Review Paper

Detection of noroviruses in water: current status and future directions

Mohammad R. Karim and Mark W. LeChevallier

ABSTRACT

Noroviruses are recognized as an important cause of outbreaks of acute nonbacterial gastroenteritis. This review deals with the current status and future directions for noroviruses detection in water. Currently there are no standard methods available for the concentration of noroviruses from water or other environmental samples. Noroviruses cannot be cultivated in cell culture nor passed in animal models. RT-PCR assays are currently the most widely used method for detection of noroviruses in water and other environmental samples. However, the analytical sensitivity for molecular methods is unknown. Molecular methods need to be refined and standardized through further development of procedures to simplify viral nucleic acid extraction, the RT-PCR process, and the development of primers and probes that can detect a majority of noroviruses present in environmental samples. Moreover, methods need to be developed to determine the infectious virus.

Key words | drinking water, norovirus, RT-PCR

Mohammad R. Karim (corresponding author)
American Water,
Quality Control and Research Laboratory,
1115 South Illinois Street,
Belleville, IL 62220,
USA
E-mail: *mkarim@amwater.com*

Mark W. LeChevallier
American Water,
1025 Laurel Oak Road,
Voorhees, NJ 08043,
USA

INTRODUCTION

Since the discovery of the Norwalk virus in 1972, noroviruses (NVs), previously called small, round, structured viruses (SRSVs), human caliciviruses and 'Norwalk-like viruses' (NLVs), have emerged as the single most common cause of acute nonbacterial gastroenteritis in humans. NVs are the major cause of outbreaks in all age groups, whereas Sapoviruses (SVs), previously called classic human calicivirus, 'Sapporo-like viruses' (SLVs), have been primarily associated with paediatric gastroenteritis and are rarely associated with outbreaks. The Center for Disease Control and Prevention (CDC) determined that NVs account for 93% of reported outbreaks of nonbacterial gastroenteritis that have been examined over a period of 3 years in the United States (Fankhauser *et al*. 2002). NVs have been implicated in 82% of 184 outbreaks reported to municipal public health centres in the Netherlands during 1991–1999 (Koopmans *et al*. 2000), 68% of outbreaks (64 of 94) during 1996–1997 in East Anglia, United Kingdom (Maguire *et al*. 1999), and 89% of 455 outbreaks in Sweden during 1994–1998 (Hedlund *et al*. 2000), confirming the aetiological significance of these viruses.

The family *Caliciviridae* has recently been divided into four genera: *Norovirus* (type strain Norwalk virus), *Sapovirus* (type strain Sapporo virus), *Vesivirus* (type strain vesicular exanthema of swine virus) and *Lagovirus* (type strain rabbit haemorrhagic disease virus) (ICTV meeting, Paris 2002). *Norovirus* and *Sapovirus* are the two genera that have been implicated in human diseases and are generally viewed as human caliciviruses, although members of both genera have been detected in husbandry animals as well (Sugieda *et al*. 1998; Dastjerdi *et al*. 1999; Guo *et al*. 1999; Liu *et al*. 1999). The other two genera, *Lagovirus* and *Vesivirus*, are known to cause diseases in animals. However, a recently isolated *Vesivirus* (SMSV-5) has been demonstrated to cause pathogenic lesions in a human (Smith *et al*. 1998a). Humans were thought to be the only reservoir of NVs; however, recent reports of NVs in farm animals (van der Poel *et al*. 2000) and the possible

zoonotic transmission of NV (Smith *et al*. 1998b) suggest that animals may also serve as a reservoir.

Based on sequence analysis, NVs can be divided into four genetic groups, called genogroups (G), GI–GIV, of which genogroup III has so far been found to cause disease only in cattle (Ando *et al*. 2000). NVs within genogroups can be further divided into at least 18 genetic clusters or genotypes (Table 1). Similarly, SVs can be divided into at least three genogroups (GI–GIII), comprising five genotypes; genogroup I and II strains have been detected in humans and genogroup III comprises one porcine (Cowden) strain (Schuffenecker *et al*. 2001).

The genome of NVs possesses a positive sense, single-stranded RNA of approximately 7.6 to 7.7 kb in length and is composed of three open reading frames (ORFs) (Jiang *et al*. 1993). For Norwalk virus, the largest ORF, ORF1, located at the 5′ end of the genome, encodes a large polyprotein of 1,738 amino acids (aa). This polyprotein contains short motifs of amino acid that are analogous to the helicase, cysteine protease, and RNA-dependent RNA polymerase proteins of picornaviruses (Jiang *et al*. 1990, 1993). The second ORF, ORF2, encodes the viral capsid protein (Jiang *et al*. 1993). The third and the smallest ORF, ORF3, encodes a minor structural protein (Glass *et al*. 2000). The genome of the SVs is organized differently from NVs and is more similar to animal calicivirus belonging to the genus *Lagovirus*. The single stranded RNA genome is about 7.5 kb long with three predicted ORFs and a 3′ polyadenylated tail (Liu *et al*. 1995, 1997; Numata *et al*. 1997). Unlike NVs, ORF1 of SVs codes for both putative nonstructural proteins (helicase, cysteine protease and RNA-dependent RNA polymerase) as well as the capsid protein (Liu *et al*. 1995; Numata *et al*. 1997). ORF2, corresponding to the ORF3 of NVs, situated at the 3′ end of the genome, encodes a basic protein of unknown function. SVs contain a third ORF overlapping the capsid coding region, which encodes a small basic protein (Liu *et al*. 1995; Numata *et al*. 1997). ORF3 of SVs has no corresponding ORF in NVs. The significance of this ORF3 is unclear.

Unlike many bacterial pathogens, which have been controlled largely by water and wastewater treatment practices, the incidence of water-related viral diseases has remained virtually unchanged over the past several decades (Metcalf *et al*. 1995). Commonly used bacterial indicators are not considered adequate indicators of viral contamination and the use of bacteriophages (viruses that infect bacteria) as viral indicators is still under evaluation. NVs are on the USEPA Contaminant Candidate List

Table 1 | Grouping of noroviruses by genetic relatedness

Genogroup	Cluster	Fields prototype[1]	CDC[2] cluster
I	1	Norwalk	GI.1
I	2	Southampton	GI.2
I	3	Desert Shield	GI.3
		[Stavanger]*	GI.3b
I	4	Chiba	GI.4
I	5	Musgrove	GI.5
I	6	Hesse	GI.6
I	7	Winchester	GI.7
II	1	Hawaii	GII.1
II		[Wortley]*	GII.1b
II	2	Melksham	GII.2
II	3	Toronto	GII.3
II	4	Bristol	GII.4
II	5	Hillingdon	GII.5
	6	Seacroft	GII.6
II	7	Leeds	GII.7
II	NA	Amsterdam	GII.8
II		[Virginia Beach]*	GII.9
NA	NA	Jena	GIII.1
NA	NA	Bo/NLV/CH126/1998/NL	GIII.2
NA	NA	Alphatron	GIV.1

NA=not assigned
[1]Green *et al*. (2001)
[2]Center for Disease Control and Prevention
*Pairs with uncorrected amino acid differences in the 15–20% range: Desert Shield and Stavanger (GI.3 & GI.3b) Hawaii and Wortley (GII.1 & GII.1b)
Source: CDC, personal communication

for regulatory consideration for drinking water (USEPA 1998). Because methods for detecting NVs in water samples are still under development, the prevalence of NV contamination of drinking water and other water types and the impact of such contamination on consumers and the water industry remain unclear. Therefore, there is a need to develop a detection method that is rapid, sensitive and selective for infectious NVs. This paper reviews the current methods available for the detection of NVs in water and environmental samples and provides future research directions.

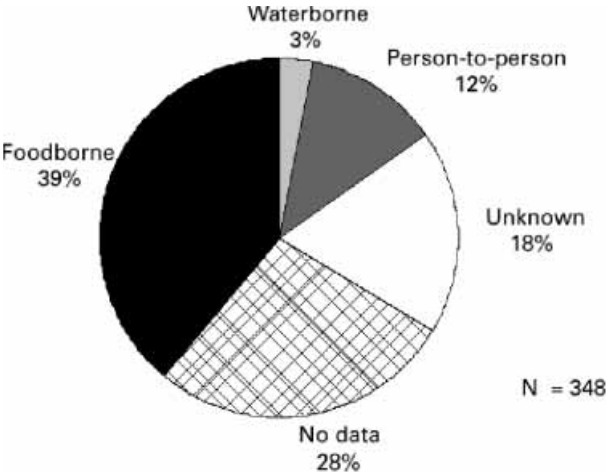

Figure 1 | Mode of transmission of 348 outbreaks of norovirus gastroenteritis reported to CDC during January 1996 to November 2000 (source: *MMWR Weekly Report* 2001).

EPIDEMIOLOGY

NVs and SVs are recognized as an important cause of acute nonbacterial gastroenteritis. Numerous food and waterborne outbreaks of NVs have been reported throughout the world (Vinjé *et al*. 1997; Maguire *et al*. 1999; Bon *et al*. 1999; Hedlund *et al*. 2000; Koopmans *et al*. 2000; O'Ryan *et al*. 2000; Nakata *et al*. 2000; Pang *et al*. 2000). A Norwalk-like virus strain (95/96-US) with global distribution has been identified (Noel *et al*. 1999). NVs caused 93% and 73.4% of nonbacterial gastroenteritis outbreaks in the United States and Japan, respectively, over a 3-year period (Iritani *et al*. 2000; Fankhauser *et al*. 2002).

NVs outbreaks have been reported in many different settings, such as schools (Adler and Zickl 1969), nursing homes (Green *et al*. 2002), hospitals (Hedlund *et al*. 2000), cruise ships (Herwaldt *et al*. 1994; Khan *et al*. 1994; MMWR 2002), tourist resorts (Boccia *et al*. 2002), child-care centres (Hedlund *et al*. 2000) and Navy ships (McCarthy *et al*. 2000). NVs are primarily transmitted through the faecal–oral route, although airborne transmission has been suggested (Sawyer *et al*. 1988). Outbreaks caused by the consumption of shellfish are common (Shieh *et al*. 2000). During an outbreak, primary cases usually result from exposure to a faecally contaminated vehicle such as food or water (Daniels *et al*. 2000; Deneen *et al*. 2000; Boccia *et al*. 2002), whereas secondary and tertiary cases among contacts of primary cases result from person-to-person transmission (Becker *et al*. 2000). Despite the transmission of NVs throughout the year, studies from 12 year-round surveys conducted during 1978 to 1998 in eight countries indicate that the peak incidence of NVs occurs during the winter months (Mounts *et al*. 2000).

