

## Enteric viruses in inlet and outlet samples from sewage treatment plants

M. Myrmel, E. M. M. Berg, B. Grinde and E. Rimstad

### ABSTRACT

Samples collected every two weeks from the inlet and outlet of three sewage treatment plants were screened for the presence of noro-, rota-, astro-, adeno-, hepatitis A- and circoviruses by (RT)-nested PCR, and for F-specific bacteriophages by isolation in *Escherichia coli* Famp. Plants A and B were secondary treatment plants and plant C used primary treatment. Noroviruses were detected in 43%, 53% and 24% of the inlet samples and 26%, 40% and 21% of the outlet samples from plants A, B and C, respectively. Astroviruses, rotaviruses and adenoviruses were more prevalent. Adenoviruses were detected in 96% of inlet and 94% of outlet samples, supporting the potential of these viruses as indicators of viral contamination from sewage. Hepatitis A virus and circoviruses were found only rarely. Reduction of infective viral particles during sewage treatment was evaluated using F-specific bacteriophages. The phages were reduced by, respectively, 99%, 87% and 0% in plants A, B and C, which corresponded to the observed differences in reduction of norovirus positive samples between the same plants. The study shows that the high viral load in sewage results in a discharge to the environment of a large amount of virus despite sewage treatment. On the other hand, the advantage of a more advanced treatment is demonstrated.

**Key words** | circovirus, enteric viruses, F-specific bacteriophages, norovirus, real-time PCR, sewage

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### INTRODUCTION

Dissemination of enteric viruses occurs directly by person-to-person contact or indirectly through food, water and the environment. The modes of spread, and the susceptibility of the population to infection, vary between types of virus. Viral food- and waterborne outbreaks of gastroenteritis, which occur worldwide, are most often caused by noroviruses (NV) and persons of any age may get infected (Hedberg & Osterholm 1993; Hedlund *et al.* 2000; Miettinen *et al.* 2001). Hepatitis A virus (HAV) is less common in developed countries; however, outbreaks of hepatitis due to contamination of drinking water and shellfish by HAV have been recorded in the US and Europe (Mele *et al.* 1989; Bloch *et al.* 1990; Desenclos *et al.* 1991; De Serres *et al.* 1999). In these populations, where HAV is not endemic, adults as well as children become infected. Rota- (RV),

astro- (AV) and enteric adenovirus (AdV) are rarely associated with food- or waterborne disease and mainly cause gastroenteritis in children (O'Neill *et al.* 2002), but adults (i.e. mostly elderly) may also be susceptible to infections with RV and AV (Lewis *et al.* 1989; Timenetsky *et al.* 1996; Svenungsson *et al.* 2000). Rotavirus can cause severe gastroenteritis in children and is the main cause of infantile morbidity worldwide (Desselberger 2000). In some countries enteric AdV (subtypes 40 and 41) are registered as second only to rotavirus as aetiological agents of infantile gastroenteritis (Uhnnoo *et al.* 1990).

There is a correlation between severity of the disease caused by enteric viruses and laboratory diagnosis of the aetiological agent. Mild infections are more prone to pass unnoticed (i.e. be underestimated), while severe illnesses

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are more likely to be diagnosed and registered. NV, AV and AdV infections most often cause mild gastroenteritis and the majority of cases, except large outbreaks of NV-induced gastroenteritis, may pass unregistered. Infections with RV can be more severe, sometimes require hospitalisation, and therefore are more likely to be diagnosed. Hepatitis A virus infections are notifiable in Norway, thus most cases with clinical illness are presumably recorded.

Enteric viruses are shed in faeces and the content of these viruses in sewage therefore reflects the infectious status of the population. Moreover, sewage is an important source for viruses which can contaminate drinking water, shellfish and recreational water (Timenetsky *et al.* 1996; Kukkula *et al.* 1997, 1999; Hafliker *et al.* 2000; Lee *et al.* 2002). There is limited knowledge about the occurrence and viability of these viruses in aquatic environments. Studies of the presence of enteric viruses in sewage, and the efficacy of virus removal by various sewage treatment systems, is therefore of interest.

Present water quality assessments rely on the use of bacterial indicators, which do not sufficiently reflect the occurrence of enteric viruses (Gerba *et al.* 1979; Keswick *et al.* 1984; Bosch 1998). Monitoring specific virus pathogens in water samples would provide more reliable information for risk assessments of waterborne viral infections. Direct monitoring of several viral pathogens in water is, however, impractical. AdV have been proposed as an indicator for enteric viruses due to their high prevalence in sewage (Pina *et al.* 1998b). Like AdV, human circoviruses (huCV) are small, non-enveloped DNA viruses. The two main types of huCV, TT virus (TTV) and TTV-like-mini-virus (TLMV), give persistent infections with continuous viral replication. Moreover, these viruses are shed in faeces, and appear to be present in the majority of people worldwide (Takahashi *et al.* 1998; Huang *et al.* 2001; Moen *et al.* 2002). These qualities suggest that huCV may also be suitable as indicators of viral faecal contamination.

