogenic viruses in aquatic environment, especially in drinking water, are a global problem for public health. In drinking water treatment plants (DWTPs), stringent regulations for monitoring the water quality by bacterial indicators are implemented in order to supply safe, reliable drinking water to the communities. The bacteria used as fecal indicators in the processes for monitoring the purification from microorganisms are often *Escherichia coli* and Enterococci (Figueras and Borrego, 2010). However, bacteria have been questioned as indicators for removal and inactivation of viruses and protozoa cysts (Gerba et al., 1979; Harwood et al., 2005). The World Health Organization (WHO) recommends multi-barrier approaches to prevent distribution of contaminated drinking water and reduce the contaminations to levels not hazardous for health (WHO, 2017). In many DWTPs the raw water is treated with coagulation, flocculation, sedimentation, rapid-filtration, and additional chlorine and/or UV-disinfection before the water is distributed. Concerns about by-products from chlorine have arisen and consequently less or no chlorine treatment is the trend for current disinfection of the water (Kim et al., 2003; Li and Mitch, 2018; Richardson, 2003). Instead alternative disinfection and removal technologies have been developed and are now implemented, as nanofiltration, ultrafiltration (UF), and ozonation, which

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Abbreviations

DWTP	drinking water treatment plant
HEV	hepatitis E virus
NA	nucleic acids
UF	Ultra filtration
UV	ultraviolet light

can be applied together with conventional treatment (Boleda et al., 2011; Lopes et al., 2013; Meunier et al., 2006; Shin and Sobsey, 2003; Xia et al., 2004).

The survival rate of viruses in environmental waters is affected by various conditions, as temperature and pH. Many viruses are stable and can survive for long periods in groundwater or drinking water, as hepatitis A virus, for which 99% inactivation takes about 56 days (Rzezutka and Cook, 2004). For adenovirus type 41 it is up to 304 days at 4 °C in water (Enriquez et al., 1995; Rzezutka and Cook, 2004). In addition, some viruses, like adenovirus, are resistant to UV disinfection, which is commonly used in drinking water treatment plants (Linden et al., 2007; Silva et al., 2011).

The bacteriophages MS2 and Qβ are often used as surrogate viruses in studies of removal and inactivation of viruses during water treatment, since they have similar size, composition, morphology and structure with human enteric viruses, and also share similar persistence in multiple water environments (Arraj et al., 2005; Nasser et al., 1995). Other bacteriophages, as gokushoviruses, are more abundant in many different waters globally as in seas, rivers and oceans (Labonte et al., 2015). These viruses were the most common virus found in the water at a local sewage treatment plant in Sweden (Wang et al., 2018). Considering their high abundance in aquatic environment and specific geographical distribution, these viruses may be used as a model indicator for the presence of and purification efficiency from viruses in treated water at local level.

Hepatitis E virus (HEV) is a small RNA virus associated with numerous outbreaks linked to contaminated water supplies in Asia and Africa, where genotypes HEV1 and HEV2 are prevalent (Rab et al., 1997; Sailaja et al., 2009; Swain et al., 2010). Currently, five genotypes infect humans, HEV1-4 and HEV7 (Purdy et al., 2017; Sridhar et al., 2017). HEV3 and HEV4 are zoonotic, and have wild boar and domestic pigs as the major reservoirs (Hammerschmidt et al., 2017; Roth et al., 2016; Rutjes et al., 2010). The role of water for transmission of these subtypes is still unknown.

This study was performed to investigate the virome in different waters by metagenomics sequencing and to evaluate the efficacy of removing viruses during the purification process from raw to drinking water.

2. Materials and methods

2.1. Sample collection sites

Gothenburg is located at the estuary of the River Göta älv on the west coast of Sweden. It is the second largest Swedish city with about 550,000 inhabitants in the urban area. The raw water from the River Göta älv serves as the main source for drinking water, which is produced at two major DWTPs, Alelyckan and Lackarebäck. Alelyckan supplies water mainly to northeast Gothenburg as well as to central and eastern Hisingen, an island north of central Gothenburg. Lackarebäck supplies water mainly to southern and south-eastern Gothenburg. The central city and western Hisingen are supplied from both DWTPs.

The raw water is treated through chemical flocculation, sedimentation, and activated carbon filtration followed by inactivation by chlorine and chlorine dioxide at both DWTPs. The choice of raw water is based on quality criteria. During periods of actual or suspected faecal contamination raw water from the reservoir lake Delsjön is used instead from the river directly.

At Alelyckan DWTP, the treated water is disinfected by ultraviolet light (UV light) with a UV dose of 40 mJ/cm^2. The microbiological barriers are followed-up with removal of micro-algae and on-line turbidity. Chemical inactivation is controlled to achieve enough CT-values for virus inactivation (CT is the disinfectant concentration multiplied by the contact time) and checked with live cell staining as well as bacterial indicators. There is certification of virus inactivation for the type of UV-equipment based on seeding test with UV-resistant micro-organisms, but the monitoring during operation relies on in-direct measurements of UV254 transmittance and light intensity.