NVs have been detected in surface water (Jiang *et al*. 2001), groundwater (Abbaszadegan *et al*. 2003), drinking water (Kukkula *et al*. 1999), mineral water (Beuret *et al*. 2002), and are a well-documented cause of waterborne outbreaks of acute gastroenteritis (Kaplan *et al*. 1982; Kukkula *et al*. 1997; Beller *et al*. 1997; Brugha *et al*. 1999; Boccia *et al*. 2002). Contaminated drinking water consumption has been reported to be associated with NV outbreaks (Taylor *et al*. 1981; MMWR 1982; Kukkula *et al*. 1997, 1999; Beller *et al*. 1997; Boccia 2002; Anderson *et al*. 2003). Contamination of untreated groundwater due to geological conditions, overloaded sewage disposal systems, back-siphonage or backflow, inadequate chlorination of municipal drinking water supplies or breakdowns in the water system have been implicated in the outbreaks. Outbreaks associated with swimming have also been reported (Kappus *et al*. 1982; Baron *et al*. 1982).

For 348 outbreaks of NV gastroenteritis reported to CDC during January 1996–November 2000, food was implicated in 39%, person-to-person contact in 12%, and water in 3%; 18% could not be linked to a specific transmission mode (Figure 1). However, owing to the lack

of a reliable detection method and a reliable surveillance system, the actual disease burden of water-related norovirus infection might have been under-reported. Collaboration among local, state and national public health authorities and development of a reliable detection method would help to define the actual disease burden that might be attributed to waterborne noroviruses. Immunity to NV is short-lived (Estes *et al*. 2000) and vaccine development studies using recombinant Norwalk virus-like particles (rVLP) are in the early stages of development (Guerrero *et al*. 2001; Harrington *et al*. 2002).

CONCENTRATION OF NOROVIRUSES FROM WATER

Even though methods for concentration of many enteric viruses have been developed, limited studies have been done into concentrating NVs from water (Table 2). One of the major limitations in NV concentration methods development is the lack of a supply of virus stock. At present NVs cannot be cultivated in cell culture nor passed in animal models. Clinical sources of NVs are not available to many laboratories interested in environmental methods developments. Hence, animal caliciviruses, such as primate calicivirus Pan-1 and feline calicivirus have been used as surrogates for NVs in some concentration studies (Myrmel *et al*. 1999; Huang *et al*. 2000).

Huang *et al*. (2000) described a method for concentration of NV from 40 l of water using electropositive filters where Pan-1 was used as a model organism. Deionized, finished, ground and surface water samples were seeded with infectious Pan-1 and then filtered using 1MDS filters, eluted with beef extract, and reconcentrated by polyethylene glycol (PEG) precipitation. Pan-1 was found to be sensitive to high pH. At pH 9.0, a beef extract solution recovered 35% more infectious Pan-1 virus compared with same the method at pH 9.5. Using this method, Pan-1 was recovered from small volumes (200 ml) of deionized, finished, ground and surface water at efficiencies of 94, 73, 67 and 64%, respectively, when samples were assayed after elution without further concentration. When larger volumes of water (up to 40 l) were tested, 38, 19 and 14% of the seeded Pan-1 were recovered from finished, ground and surface waters, respectively (Huang *et al*. 2000). A similar method using an organic flocculation procedure for reconcentration of viruses was used to detect NVs in a large volume (1,512 l) of groundwater (Abbaszadegan *et al*. 2003).

A three-step method has been developed for concentrating NVs from 1 l of freshwater or river water using electropositive filters (Gilgen *et al*. 1997). Briefly, 1 l of water was filtered through a positively charged membrane by use of a fibreglass prefilter. Viruses were eluted from the membrane with 50 mM glycine-NaOH, pH 9.5, containing 1% beef extract. Further concentration to 100 μl was achieved by use of a Centricon-100 microconcentrator. Following RNA extraction from the sample concentrate, NVs were detected by reverse transcriptase polymerase chain reaction (RT-PCR). The method was used to recover NVs from untreated water, treated water and tap water (Kukkula *et al*. 1999).

NVs were concentrated from 200 l of surface water using 1MDS electropositive filters followed by elution with 1 l of 1% beef extract glycine (Schwab *et al*. 1996). The eluate was further concentrated and purified with PEG precipitation, ProCipitate (a commonly available protein precipitating reagent) precipitation and a second PEG precipitation. Aliquots of the second PEG precipitate were further processed by the antibody capture method for subsequent RT-PCR. The detection limit for this method was 10^3 PCR units of Norwalk virus (Schwab *et al*. 1996).

In another study, Norwalk-like viruses were concentrated from 10 l of tap water, river water, seawater and sewage effluent using 0.45 μm negatively charged cellulose nitrate membranes (Wyn-Jones *et al*. 2000). The pH of the water was preconditioned to 3.5 and was passed through a composite filter stack consisting of a glass fibre prefilter before passing through the cellulose nitrate membrane filter. Viruses were eluted from the filter with 200 ml of 0.1% (w/v) skimmed milk at pH 9.5 and then further concentrated by acid flocculation at pH 4.5 and brought to a final volume of 10 ml. Viruses were detected in the sample concentrate using RT-PCR (Wyn-Jones *et al*. 2000).

Recently, a new procedure for concentration of enteroviruses and Norwalk virus using a negatively

Table 2 | Summary of methods used for concentrating noroviruses from water and environmental samples

Filter absorption-elution method	Type of filter	Initial volume of water	Type of water	Method of detection	Percentage recovery/detection limit	References
Positively charged filters	1MDS disk filter	40 l	Finished water, ground water and surface water	PA[1]/RT-PCR[2]	14–8% (using primate calicivirus Pan-1 as model organism)	Huang *et al.* 2000
	Membrane filter	1 l	Fresh water, river water, untreated and treated water, tap water	RT-PCR	10^5 dilution of NLV stool sample per litre of water (the actual titre of virus in the stool sample is not known)	Gilgen *et al.* 1997
	1MDS cartridge filter	1,512 l	Groundwater	RT-PCR	Not tested	Abbaszadegan *et al.* 1999
	1MDS cartridge filter	200 l	Raw surface water	Antibody capture – RT-PCR	2 PFU of poliovirus-1, 10^3 PCR units of Norwalk virus	Schwab *et al.* 1996
Negatively charged filters	Cellulose nitrate membrane	20–100 ml raw sewage, 10 l of water	Tap water, river water, seawater and raw sewage	RT-PCR	Not tested	Wyn-Jones *et al.* 2000
	Membrane filter	1–2 l	Seawater	RT-PCR	61–73% (using poliovirus-1 as a model organism)	Katayama *et al.* 2002
	Membrane filter		Deionized water, raw water	RT-PCR	5–10% (using feline calicivirus as a model organism)	Myrmel *et al.* 1999
	Membrane filter	10 l	Raw sewage	RT-PCR	10 to 100 RNA containing particles	Lodder *et al.* 1999

[1]Plaque assay; [2]Reverse transcription PCR

charged membrane filter was described by Katayama *et al*. (2002). Briefly, 2 l water samples were filtered through a 90 mm diameter negatively charged membrane filter with 0.45 μm pore size followed by an acid rinse (0.5 mM H_2SO_4, pH 3.0) and elution with 1 mM NaOH (pH 10.5). The eluates were neutralized with 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of 100 × Tris-EDTA buffer followed by further concentration to 1 ml by ultrafiltration and detection by RT-PCR. The recovery efficiency of this method using seeded poliovirus from natural seawater was 61 to 73%. Using this method, Norwalk virus was detected in 2 l of unseeded seawaters. The method is free from beef extract elution, which could have an inhibitory effect in the subsequent viral genome detection by RT-PCR.

NVs were recovered from 10 l of raw sewage using the conventional filter adsorption-elution method followed by a modified two-phase separation method by Dextran T40 and 10% PEG 6000 (Lodder *et al*. 1999). After separation, the bottom phase and the interphase were further purified by spin column gel chromatography using Sephadex G200 and by ultrafiltration. Using this protocol Norwalk-like virus RNA was detected by RT-PCR in sewage water concentrate (Lodder *et al*. 1999).

A filter-elution method using 47 mm diameter flat electronegative filters (0.45 μm pore size) for NLV concentration from 500 ml of deionized water, raw drinking water and bog water has been described by Myrmel *et al*. (1999). Feline calicivirus (FCV) was used as a model organism for the seeding experiments. Samples were pretreated with $MgCl_2$ and pH adjusted to 5 to adsorb viruses to the filter. Filters were eluted with a urea-arginine-phosphate buffer, viruses in the eluates were flocculated, the floc was centrifuged and RNA was extracted for RT-PCR. The estimated recovery of the FCV using this method was 5–10%. Humic acid and metal ions prevented detection of NLV in the bog water.

One of the problems associated with virus concentration methods is the co-concentration of unrelated material, such as humic substances, which may interfere with the methods used for virus detection in the sample concentrates (Wilson 1997). This is especially important because NVs are currently not cultivable and the co-concentration of inhibitory substances may prevent the detection of NVs by nucleic acid amplification. Sobsey (1999) examined three different amino acid (lysine, glycine and arginine) eluants to recover adsorbed Norwalk virus from positively charged filters (virosorb 1MDS). Uses of the amino acid eluants resulted in efficient recoveries of Norwalk virus from seeded tap water samples and were less inhibitory than beef-extract containing eluants for the detection of NVs by RT-PCR. Although there have been several methods for the concentration of viruses from water, currently no standardized protocol exists for the concentration of NVs from water and other environmental samples.

DETECTION METHODOLOGIES

NVs cannot currently be cultivated in cell culture nor passed in animal models. Studies attempting to induce illness in several types of animal have not been successful (Kapikian and Channock 1985). However, animal caliciviruses such as the porcine enteric calicivirus (PEC) have been successfully grown in cell cultures (Flynn and Saif 1988; Parwani *et al*. 1991).