The present study was conducted to obtain information about: the circulation of enteric viruses in the Norwegian population; seasonal differences in the occurrence of various viruses; to what extent enteric viruses are released into the environment from different types of sewage plant; and to indicate which viruses may be suited as indicators of viral contamination from sewage. NV were genotyped to

map seasonal or geographical distribution of different strains. AdV and huCV were evaluated as indicators and the reduction of infective viruses in the treatment plants was evaluated by quantification of F-specific bacteriophages (F-phages). Three treatment plants were included: two serving a densely populated area, and a less advanced plant serving a small municipality. Treated sewage from the latter was discharged in the vicinity of shellfish harvest areas.

## MATERIALS AND METHODS

### Sewage treatment plants

Plant A receives approximately 280,000 person equivalents (p.e.) and treats 35–40 million m<sup>3</sup> sewage annually. Plant B serves a population of 437,000 and treats 110–130 million m<sup>3</sup> sewage annually. Both are secondary treatment plants, using coagulation and biological treatment, serving the Oslo area. Plant A uses activated sludge and includes a sedimentation step at the end of the process. Plant B uses a biofilm process. The operation period in plant A is 17 h and 3 h in plant B. Plant C is a primary treatment plant with a screen, receiving sewage from approximately 1,800 inhabitants of a rural community in the mid-region of Norway.

### Sewage samples

Samples of raw and treated sewage were collected at the same time of day approximately every two weeks between October 2001 and October 2003. A total of 145 raw and 118 treated sewage samples were collected: 49 raw and 47 treated samples from plant A, 51 and 47 samples from B, and 43 and 24 samples from plant C. In plants A and B, samples of inlet and outlet sewage were automatically collected every 5 to 10 minutes and mixed to represent a period of 24 hours. Fifty millilitres of these 24-hour composite samples were collected on the same days and were kept at 4 to 10°C until tested for F-phages, no more than 6 h after sampling. Samples from plant C were shipped overnight and analysed for F-phages the following day. All samples were frozen and kept at –20°C prior to the molecular detection of enteric viruses. Samples were tested for the presence of various viruses as detailed below.

A sufficient number of samples were included for each virus in order to indicate the prevalence of the virus in question, and its suitability as an indicator of faecal contamination.

### F-phages

The *Escherichia coli* host bacterium HS(pFamp)R and the double agar layer method were used for the detection of F-phages (Debartolomeis & Cabelli 1991). The *E. coli* HS(pFamp)R was kindly provided by Dr M. D. Sobsey, University of North Carolina, USA. The HS(pFamp)R host bacterium is relatively resistant to infection with somatic DNA phages; phages plaquing on HS(pFamp)R are mainly F-RNA or F-DNA phages (Debartolomeis & Cabelli 1991). Sewage samples were tested undiluted, except for the raw sewage from plants A and B, which was diluted 1:10 in sterile water. Briefly, one ml aliquots were mixed with 5 ml Tryptic Soy Broth (TSB) semisolid agar (0.7%), containing 0.015% each of ampicillin and streptomycin, and 80 µl Famp in exponential growth phase. After mixing, the samples were poured onto TSB solid agar (1.5%) and incubated at 37°C for 18 h. The total number of plaques made by F-phages (RNA and DNA phages) was counted. Parallel samples were incubated with RNase to select for F-specific DNA bacteriophages (F-DNA phages), and thereby to estimate the concentration of F-specific RNA bacteriophages (F-RNA phages).

### Recovery of viral particles for nucleic acid detection

A modified version of a method previously described was used (Puig *et al.* 1994). Sewage samples (13 ml) were centrifuged at  $135,000 \times g$  for 90 min at 4°C using a SW40 rotor in a Beckman ultra centrifuge. The pellets were dissolved in 5 ml of glycine buffer (0.25 M glycine, 0.15 M NaCl, pH 9.5) by stirring for 16 h at 4°C. The samples were diluted to 13 ml with phosphate buffered saline (PBS) and centrifuged at  $4,300 \times g$  for 15 min to remove particulate material. The supernatants were then centrifuged at  $135,000 \times g$  for 90 min at 4°C and the pellets (viral concentrates) dissolved in 200 µl PBS. The viral concentrates were kept at -70°C until extraction of nucleic acids. Negative controls (i.e. PBS) were included for every fifth sample and processed like the sewage samples. Positive

controls were not included at this step. Outlet samples from plants A and B had low contents of particulate material, which allowed for the extraction of nucleic acids from the pellets originating from the first ultra centrifugation.

### Extraction of RNA and DNA

RNA was extracted from 100 µl of viral concentrates after addition of 900 µl of a guanidithiocyanate (GuSCN) lysis buffer containing silica particles (Boom *et al.* 1990). The samples were incubated for 10 min at room temperature, vortexed and centrifuged ( $12,000 \times g$  for 15 s). The silica particles were subsequently washed twice with washing buffer (GuSCN in 0.1 M Tris hydrochloride, pH 6.4), twice with 70% ethanol, and once with acetone. The particles were then dried at 56°C for 10 min, and the RNA eluted in 80 µl diethyl pyrocarbonate-treated water with 160 µM RNase inhibitor (ribonucleoside vanadyl complexes; Sigma). The RNA was stored at -70°C until use in reverse transcription (RT)-PCR. Viral DNA was extracted from 100 µl of viral concentrates supplemented with 100 µl of ddH<sub>2</sub>O using the High Pure Viral Nucleic Acid Kit (Roche). The DNA was eluted in 50 µl of the provided elution buffer, and either used immediately or stored at -70°C.

### RT nested PCRs for NV, AV, RV and HAV

The OneStep RT-PCR Kit (Qiagen) was used. A 5 µl sample of RNA extract, corresponding to 400 µl of sewage, was included in each of the four separate 50 µl RT-PCRs. RV ds-RNA was heat denatured at 95°C for 5 min and rapidly cooled on ice prior to addition to the RT-PCR mix. The primers and cycling conditions for the RT nested PCRs are listed in Tables 1 and 2. In order to increase the specificity, touch down procedures were used for the NV, AV and RV PCRs. An elevated annealing temperature was used in the first cycle. Then the temperature was reduced by 0.5°C (NV) or 1°C (RV and AV) per cycle for the next 14 cycles, thereby reaching the annealing temperature used in the last 25 (NV), 10 (RV) or 5 (AV) cycles. Each run included negative (water) and positive controls. The positive controls consisted of an HAV positive serum sample or faecal samples with either NV, AV or RV. A nested (NV and HAV) or semi-nested (AV and RV) real time PCR was performed in

**Table 1** | Primers used for the various (RT)-PCRs and (semi)nested PCRs

Virus	Primer	Sequence	Location <sup>a</sup>	Amplicon (bp)	Reference
<b>NV</b>					
RT-PCR	MJV12	tay cay tat gat gch gay ta	4553–4572	327	(Vinje <i>et al.</i> 2004)
	Reg A	ctc rtc atc icc ata raa iga	4859–4879		
nested PCR	p290	gat tac tcc aag tgg gac tcc ac	4568–4590	204	(Jiang <i>et al.</i> 1999)
	Mp290	gat tat act ssm tgg gay tcm ac	4568–4590		(Myrmel <i>et al.</i> 2004)
	rev SR46	cca gtg ggc gat gga att cca	4754–4773		(Ando <i>et al.</i> 1995)
	rev SR48-52	cca rtg rtt tat rct gtt cac	4754–4773		(Ando <i>et al.</i> 1995)
semi nested (for RLB typing)	p290/Mp290 Reg A	biotinylated			
<b>AV</b>					
RT-PCR	Mon 340	cgt cat tat ttg ttg tca tac t	1182–1203	289	(Belliot <i>et al.</i> 1997)
	Mon 348	aca tgt gct gct gtt act atg	1450–1470		
semi nested	Mon 394 Mon 348	gar atc cgt gat gct aat gg	1250–1269	220	(Belliot <i>et al.</i> 2001)
<b>RV</b>					
RT-PCR	Beg 9	ggc ttt aaa aga gagaat ttc cgt ctg g	1–28	392	(Gouvea <i>et al.</i> 1990)
	R4	gat cct gtt ggc cat cc	376–392		
semi nested	RFP5 R4	gta tgg tat tga ata tac cac	51–71	342	(Flores <i>et al.</i> 1990)
<b>HAV</b>					
RT-PCR	HAVextF	gtt aat gtt tat ctt tca gca at	2132–2154	310	This study
	HAVextR	gat ctg atg tat gtc tgg att ct	2419–2441		
nested PCR	HAVintF	gtt ttg ctc ctc ttt atc atg cta tg	2167–2192	247	(Robertson <i>et al.</i> 1991)
	HAVintR	gga aat gtc tca ggt act ttc ttt g	2389–2413		
<b>AdV</b>					
PCR	AdVof	gac atg act ttt gag gtg gac cc	21545–21567	140	(Myrmel <i>et al.</i> 2004)
	AdVor	ccg gcc gag aag ggc gt	21668–21684		
nested PCR	AdVif	ttt gag gtg gac ccc atg ga	21554–21573	125	
	AdVir	gag aag ggc gtg cgc agg ta	21659–21678		
<b>TTV and TLMV</b>					
PCR	TTV/TLMVf	tcc gaa tgg ctg agt tt	102–118	118	(Myrmel <i>et al.</i> 2004)
	TTV/TLMVr	cga att gdd cct tga ct	203–219		
TTV nested (all four primers included in each reaction)	TTVfa	gtt ttc tac gcc cgt cc	115–131	96	
	TTVfb	gtt ttc yac gcc cgt cc			
	TTVra	cct tga ctc cgg tgt gta a	192–210		
	TTVrb	cct tga ctb cgg tgt gta a			
TLMV nested	TLMVf	agt tta tgc cgc cag acg	193–210	95	
	TLMVr	ccc tag act tcg gtg gtt tc	268–287		

<sup>a</sup>Nucleotide positions are in reference to Norwalk virus (M87661), human AV-2 (L13745), RV serotype G1 strain Wa (M21843), HAV strain HM175 (M16632), human AdV (M73260), TTV genome TA278 (AB008394), and TLMV reference strain CBD231 (AB026930).

**Table 2** | Reaction and cycling conditions used for the detection of enteric RNA viruses

Virus	Primer concentrations	Cycling conditions
<b>NV</b>		
RT-PCR	0.6 $\mu$ M each	37°C (30 min), 95°C (15 min) 94°C (60 s), 50°C (– 0.5°C/cycle) (90 s), 72°C (60 s) $\times$ 15 94°C (60 s), 37°C (90 s), 72°C (60 s) $\times$ 25 72°C (7 min)
Nested PCR	0.32 $\mu$ M each	95°C (15 min) 94°C (20 s), 49°C (90 s), 72°C (30 s) $\times$ 40 Optical read at 78°C
Semi nested	0.3 $\mu$ M each	Like nested, except 72°C (7 min) instead of optical read
<b>AV</b>		
RT-PCR	0.3 $\mu$ M each	50°C (30 min), 95°C (15 min) 94°C (20 s), 65°C (– 1.0°C/cycle) (30 s), 72°C (30 s) $\times$ 15 94°C (20 s), 50°C (30 s), 72°C (30 s) $\times$ 5 72°C (7 min)
Semi nested	0.3 $\mu$ M each	95°C (15 min) 94°C (20 s), 50°C (45 s), 72°C (30 s) $\times$ 40 Optical read at 75°C
<b>RV</b>		
RT-PCR	0.3 $\mu$ M each	Like AV, except 10 cycles of annealing at 50°C
semi nested	0.3 $\mu$ M each	Like AV, except annealing at 54°C and optical read at 74°C
<b>HAV</b>		
RT-PCR	0.4 $\mu$ M each	45°C (30 min), 95°C (15 min) 94°C (30 s), 45°C (60 s), 72°C (30 s) $\times$ 40 72°C (7 min)
Nested PCR	0.4 $\mu$ M each	95°C (15 min) 94°C (20 s), 50°C (65 s), 72°C (30 s) $\times$ 40 Optical read at 76°C

order to improve detection. The QuantiTect SYBRGreen PCR Kit (Qiagen) was used in the nested reactions, in which 0.5  $\mu$ l aliquots of RT-PCR products were included in a 25  $\mu$ l PCR mix. Real time PCR was performed in a SmartCycler (Cepheid). Each run ended with a melting curve analysis.

#### Nested PCRs for AdV, TTV and TLMV

The nested PCRs for AdV, TTV and TLMV were performed as previously described (Myrmel *et al.* 2004). The primers employed, designed to detect human variants, are included

in Table 1. The PCR products were analysed by agarose gel electrophoresis (2% agarose with ethidium bromide). Each run included negative (water) and positive controls. The positive controls consisted of AdV obtained from cell culture, or serum samples containing either TTV or TLMV. All controls were diluted to 1–2 logs above endpoint in PCR titration.

#### Seasonal variation

Seasonal variations in the prevalence of NV, AV and RV in raw sewage were tested by separating all samples from the

three plants into two groups according to the date of collection. The summer period included samples collected in April–September and the winter period included samples from October–March. The significance of the correlations was tested with the Pearson Chi-square (2-sided) test.

### Subtyping and sequencing

A subset of 64 samples that were positive in the NV nested real time PCR were examined by reverse line blot hybridisation (RLB) (Vinje & Koopmans 2000) for verification and genotyping, as previously described (Myrmet *et al.* 2004). The products from the nested real-time PCR were too short to include all the binding sites for the 18 different probes used in the hybridisation procedure. Therefore, the outer RT-PCR products were used in a semi nested PCR (primers p290/Mp290 and biotinylated RegA) to produce DNA fragments of sufficient length for RLB.

The real time PCR products from seven NV positive samples, which could not be genotyped by RLB, as well as seven AV, eight RV and five HAV positive samples were sequenced in order to verify the authenticity of the PCR products. The products were sequenced in both directions using the MegaBACE 1000 Sequencing System (Amersham Biosciences), the ABI BigDye Terminator Cycle Sequencing

kit (Applied Biosystems) and the (semi)nested primers referred to in Table 1. The nucleotide sequences were analysed using Vector NTI (InforMax) and aligned to sequences available in the GenBank.

## RESULTS

### Viral prevalence

The detection rates of NV, RV, AV, AdV, HAV, TTV, TLMV and F-phages in raw and treated sewage samples from the three plants are displayed in Table 3. As can be seen, the number of NV, AV and RV positive samples were reduced upon treatment in plants A and B, but the reductions were not statistically significant. In plant C there was no reduction in positive samples between inlet and outlet sewage. HAV was found in two inlet samples from each of plants A and B, and in one outlet sample from plant B. Two of the HAV positive samples were collected 14 days apart, from plants A and B, while the remaining three were separated by 5–7 months. AdV was found in 24 of a total of 25 (96%) raw sewage samples and in 15 of 16 (94%) treated samples. No TLMV and only three TTV positive samples were found in the 24 samples tested.

F-phages were found in all the inlet samples from the three plants, in 26 of 31 outlet samples (84%) from plant A,

**Table 3** | The presence of F-bacteriophages and enteric viruses in raw and treated sewage samples from plants A, B and C collected between October 2001 and October 2003<sup>a</sup>

Plant	Pos/n <sup>b</sup>	F-phages		DNA <sup>d</sup>	No. of positive samples/no. tested (%)						
		Total <sup>c</sup>	(Range)		NV	AV	RV	HAV	AdV	TTV	TLMV
<b>A</b>											
Inlet	31/31	611	(75–1,800)	502	21/49 (43)	33/35 (94)	29/35 (83)	2/25	11/11	3/10	0/10
Outlet	26/31	6	(0–18)	2	12/47 (26)	17/24 (71)	21/31 (68)	0/20	9/9	0/2	0/2
<b>B</b>											
Inlet	30/30	271	(70–1,100)	149	27/51 (53)	28/34 (82)	26/36 (72)	2/21	9/9	0/6	0/6
Outlet	30/30	36	(5–88)	18	19/47 (40)	16/20 (80)	15/27 (56)	1/23	6/7	0/3	0/3
<b>C</b>											
Inlet	15/15	86	(6–270)	110	10/41 (24)	12/26 (46)	12/32 (38)	0/19	4/5	0/3	0/3
Outlet	15/15	86	(3–280)	109	5/24 (21)	6/13 (46)	5/14 (36)	0/7	nd <sup>e</sup>	nd	nd

<sup>a</sup>The samples were analysed by plaque assay (F-phages) and (RT)-nested PCR (NV, AV, RV, HAV, AdV, TTV and TLMV).

<sup>b</sup>No. of samples positive for F-phages/no. tested.

<sup>c</sup>Mean no. of PFU of F-RNA and F-DNA phages per ml sewage.

<sup>d</sup>Mean no. of PFU of F-DNA phages per ml sewage.

<sup>e</sup>Not done.



and in all the outlet samples from plants B and C. There was a 2 log reduction, a 0.89 log, and no reduction in the concentration of F-phages during treatment in plants A, B and C, respectively. The estimated mean PFU of F-RNA phages per ml raw and treated sewage was, respectively, 109 and 4 in plant A, compared with 122 and 36 in plant B. In plant C there appeared to be only F-DNA phages, but RNase treatment of plated samples actually increased the number of plaques.

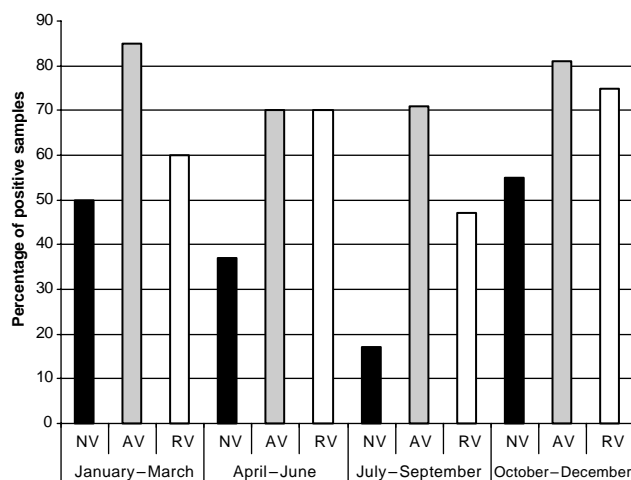
### Seasonal variation

A statistically significant seasonal variation ( $p \leq 0.01$ ) was found for NV in raw sewage. The prevalence was higher (53%) in the cold season, October–March, than in the summer (28%), April–September. The prevalence of AV and RV did not vary significantly between the summer and winter period. A quarterly distribution of NV, AV and RV is displayed in Figure 1.

### Genotyping and verification of PCR results

The (semi)nested NV, AV, RV and HAV real time PCRs all gave distinct melting curve diagrams, although a variability in melting point temperatures ( $T_m$ s) were registered for each group of viruses (Figure 2). Primer dimers or other non-specific products were not observed.

Of the 64 NV semi nested PCR products tested in RLB, 47 were positive for genogroup II. Only 33 of these 47



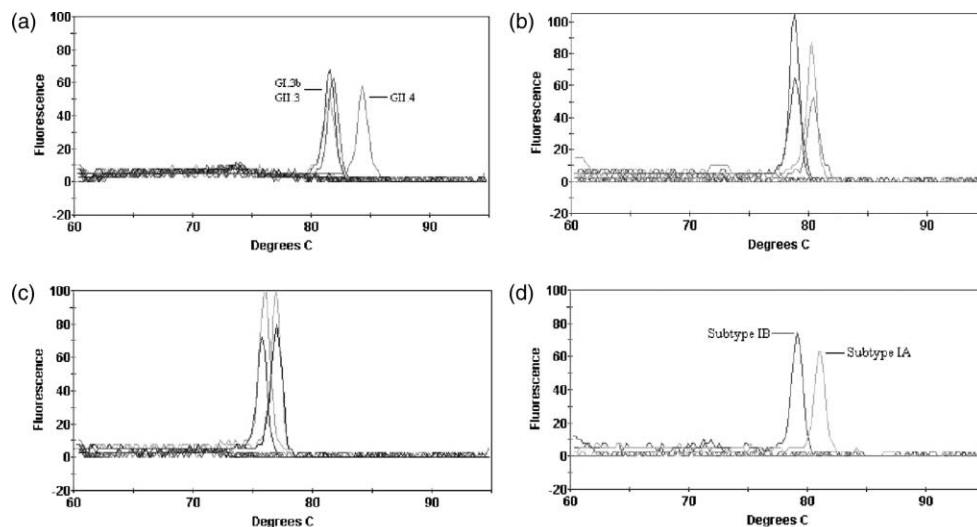
**Figure 1** | Seasonal distribution of noroviruses (NV), astroviruses (AV) and rotaviruses (RV) in raw sewage samples.

products reacted with one of the probes designed to distinguish between genotypes within this genogroup. The Lordsdale genotype was the most prevalent strain (detected in 26 samples) and was found regularly during the 2-year period. The Melksham genotype was found sporadically in nine samples, while the six samples positive for the Leeds genotype clustered in March and April 2003. The Wortley genotype (16 samples) was found in two clusters, one in November and December 2002, the other in March and April 2003. Genogroup I strains were detected in 34 samples, but only eight could be genotyped (as belonging to either the Norwalk, Desert Shields or Sindlesham genotype). Multiple genotypes (2–5) were detected in 31 samples. The same spectrum of strains were detected in all three plants. Interestingly, during a particular period the Wortley strain was found in all three plants.

Seven of the 64 NV PCR products did not hybridise with any of the group or strain-specific probes. Sequencing revealed that five of them belonged to genogroup II (GII.1, GII.3 and GII.4), and two belonged to genogroup I (GI.3b), when compared with the genotypes outlined by Vinje *et al.* (2004). The above NV sequences represented genotypes with different melting points in the real time PCR (Figures 1 and 2). Similarly, seven AV, eight RV and five HAV nested PCR products, representing different  $T_m$ s of each virus group, were sequenced. All sequences confirmed the expected viral origin of the amplicons, and that the differences in  $T_m$  reflected variations in nucleotide sequences. Four of the five HAV positive samples had unique sequences; the single HAV positive outlet sample from plant B contained a genotype IB strain, whereas the other strains were classified as IA, as outlined by Costa-Mattioli *et al.* (2003). The IA strains showed a variability of 2–6%, while the IB genotype differed from the IA strains in 9–10% of the base positions. Two samples collected two weeks apart from plants A and B contained HAV strains, subtype IA, with identical sequences (190 bp).

## DISCUSSION

NV are the main agents associated with waterborne outbreaks of viral gastroenteritis in Norway (Nygard *et al.* 2003). The relatively high prevalence (24–53%) of this virus



\*The observed variations in  $T_m$  (melting point temperature) within each group of viruses were found to reflect nucleotide sequence differences, as specified for NV and HAV.

**Figure 2** | Real time PCR melting curve analyses for: (a) NV; (b) AV; (c) RV and (d) HAV\*.

in raw sewage presumably reflects the frequency of NV-caused gastroenteritis. Although the number of positive samples was reduced upon treatment in plants A and B, the output from the plants still contained viral nucleic acids. In plant C, a small primary treatment plant, there was no appreciable reduction in either F-phage numbers or NV positive samples, while in the more advanced plants there was a 0.89–2.0 log reduction of the F-phages. Most likely some of the NV detected in outlet samples from all three plants reflect infectious virus particles. This assumption is based on the view that the RNA is easily degraded if the viral particles disintegrate, and on the fact that infective F-RNA phages were isolated from treated sewage samples. Yet, one would expect, as has been shown in a study on enterovirus in treated sewage (Gantzer *et al.* 1998), that the number of samples positive by RT-PCR is significantly higher than the number of samples positive by cell culture. Currently the question of infectivity cannot be tested for NV owing to the lack of a cell culture system (Atmar & Estes 2001). The method used to enrich for viruses included ultra centrifugation and elution. We did not test the method for yield, but assume that the previously published figure of 70% recovery for adenoviruses (Puig *et al.* 1994; Pina *et al.* 1998b) is relevant in the case of noroviruses as well. This method has also been employed in the recovery of hepatitis

A virus, hepatitis E virus and polyomaviruses from sewage (Pina *et al.* 1998a, 2001; Bofill-Mas *et al.* 2000) with an equal sensitivity as for the detection of adenoviruses. Hepatitis A, hepatitis E, polyoma- and noroviruses are all none enveloped viruses with a particle size in the same range.

Although real time protocols were used for the final PCR detection of NV, AV, RV and HAV, we did not consider the data quantitative as the real time PCR was the second step in nested PCRs.

The results on NV and F-phages suggest that plant A was more effective than plant B in virus reduction. In plant B there was an equal distribution of F-RNA and F-DNA phages in inlet and outlet samples. The reduction in the amount of both F-phages was 87%. In plant A, however, the F-DNA phages were reduced by 99%, and the estimated reduction of F-RNA phages was 96%. The higher resistance of F-RNA phages than F-DNA phages to treatment in plant A was confirmed by the relative amount of plaques from F-RNA-phages in raw (18%) compared with treated sewage (80%).

GII was the dominating NV genogroup (found in 52 of 64 samples), and Lordsdale the dominating NV genotype, a result also reflected in a previous study on Norwegian faecal samples (Vainio *et al.* 2001). More surprisingly, GI strains were detected in 36 of 64 samples, a prevalence appreciably higher than what might be expected based on NV in clinical



samples (Vainio *et al.* 2001; Fankhauser *et al.* 2002; Reuter *et al.* 2002). This discrepancy could indicate that GI strains more often cause sub-clinical infections. The finding of the same NV genotype (i.e. Wortley strain) in all three plants during one month suggests that NV has an ability to spread rapidly in a population.

Waterborne transmission of gastroenteritis viruses, other than NV, has not been reported in Norway. The lack of reports, however, does not necessarily imply that such transmissions are excluded. During 2001 and 2002, the Norwegian Institute of Public Health registered a yearly average of respectively 576, 266, 0, 530 (of which 300 were gastroenteritis) and 76 cases of RV, NV, AV, AdV and HAV infections. The majority of infections caused by these viruses are probably relatively mild or asymptomatic, and therefore normally not reported. An exception to this may be HAV infections, RV infections in infants and possibly outbreaks of gastroenteritis caused by NV. NV induce a relatively short-lived immunity causing individuals to remain susceptible throughout life (Parashar & Glass 2003), while RV, AV and AdV cause clinical symptoms primarily in specific segments of the population: infants, the elderly or immunosuppressed individuals. Consequently, the likelihood of bringing attention to non-NV viral gastroenteritis may be restricted. Moreover, the number of geographically related cases is less likely to be sufficient to consider water as a vehicle of transmission, or to warrant reports to health authorities.

The prevalence of positive PCRs for AV, RV and AdV in raw sewage was high (38–100%), particularly in the samples from the urban area (Oslo) (72–100%), indicating that these viruses are continuously present in densely populated communities. Astroviruses are increasingly being recognised as gastrointestinal pathogens (Palombo & Bishop 1996; Dennehy *et al.* 2001). However, the absence of reported cases of AV induced gastroenteritis in Norway presumably reflects that this agent rarely causes severe disease. In a study from The Netherlands (Lodder *et al.* 1999), the concentration of NV in sewage was higher than that of RV. The discrepancy between this study and the present results may be due to methodological differences.

Hepatitis A virus was detected in only 4 of 65 raw sewage samples and in 1 of 50 treated samples. The

infrequent detection corresponds to the low number of HAV infections registered. The strain variability indicates different origins of the strains, and supports the observation that the majority of cases are contracted abroad, although infections through sharing of needles among drug abusers are also common (Stene-Johansen *et al.* 1998). However, the finding of HAV in treated sewage emphasises the fact that water contaminated with sewage may constitute a risk of infection, particularly in a population, such as the Norwegians, with a low HAV immunity (Pebody *et al.* 1998). Moreover, an increase of infected asymptomatic children travelling from endemic areas may augment the problem (Wilson & Kimble 2001).

The almost universal presence of AdV in both raw and treated sewage samples (39 of 41) supports the proposal to use this group of viruses as an indicator for sewage contamination. The present PCR assay was designed to detect only human AdV. Enteric strains will probably dominate in sewage samples and it is assumed that the present results primarily reflect human enteric AdV (subtypes 40 and 41).

The number of samples tested for TTV and TLMV was low, but the present prevalence (12.5% for TTV) is close to the prevalence (12.7%) found in a study on sewage from a treatment plant in India (Vaidya *et al.* 2002). The low prevalence of TTV is somewhat surprising, considering the ubiquity of the infection in the population (Huang *et al.* 2001). The result may reflect the fact that most people shed a low number of viruses in faeces, or that the viral particles are unstable in the sewage environment.

Data from the present study, and from a previous study on enteric viruses in Norwegian shellfish (Myrmet *et al.* 2004), suggest that AdV or F-RNA phages are better choices as sewage indicators than human circoviruses. As proposed by others (Havelaar 1987a, b), F-RNA phages may be particularly useful owing to their high prevalence in sewage, their resistance to environmental degradation and their ease of detection. However, the F-RNA phages may originate from the intestines of both humans and animals. In the present study F-RNA phages were found in all the inlet samples and in 92% of the treated samples from plants A and B. In plant C, RNase treatment of plated samples actually resulted in an increased number of plaques, which may indicate that the F-RNA phages

interfere with the replication of F-DNA phages. Little is known about the reservoir and environmental resistance of F-DNA phages; however, they were ubiquitous in the sewage samples examined.

The present viral prevalence was relatively high compared with some previous studies. Variations in sensitivity regarding virus recovery and detection may contribute to the difference. For example, two studies from Bangkok reported that, respectively, 8% and 0% of raw sewage samples were ELISA positive for RV (Kittigul *et al.* 2000, 2001); in Sao Paulo 21% of the samples were positive for RV using indirect immunofluorescence (Mehnert & Stewien 1993). Moreover, although an RT-semi nested PCR was used in a study from Barcelona, only 4 of 15 sewage samples were positive for RV (Gajardo *et al.* 1995). A similar study in France, however, reported a prevalence of RV in raw sewage of 42% (Dubois *et al.* 1997), which is closer to the present results. A study on AV in France and Spain also showed a relatively low prevalence (Pinto *et al.* 2001).

The period of sample collection may also contribute to the results. The present study showed a higher prevalence of NV, RV and AV during the cold season, but the difference was statistically significant only for NV. Although most types of viral gastroenteritis appear to be more common in winter, this seasonal distribution is best documented in the case of NV and RV (Koopmans & Brown 1999; Hedlund *et al.* 2000; Mounts *et al.* 2000; Vainio *et al.* 2001). The dominance of viral gastroenteritis during the cooler months, which resembles that of viral infections spread by the respiratory route, is not fully explained. However, increased viral stability in the environment due to lowered temperature, as found in studies on poliovirus, HAV and astrovirus (Bosch 1995; Abad *et al.* 1997), could promote waterborne gastroenteritis during winter, and thereby a higher viral load in sewage.

The present results demonstrate the benefits of employing a more advanced treatment of sewage. The frequent detection of gastroenteritis viruses in small volumes of both raw and treated sewage indicates that sewage may be a source of viral dissemination. Current treatment of drinking water in well operated water plants is presumably sufficient to inactivate most viruses, but the environmental spread of enteric viruses may still contribute to an endemic situation of viral gastroenteritis (Payment 1999; Baggi & Peduzzi 2000; Gofti-Laroche *et al.* 2003). The release of infectious

viruses into the environment is a concern, particularly with regard to the use of contaminated water in food production, i.e. the use of fresh water for irrigation, the use of marine water for culturing of shellfish, as well as the use of water for recreation.

## CONCLUSIONS

Noro-, astro-, rota- and enteric adenovirus were frequently detected by (RT)-PCR in small volumes of raw and treated sewage from two secondary treatment plants in the Oslo area and from a primary plant in a small rural community. Hepatitis A virus was found sporadically in the sewage from the Oslo area. Reduction of F-specific bacteriophages was used to estimate the efficiency of sewage treatment. The two secondary treatment plants, including either coagulation/activated sludge or coagulation/biofilm, reduced the concentration of F-specific bacteriophages by 99% and 87%, respectively. No reduction was found in the small primary treatment plant. The viral load in raw and treated sewage is high and may represent a source of low-level transmission of enteric viruses contributing to an endemic situation of gastroenteritis.

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