In Lackarebäck, UF membranes are added as an extra microbial barrier instead of UV disinfection. The pores of the membranes have a nominal size of 20 nm, which is less than the size of most target pathogens. Pore size typically gets smaller by fouling/scaling during operation. When coagulation is applied before UF, most viruses are believed to adsorb to larger flocs before UF or on flocs accumulating on the membrane surface (Bratby, 2006). Before installation of full-scale modules, removal of small particles was tested on selected membranes from different batches of membranes. MS2 phages and virus like particles (VLPs) met the criteria of more than 4-log10 removal. The integrity control of the UF membranes is performed daily by pressurizing the membranes with air, and the ability to retain air is recorded to check for single defect fibres. Failure to meet these requirements automatically leads to removal of the unit. Low range on-line particle counters are also used to monitor for major damage and changes in pore size distribution. Removal of fluorescent VLPs are analysed by fluorescence microscopy. These tests showed more than 4-log10 removal of VLPs larger than 20 nm by the UF membranes. All measured parameters of the waters during the sampling period are given in A1.1 Table S1.

Before pumping the water into the supply network, chlorine and chlorine dioxide are added, at Alelyckan as part of the multiple microbial barriers, and at Lackarebäck mainly to inhibit bacterial growth and to control lake related odour. The CT-value for chlorination at Alelyckan DWTP was approximately 35 min × mg/l and 18 min × mg/l at Lackarebäck DWTP. The pH is thereafter adjusted to 8.0–8.1 and alkalinity to 1.0–1.1 mmol/l. The water passes through a 176-km-long water supply network to 13 water towers around the city's heights and 68 drinking water pump stations before being distributed to the consumers. The distribution to the tap water sampling point in this study is shown in B1.1 Fig. S1. The transport time from Lackarebäck DWTP is estimated to have been 8 h and from Alelyckan DWTP 12 h. Some water may have prolonged retention time due to the drinking-water storage in the same pressure zone as the sampling point.

2.2. Sample collection and concentration of viruses in water

The sampling place, date and volume of eight water samples analysed are given in Table 1. The sampling period was over a three-week period with stable raw water quality and treatment at Lackarbäck DWTP. The samples at Alelyckan before and after UV-treatment were collected the same day.

One sample of 20 L raw water to be treated at Lackarebäck DWTP was filtered twice through a Nano-Ceram cartridge filter (Argonide, Sanford, Florida, USA) at an average flow rate of 2.5 L/min, as previously described (Wang et al., 2018). Another two

Table 1
Date and volume of water samples collected from Lackarebäck and Alelyckan DWTPs, tap water and environment water.

Sample	Collection Date	Volume (L)
Raw water (Lackarebäck)	01/02/2017	20
After carbon filtration (Lackarebäck)	12/28/2016–12/29/2016	5,050
Permeate after UF (Lackarebäck)	12/28/2016–1/2/2017 (119 h)	10,350
After carbon filtration (Alelyckan)	01/05/2017	6,000
After UV treatment (Alelyckan)	01/05/2017	7,100
Tap water -day 1	12/19/2016–12/20/2016	1,428
Tap water -day 2	12/20/2016–12/21/2016	1,168
Tap water -day 3	12/21/2016–12/22/2016	1,383

samples were from Lackarebäck DWTP. One was a Nano-Ceram filter connected to the flow of water after activated carbon treatment during 29 h, when 5,050 L water was filtered at a flow rate of 2.9 L/min. The other sample was a Nano-Ceram filter connected to the flow of water after UF treatment during 119 h, when 10,350 L of water were filtered at a flow rate of 1.45 L/min (Table 1).

In Alelyckan DWTP the same sampling method was applied, with 6,000 and 7,100 L of water before and after UV treatment were passed through Nano-Ceram filters for 48 h with flow rates of 2.08 L/min and 2.48 L/min, respectively (Table 1).

In the Department of Clinical Microbiology-Virology (CML), located in the centre of Gothenburg, the tap water is supplied from both of DWTPs. A Nano-Ceram filter was connected to the tap, and between 1,168 and 1,438 L water were filtered at an average flow rate of 2 L/min overnight during three consecutive nights (Table 1).

After on-site sampling, all the above-mentioned Nano-Ceram filters were kept moist in plastic bags until elution of bound particles at CML. The viruses concentrated on the Nano-Ceram cartridge filters were eluted from the filter and further concentrated by ultracentrifugation as previously described (Wang et al., 2018). The pellet was suspended in 2.4 mL 10 mM Tris-HCl (pH 8.0) buffer and stored at −80 °C until analysis.

2.2.1. Sample preparation for NGS

Two-hundred μL of the dissolved pellet after ultracentrifugation were treated with 50 U Benzonase nuclease (Sigma-Aldrich, St. Louis, MO, USA), with 1.25 mM $MgCl_2$ (Applied Biosystems, Foster City, CA, USA) to degrade free DNA and RNA. After 1 h incubation at 37 °C, a final concentration of 50 mM EDTA was added to inhibit the nuclease activity. The nucleic acids were extracted using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturers' protocol and eluted in 100 μL elution buffer.

Total RNA was reverse transcribed into cDNA in a 20-μL reaction mix using Omniscript Reverse Transcription Kit (Qiagen). Six microliters of extracted nucleic acids were incubated at 65 °C for 5 min and a master mix containing 1 × Buffer RT (Qiagen), 0.5 mM dNTP mix (Qiagen), 40 U RNaseOUT™ (Invitrogen), 4 μM of SIS3 (A1.1 Table S2) and Oligo-dT (Invitrogen) primer mix, and 4 U Omniscript Reverse Transcriptase (Qiagen) were added. cDNA synthesis was performed at 37 °C for 60 min, and the cDNA was stored on ice before proceeding to PCR amplification.

Each extracted DNA and cDNA were amplified by nested PCR in triplicate. For nucleic acids directly from extraction, a touch-down gradient PCR was firstly applied. The 50 μL reaction mix contained 10 μL template, 1 × Taq buffer (Applied Biosystems), 4 mM $MgCl_2$ (Applied Biosystems), 0.5 mM dNTP (Sigma-Aldrich), 5 U Taq DNA polymerase (Roche Diagnostics), and 1 μM of SISP3 primers A1.1 Table S2). The PCR reaction was performed for one cycle at 95 °C for 3 min and 37 °C for 90 s, followed by 38 cycles touch-down PCR with 95 °C for 30 s, 58 °C for 30 s (1 °C decrease per cycle) and 72 °C for 2 min, followed by 7 cycles of 95 °C for 30 s, 37 °C for 30 s and 72 °C for 2 min, and with 5 min final extension at 68 °C. The cDNA was also amplified by a touch-down gradient PCR. The reaction mix was the same as for total nucleic acids apart for the primers, which were 1 μM of SIS3 primer (A1.1 Tables S2) and 1 μM of Oligo-dT. The PCR reaction was the same as for total nucleic acids. The primers used were modified SISPA primers (A1.l Table S2). The amplicones obtained were further amplified by nested PCR for both amplified total nucleic acids and cDNA with the same reaction mix except for the primers. The product for amplified total nucleic acids were nested with primer SISP2 and the product from amplified cDNA was nested with primer SIS2 (A1.1 Table S2). The mix for the nested PCR reaction contained 5 μL of the first round PCR product, 1 × Taq buffer (Applied Biosystems), 4 mM $MgCl_2$ (Applied Biosystems), 0.5 mM dNTP (Sigma-Aldrich), 5 U Taq DNA polymerase (Roche Diagnostics), 0.8 μM of each primer. The PCR cycling was performed by an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and a final extension at 68 °C for 5 min.

Six tubes of PCR products from both amplified total nucleic acids and cDNA were pooled and purified with QIAquick PCR purification kit (Qiagen). Libraries for Illumina sequencing were built by Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA, USA) according to manufacturers' protocol. The concentration and size distribution of the libraries were analysed using Qubit 4 Fluorometer (ThermoFisher, Waltham, MA, USA) and Agilent High Sensitivity D1000 ScreenTape System on TapeStation 2200 (Agilent, Santa Clara, CA, USA). The final libraries were sent to Eurofins Genomics (Eurofins Genomics Germany GmbH, Ebersberg, Germany) for sequencing performed on a HiSeq 4000 platform (Illumina) to produce 150-bp paired-end reads. In total 1.1% of the original volume of the sample was used for the sequencing.

2.2.2. NGS data analysis

Raw data from the Illumina sequencing was imported to CLC Genomic Workbench 11.0.1 (Qiagen, Hilden, Germany) for analysis. For accepted sequence reads, the quality score was 0.05, the primer sequences were removed by trimming, and all reads longer than 50 bp were accepted. The reads were *de novo* assembled using the built-in CLC *de novo* assembler with a word size of 20 and an automatic bubble size of 50 bp. All viral sequences were downloaded from NCBI GenBank to build a local genomic viral database, and the contigs and unassembled singleton reads longer than 100 bp were blasted against this local viral database using BLASTn. The reads with a cut-off for E value $< 10^{-5}$ and HSP lengths >100 bp were considered as possible viral hits. These hits were used for a second blast against all genomes in the NCBI GenBank non-redundant nucleotide database (nt/nr) using BLASTn. The reads that satisfied the same criteria as in the first blast were considered as significant viral hits. These identified viral reads were further classified into virus family level for subsequent analysis. The identified sequences of HEV and gokushovirus were mapped to reference sequence using CLC Genomic Workbench. Consensus sequences for both viruses were extracted and used for further analysis.

2.3. Validation of NGS results by nested PCR

Nested PCR primers were designed and used based on the assembled sequences from NGS for amplification of gokushovirus (A1.1 Table S2). For the first round amplification, a 50-μL reaction mix containing 5 μL cDNA or nucleic acids, 1 × Taq buffer (Applied Biosystems), 2.25 mM $MgCl_2$ (Applied Biosystems), 0.2 mM dNTP (Sigma-Aldrich), 1 U Taq DNA polymerase (Roche Diagnostics), 0.3 μM of each primer were used (A1.1 Table S2). The PCR reaction was performed for one cycle at 94 °C for 3 min, followed by 40 cycles of 94 °C for 20 s, 55 °C for 30s and 72 °C for 1 min, and with 5 min final extension at 68 °C. The nested PCR amplification used 5 μL PCR products from first round as template, and the reaction mix was same as first round except for a change of the $MgCl_2$ concentration to 2.75 mM. The cycling condition was same as for the first round of PCR. The amplified products were purified using QIAquick PCR purification kit (Qiagen) according to manufacturer's protocol and sent to Eurofins Genomics (Germany) for Sanger sequencing.

2.4. HEV quantification by real-time PCR

A real-time PCR was used to quantify the amount of HEV RNA in the water samples. The World Health Organization International Standard (IS) for HEV (Paul-Ehrlich-Institut, Langen, Germany) with a 250,000 International Units (IU) of HEV RNA/mL was serially diluted in eight 5-fold-steps (1/5 to 1/390,625) and used as a standard for quantification. The extracted RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription kit (ThermoFisher). A 16-μL pre-reaction mix containing 10 μL RNA, 4 mM dNTP, and 1 × RT random primers was preheated at 65 °C for 7 min. Thereafter, 1 × RT buffer, 20 U RNase inhibitor and 50 U MultiScribe™ Reverse Transcriptase were added and the reaction was performed at 25 °C for 10 min, followed by 37 °C for 2 h, and 85 °C for 5 min. The cDNA was used immediately as template in a real-time PCR reaction, with primers and probe given in A1.1 Table S2 and reaction conditions as previous described (Roth et al., 2016).

2.5. Sequence analysis

The amplified and sequenced 357 nucleotides of the VP1 of gokushovirus, and 820 nucleotides of the junction region of ORF1-ORF2-ORF3 of the HEV consensus sequences from the metagenomics analysis were analysed in the program SeqMan in the DNAStar program package version 10.1.2 (DNA Star Inc, Madison, WI 53705, USA). For the phylogenetic analysis of partial VP1 of gokushovirus, a total of 204 sequences from viruses belonging to the *Microviridae* family in GenBank were aligned with sequences obtained from the water samples in this study. For HEV the corresponding region of 133 HEV3 sequences and two HEV1 sequences obtained from NCBI database were aligned with the HEV sequences obtained in this study. Phylogenetic analysis was carried out with the PHYLIP package version 3.65 (Felsenstein, 1996). The evolutionary distances were calculated using Hasegaw-Kishino-Yano (HKY) algorithm in DNADIST program in the PHYLIP package, and phylogenetic trees were constructed using unweighted pair-group method using arithmetic averages (UPGMA) and neighbor-joining (NJ) methods in the PHYLIP package. The trees were visualized with the program TreeView version 1.6.6 (Page, 2002). All sequences obtained from this study are deposited in GenBank with accession numbers MN51833-MN518340.

3. Results

3.1. Number of virus reads identified by NGS

A total of 8.5×10^6 to 31×10^6 raw reads were obtained for the samples sequenced (Table 2). After trimming and reassembling, the sequences were analysed for homology by blast first to all viral sequences in GenBank. Thereafter the sequences obtained were investigated by a second blast for homology to all sequences available in GenBank (the non-redundant nucleotide database; Table 2). This resulted in 7.5×10^3 to 435×10^3 reads or contigs classified as viral reads (0.02%–3.54% of all reads). All reads similar to viral sequences were between 110 bp and 900 bp.

The highest number of reads corresponding to viral sequences per L sample was in the raw water for Lackarebäck DWTP (Table 2). The number of viral reads per L water was substantially reduced 2.2 to 2.7-log10 after carbon filtration treatment at Lackarebäck DWTPs (Table 2). In Alelyckan DWTP, additional UV disinfection lowered the viral number of reads 3.2-log10, and in Lackarebäck DWTP the passage through UF membranes lowered the number 4.3-log10. In tap water sampled for three consecutive days, the number of viral sequence reads varied between 540 and 2,211 per litre, corresponding to 0.02–0.11% of the total reads in these samples (Table 2).

Sequences found homologous to viruses belonged to 12–28 known viral families and to about 650 non-classified viruses in the eight different samples (Table 2; A1.2 Tables S3–S4). The most abundant sequences were homologous to bacteriophages classified into the family *Microviridae* and accounted for 57% of the viral reads in water after carbon filtration at Lackarebäck DWTP, followed by *Myoviridae*, and *Siphoviridae*. Sequenced homologous to protist viruses as *Mimiviridae* and *Phycodnaviridae* were also abundant, and the most common viruses infecting vertebrates belonged to the *Hepeviridae*, *Parvoviridae* and *Circoviridae* families (A1.2 Table S3).

The number of viral reads was substantially higher in raw water than in tap water. For some phages the number increased after carbon filtration at the DWTPs but decreased considerably after UF. For *Mimiviridae*, a group of giant viruses, the viral reads found in raw water was reduced by 40–45% after carbon filtration in the two DWTPs, and to undetectable levels after UF, and by 90% after UV (A1.2 Table S3). For some other viruses belonging to the viral family *Virgaviridae*, a family of small viruses infecting plants, the viral reads were relatively stable with 80–240 reads in all samples except in one tap water sample where it was missing (A1.2 Table S3). It is noteworthy that a virus identified the water samples in this study, pepper mild mottle virus (PMMoV) in the *Virgaviridae* family, was recently proposed as a potential indicator of human fecal contamination of environmental waters (Shirasaki et al., 2017).

3.2. Detection and phylogenetic analysis of HEV in water samples

There were 1×10^3 to 33×10^3 viral reads of *Hepeviridae* sequences identified in all eight water samples. The number of reads of HEV sequences per L water was reduced with about 3-log10 and 3.6-log10 after UV or UF treatment in the DWTPs (A1.2 Table S3). The number of reads of HEV sequences in tap water varied from 1,900 to 27,600.

The presence of HEV sequences in the waters was confirmed by qPCR, which also was used to quantify the HEV genomes. HEV RNA could be quantified in all except two samples, the raw water and before UV treatment (A1.4, Table S5). The water samples contained between 6 and 130 IU HEV RNA/mL, with the effluent from UF having the lowest amount and one tap water the highest (A1.4, Table S5). This calculation, shown in Table S5, is based on the ct-values of the water samples and on the regression line obtained

Table 2
Number of total reads of sequences and those corresponding to viral genomes obtained by NGS in the different water samples, not considering the recovery efficiency of viruses by the concentration method used.

Sample	Total reads	No. of identified viral families	Viral reads	Percentage viral reads	Viral reads/L original sample
Raw water (Lackarebäck)	8,544,764	26	302,291	3.54%	1,374,050
After carbon filtration (Lackarebäck)	28,075,226	26	435,500	1.55%	7,839
Permeate after UF (Lackarebäck)	23,766,188	16	7,574	0.03%	67
After carbon filtration (Alelyckan)	26,492,646	28	164,261	0.62%	2,488
After UV treatment (Alelyckan)	20,622,742	23	71,535	0.35%	916
Tap water - D1	25,895,686	18	23,688	0.09%	1,508
Tap water - D2	29,825,506	13	6,942	0.02%	540
Tap water - D3	31,253,164	12	33,633	0.11%	2,211

from log10-dilution and ct-values of the WHO standard, assuming a 10%–20% recovery of viruses during concentration by Nano-Ceram filtration as shown previously (Wang et al., 2018).

Phylogenetic analysis of consensus sequences of 820 nucleotides in the junction region of ORF1-ORF2-ORF3 revealed that seven of the HEV sequences belonged to subtype HEV3c/i and one to HEV3a. The HEV3c/i sequences were found in tap water (three strains), in water treated after carbon filtration (two strains), and one strain each was in raw water and in water after UF at Lackarebäck DWTP (Fig. 1). Six of these strains formed one separate clade on the HEV3c/i branch in the phylogenetic tree. Another HEV3c/i strain from raw water was similar to a strain from a Swedish blood donor (Fig. 1). The HEV strain from water after carbon filtration treatment in Alelyckan DWTP was on the branch formed by HEV3a strains, which are often found in Asia and the USA (Fig. 1) and was similar to a strain from a Canadian swine. None of these strains were similar to the HEV3f or 3e strains, which are usually isolated from Swedish pigs and wild boar.

3.3. *Phylogenetic analysis of bacteriophages from Microviridae identified in water samples*

To validate the NGS results and to understand the phylogenetic relationships between viruses identified in the different types of waters, a semi-nested PCR was developed for detection and sequencing of gokushovirus within the *Microviridae* family. Strains in seven samples could be amplified and sequenced in the VP1 region (B1.1 Fig. S2). Phylogenetic analysis revealed that all gokushovirus strains belonged to the subfamily *Gokushovirinae* in the *Microviridae* family (Fig. 2). The strains from raw and treated water in both DWTPs were similar and formed one clade in the phylogenetic tree. They were on the same branch as strains in fresh water from France. The strain identified in a tap water sample was divergent and found in another clade with strains isolated from freshwater fish in the USA.

4. Discussion

This study showed a substantial removal of most viruses from raw water treated with conventional methods and additional barriers at two Swedish DWTPs. Larger viruses were efficiently removed by UF. However, genomes of some smaller viruses, as HEV and some bacteriophages and plant viruses, were detected after UF and UV treatment. Although UV has been shown to reduce the infectivity on recombinant HEV1/HEV3 virus adapted to cell cultures (Guerrero-Latorre et al., 2016), the inactivation efficiency for wild type HEV is not known (Fenaux et al., 2019). The HEV strains in this study could enter the water supply network despite that all standards were fulfilled for routine monitoring of small particles in the outlet water at the DWTPs. The pores in the UF membranes used are smaller than those recommended by the Swedish authorities, which have stated that membranes with pore size 100 nm can be a used microbial barrier, and the Norwegian guide for barrier analyses recommend 40 nm nominal pore size for UF (Svenskt-Vatten, 2015). These larger pore sizes would probably not remove smaller viruses as efficiently as the membranes in this study. Common human viruses with fecal/oral spread that may not have been removed by larger pores are e.g. norovirus (38–40 nm), rotavirus (45 nm) and adenovirus (90–100 nm). Several other viruses with fecal/oral spread are as smaller as HEV, about 30 nm in diameter, and may pass through the membranes, as those belonging to the *Picornaviridae* family, for example hepatitis A virus, enterovirus, Aichivirus and parechovirus. This should be considered during outbreaks of these viruses in the community. However, the retention of viruses by UF may not only depend on virus size but also on other physiochemical factors of the viral capsid influencing on the flocculation of the virus during purification at the DWTPs. The results from this study indicate that the current methods are sensitive for reducing almost all larger viruses and most of the smaller size viruses in water. Although HEV was found in treated water before disinfection at the DWTPs and in tap water, the amount per L tap water was comparable low. Therefore, the risk of infection by consumption of the drinking water is probably negligible. However, further studies on viral infectivity and lowest infectious dose are needed.

The decrease of number of viral reads with more than 3-log10 by conventional treatment of raw water indicate that the treatment has high virus removal. The findings of viruses including HEV in water leaving the DWTPs should be of concern to drinking water producers that do not practice the multibarrier approach recommended by the World Health Organization. It should be noted that the analysing methods used in this study do not separate between active and inactivated viruses, the viability of the viruses needs to be further studied.

It was somewhat surprising to find from 10 to 130 IU HEV3 sequences per mL raw, treated and tap water in this study. It is well known that HEV1 and HEV2 cause waterborne outbreaks in developing countries in Asia and Africa (Chen et al., 2016; Hazam et al., 2010; Naik et al., 1992; Rab et al., 1997). However, in Europe, HEV3 is the most common genotype and has mainly zoonotic spread through consumption of contaminated meat. HEV3 has been detected in shellfish grown in coastal waters in Europe, showing that HEV is prevalent in the water, and that consumption of shellfish may also be a potential route for HEV transmission (Crossan et al., 2012; Mesquita et al., 2016; Said et al., 2009). The role of drinking water for its spreading is not well understood. Only one recent study from France has shown that infections with HEV3 may be associated with drinking tap water (Mansuy et al., 2016). The microbial quality of drinking water in developed countries is usually well monitored, but some studies have shown the presence of adenovirus, enteroviruses, and norovirus in tap water (Cho et al., 2000; Haramoto et al., 2004). One study in Korea showed that

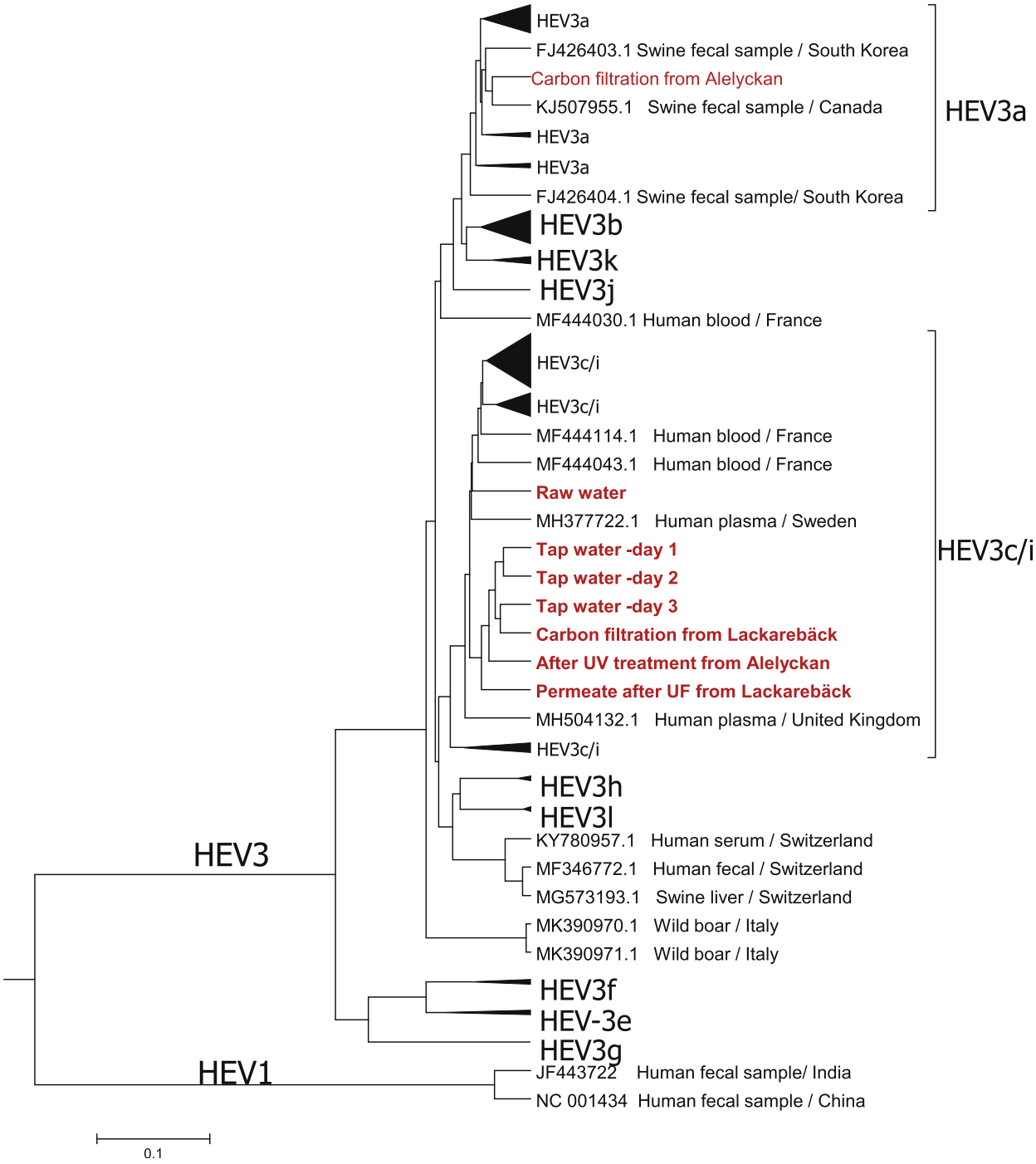

Fig. 1. Phylogenetic tree of 820 nucleotides of the junction region of ORF1-ORF2- ORF3 in HEV3 strains with HEV1 as outgroup. Accession number and origin of the strains are given at the nodes. The strains from this study are marked in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

enterovirus and adenovirus identified in tap water were still viable (Lee and Kim, 2002). It is not known if HEV at low levels in the drinking water is infectious, since the human infectious dose for HEV is not known. For macaques it has shown to be more than $10^{4.5}$ particles for oral infection (Tsarev et al., 1994), which would correspond to consumption of more than 1 L of the tap water with the highest amount of HEV in this study. Even if the viability of the virus is not known, it is shown that viruses are viable for longer periods in purified waters(Rzezutka and Cook, 2004). The finding of HEV3 in tap water indicates that the route of waterborne HEV3 infections should be considered also in developed countries, and the presence of this virus in the waters may be monitored if outbreaks are suspected.

Phylogenetic analysis of the HEV sequences showed that the strains belonged to subtype HEV3c/i. This subtype is not found in Swedish pigs or wild boars, which are infected with subtypes HEV3f or 3e (Roth et al., 2016; Wang et al., 2019). HEV3c/i often causes chronic HEV infection with prolonged excretion by the

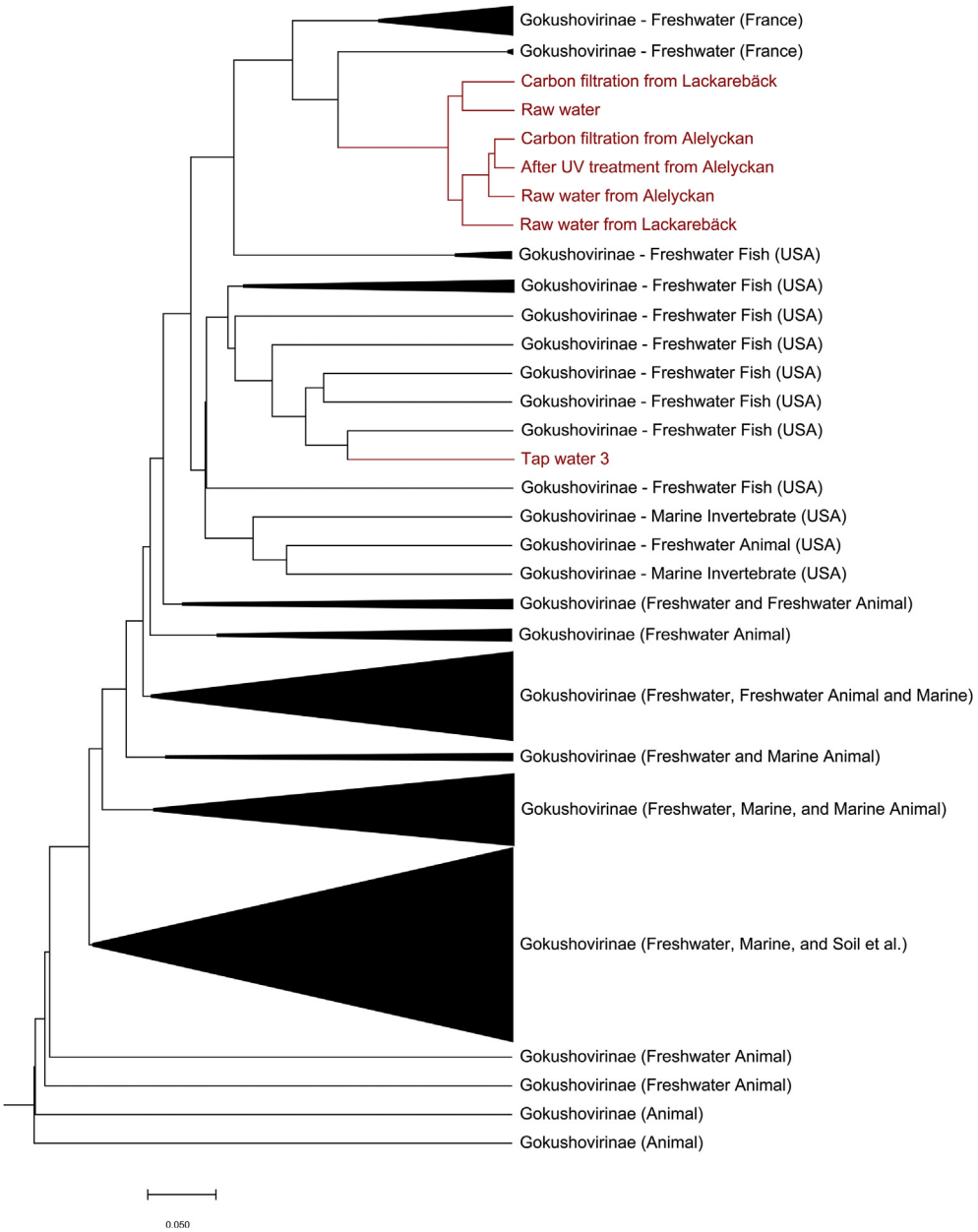

Fig. 2. Phylogenetic tree of 357 nucelotides of partial VP1 in gokushoviruses. Origin and accession number of the strains are given at the nodes. The strains sequenced in this study are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

carrier (Norder et al., 2018). It has been isolated from humans with unknown source of infection in Sweden, although imported food items have been suspected (Norder et al., 2018). The results in this study indicate that water may also be a source for HEV3c/i infections. The reason for the high prevalence of this subtype in water needs further investigation. Different HEV3 subtypes may have different sensitivity to treatment, or HEV3c/i, may spread into the waters more often than other subtypes. HEV3 has also been found at low levels in sewage samples in the Gothenburg area (Hellmer et al., 2014), but the genetic relations between HEV strains in sewage, and raw and treated water is still unclear, and further investigations of their possible transmission routes are needed.

Apart from HEV, a high diversity of other viruses was found in the waters before and after treatments, as well as in tap water. Many viruses were bacteriophages and plant viruses. This is in agreement with another study on the virome in reclaimed and potable water in the US (Rosario et al., 2009). Some bacteriophages could originate from normal growth of their bacterial hosts in the water. The identification of abundant bacteriophages is promising for development of techniques for monitoring removal or intrusion of viruses at DWTPs when the number of pathogenic viruses is low. The current monitoring of microbiota in the waters at the Swedish DWTPs is mainly based on faecal indicator bacteria as *E. coli* and Enterococci and in Gothenburg also Clostridium and coliphages. However, the commonly used surrogate bacteriophages were not found in the water samples in this study. The use of another widely distributed surrogate phage present in both natural aquatic environments and in treated water could be helpful to better understand the removal processes and efficiency. One of the most common sequences identified in this study were homologous to gokushoviruses. This virus has also been found in abundance in different waters globally and from sewage treatment plants, also in

Sweden (Diemer and Stedman, 2016; Labonte and Suttle, 2013; Wang et al., 2018; Zhong et al., 2015). Further studies will explore the possible use of this virus for monitoring the quality of drinking water.

This study identified numerous sequences homologous to viruses that could not be classified into a known viral family. Most could not be cultured by current techniques. This shows that metagenomics is useful for identification of both known and unknown viruses in environmental samples, although it cannot distinguish between viable and non-infectious viruses. Other drawbacks of this technique are the sensitivity of the PCR used for amplifying the sequences before NGS, and the workflow used for sequence analysis. These methods may underestimate the viral diversity, as has been shown in sewage samples, in which viruses could be identified by qPCR but not by NGS (Cantalupo et al., 2011; Wang et al., 2018). The analysis of the sequences may also overestimate the number of identified viruses, since the assembled sequences may give false hits when they are used for blast against a database (Bibby et al., 2019; Rosseel et al., 2014). To reduce the possible false-positive viral reads, the assembled sequences were used at two blast occasions in this study. First against a database with viral sequences, and the hits obtained were searched against the whole nucleotide database in GenBank. This optimized workflow reduced the number of viral sequences classified into different viral families from 62 to 28 and accelerated the process. However, both false negative and positive results could be obtained by NGS. Subsequent confirmations by specific qPCR, Sanger sequencing and phylogenetic analyses are needed to provide robust classification of the viral genomes identified, which was performed for HEV and gokushkovirus in this study.

5. Conclusion

This study showed that even if the number of viruses were reduced 3-4-log10 at the two DWTPs, there were sequences representing many different virus families, including HEV, in the effluent after UF and in tap water. The total number of viral reads in tap water was about 0.1% of that in raw water, but for HEV it was 2.2%. Despite this, the risk for getting infected by viruses in these concentrations in the drinking water is probably negligible. However, there may be a risk of transmission during outbreaks with large number of infected persons excreting high concentrations of small fecal/oral transmitted viruses. The virome and reduction of many different small RNA and DNA viruses in water could be monitored using an indicator virus, as gokushovirus, which was found in all water samples. Further studies are necessary to investigate the viability of the viruses identified in water. To achieve better understanding of the efficiency of virus removal and inactivation there may be a need for routine monitoring for viral indicators in waters at the DWTPs and in the distribution network. This knowledge will help tracking possible transmissions of viruses from raw water to tap water, and thereby ultimately reduce potential risks of viral infections from drinking tap water during outbreaks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.115141.

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