Knowledge of the virus–cell receptor interaction for poliovirus has provided insight into many aspects of virus infection. The protein capsid that surrounds many animal viruses plays an important role in viral infection. Upon encountering a target cell, the capsid must interact with a cell surface receptor and release the viral nucleic acid into the cell. Studies for identification of the NV binding site in the capsid protein and cell surface receptors are in the early stage of development. Experiments with recombinant Norwalk virus (rNV) capsids suggest that the C-terminal region of the capsid protein is involved in specific binding of rNV virus-like proteins (VLPs) to human Caco2 cells (White *et al*. 1996). The binding of VLPs on cell surfaces is mediated by a protein–protein interaction and VLPs appear to bind to a 105-kDa cellular binding protein in Caco-2 cells, which is termed the NV attachment (NORVA) protein (Tamura *et al*. 2000). A recent human volunteer study suggests that histo-blood group antigens may serve as receptors for noroviruses (Lindesmith *et al*. 2003). Identification and characterization of NV receptors in human cells may help to develop

recombinant continuous cell lines that are capable of facilitating NV replication. Despite the lack of a cell culture system, several methods have been developed for the detection of NVs. These include electron microscopy, immunological methods and molecular methods.

Electron microscopy

Under the electron microscope (EM), definite identification of NVs in faecal specimens is difficult because of the lack of a distinctive surface morphology. Immune electron microscopy (IEM), which utilizes antigen–antibody interactions to aggregate virus particles, improves the sensitivity of EM, but still the virus titre should be 10^5–10^6 ml^{-1}. The application of the IEM technique resulted in the discovery of Norwalk virus (Kapikian 1972). Solid-phase IEM, a variation of IEM, increases the sensitivity of the diagnosis of EM up to 100-fold (Biel and Gelderblom 1999) and has been used for the classification of NVs into antigenic groups (Lewis *et al*. 1995).

The advantages of using the EM for detection of NVs are rapid diagnosis and having a catch-all method for other enteric viruses with a distinct morphology (e.g. rotavirus, adenoviruses and astroviruses). The disadvantages of the EM techniques are the requirement for a high-titre purified sample and in the case of IEM the lack of readily available sera that are reactive to all antigenic types of NVs. Moreover, EM requires a skilled microscopist and expensive equipment. For these reasons, EM techniques have little usefulness for detection of NVs in water and other environmental samples and are better suited for diagnosis of clinical illness and epidemiological investigations.

Immunological methods

Immune adherence hemagglutination assay (IAHA) can also be used for the detection of NVs and its antibodies. However, this technique is not efficient for the detection of viruses or antigens in stool samples (Kapikian *et al*. 1978). Radioimmunoasay (RIA) is more efficient and sensitive than IEM and detects both particulate and soluble antigens (Kapikian and Chanock 1990). RIA has been used to perform seroprevalence studies and to investigate outbreaks of gastroenteritis (Goodman *et al*. 1982; Baron *et al*. 1984).

Enzyme immunoassasy (EIA) and enzyme linked immunosorbent assay (ELISA) are more sensitive than RIA. Early enzyme immunoassays were reliant on human sera and viral antigen from infected individuals. Later, expression of the capsid proteins of NVs in baculoviruses (Jiang *et al*. 1992) that self-assemble into stable recombinant virus-like particles (VLPs) has allowed the production of hyper-immune antibodies in laboratory animals. VLPs are also used as a source of antigen. VLPs have been used to generate polyclonal and monoclonal antibodies. This allowed the development of antigen and antibody detection EIAs for use in clinical and epidemiological studies. Both the antigen and antibody detection EIAs are highly sensitive and have been invaluable for the detection of virus infections (Herrmann *et al*. 1995; Brinker *et al*. 1999) and antibody and antigen detection in epidemiological studies (Smit *et al*. 1997; Honma *et al*. 1998; Nakata *et al*. 1998). However, the high specificity of antigen detection EIA limits its application because of the antigenic diversity of the NVs. The more broadly reactive antibody detection EIA has important applications in epidemiological investigations, in particular for determining seroprevalence, geographic and temporal distribution. Like EM methods, immunological methods are very useful for clinical diagnosis of NV infections and for epidemiological studies; however, they lack the sensitivity to reliably detect low levels of NVs in water and environmental samples.

Molecular methods

Knowledge of the sequence of the NV genome has led to the development of molecular methods such as RT-PCR assays for the detection of NVs in clinical specimens as well as in food, water and other environmental samples. The RT-PCR assay is more sensitive than microscopic and immunological methods and if primers are carefully chosen may be broadly reactive to a large number of strains. However, a number of factors may affect the sensitivity and specificity of RT-PCR. These factors include the types of sample processed, methods used for

the extraction/purification of viral nucleic acids, the presence of inhibitors, primer selection, use of appropriate enzymes and the confirmation of PCR products. Currently there is no standard method for the detection of NVs from water and other environmental samples.

RNA extraction methods

Environmental samples may contain humic acids and other substances that can inhibit enzymes used in the RT-PCR reaction. Thus, it is necessary to purify the viral nucleic acid prior to RT-PCR. The important considerations in selecting an extraction method are the ability to remove PCR inhibitory substances, the efficiency of viral nucleic acid recovery, the ease of performance and the number of samples that can be processed at one time (Atmar and Estes 2001). Several approaches for the extraction/purification of NV RNA have been reported (Boom *et al*. 1990; Schwab *et al*. 1997; Myrmel *et al*. 2000; Jiang *et al*. 2001). Most methods developed to recover or purify viral RNA from clinical or environmental samples involve multistep extraction and elution procedures using a combination of reagents such as guanidinium thiocyanate (GITC), cetyltrimethylammonium bromide (CTAB), trizol, polyethylene glycol, silica, Sephadex, Chelex, phenol-chloroform and ethanol (Tables 3, 4 and 5). Currently there is no standard method available for sample purification and RNA extraction from water and other environmental samples.

The GITC-silica method was originally developed for purification of viral RNA from clinical samples (Boom *et al*. 1990). This method is based on lysing the virus particles by guanidinium thiocyanate followed by separation of nucleic acids using size-fractionated silica particles. The GITC-silica method has been used for extraction of NV RNA from environmental samples (Wyn-Jones *et al*. 2000). In a comparative study between four different RNA extraction methods (RNA extraction using Chelex-100, Sephadex G200 column chromatography, GITC-silica method and CTAB method) for the detection of NVs in faecal specimens, the GITC-silica method was found to be the best method for the extraction of viral RNA from faecal samples (Hale *et al*. 1996).

Jiang *et al*. (2001) evaluated a number of viral extraction methods to remove PCR inhibitory substances from water concentrates. These included the CTAB method (a cationic detergent), the trizol method, a silica gel-membrane method and a high-salt trizol method. CTAB was added to samples after concentration by polyethylene glycol (PEG) precipitation. The trizol method relies on GITC to lyse the viral capsid followed by phenol/chloroform extraction of viral RNA. The silica-membrane method selectively adsorbs nucleic acids, after lysis of the virion by GITC, effectively removing polysaccharides and proteoglycans. The high-salt trizol method removes polysaccharides and proteoglycans by selectively precipitating the RNA in the presence of a high salt concentration. The trizol method was effective and simple for removal of RT-PCR inhibitors from water samples. The silica-membrane and high-salt trizol method were useful for extraction of viral RNA from more polluted water samples (Jiang *et al*. 2001). Commercial viral RNA extraction kits are now available which are convenient and reduce contamination. These kits are based on the Boom principle of adsorption to silica but are much easier to use than previous methods.

Size exclusion chromatography is another method that can be used for removing PCR inhibitory substances from environmental samples. Abbaszadegan *et al*. (1993) compared five different methods for removing the PCR inhibitory substances from sample concentrates, including ethylene glycol-bis (β-aminoethyl ether)-N,N,N^1, N^1-tetraacetic acid (EGTA), and size exclusion chromatography using Sephadex G-200, Sephadex G-100 + Chelex-100, Cheles-100, and mixed bed resin. The best results were obtained when Sephadex G-100 in combination with Chelex-100 was used for the treatment. However, recovery efficiency of these methods has not been tested and virus loss may occur during the purification process.

Heat release of viral RNA is accomplished by heating virus-containing samples at 95°C for 5 min (Schwab *et al*. 1997). When compared with the conventional CTAB RNA extraction method in stool samples, heat release had similar detection sensitivity to the CTAB method (Schwab *et al*. 1997). However, this method may not be suitable for environmental samples.

Table 3 | Sample purification/RNA extraction and PCR product confirmation methods used for water and other environmental samples

Sample type	Sample purification/RNA extraction	Primers/primer set designations used	DNA polymerase used	Method of conformation/ probes used	References
Sea water, river water and sewage	GITC-silica	CDR 1U and CDR L1B	*Taq* polymerase	DNA sequencing	Wyn-Jones *et al.* 2000
Surface water	PEG precipitation followed by ProCipitate and a second PEG precipitation. Further purified either by guanidine extraction of RNA or antibody capture followed by guanidine RNA extraction	5′CAAATTATGACAGAAT CCTTC 3′GAGAAATATGACATGG ATTGC	*Taq* polymerase	Southern hybridization	Schwab *et al.* 1996
Beef extract mock elutes	PEG precipitation followed by ProCipitate and a second PEG precipitation. Further purified by spin column gel chromatography using Sephadex G200 followed by ultrafiltration. Viral RNA was released by heating 99°C for 5 min	5′CAAATTATGACAGAAT CCTTC 3′GAGAAATATGACATGG ATTGC	*Taq* polymerase	Southern hybridization	Schwab *et al.* 1995
Deionized, finished, ground and surface water	Trizol method	Pan-1 36, and Pan-1 35	*Taq* polymerase	Southern hybridization using pan-1 genome specific probe	Huang *et al.* 2000
Water concentrate	SepaGene RV-R	COG1F, COG1R, COG2F, COG2R	Not known	Taqman probes (TET)AGATYGCGATCYCCTGT CCA(TAMRA) (FAM)TGGGAGGGCGATCGC AATCT(TAMRA)	Katamaya *et al.* 2002
10% stool suspensions, sewage or concentrated water	Phenol containing tripure reagent and precipitated with ethanol	NVp 110, NVp 69 and NI	Not known	PCR product was confirmed with the microplate hybridization method by use of streptavidin-coated white microplates and luminometric detection	Kukkula *et al.* 1999
Mineral water	QIAmp HCV viral extraction kit	SRI-2, SRI-3, SRII-2, SRII-3 Mon 431, 432, 433, 434	*Taq* polymerase	DNA sequencing	Beuret *et al.* 2002
Sewage	Spin column gel chromatography using Sephadex G200 and ultrafiltration in a Centricon 100 microconcentrator followed by GTIC-silica method	JV 12 and JV 13	Ampli*Taq*	DNA sequencing	Lodder *et al.* 1999
Tap water, river water	QIAmp HCV kit	SRI-1, SRI-2, SRI-2, SRII-1, SRII-2, SRII-3	*Taq* polymerase	Nested PCR	Gilgen *et al.* 1997

Table 4 | Sample purification/RNA extraction and PCR product confirmation methods used for food and clinical samples

Sample type	RNA extraction/sample purification	Primers used	DNA polymerase used	Method of conformation/probes used	Reference
Shellfish	Proteinase K- phenol chloroform extraction followed by ethanol precipitation and CTAB-NaCl extraction	NVp35 and NVp 36	*Taq* polymerase	Slot blot/southern hybridization	Atmar *et al.* 1995
Shellfish	Glycine-PEG-Tri-reagent-magnetic poly(dt) beads (GPTT extraction procedure)	M5 and M3	*Taq* polymerase	DNA sequencing	Kingsley and Richards 2001
Food	Trizol–phenol chloroform extraction–alcohol precipitation	NVp35 and NVp36	*Taq* polymerase	Southern blot hybridization/sequencing	Schwab *et al.* 2000
Shellfish	GITC-silica	NI and E3	*Taq* polymerase	Southern blot hybridization/sequencing	Lees *et al.* 1995
Shellfish	GITC-silica	G1, G2, SM31, N1 and E3	*Taq* polymerase	Nested PCR	Green *et al.* 1998
Oyster	Silica gel membrane	SR33, SR46, SR48, SR50, SR52	Not known	Southern hybridization	Shieh *et al.* 1999
Food	Trizol–chloroform extraction–alcohol precipitation	G1 and G2 primer set, mon 381 and mon 383	Not known	DNA sequencing	Daniels *et al.* 2000
Stool	GITC-silica	JV12 and JV 13	Ampli*Taq*	Southern hybridization	Vinjé *et al.* 1997
Stool	PEG-CTAB	JV12 and JV 13	Ampli*Taq*	Southern hybridization	Vinjé and Koopmans 1996

Table 4 | *Continued*

Sample type	RNA extraction/sample purification	Primers used	DNA polymerase used	Method of conformation/probes used	Reference
Oyster, stool	CTAB-NaCl extraction followed by ethanol precipitation	NVp 110, NVp36, NVp69, NI, SR33, SR48, SR50 and SR52	*Taq* polymerase	Slot blot hybridization/sequencing	Le Guyader *et al.* 1996
Stool, shellfish	Protinease K digestion-CTAB–ethanol precipitation	NVp35 and NVp36	r*Tth* polymerase	Southern hybridization/DNA enzyme immunoassay	Schwab *et al.* 2001
Stool	Sephadex G 200 chromatography	5′CAAATTATGACAGAATCCTTC 3′GAGAAATATGACATGGATTGC	Ampli*Taq*	Oligoprobe hybridization	DeLeon *et al.* 1992
Stool	GITC-silica	NV35, NV36, JV2, JV3, SR33, SR80 JV33	Ampli*Taq*	Southern hybridization and sequencing	Vinjé *et al.* 2000
Faecal specimen from animals	GITC-silica	JV 12 and JV 13	Ampli*Taq*	Southern blot and sequencing	Van der Poel *et al.* 2000
Stool	Virus particles were isolated by use of Sephadex G-200 spin column. Viral RNA was purified by guanidine-urea-phenol extraction	JV 12 and JV 13	Not known	DNA sequencing	Deneen *et al.* 2000
Stool	Ultraspec-3 isolation system	G1 and G2 primers SR33, SR 48, SR50, SR52	*Taq* polymerase	Southern hybridization/sequencing	Iritani *et al.* 2000

Table 5 | Sample purification/RNA extraction and PCR product confirmation methods used for clinical samples

Sample type	RNA extraction/sample purification	Primers used	DNA polymerase used	Method of conformation/probes used	Reference
Stool	GITC-silica	SVs- SLV-r-c and Sapp 36	*Taq* polymerase	Southern hybridization	Pang *et al.* 2000
Stool	QIA Amp RNA kit	Degenerate primer NVp 110 NVp36, NVp 69, SR 48-50-52 and NI		Sequencing	Bon *et al.* 1999
Stool	Trizol method	P 289, p 290	*Taq* polymerase	Gel electrophoresis	O'Ryan *et al.* 2000
Stool	QIA Amp RNA kit	NV4562, NV 4611, NV 4692, NV5296, NV5298, NV5366	Superscript II RT/*Taq* mix	DNA sequencing	Yuen *et al.* 2001
Stool	GITC-silica	JV 12, JV 13, NVp69 and NVp110	Not known	Reverse-line blot hybridization/sequencing	Johansson *et al.* 2002
Stool	Ultraspec-3	SR33, SR 46, SR 50, SR52 CVF, CVR	Tth DNA polymerase	DNA sequencing	Gonin *et al.* 2000
Stool	Phenol-chloroform extraction followed by GTIC-silica extraction and ethanol precipitation	Oligo $(dT)_{30}$ latex, VN, TT, Mon 189, SR33, SR46, SR 48, SR 50, SR 52, CL6, CL10, CL14, CL18, CL22, CL26, CL28, SR34, SR62, SR60	*Taq* and *Pwo* DNA polymerase mixture	Southern hybridization	Ando *et al.* 1997
Stool	Heat release	NVp35 and NVp36	*Taq* polymerase	Hybridization and detection by slot blot	Schwab *et al.* 1997
Stool	PEG-CTAB	JV 12 and 13	Ampli*Taq*	Reverse line blot hybridization (RLB) using 18 different probes for simultaneous confirmation and genotyping	Vinjé and Koopmans 2000
Stool	Phenol-chloroform extraction and purified by GTIC-silica method. Purified RNA was concentrated by ethanol precipitation	SR33, SR46, SR48, SR50, SR52	Ampli*Taq*	Southern hybridization by five different probes	Ando *et al.* 1995
Stool	GITC-silica	NI and E3	*Taq* polymerase	Sequencing	Maguire *et al.* 1999

Immunomagnetic separation (IMS) (also known as antibody capture (AbCap), immunomagnetic capture (IMC) or immunocapture) has been demonstrated to be an effective method to capture and concentrate different enteric viruses in samples and separate them from inhibiting substances for subsequent RT-PCR. Immunomagnetic separation utilizes paramagnetic beads coupled to a virus-specific antibody that allows separation of virus from a sample concentrate. Rabbit polyclonal antibodies, raised against a recombinant capsid protein from a genogroup I NV, were used to separate NVs from water concentrate (Myrmel *et al*. 2000). The advantage of the IMS method is that free viral RNA present in a sample is not likely to be detected by IMS. Therefore, the IMS method detects intact virus particles and hence potentially infectious viruses indicating the public health significance. Moreover, it reduces the RT-PCR inhibitory substances in the samples and increases the efficiency of detection (Schwab *et al*. 1996; Sunen and Sobsey 1999; Gilpatrick *et al*. 2000). The AbCap method was found to be more efficient than direct RNA extraction and the cell culture infectivity method for enteroviruses. When compared with direct RNA extraction and the cell culture method, the antibody capture method resulted in significantly more enterovirus-positive field samples (Schwab *et al*. 1996).

The antigenic diversity among NVs makes it difficult to detect all NVs in a sample owing to the lack of broadly reactive antibodies. An antibody raised against genogroup I NVs was found to be less effective in capturing genogroup II NVs (Myrmel *et al*. 2000). However, the AbCap method with broadly reactive polyclonal antisera using pooled human immunoglobulins (HSIG) was shown to isolate a number of different human enteric viruses simultaneously (Schwab *et al*. 1996). The sensitivity of the AbCap method for poliovirus 1 was shown to be 0.1 PFU (Schwab *et al*. 1996).

Despite multi-step viral RNA purification and extraction, RT-PCR inhibitors may still persist in the purified samples and thus produce false negative results. Use of an RNA internal standard permits the detection of inhibitors present in the sample, allowing the identification of false negatives (Atmar *et al*. 1995, 1996; Schwab *et al*. 1997, 2000; Daniels *et al*. 2000). However, the currently developed internal controls are genotype specific and may not be suitable for use in RT-PCR amplification of genetically diverse NVs.

RT-PCR

The use of different DNA polymerases during the PCR amplification of NVs has been reported. Usually RT-PCR protocols for NV amplify targets of a few hundred base pairs using a single DNA polymerase. However, Ando *et al*. (1997) used a mixture of *Taq* DNA polymerase and *Pwo* DNA polymerase that could amplify a 3-kb product from 24 NLV strains from stool samples and overcome the secondary structures between the RNA polymerase gene and the 5′ end of the second open reading frame. Use of an r*Tth* polymerase, which functions as both a reverse transcriptase and DNA polymerase, was found to be comparable to that of two enzymes containing both avian myeloblastosis reverse transcriptase and *Taq* polymerase (Schwab *et al*. 1998).

For environmental samples it is important to prevent the carryover contamination of samples by previously amplified PCR products. The use of a single enzyme allows the use of thermo-liable uracil N-glycosylate (HK-UNG) for prevention of carryover contamination in the RT-PCR (Schwab *et al*. 1998, 2001). dUTP is used in place of dTTP in the RT-PCR reaction mix. Samples are treated with HK-UNG before RT-PCR to destroy any dUTP containing amplicons that may have contaminated the sample during preparation. Then the HK-UNG enzyme is inactivated by heating and the viral RNA is amplified. This procedure prevents contaminating amplicons from being amplified (Schwab *et al*. 2001).

Selection of primers

The sensitivity and specificity of RT-PCR assays largely depends on the selection of primers. RT-PCR assays targeting the capsid protein of NVs have been described (Green *et al*. 1997; Noel *et al*. 1997; Hafliger *et al*. 1997; Shieh *et al*. 2000). However, the majority of primers have been designed to amplify the most conserved region of the genome, the RNA-dependent RNA polymerase region. Although a number of primer pairs have been developed

(Tables 3, 4 and 5), few studies have reported the detection sensitivity of primers, and the genetic diversity among NVs has made it difficult to select a single primer pair to detect all NVs in clinical or environmental samples (Atmar and Estes 2001). Several approaches have been described for broad detection of NVs. These include the use of primers from a highly conserved region of NVs, use of primers with equi-molar degeneracies to facilitate less specific template annealing, and incorporation of inosine into the primers to allow mismatches at specific locations of NVs (LeGuyader *et al*. 1996; Jiang *et al*. 1999; Foley *et al*. 2000).

Semi-nested and nested RT-PCR assays for specific detection of genogroup I and II have been described (Gilgen *et al*. 1997; Green *et al*. 1998; Myrmel et al. 1999). A hemi-nested multiplex RT-PCR has been described that could simultaneously detect and differentiate genogroup I and II in clinical samples (Yuen *et al*. 2001). Seeding experiments showed a nested RT-PCR assay to be 10 to 1,000 times more sensitive than the single-round RT-PCR (Green *et al*. 1998).

Confirmation of RT-PCR results

Gel electrophoresis results are considered as presumptive positive and are usually confirmed by a second method (Tables 3, 4 and 5). Oligoprobe hybridization is a convenient and sensitive way to confirm presumptive RT-PCR positives by gel electrophoresis. The sequence of the virus genome between the primers used in the RT-PCR assay is targeted to develop hybridization probes. Both radiolabelled and non-radiolabelled probes have been used and a number of probes have been developed for the detection of NVs (Ando *et al*. 1995; Vinjé and Koopmans 2000; Jiang *et al*. 2001). Different formats of oligoprobe hybridization such as dot or slot blot hybridization, liquid hybridization, and southern hybridization have been described. Besides confirming the RT-PCR products, hybridization may also increase the sensitivity of detection. RT-PCR amplicons not visible by gel electrophoresis have been detected by hybridization (Atmar *et al*. 1996). The drawback of hybridization assays is that the genetic diversity of NVs makes it difficult to select a single or even a small number of probes that can detect all possible NV sequences (Le Guyader *et al*. 1996).

Overcoming the genetic diversity has been attempted by using multiple probes. A reverse-line blotting (RLB) strategy using multiple probes (18 probes) has been described (Vinjé and Koopmans 2000). Norovirus RNA was amplified using biotinylated primers and virus-specific amplicons were captured during hybridization by one of the multiple probes linked to the membrane in individual dots on a blot. This procedure simultaneously confirmed the RT-PCR product and was able to discriminate between 13 NV genotypes. Compared with southern hybridization, RLB was equally sensitive for genogrouping but slightly less sensitive for genotyping (Vinjé and Koopmans 2000).

Restriction fragment length polymorphism (RFLP) has been used to confirm positive RT-PCR results (Gilgen *et al*. 1997). A heteroduplex mobility assay (HMA) has been described for identification of PCR amplicons among the commonly circulating NV strains without DNA sequencing (Mattick *et al*. 2000). Amplicons were mixed with, and annealed to, similar amplicons from reference strains. Those of less than 90% sequence identity formed visible heteroduplexes, allowing the strains to be categorized (Mattick *et al*. 2000).

DNA sequencing of the amplicon is another approach used to interpret RT-PCR assays. Sequencing not only identifies the specific virus, but the sequences can be compared with other strains for phylogenetic analysis. As it is not unusual when amplification of field samples results in multiple bands or a smear when visualized on agarose gels, sequencing can only be performed after cloning of gel purification of the products.

Quantitative PCR

Real-time RT-PCR is an attractive alternative to conventional PCR because of its greater rapidity, sensitivity and reproducibility. Moreover, real-time PCR would allow for quantitative interpretation of field data for NVs in environmental samples. Increasingly laboratories have the ability to employ real-time PCR assays and the commonly used Taqman chemistry is very well standardized. It typically incorporates a probe confirmation step into a

one-tube single assay. However, due to the extreme genetic variability of NV strains the design of 'catch-all' Taqman primers/probes for NVs is a real challenge and may not be possible. Quantitation of virus requires a known standard, which, owing to lack of a cell culture system for NVs, is difficult to assess. However, synthetic RNA as run-off transcript of a cloned NV strain could potentially be used.

DETECTION OF NOROVIRUSES IN WATER: THE WAY FORWARD

Sample concentration and processing

To overcome the limitation of the lack of a virus stock for development of concentration methods, there needs to be an innocuous surrogate virus that closely mimics the properties of NVs and can be quantified. Such a surrogate would be beneficial because methods development with human pathogens may be impractical owing to possible biosafety issues including contamination to laboratory personnel or release of the pathogen to the environment. Also for field and pilot studies of water treatment plant performance, the introduction of pathogens into these conditions would be prohibited.

Options for surrogate development are varied. It has been demonstrated that animal caliciviruses may be appropriate surrogate models for some aspects of NV behaviour. Some investigators have used feline caliciviruses for disinfection studies, and primate caliciviruses have been used for evaluation of water concentration methods (Keswick *et al*. 1985; Doultree *et al*. 1999; Huang *et al*. 2000). However, questions remain whether respiratory animal caliciviruses are appropriate models for NVs. Other viruses, for example attenuated poliovirus, virus-like particles or recombinant virus constructs may also be applicable surrogates (Redman *et al*. 1997; Katamaya *et al*. 2002). However, poliovirus research may be curtailed shortly because of the global eradication programme; thus different enterovirus surrogates may be needed.

VLPs can be a powerful tool as surrogates for NVs since they are morphologically and antigenetically similar to the native virus (Jiang *et al*. 1992; Green *et al*. 1993). Moreover, they are essentially harmless particles that can be employed in environmental studies without posing any human health threat. VLPs have been used as a surrogate in a filtration study (Redman *et al*. 1997). Because VLPs only contain the capsid viral protein, they may be employed to study capsid degradation during concentration procedures, which would be important for understanding how sample processing might affect viral infectivity. To mimic the actual virus particles, VLPs would need to be constructed to contain a well-defined non-related RNA in order to differentiate the VLPs from the actual NVs. These virus constructs would be useful as internal positive controls for use in virus concentration procedures and molecular assays. However, the physicochemical characteristics (such as density, geometry, integrity, surface electric charge) and stability of VLPs could be different from those of the NVs of concern and these factors must be taken into consideration for any method development.

Once the selection of the test organism or surrogate has been resolved, it will be necessary to determine the optimum filter materials and conditions for the adsorption and elution of NVs in a range of water matrices. In addition, a suitable method for purification of NVs in sample concentrates prior to analysis will be necessary. Finally, a round robin testing of methods will be required to standardize NV analysis and increase confidence in the water quality results.

Molecular methodologies for detection and confirmation

An optimized sample preparation and viral RNA purification scheme needs to be developed prior to assay by molecular methods. The viral RNA purification/extraction approach(es) should address inhibitor and interfering substance removal, low template detection, competing nucleic acids, sample volume, reproducibility, sequence fidelity, robustness and recovery efficiency.

Genetic diversity among NVs is a major challenge in developing a broadly reactive RT-PCR assay to detect all or the majority of NVs. Molecular assays should be evaluated for their ability to detect a wide diversity of NVs at

low levels, distinguish or differentiate human from animal and non-enteric caliciviruses, and be applicable to a wide range of environmental samples and matrices (i.e. drinking water, wastewater, recreational water, shellfish and other foods).

The genetic diversity also makes it difficult to select a single, or even a small number, of probes that can detect all possible NV sequences for confirmation of PCR products. A confirmation and characterization scheme should be developed that incorporates differentiation of major genogroups of human, animal and non-enteric caliciviruses.

Detection of infectious noroviruses

Because NVs currently cannot be grown in cell culture, determining the infectivity of NVs remains a major challenge. Although there is little published data, anecdotal reports indicate that many researchers have attempted to cultivate NVs in cell and organ culture without success. The only currently available model for directly assessing NV infectivity requires the use of human subjects. The cost and risk associated with these experiments severely limits their usefulness for inactivation studies and prohibits their use for routine monitoring. Several researchers have used cultivatable animal caliciviruses as surrogates for NV infectivity, but the biophysical properties of these viruses are sufficiently different to raise questions about extrapolating these results to human NVs. In the absence of a true infectivity assay, a molecular surrogate for infection could be a useful model for studying neutralization, disinfection/inactivation, and for measuring infectious virus in environmental samples. Approaches to distinguish between inactivated and infectious NVs might include:

- a reporter system that uses an enzymatic or fluorescent signal that is produced after exposure to the infectious virus (Olivo *et al*. 1994);
- differential induction of a cellular response to infectious virus (e.g. microarray detection of changes in specific mRNA levels);
- transfection of the viral genome in the infected cell; or
- receptor ligand binding or immunocapture of virus combined with nucleic acid detection.

It should be noted that it has been demonstrated that non-infectious recombinant virus-like particles (VLPs) can bind to and be internalized in human cells (White *et al*. 1996); thus, an assay for viable virus must depend on more than ligand binding alone.

Even though previous attempts to develop a cell culture system have failed, a permissive cell culture system for NVs should remain as an important goal. Recently, there have been a number of important developments that increase the likelihood of success for a NV cell culture system. Some of these developments include more sensitive assays to monitor cell culture assays (RT-PCR, confocal and digital imaging capture systems for real-time analyses, a potential Norwalk virus receptor identification, newer cloning techniques to attach ligand to a cell line, etc.). The identification of a potential NV receptor (Tamura *et al*. 2000) and new cloning techniques to attach such ligands to cell lines offer the possibility of constructing permissive cells. Other approaches to consider might include:

- design of cell culture systems that mimic target organs (biocultures, primary intestinal biopsies, or raft cultures);
- design of genetic based replicon or reporter systems to indicate the presence of infectious viruses.

As a stepping-stone to the development of a NV cell culture system, a better understanding of animal enteric caliciviruses could yield important insights. For example, although porcine calicivirus has been grown in cell culture, the growth requires undefined factors present in the animal intestinal contents (Flynn *et al*. 1988). Efforts are under way to characterize these growth factors, but it is not known whether such factors are important for human or other animal enteric caliciviruses. A more thorough understanding of the infectivity and pathogenesis of enteric caliciviruses in animals may lead to breakthroughs in the understanding of NVs, and systems to study them.

The ability to better assess the infectivity of NVs is important because there is conflicting data on susceptibility of NVs to chlorination and water treatment. A study of Norwalk virus inactivation by chlorine, which assessed infectivity in human volunteers, suggested that the virus was relatively resistant to chlorination (Keswick *et al*.

1985). Recent data demonstrated that feline calicivirus is more sensitive to chlorine than poliovirus-1 (Shin *et al*. 1998; Thruston-Enriquez *et al*. 2003). Moreover, data from waterborne NV outbreaks implies that interruption of chlorination or contamination of the distribution system were associated with illness. Uncertainty over the effectiveness of basic water treatment processes underscores the need for NV infectivity methods to better ascertain risks to public health.

CONCLUSIONS

Noroviruses are recognized as the most important cause of acute nonbacterial gastroenteritis. Currently there are no standard methods available for the concentration of caliciviruses from small or large volumes of water or other environmental samples. Development of a cell culture model would result in the detection of infectious NVs in environmental samples as well as increase the understanding of virus replication and the environmental factors that lead to virus inactivation. Experiments with recombinant Norwalk virus (rNV) capsids suggest that the C-terminal region of the capsid protein is involved in the specific binding of rNV virus-like proteins to human cells. Identification and characterization of NV receptors in human cells may help to develop a recombinant continuous cell line capable of facilitating NV replication. RT-PCR assays are currently the most widely used methods for the detection of NVs in water samples. However, the methods need to be refined and standardized through the further development of procedures to simplify viral nucleic acid extraction, RT-PCR processes, and the development of primers and probes that can detect the majority of NVs present in low numbers in environmental samples. Moreover, methods need to be developed to determine the infectivity of the viruses. Finally, because methods for detecting NVs in water samples are still under development, the prevalence of NV contamination of drinking water and other water types remains unclear. These methods are necessary to evaluate the occurrence, risk and efficacy of current treatment barriers for NVs in drinking water and other environmental samples.

ACKNOWLEDGEMENTS

The study was funded by the AWWA Research Foundation and the subsidiary utilities of American Water, Voorhees, New Jersey. The authors thank Kathryn Martin, American Water Works Research Foundation project officer, and the following members of the project advisory committee: Dr Kellogg Schwab (Johns Hopkins University), Dr Peter Wyn-Jones (University of Sunderland, UK) and Dr Jan Vinjé (University of North Carolina). The authors also thank the following individuals for their participation in a norovirus workshop to provide the future research directions: Dr Mark D. Sobsey, Dr Charles P. Gerba, Dr Mary K. Estes, Dr Jason Jiang, Dr Linda Saif, Dr Kathleen Henshilwood, Dr Marion Koopmans, Dr Steve Monroe, Dr Kim Green, Dr Jane Sellwood, Dr Soizick LeGuyader, Dr Christine Moe, Dr Ricardo De León, Dr Shay Fout, Dr Albert Bosch, Dr Robert Atmar and Dr David Lees.

REFERENCES

Abbaszadegan, M., Huber, M. S., Gerba, C. P. & Pepper, I. L. 1993 Detection of enteroviruses in groundwater with the polymerase chain reaction. *Appl. Environ. Microbiol.* **59**, 1318–1324.

Abbaszadegan, M., Stewart, P. W., LeChevallier, M. W., Rosen, J. S. & Gerba, C. P. 2003 Occurrence of viruses in US groundwaters. *J. Am. Wat. Wks Assoc.* **95**(9), 107–120.

Adler, I. & Zickl, R. 1969 Winter vomiting disease. *J. Infect. Dis.* **119**, 668–673.

Anderson, A. D., Heryford, A. G., Sarisky, J. S., Higgins, C., Monroe, S. S., Beard, R. S., Newport, C. S., Cashdollar, J. L., Fout, G. S., Robbins, D. E., Seys, S. A., Musgrave, K. J., Medus, C., Robbins, D. E., Seys, S. A., Musgrave, K., Medus, C., Vinjé, J., Bresee, J. S., Mainzer, H. M. & Glass, R. I. 2003 A waterborne outbreak of Norwalk-like virus among snowmobilers-Wyoming, 2001. *J. Infect. Dis.* **187**, 303–306.

Ando, T., Monroe, S. S., Gentsch, J. R., Jin, Q., Lewis, D. C. & Glass, R. I. 1995 Detection and differentiation of antigenetically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridization. *J. Clin. Microbiol.* **33**, 64–71.

Ando, T., Monroe, S. S., Noel, J. & Glass, R. I. 1997 A one-tube method of reverse transcription-PCR to efficiently amplify a 3-kilobase from the RNA polymerase gene to the poly (A) tail of small round-structured viruses (Norwalk-like viruses). *J. Clin. Microbiol.* **35**, 570–577.

Ando, T., Noel, J. S. & Fankhauser, R. L. 2000 Genetic classification of 'Norwalk-like viruses'. *J. Infect. Dis.* **81**, S336–348.

Atmar, R. & Estes, M. K. 2001 Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. *Clin. Microbiol. Rev.* **14**, 15–37.

Atmar, R. L., Neill, F. H., Romalde, J. L., LeGuyader, R., Woodley, C. M., Metcalf, T. G. & Estes, M. K. 1995 Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. *Appl. Environ. Microbiol.* **61**, 3014–3018.

Atmar, R. L., Neil, F. H., Woodley, C. M., Manger, R., Fout, G. S., Burkhardt, W., Leja, L., McGovern, E. R., Le Guyader, F., Metcalf, T. G. & Estes, M. K. 1996 Collaborative evaluation of a method for the detection Norwalk virus in shellfish tissues by PCR. *Appl. Environ. Microbiol.* **62**, 254–258.

Baron, R. C., Murphy, F. D., Greenberg, H. B., Davis, C. E., Bregman, D. J., Gary, G. W., Hughes, J. M. & Schonberger, L. B. 1982 Norwalk gastrointestinal illness: An outbreak associated with swimming in a recreational lake and secondary person-to-person transmission. *Am. J. Epidemiol.* **115**(2), 163–172.

Baron, R. C., Greenberg, H. B., Cukor, G. & Blacklow, N. R. 1984 Serological responses among teenagers after natural exposure to Norwalk virus. *J. Infect. Dis.* **150**, 531–534.

Becker, K. M., Moe, C. L., Southwick, K. L. & MacCormack, J. N. 2000 Transmission of Norwalk virus during a football game. *N. Engl. J. Med.* **343**, 1223–1227.

Beller, M., Ellis, A., Lee, S. H., Jenkerson, S. A., Funk, E., Sobsey, M. D., Simmons, O. D., Monroe, S. S., Ando, T., Noel, J., Petric, M., Middaugh, J. P. & Spika, J. S. 1997 Outbreak of viral gastroenteritis due to a contaminated well. *J. Am. Med. Assoc.* **278**, 563-568.

Beuret, C., Kohler, D., Baumgartner, A. & Luthi, T. M. 2002 Norwalk-like virus sequences in mineral waters: One-year monitoring of three brands. *Appl. Environ. Microbiol.* **68**, 1925–1931.

Biel, S. S. & Gelderblom, H. R. 1999 Diagnostic electron microscopy is still a timely and rewarding method. *J. Clin. Virol.* **13**, 105–119.

Boccia, D., Eugenio, A. E., Cotter, B., Rizzo, C., Russo, T., Buttinelli, G., Caprioli, A., Luisa Marziano, M. & Ruggeri, F. M. 2002 Waterborne outbreaks of Norwalk-like virus gastroenteritis at a tourist resort, Italy. *Emerg. Infect. Dis.* **8**, 563–568.

Bon, F., Fascia, P., Dauvergne, M., Tenenbaum, D., Planson, H., Petion, A. M., Pothier, P. & Kohli, E. 1999 Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France. *J. Clin. Microbiol.* **37**, 3055–3058.

Boom, R., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M. & van der Noordaa, J. 1990 Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**, 495–503.

Brinker, J. P., Blaclow, N. R., Jiang, X., Estes, M. K., Moe, C. L. & Herrmann, J. E. 1999 Immunoglobulin M antibody test to detect genogroup II Norwalk-like virus infection. *J. Clin. Microbiol.* **37**, 2983–2986.

Brugha, R., Vipond, I. B., Evans, M. R., Sandifer, Q. D., Roberts, R. J., Salmon, R. L., Caul, E. O. & Mukerjee, A. K. 1999 Community outbreak of food-borne small round structured virus gastroenteritis caused by a contaminated water supply. *Epidemiol. Infect.* **122**, 145–154.

Daniels, N. A., Bergmire-Sweat, D. A., Schwab, K. J., Hendricks, K. A., Reddy, S., Rowe, S. A., Fankhauser, R. L., Monroe, S. S., Atmar, R. L., Glass, R. I. & Mead, P. 2000 A foodborne outbreak of gastroenteritis associated with Norwalk-like viruses: first molecular traceback to deli sandwiches contaminated during preparation. *J. Infect. Dis.* **181**, 1467–1470.

Dastjerdi, A. M., Green, J., Gallimore, C. I., Brown, D. W. G. & Bridger, J. C. 1999 The bovine Newbury agent-2 is genetically more closely related to human SRSVs than to animal caliciviruses. *Virology* **254**, 1–5.

De Leon, R., Matsui, S. M., Baric, R. S., Herrmann, J. E., Blacklow, N. R., Greenberg, H. B. & Sobsey, M. D. 1992 Detection of Norwalk virus in stool specimen by reverse transcriptase-polymerase chain reaction and nonradioactive oligoprobes. *J. Clin. Microbiol.* **30**, 3151–3157.

Deneen, V. C., Hunt, J. M., Paule, C. R., James, R. I., Johnson, R. G., Raymond, M. J. & Hedberg, C. W. 2000 The impact of foodborne calicivirus disease: the Minnesota experience. *J. Infect. Dis.* **181**, S281–283.

Doultree, J. C., Druce, J. D., Birch, C. J., Bowden, D. S. & Marshall, J. A. 1999 Inactivation of feline calicivirus, a Norwalk virus surrogate. *J. Hosp. Infect.* **41**, 51–57.

Estes, M., Ball, J. M., Guerrero, R. A., Opekun, A. R., Gilger, M. A., Pacheco, S. S. & Graham, D. Y. 2000 Norwalk virus vaccines: challenges and progress. *J. Infect. Dis.* **181**, S367–373.

Flynn, W. & Saif, L. 1988 Serial propagation of porcine enteric calicivirus-like virus in primary porcine kidney cell cultures. *J. Clin. Microbiol.* **26**, 206–212.

Foley, B., O'Mahony, J., Morgan, S. M., Hill, C. & Morgan, J. G. 2000 Detection of sporadic case of Norwalk-like virus (NLV) and astrovirus infection in a single Irish hospital from 1996 to 1998. *J. Clin. Virol.* **17**, 109–117.

Frankhauser, R. L., Monroe, S. S., Noel, J. S., Humphrey, C. D., Bresee, J. S., Parashar, U. D., Ando, T. & Glass, R. I. 2002 Epidemiologic and molecular trends of 'Norwalk-like viruses' associated with outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* **186**, 1–7.

Gilgen, M., Germann, D., Luthy, J. & Hubner, P. 1997 Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples. *Int. J. Food Microbiol.* **37**, 189–199.

Gilpatrick, S. G., Schwab, K. J., Estes, M. K. & Atmar, R. L. 2000 Development of an immunomagnetic capture reserves transcription-PCR assay for the detection of Norwalk virus. *J. Virol. Meth.* **90**, 69–78.

Glass, P., White, L. J., Ball, J. M., Leparc-Goffart, I., Hardy, M. E. & Estes, M. K. 2000 Norwalk virus open reading frame 3 encodes a minor structural protein. *J. Virol.* **74**, 6581–6591.

Gonin, P., Couillard, M. & d'Halewyn, M. 2000 Genetic diversity and molecular epidemiology of Norwalk-like viruses. *J. Infect. Dis.* **182**, 691–697.

Goodman, R. A., Buehler, J. W., Greennberg, H. B., McKinley, T. W. & Smith, J. D. 1982 Norwalk gastroenteritis associated with a water system in a rural Georgia community. *Arch. Environ. Health* **37**, 358–360.

Green, J., Henshilwood, K., Gallimore, C. I., Brown, D. W. G. & Lees, D. N. 1998 A nested reverse transcriptase PCR assay for detection of small round-structured viruses in environmentally contaminated molluscan shellfish. *Appl. Environ. Microbiol.* **64**, 858–863.

Green, K. Y., Lew, J. F., Jiang, X., Kapikian, A. J. & Estes, M. K. 1993 Comparison of reactivities of baculovirus-expressed recombinant Norwalk-virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. *J. Clin. Microbiol.* **31**, 2185–2191.

Green, K. Y., Chanock, R. M. & Kapikian, A. 2001 *Fields Virology*, 4th edn. In: Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B. & Straus, S. E. (eds). Lippincott Williams & Wilkins, Philadelphia.

Green, K. Y., Belliot, G., Taylor, J. L., Valdesuso, J., Lew, J. F., Kapikian, A. Z. & Lin, F. C. 2002 A predominant role for Norwalk-like viruses as agents of epidemic gastroenteritits in Maryland nursing homes for the elderly. *J. Infect. Dis.* **185**, 133–146.

Green, S. M., Lambden, P. R., Caul, E. O. & Clarke, I. N. 1997 Capsid sequence diversity in small round-structured viruses from recent UK outbreaks of gastroenteritis. *J. Med. Virol.* **52**, 14–19.

Guerrero, R. A., Ball, J. M., Krater, S. S., Pacheco, S. E., Clements, J. D. & Estes, M. K. 2001 Recombinant Norwalk virus-like particles administered intranasally to mice induce systemic and mucosal (fecal and vaginal) immune responses. *J. Virol.* **75**, 9713–9722.

Guo, M., Chang, K. O., Hardy, M. E., Zhang, Q., Parwani, A. V. & Saif, L. J. 1999 Molecular characterization of a porcine enteric calicivirus genetically related to sapporo-like human calicivirus. *J. Virol.* **73**, 9625–9631.

Hafliger, D., Gilgen, M., Luthy, J. & Hubner, P. 1997 Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood. *Int. J. Food Microbiol.* **37**, 27–36.

Hale, A. D., Green, J. & Brown, D. W. G. 1996 Comparison of four RNA extraction methods for the detection of small round structured viruses in fecal specimens. *J. Virol. Meth.* **57**, 195–201.

Harrington, P. R., Yount, B., Johnston, R. E., Davis, N., Moe, C. & Baric, R. S. 2002 Systemic, mucosal and heterotypic immune induction in mice inoculated with Venezuelan equine encephalitis replicons expressing Norwalk virus-like particles. *J. Virol.* **76**, 730–742.

Hedlund, K. O., Rubilar-Abreu, E. & Svensson, L. 2000 Epidemiology of calicivirus infections in Sweden, 1994–1998. *J. Infect. Dis.* **181**, S275–280.

Herrmann, J. E., Blacklow, N. R., Matsui, S. M., Lewis, T. L., Estes, M. K., Ball, J. M. & Brinker, J. P. 1995 Monoclonal antibodies for detection of Norwalk virus antigen in stools. *J. Clin. Microbiol.* **33**, 2511–2513.

Herwaldt, B. L., Lew, J. F., Moe, C. L., Lewis, D. C., Humphrey, C. D., Monroe, S. S., Pon E. W. & Glass, R. I. 1994 Characterization of a variant strain of Norwalk virus from a food-borne outbreak of gastroenteritis on a cruise ship in Hawaii. *J. Clin. Microbiol.* **32**, 861–866.

Honma, S., Nakata, S., Numata, K., Kogawa, K., Yamashita, T., Oseto, M., Jiang, X. & Chiba, S. 1998 Epidemiological study of prevalence of genogroup II human calicivirus (Mexico virus) infections in Japan and Southeast Asia as determined by enzyme-linked immunosorbent assays. *J. Clin. Microbiol.* **36**, 2481–2484.

Huang, P. W., Laborde, D., Land, V. R., Matson, D. O., Smith, A. W. & Jiang, X. 2000 Concentration and detection of caliciviruses in water samples by reverse transcription-PCR. *Appl. Environ. Microbiol.* **66**, 4383–4388.

Iritani, N., Seto, Y., Haruki, K., Kimura, M., Ayata, M. & Ogura, H. 2000 Major change in the predominant type of 'Norwalk-like viruses' in outbreaks of acute nonbacterial gastroenteritis in Osaka City, Japan, between April 1996 and March 1999. *J. Clin. Microbiol.* **38**, 2649–2654.

Jiang, X., Graham, D. Y., Wang, K. & Estes, M. K. 1990 Norwalk virus genome cloning and characterization. *Science* **250**, 1580–1582.

Jiang, X., Wang, M., Graham, D. Y. & Estes, M. K. 1992 Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* **66**, 6527–6532.

Jiang, X., Wang, M., Wang, K. & Estes, M. K. 1993 Sequence and genomic organization of Norwalk virus. *Virology* **195**, 51–61.

Jiang, X., Huang, P. W., Zhong, W. M., Farkas, T., Cubitt, D. W. & Matson, D. O. 1999 Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. *J. Virol. Meth.* **83**, 145–154.

Jiang, X., Huang, P. W., Laborde, D., Land, V. R. & Matson, D. O. 2001 *Detection of Caliciviruses in Water Samples*. American Water Works Association Research Foundation, Denver, Colorado.

Johansson, P. J. H., Torven, M., Hammarlund, A. C., Bjorne, U., Hedlund, K. O. & Svensson, L. 2002 Food-borne outbreak of gastroenteritis associated with genogroup I calicivirus. *J. Clin. Microbiol.* **40**, 794–798.

Kapikian, A. Z. 1972 Visualization by immune electron microscopy of a 27 nm particle associated with acute infectious non-bacterial gastroenteritis. *J. Virol.* **10**, 1075–1081.

Kapikian, A. Z. & Channock, R. M. 1985 *Virology*. Raven Press, New York.

Kapikian, A. Z. & Chanock, R. M. 1990 *Virology*. Raven Press, New York.

Kapikian, A. Z., Greenberg, H. B., Cline, W. L., Kalica, A. R., Wyatt, R. G., James, Jr, H. D., Lloyd, N. L., Chanock, R. M., Ryder, R. W. & Kim, H. W. 1987 Prevalence of antibody to the Norwalk agent by a newly developed immune adherence hemagglutination assay. *J. Med. Virol.* **2**, 281–294.

Kaplan, J. E., Goodman, R. A., Schonberger, L. B., Lippy, E. C. & Gary, G. W. 1982 Gastroenteritis due to Norwalk virus: An outbreak associated with a municipal water system. *J. Infect. Dis.* **146**, 190–197.

Kappus, K. D., Marks, J. S., Holman, R. C., Bryant, J. K., Baker, C., Gary, G. W. & Greenberg, H. B. 1982 An outbreak of Norwalk gastroenteritis associated with swimming in a pool and secondary person-to-person transmission. *Am. J. Epidemiol.* **116**(5), 834–839.

Katayama, H., Shimasaki, A. & Ohgaki, S. 2002 Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. *Appl. Environ. Microbiol.* **68**, 1033–1039.

Keswick, B. H., Satterwhite, T. K., Johnson, P. C., Dupont, H. L., Secor, S. L., Bitsura, J. A., Gary, G. W. & Hoff, J. C. 1985 Inactivation of Norwalk virus in drinking water by chlorine. *Appl. Environ. Microbiol.* **50**, 261–264.

Khan, A. S., Moe, C. L., Glass, R. I., Monroe, S. S., Estes, M. K., Chapman, L. E., Jiang, X., Humphrey, C., Pon, E. & Iskander, J. K. 1994 Norwalk virus-associated gastroenteritis traced to ice consumption aboard a cruise ship in Hawaii: comparison and application of molecular method-based assays. *J. Clin. Microbiol.* **32**, 318–322.

Kingsley, D. H. & Richards, G. P. 2001 Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish. *Appl. Environ. Microbiol.* **67**, 4152–4157.

Koopmans, M. G., Vinje, J., de Wit, M., Leenen, I., van der Poel, W. & van Duynhoven, Y. 2000 Molecular epidemiology of human enteric caliciviruses in the Netherlands. *J. Infect. Dis.* **181**, S262–269.

Kukkula, M., Arstila, P., Klossner, M. L., Maunula, L., Bonsdorff, C. V. & Jaatinen, P. 1997 Waterborne outbreak of viral gastroenteritis. *Scand. J. Infect. Dis.* **29**, 415–418.

Kukkula, M., Maunula, L., Silvennoinen, E. & von Bonsdorff, C. 1999 Outbreak of viral gastroenteritis due to drinking water contaminated by Norwalk-like viruses. *J. Infect. Dis.* **180**, 1771–1776.

Lees, D. N., Henshilwood, K., Green, J., Gallimore, C. I. & Brown, D. W. G. 1995 Detection of small round structured viruses in shellfish by reverse transcription-PCR. *Appl. Environ. Microbiol.* **61**, 4418–4424.

Le Guyader, F., Estes, M. K., Hardy, M. E., Neill, F. H., Green, J., Brown, D. & Atmar, R. L. 1996 Evaluation of a degenerate primer for the PCR detection of human caliciviruses. *Arch. Virol.* **141**, 2225–2235.

Lewis, D., Ando, T., Humphrey, C. D., Monroe, S. S. & Glass, R. I. 1995 Use of solid-phase immune microscopy for classification of Norwalk-like viruses into six antigenic groups from 10 outbreaks of gastroenteritis in the United States. *J. Clin. Microbiol.* **33**, 501–504.

Lindesmith, L., Moe, C., Marionneau, S., Ruvoen, N., Jiang, X., Lindblad, L., Stewart, P., Lependu, J. and Baric, R. 2003 Human susceptibility and resistance to Norwalk virus infection. *Nat. Med.* **9**, 548–553.

Liu, B. L., Clarke, I. N., Caul, E. O. & Lambden, P. R. 1995 Human enteric caliciviruses have a unique genome structure and are distinct from the Norwalk-like viruses. *Arch. Virol.* **140**, 1345–1356.

Liu, B., Clarke, I. N., Caul, E. O. & Lambden, P. R. 1997 The genomic 5' terminus of Manchester calicivirus. *Virus Genes* **15**, 25–28.

Liu, B. L., Lambden, P. R., Gunther, H., Otto, P., Elschner, M. & Clarke, I. N. 1999 Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses. *J. Virol.* **73**, 819–825.

Lodder, W. J., Vinje, J., Heide, R. V. D., Husman, A. M. D. R., Leenen, E. J. T. M. & Koopmans, M. P. G. 1999 Molecular detection of Norwalk-like caliciviruses in sewage. *Appl. Environ. Microbiol.* **65**, 5624–5627.

Maguire, A., Green, J., Brown, D. W. G., Desselberger, U. & Gray, J. J. 1999 Molecular epidemiology of outbreaks of gastroenteritis associated with small round-structured viruses in East Anglia, United Kingdom, during 1996–1997 seasons. *J. Clin. Microbiol.* **37**, 81–89.

Metcalf, T. G., Melnick, J. L. & Estes, M. K. 1995 Environmental virology: from detection of virus in sewage and water by isolation to identification by molecular biology – a trip of over 50 years. *Ann. Rev. Microbiol.* **49**, 461–487.

Mattick, K. L., Green, J., Punia, P., Belda, F. J., Gallimore, C. I. & Brown, D. W. G. 2000 The heteroduplex mobility assay (HMA) as a pre-screening screen for Norwalk-like viruses. *J. Virol. Meth.* **87**, 161–169.

McCarthy, M., Estes, M. K. & Hyams, K. C. 2000 Norwalk-like virus infection in military forces: epidemic potential. sporadic disease, and the future direction of prevention and control efforts. *J. Infect. Dis.* **181**, S387–391.

MMWR Weekly Report 1982 Community outbreak of Norwalk Gastroenteritis – Georgia *MMWR Weekly Report* **31**(30), 405–407.

MMWR Weekly Report 2001 'Norwalk-like viruses' public health consequences and outbreak management. *MMWR Weekly Report* **50**(9), 1–17.

MMWR Weekly Report 2002 Outbreaks of gastroenteritis associated with noroviruses on cruise ships – United States. *MMWR Weekly Report* **51**(49), 1112–1115.

Mounts, A. W., Ando, T., Koopmans, M., Bresee, J. S., Noel, J. & Glass, R. I. 2000 Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses. *J. Infect. Dis.* **181**, S284–287.

Myrmel, M., Rimstad, E., Wasteson, Y. & Skjerve, E. 1999 Detection of small round structured viruses in artificially contaminated water using filter absorption and reverse transcription polymerase chain reaction. *Int. J. Food Microbiol.* **49**, 85–94.

Myrmel, M., Espen, R. & Wasteson, Y. 2000 Immunomagnetic separation of a Norwalk-like virus (genogroup I) in artificially contaminated environmental samples. *Int. J. Food Microbiol.* **62**, 17–26.

Nakata, S., Honma, S., Numata, K., Kogawa, K., Ukae, S., Adachi, N., Jiang, X., Estes, M. K., Gatheru, Z., Tukel, P. M. & Chiba, S. 1998 Prevalence of human calicivirus infections in Kenya as determined by enzyme immunoassays for three genogroups of the virus. *J. Clin. Microbiol.* **36**, 3160–3163.

Nakata, S., Honma, S., Numata, K., Kogawa, K., Ukae, S., Morita, Y., Adachi, N. & Chiba, S. 2000 Members of the family

Caliciviridae (Norwalk virus and Sapporo virus) are the most prevalent cause of gastroenteritis outbreaks among infants in Japan. *J. Infect. Dis.* **181**, 2029–2032.

Noel, J. S., Ando, T., Leite, J. P., Green, K. Y., Dingle, K. E., Estes, M. K., Seto, Y., Monroe, S. S. & Glass, R. I. 1997 Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990–1995. *J. Med. Virol.* **53**, 372–383.

Noel, J. S., Fankhauser, R. L., Ando, T., Monroe, S. S. & Glass, R. I. 1999 Identification of a distinct common strain of 'Norwalk-like viruses' having a global distribution. *J. Infect. Dis.* **179**, 1334–1344.

Numata, K., Hardy, M. E., Nakata, S., Chiba, S. & Estes, M. K. 1997 Molecular characterization of morphologically typical human calicivirus Sapporo. *Arch. Virol.* **142**, 1537–1552.

Olivo, P. D., Frolov, I. & Schlesinger, S. 1994 A cell line that expresses a reporter gene in response to infection by sindbis virus: a prototype for detection of positive strand RNA viruses. *Virology* **198**(1), 381–384.

O'Ryan, M. L., Mamani, N., Gaggero, A., Avendano, L. F., Prieto, S., Pena, A., Jiang, X. & Matson, D. O. 2000 Human caliciviruses are a significant pathogen of acute sporadic diarrhea in children of Santiago, Chile. *J. Infect. Dis.* **182**, 1519–1522.

Pang, X., Honma, S., Nakata, S. & Vesikari, T. 2000 Human caliciviruses in acute gastroenteritis of young children in the community. *J. Infect. Dis.* **181**, S288–294.

Parwani, A. V., Flynn, W. T., Gadfield, K. L. & Saif, L. 1991 Serial propagation of porcine enteric calicivirus in a continuous cell line. *Arch. Virol.* **120**, 115–122.

Redman, J. A., Grant, S. B. & Olson, T. M. 1997 Filtration of recombinant Norwalk virus particles and bacteriophage MS2 in quartz sand: Importance of electrostatic interactions. *Environ. Sci. Technol.* **31**, 3378–3383.

Sawyer, L. A., Murphy, J. J., Kaplan, J. E., Pinsky, P. F., Chacon, D., Walmsley, S., Schonberger, L. B., Phillips, A., Forward, K. & Goldman, C. 1988 25 to 30 nm virus particles associated with hospital outbreak of acute gastroenteritis with evidence for airborne transmission. *Am. J. Epidemiol.* **127**, 1261–1271.

Schuffenecker, I., Ando, T., Thouvenot, D., Lina, B. & Aymard, M. 2001 Genetic classification of 'Sapporo-like viruses'. *Arch. Virol.* **146**, 2115–2132.

Schwab, K. J., DeLeon, R. & Sobsey, M. D. 1995 Concentration and purification of beef extract mock elutes from water samples for the detection of enteroviruses, hepatitis A virus, and Norwalk virus by reverse transcription-PCR. *Appl. Environ. Microbiol.* **61**, 531–537.

Schwab, K. J., De Leon, R. & Sobsey, M. D. 1996 Immunoaffinity concentration and purification of waterborne enteric viruses for detection by reverse transcriptase PCR. *Appl. Environ. Microbiol.* **62**, 2086–2094.

Schwab, K. J., Estes, M. K., Neill, F. H. & Atmar, R. L. 1997 Use of heat release and an internal RNA standard control in reverse transcription-PCR detection of Norwalk virus from stool sample. *J. Clin. Microbiol.* **35**, 511–514.

Schwab, K. J., Neil, F. H., Estes, M. K. & Atmar, R. L. 1998 Improvements in the RT-PCR detection of enteric viruses in environmental samples. *Wat. Sci. Technol.* **38**, 83–86.

Schwab, K. J., Neill, F. H., Fankhauser, R. L., Danniels, N. A., Monroe, S. S., Bergmire-Sweat, D. A., Estes, M. K. & Atmar, R. L. 2000 Development of methods to detect 'Norwalk-like viruses' (NVs) and hepatitis A virus in delicatessen foods: application to a food-borne NLV outbreak. *Appl. Environ. Microbiol.* **66**, 213–218.

Schwab, K. J., Neill, F. H., LeGuyader, F., Estes, M. K. & Atmar, R. L. 2001 Development of a reverse transcription-PCR-DNA enzyme immunoassay for detection of 'Norwalk-like' viruses and hepatitis A virus in stool and shellfish. *Appl. Environ. Microbiol.* **67**, 742–749.

Shieh, Y. S. C., Calci, K. R. & Baric, R. S. 1999 A method to detect low levels of enteric viruses in contaminated oysters. *Appl. Environ. Microbiol.* **65**, 4709–4714.

Shieh, Y. S. C., Monroe, S. S., Fankhauser, R. L., Langlois, G. W., Burkhardt III W. & Baric, R. S. 2000 Detection of Norwalk-like virus in shellfish implicated illness. *J. Infect. Dis.* **181**, S360–366.

Shin, G. A., Battigelli, D. & Sobsey, M. D. 1998 Reduction of Norwalk virus, poliovirus 1, and coliphage MS2 by free chlorine, chlorine dioxide, and ozone disinfection of water. Water Quality and Technology Conference, San Diego, California.

Smit, T. K., Steele, A. D., Peenze, I., Jiang, X. & Estes, M. K. 1997 Study of Norwalk virus and Mexico virus infections at Ga-Rankuwa Hospital. Ga-Rankuwa, South Africa. *J. Clin. Microbiol.* **35**, 2381–2385.

Smith, A. W., Berry, E. S., Skilling, D. E., Barlough, J. E., Poet, S. E., Berke, T., Mead, J. & Matson, D. O. 1998a In vitro isolation and characterization of a calicivirus causing vesicular disease of the hands and feet. *Clin. Infect. Dis.* **26**, 434–439.

Smith, A. W., Skilling, D. E., Cherry, N., Mead, J. H. & