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Immunomagnetic separation of a Norwalk-like virus (genogroup I) in artificially contaminated environmental water samples

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Abstract

Rabbit polyclonal antibodies were raised against a recombinant capsid protein from a genogroup I Norwalk-like virus (NLV). Magnetic beads coated with these antibodies were used in immunomagnetic separation (IMS) of the NLV. After capture of the NLV and washing of the beads, viral RNA was heat released and detected by RT-PCR. This IMS procedure was shown to have high sensitivity for detection of homologous NLV, while capture of a genogroup II NLV was less efficient. Antigen capture was not influenced by the content of humic acids in the samples. The combination of IMS and heat release was found to be more efficient than organic extraction of RNA from water contaminated with humic acids. The efficacy and simplicity of IMS/heat release render this combination a feasible tool for the preparation of NLV RNA from environmental samples, although the antigenic diversity of NLV may be a complicating factor.

Keywords: Norwalk virus; Norwalk-like viruses; human calicivirus; immunomagnetic separation; RNA-extraction.

1. Introduction

Norwalk-like viruses (NLV) have a worldwide distribution and are common causes of gastroenteritis in humans (Estes and Hardy, 1995). NLV are transmitted via the fecal-oral route, and contaminated food and water, and person-to-person contact, are major sources of infection (Kaplan et al., 1982). Watersheds may get contaminated by virus through wastewater effluents from treatment plants and other sources of feces. Fecally contaminated drinking water and recreational waters have been implicated in several outbreaks of NLV gastroenteritis (Morens et al., 1979; Taylor et al., 1981; Beller et al., 1997; Baron et al., 1982; Kappus et al., 1982).

Taxonomically, the NLV belong to the human caliciviruses (HuCV) (Jiang et al., 1990; Jiang et al., 1993; Lambden et al., 1993; Lambden et al., 1995; Hardy and Estes, 1996). Sapporo-like viruses (SLV) make up the other HuCV subgroup, differing from

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the NLV in organization of the virus genome and epidemiology (Liu et al., 1995; Nakata et al., 1996). The genome of NLV consists of three open reading frames (ORFs) with a high degree of genetic variability. Based on nucleotide differences, NLV are divided into genogroups I and II (Wang et al., 1994; Ando et al., 1994; Lew et al., 1994; Cauchi et al., 1996; Berke et al., 1997; Wright et al., 1998). At present, genogroup II viruses seem to be most prevalent. A single structural protein makes up the capsid and is encoded by ORF2. The capsid protein contains highly variable regions which probably account for the antigenic diversity of NLV (Green et al., 1995b). There is no consensus antigenic classification system, but at least four antigenic types have been described (Lambden et al., 1993; Lewis et al., 1995).

Epidemiological investigations may indicate the source of an outbreak of viral gastroenteritis. Detection of enteric viruses in environmental samples has, however, been cumbersome and has not been routinely performed. As there is no in vitro cultivation system for NLV, the dominant tool for detection has been RT-PCR. However, organic and inorganic RT-PCR inhibitors in the environmental samples limit the application of this method (Tsai and Olson, 1992). Several steps of concentration of virus from environmental samples are usually required to achieve a volume suitable for RT-PCR. The product is a virus concentrate which may contain RT-PCR inhibitory substances. Removal of inhibitory compounds can be achieved by antigen capture with specific antibodies bound to a solid phase (Grinde et al., 1995; Schwab et al., 1996). Antigen capture also reduces the possibility of detecting non-infective virus particles or free RNA by subsequent RT-PCR. Immunomagnetic separation (IMS) utilizes magnetizable beads as the solid phase, and combines simple purification with concentration of the antigen into small volumes, which are suitable for RT-PCR.

The objective of this study was to raise antibodies against the capsid protein of a NLV and to establish a procedure for IMS of this virus. Viral RNA was heat released from antigen bound to the beads, and detected by RT-PCR. The efficacy of antigen capture was evaluated in water contaminated with humic acids and in a concentrate of water from a drinking water reservoir. Secondly, the sensitivity of IMS combined with RNA heat release was compared to that of organic extraction of viral RNA.

2. Materials and methods

2.1. Virus and virus antigen

The virus used as antigen and as origin for recombinant antigen production, originated from a fecal sample collected from a patient during an outbreak of gastroenteritis in a hospital in Stavanger, Norway, in 1995 (Hu/NLV/Stav/95/Nor). The feces was confirmed to contain a NLV by electron microscopy and was kindly provided by the National Institute of Public Health (NIPH). The fecal sample was diluted 1:10 in Hanks' salt solution (GibcoBRL, N.Y., NY) with antibiotics, stirred for 15 min and cleared by centrifugation (45 min at 1000 g). The supernatant (fecal extract) was stored in -70°C. If not otherwise specified, 'fecal extract' refers to the extract containing Hu/NLV/Stav/95/Nor.

The expression of a recombinant capsid antigen from Hu/NLV/Stav/95/Nor (rStav) in baculovirus has been described previously (Myrmel and Rimstad, 2000). The recombinant baculovirus was constructed by insertion of ORF2 from Hu/NLV/Stav/95/Nor. Nucleotide sequence analysis of ORF2 placed Hu/NLV/Stav/95/Nor into NLV genogroup I.

A second fecal extract (also provided by NIPH and prepared as described), contained a NLV which had previously been categorized as a genogroup II NLV by RT-PCR (Myrmel et al., 1999). This fecal extract was used to test the specificity of the IMS procedure.

2.2. Polyclonal antibodies against rStav

One rabbit was immunized subcutaneously with 100 μg rStav in Freund's complete adjuvant (GibcoBRL) followed by three booster injections of 70–100 μg in Freund's incomplete adjuvant, at three week intervals. Blood samples were collected prior to immunization and 5, 8, 10, 11 and 12 weeks post immunization (p.i.). Western blotting (WB) was used to evaluate the immune response and has been described earlier (Myrmel and Rimstad, 2000). An end-point dilution WB was used to identify the

serum sample with the highest antibody titer against rStav.

2.3. Purification of antibodies and coating of magnetic beads

Rabbit serum antibodies were precipitated by saturated ammonium sulphate at 4°C for 16 h (Harlow and Lane, 1988). Antibodies in pre-immunization serum were precipitated and used as a negative control. The precipitates were redissolved in PBS and dialyzed against PBS (pH 7.2) with 0.02% NaN_3 at 4°C for 16 h. The protein concentrations were estimated by the method described by Bradford (1976). Antibodies were stored at 4°C and −20°C (prolonged storage).

Magnetizable beads (Dynabeads M-280 with sheep anti-rabbit IgG) were kindly supplied by Dynal (Oslo, Norway). Five micrograms of precipitated protein were used for coating of 10^7 beads for 2 h at room temperature. Coated beads were washed four times (PBS pH 7.2, 0.02% NaN_3, 0.1% BSA) and stored at 4°C in washing buffer (6.7×10^8 beads/ml).

2.4. IMS procedure

Tenfold dilutions of fecal extract in 1 ml PBS (pH 7.2) (starting at 10^{-1} dilutions) were used to test the sensitivity of the IMS procedure. A total of 30 μl of beads was added to each sample and incubated on a roller at either 4°C for 16 h, or at room temperature for 1 or 3 h. Subsequent experiments were performed with incubation at 4°C for 16 h, as this incubation resulted in highest sensitivity. After antigen capture, the beads were magnetized and the supernatant removed. The beads were washed twice with 1 ml washing buffer and once with diethylpyrocarbonate-treated water (DEPC water) (Sigma, St. Louis, Mo) prior to the heat release of viral RNA and RT-PCR (see below).

To evaluate any influence of sample volume, tenfold dilutions of fecal extract were prepared in 10 ml and 50 ml of PBS. The same amount of beads (30 μl) was added to each sample. The beads were collected by a magnet, resuspended in 1 ml of washing buffer and transferred to a microcentrifuge tube. Centrifugation of the beads (2000 × g for 5 min) was compared to collection by magnet from the 50 ml samples.

2.5. Specificity of the anti-rStav antibody coated beads

The specificity of Hu/NLV/Stav/95/Nor binding to the anti-rStav antibody coated beads was examined. Uncoated beads and beads coated with pre-immunization antibodies were added to tenfold dilutions of fecal extract in 1 ml PBS (starting at 10^{-3} dilution). The specificity was evaluated as the ability of these beads to capture the antigen compared to beads coated with anti-rStav antibodies.

To test if the specificity was restricted to genogroup I NLV, IMS was performed using beads coated with anti-rStav antibodies or with pre-immunization antibodies, for capture of a genogroup II NLV diluted in PBS.

2.6. IMS in water contaminated with humic acids

Water contaminated with humic acids was collected from a bog pond. The PCR inhibitory effect of this water was demonstrated by the failure of amplification of 1 μl NLV positive PCR product in 30 μl of contaminated water (a total PCR volume of 50 μl). Tenfold dilutions (10^{-4}–10^{-7}) of the Hu/NLV/Stav/95/Nor fecal extract were prepared in 1 ml of humic acid-contaminated water, and used for IMS and subsequent RT-PCR.

2.7. IMS in concentrates of raw water

A procedure including adsorption to a positively charged filter and subsequently elution, flocculation and IMS, has been described for the concentration of hepatitis A virus from water and sewage (Jothikumar et al., 1998). A similar procedure utilizing adsorption to a negatively charged membrane filter, elution, flocculation and organic extraction of RNA from the virus concentrate, has previously been applied on environmental water inoculated with NLV (Myrmel et al., 1999). In the present study, IMS of NLV in the

water concentrate, i.e., the resulting product after the adsorption-elution/flocculation procedure, was tested. Raw water was collected from the drinking water reservoir supplying Oslo. Five liters were conditioned (by adding 6 g of $MgCl_2^{\cdot}.6H_2O$ and adjusting to pH5) and filtered through a negatively charged cellulose ester filter, 142 mm in diameter and 0.45-μm pore size (HAWP 142 50, Millipore, Bedford, MA). The filter was soaked in 50 ml of urea arginine phosphate elution buffer (pH9), which was subsequently forced through the filter. After flocculation with 1 M $MgCl_2^{\cdot}.6H_2O$, and centrifugation, the precipitate was solubilized in 4 ml of McIlvaines buffer (pH5). A 10^{-4} dilution of the fecal extract was prepared in this buffer (4 ml), and IMS with 30 μl of beads, and subsequently RT-PCR, was performed.

2.8. Extraction of RNA

The efficacy of IMS combined with the heat release of viral RNA, was compared to the extraction of RNA with guanidine isothiocyanate (GIT). TRIzol reagent (600 μl) (GibcoBRL) and chloroform (120 μl) were used for the extraction of RNA from 700 μl of fecal extract which was diluted tenfold in PBS and in bog pond water. After precipitation with isopropanol, the RNA was washed with 75% ethanol, dissolved in 30 μl of DEPC water, and immediately used for production of cDNA (see below).

2.9. Negative controls

All experiments were performed at least in duplicates and included a negative control consisting of a sample free of virus (buffer or water) which was treated identically to the spiked samples. During optimization of the IMS procedure, positive amplicons were registered in some of the negative controls. Contamination was suspected to occur with virus particles, not PCR products, during the washing of the beads. The following procedure was then established: beads in parallel samples were washed and resuspended in DEPC water before the same procedure was applied on the next samples. The negative control was processed at the end. A quick spin was performed, prior to magnetizing of the beads and opening of the tubes.

2.10. Heat release and RT-PCR

Prior to heat release, the washed beads were dissolved in 30 μl of DEPC water. RNA was released from the viral particles by heating at 99°C for 5 min. After chilling on ice, the beads were magnetized and the RNA-containing supernatant (like the TRIzol extracted RNA) was transferred to a tube containing a 20 μl mix of DEPC water, RNase inhibitor, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, M-MuLV Reverse Transcriptase and 2 U of *Taq* polymerase (Ready-To-Go RT-PCR beads, Amersham Pharmacia Biotech, Uppsala, Sweden). First strand synthesis was primed with 1 μM random hexamers (Amersham Pharmacia Biotech) and was run for 30 min at 42°C. Specific primers (0.5 μM) were added after the first strand synthesis to avoid non-specific annealing during the RT-reaction. The primers Stav1F/Stav1R were designed according to the sequence of Hu/NLV/Stav/95/Nor ORF2 and are listed in Table 1. A primer pair designed by Ando et al. (1995) (SR33/SR46) was used for amplification of the genogroup II NLV. Incubation at 95°C for 5 min preceded the PCR. The Hu/NLV/Stav/95/Nor PCR was run for 40 cycles of 94°C (30 s), 62°C (30 s) and 72°C (30 s), with a final elongation at 72°C for 7 min. The primer pair Stav1F/Stav1R should give an amplicon of 246 basepairs (bp). The sensitivity of the RNA purification procedures was evaluated according to the results of this PCR. Initially, the specificity of the

Table 1
The nucleotide sequences of primers used for detection of Hu/NLV/Stav/95/Nor[a]

Identification	Sequence (5′–3′)	Location
Stav 1F	CCCATCTCAATCCTTTCTTATC	260–281
Stav 1R	GCACATTCCTCACATCTTCC	505–486
Stav 2F	TATTAGTTGCATACCCCC	372–389
Stav 2R	CCAAAACCCTAACATCAGC	466–447

[a] The locations are related to the Norwalk virus capsid sequence.

amplicon was examined using a nested PCR with the primers Stav2F/Stav2R (Table 1). The PCR product was used as target DNA in the nested reaction. The $MgCl_2$ concentration was set to 2 mM and 0.25 μM of each primer were used. The temperature profile was 94°C for 3 min, 20 cycles of 94°C (30 s), 55°C (30 s), 72°C (30 s) and elongation at 72°C for 7 min. The nested reaction should give an amplicon of 95 bp. The genogroup II PCR, with primer pairs SR33/SR46, was run for 40 cycles of 94°C (30 s), 50°C (30 s) and 72°C (30 s), with a final elongation at 72°C for 7 min and should give an amplicon of 123 bp.

The PCR products were run in a 4% Nusieve 3:1 agarose gel (FMC Bio Products, Rockland, ME, USA) and visualized by UV illumination after ethidium bromide staining.

3. Results

3.1. Polyclonal antibodies

Antibodies against rStav were detected by WB in all rabbit sera collected p.i. (Fig. 1). The serum sample collected 10 weeks p.i. showed the highest end-point anti-rStav titer in WB (1:400 000) and was used in the subsequent experiments.

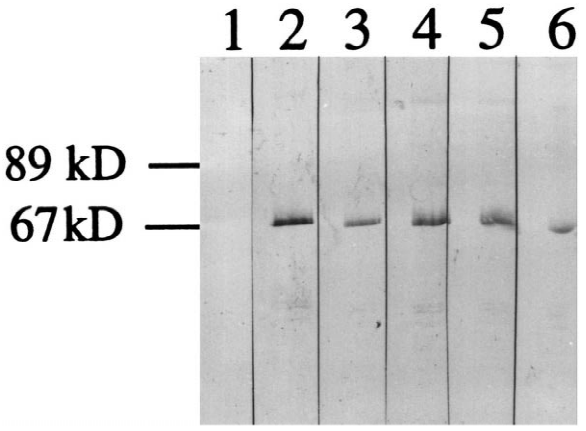

Fig. 1. Western blotting using rStav antigen (50 ng/lane) and rabbit sera (diluted 1:5000). Lane 1; pre-immunization serum, lane 2; serum collected 5 weeks p.i., lane 3; 8 weeks p.i., lane 4; 10 weeks p.i., lane 5; 11 weeks p.i., lane 6; 12 weeks p.i. Molecular sizes are indicated on the left.

3.2. IMS; incubation, sample volume and collection of beads

Antigen capture at 4°C for 16 hours was most efficient as shown by the amount of amplicons, as visualized in the agarose gel, in the subsequent RT-PCR. No difference was registered between incubation for 1 and 3 h at room temperature.

The end-point dilution of fecal extract in PBS which resulted in positive IMS from 1 and 10 ml, was 10^{-6} for both, although stronger PCR amplicons were registered from the 10 ml samples. When the sample volume was increased from 10 ml to 50 ml, with a constant concentration of fecal extract (10^{-6}) and with a tenfold dilution (10^{-7}), there was a lack of consistency and only sporadically positive PCR results were obtained (four positive samples from a total of eight). No difference was observed between magnetizing or centrifugation for collection of beads in 50 ml volumes. The results are summarized in Table 2.

3.3. Specificity of the IMS

The results displaying the specificity of the IMS are shown in Table 3. Incubation with beads coated with anti-rStav antibodies showed an endpoint at the 10^{-6} dilution and was at least 10^4 times more sensitive than incubation with beads coated with proteins from the pre-immunization serum. This difference in sensitivity shows that the antibodies have a high affinity for the genogroup I NLV. The use of uncoated beads gave an endpoint at the 10^{-5} dilution of fecal extract for positive RT-PCR and demonstrates that the virus adsorbs to the uncoated beads.

Incubation of the genogroup II NLV fecal extract with beads coated with anti-rStav antibodies showed an endpoint at the 10^{-3} dilution, while the 10^{-2} dilution was the endpoint for positive RT-PCR using beads coated with pre-immunization serum. Compared to the results with the genogroup I NLV, this shows that the antibodies have only a low affinity for the genogroup II NLV.

Table 2
Tenfold dilutions of fecal extract containing Hu/NLV/Stav/95/Nor were prepared in PBS and in humic acid-contaminated water[a]

RNA purification	Diluent	Volume (ml)	Dilutions[b]				
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
IMS	PBS	1	+	+	+	+	–
IMS	PBS	10	nd	+	+	+	–
IMS	PBS	50	nd	nd	+	+[c]	+[c]
IMS	Humic acid water	1	nd	+	+	+	–
GIT extraction	PBS	0.7	nd	+	+	+	–
GIT extraction	Humic acid water	0.7	nd	–	–	+[d]	–

[a] Viral RNA was detected by RT-PCR after IMS combined with heat release or organic (GIT) extraction of viral RNA.
[b] +, Positive RT-PCR; –, negative RT-PCR; nd, not done.
[c] Sporadically positive.
[d] One sample gave a low amount of amplicon.

Table 3
Tenfold dilutions of fecal extracts containing Hu/NLV/Stav/95/Nor (genogroup I) or a genogroup II NLV, were prepared for IMS in 1 ml of PBS[a]

Virus	Coating of beads	Dilutions[b]						
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Hu/NLV/Stav/95/Nor	Anti-rStav antibodies	+	+	+	+	+	+	–
Hu/NLV/Stav/95/Nor	Pre immunization serum	nd	nd	–	–			
Hu/NLV/Stav/95/Nor	Uncoated beads	nd	nd	+	+	+	–	–
Genogroup II NLV	Anti-rStav antibodies	+	+	+[c]	–			
Genogroup II NLV	Pre immunization serum	+	+	–				

[a] The samples were tested using beads coated either with rabbit anti-rStav antibodies or with antibodies from a negative rabbit serum or with uncoated beads (only Hu/NLV/Stav/95/Nor samples). Viral RNA was detected by RT-PCR after heat release of viral RNA.
[b] +, Positive RT-PCR; –, negative RT-PCR; nd, not done.
[c] Low amount of amplicon.

3.4. Specificity of the RT-PCRs

The results of the RT-PCRs are shown in Fig. 2. The first and nested Hu/NLV/Stav/95/Nor RT-PCRs resulted in amplicons of approximately 246 and 95 bp, respectively, while the genogroup II RT-PCR gave an amplicon of 123 bp. These results were in concordance with the expected sizes of the amplicons.

3.5. Samples containing humic acids: IMS and organic extraction of RNA

The results of the experiments in which the fecal extract was diluted in PBS and humic acid-contaminated water, respectively, are summarized in Table 2. Similar RT-PCR positive end points (10^{-6} dilutions) were found for IMS and the organic extraction of RNA when fecal extract was diluted in PBS. A 10^{-6} dilution was also the end-point when IMS was performed in water contaminated with humic acids, while the organic extraction of RNA gave inconsistent RT-PCR results. The results indicate that humic acids do not interfere with antigen capture in the IMS, and that this method is better suited than the organic extraction procedure for purification of viral RNA from samples containing humic acids.

3.6. Raw water concentrates

A positive PCR was obtained after IMS in the 4 ml concentrates of raw water, containing a 10^{-4} dilution of the fecal extract. The concentrates were obtained after filtration of 5 liters of untreated drinking water and subsequent flocculation of the filter eluate. This result indicates that IMS in water concentrates may be an alternative to application of IMS directly in a water sample.

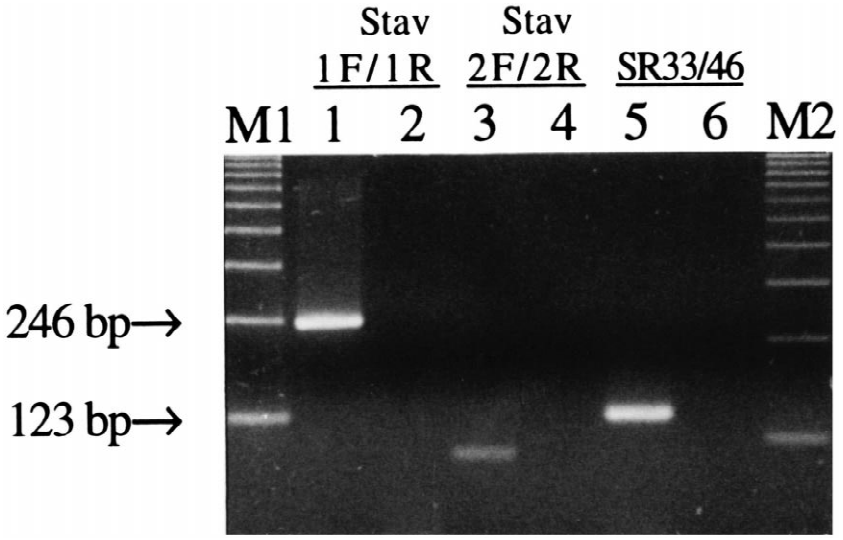

Fig. 2. Ethidium bromide-stained RT-PCR products obtained from fecal extracts containing Hu/NLV/Stav/95/Nor (genogroup I NLV) and a genogroup II NLV. Hu/NLV/Stav/95/Nor was detected by a single PCR with primer pair Stav 1F/Stav 1R (246 bp). The specificity of this product was confirmed with primer pair Stav 2F/Stav 2R (95 bp). Primer pair SR33/SR46 was used for detection of the genogroup II NLV (123 bp). M1; 123 bp ladder (Gibco BRL, N.Y., NY), Lanes 1 and 3; Hu/NLV/Stav/95/Nor, lane 5; genogroup II NLV, lanes 2, 4 and 6; negative PCR controls. M2; 100 bp ladder (Advanced Biotechnology, Surrey, UK).

4. Discussion

In the present study, a procedure for IMS based on antibodies raised against a genogroup I NLV was established. The potential of the method was demonstrated by the use of NLV-inoculated environmental samples. Detection of the virus was performed by RT-PCR after heat release of RNA from viral particles captured by magnetizable beads coated with antibodies.

The detection of NLV in clinical and environmental samples has been hampered by the lack of an in vitro cultivation system and the genomic diversity of NLV has interfered with the applicability of RT-PCR. However, conserved regions in the RNA polymerase gene have been shown to be suitable for the construction of PCR primers common for a broad variety of circulating NLV (Green et al., 1995a, Vinje et al., 1997).

The presence of RT-PCR inhibitors in environmental water has been another major obstacle to the detection of NLV. The purification of hepatitis A virus (HAV) in microcentrifuge tubes coated with antibodies has been described, however, the procedures have been restricted to sample volumes of 100 μl or less (Graff et al., 1993; Deng et al., 1994). In the present study, magnetizable beads were used as the solid phase, due to the possibility of antigen capture in larger volumes.

Polyclonal antibodies were raised in a rabbit after immunization with a purified recombinant capsid protein from a local NLV, Hu/NLV/Stav/95/Nor. The specificity of the particle-bound anti-rStav antibodies, was demonstrated by comparing IMS with anti-rStav coated beads to IMS with beads coated with proteins in serum collected prior to immunization. The results showed that the immobilized anti-rStav antibodies had retained affinity for Hu/NLV/Stav/95/Nor. The negative RT-PCR results using beads coated with pre-immunization serum indicated that at low concentrations of Hu/NLV/Stav/95/Nor, the virus did not adsorb unspecifically to coated beads. This is probably due to blocking of the surface of the beads with serum proteins, as viruses in suspensions usually stick to solids (Gerba, 1984). Adsorption of Hu/NLV/Stav/95/Nor to uncoated beads was therefore evaluated. The results show that the NLV did bind to the uncoated beads, however, antigen capture with anti-rStav antibody-coated beads was approximately 10 times as efficient. This supported the specificity of the solid phase bound anti-rStav antibodies. The relatively low difference

in antigen capture between the antibody coated beads and uncoated beads, could be due to the use of fecal extracts that had been freeze-thawed twice. Viral particles tend to be degraded under these conditions (Jiang and Estes, 1995) and any antigenic changes could be expected to reduce the ability of beads to capture antigen, although the beads are coated with high titer serum antibodies. The sensitivity of the NLV IMS was nevertheless high, with a positive result in a 10^{-6} dilution of the fecal extract (i.e. 10^{-7} dilution of the fecal sample).

The ability of the anti-rStav antibody-coated beads to capture a NLV belonging to genogroup II, and, thus, antigenically different from Hu/NLV/Stav/95/Nor, was evaluated. Due to the results with the beads coated with pre-immunization serum, the concentration of the genogroup II virus was assumed to be equal to or higher than the concentration of the genogroup I virus in the fecal samples. A low affinity of the established IMS was found for the genogroup II NLV, indicating that immunocapture of the whole range of antigenic types of NLV would require a panel of antibodies. Antigen capture of enteric viruses other than NLV was not tested. Competitive capture would result in lower sensitivity and not in false positives, due to the specificity of the RT-PCR for Hu/NLV/Stav/95/Nor.

The present IMS procedure was optimized regarding incubation time and temperature, and conditions for washing of beads with bound antigen. The yield of the captured target could be expected to increase with prolonged incubation, although with a decrease in purity (Dynal, 1996). A low incubation temperature (4°C) and stringent washing conditions probably counteracted non-specific binding to the beads. Special attention must be paid to avoidance of sample-to-sample contamination. The false positive results experienced during the optimization of IMS was probably due to contamination with virus particles in the washing process and not a consequence of the RT-PCR process. These problems were resolved by using a rigorous washing procedure.

The IMS concentrates NLV into a small volume, well suited for RT-PCR, and sample volumes of 1 ml, 10 ml and 50 ml were evaluated regarding interference with the separation efficacy. Jothikumar et al. (1998) reported an adverse influence of sample volumes above 1.5 ml. A similar result was found in the present study, as the dilution of a positive 1 ml sample (10^{-6} dilution of fecal extract) to 10 ml, resulted in a negative RT-PCR. However, an increase of sample volume from 1 ml to 10 ml, while keeping the virus concentration constant, resulted in increased intensity of the RT-PCR product. Increasing the sample volume to 50 ml gave variable RT-PCR results, and probably reflected inconsistency in collection of the beads in large volumes. The adverse effect of increased sample volumes could probably be counteracted by adding more beads. These results indicated that the sensitivity of the method is influenced by the sample volume and that the concentration of virus particles into a small volume (1 ml) prior to IMS is advantageous. However, the feasibility of IMS in 10 ml volumes was demonstrated.

Different methods for the preparation of NLV RNA from fecal samples, including the use of guanidinium thiocyanate (GTC), cetyltrimethylammonium bromide (CTAB), Chelex-100 and Sephadex G200 column chromathography have been compared (Hale et al., 1996). The results indicated a complex situation in which the amount and type of inhibitors, the amount of virus, RNA recovery and removal of inhibitory substances influenced the quality of the RNA preparation. The GTC method was the overall most efficient for the preparation of RNA for RT-PCR. In the present study, purification of viral particles by IMS combined with heat release of viral RNA was technically simple, and proved suitable for RT-PCR detection of NLV in water heavily contaminated with humic acids. The method does not imply harsh manipulation of the target and is not dependent on organic solvents. However, it was not possible to measure the recovery rate of viral RNA, and the IMS was therefore compared to guanidine extraction of RNA from the fecal extract diluted in PBS and in humic acid-contaminated water. No difference in the RT-PCR results was observed between the two methods in the PBS-diluted fecal extract. Humic acids did not influence the IMS, but did reduce the sensitivity of GIT extraction. These results indicate that the two methods give approximately equal RNA recovery, while IMS is the most efficient in removing RT-PCR inhibitors such as humic acids. Similarly, studies have shown a higher or equal sensitivity of IMS-heat release compared to guanidinium extraction in the preparation of HAV RNA from fecal extracts and tap

water concentrates (Jothikumar et al., 1998; Arnal et al., 1999). The feasibility of IMS of NLV in a 4 ml concentrate of untreated drinking water was shown in our study, indicating the possibility of using IMS as the final step in the concentration/purification of virus from concentrates of large water samples. In a study by Suñén and Sobsey (1999) on the detection of NLV in concentrates from hardshell clams, positive RT-PCR was obtained when IMS was performed in combination with guanidinium isothiocyanate extraction, but not as a single purification step. These results confirm the influence of the type of inhibitors on the efficiency of the NLV RNA purification process and indicate the need for several optimized purification methods.

The wide antigenic variation among NLV is presently an obstacle to the effective use of IMS in the detection of these viruses. If common antigen epitopes were to be revealed by antigen mapping of NLV, then the use of high affinity antibodies against these epitopes could facilitate the capture of different antigenic variants. The present study indicates that IMS combined with heat release of viral RNA and RT-PCR is an effective and simple method for the detection of genogroup I NLV in environmental water samples and in concentrates of water samples.

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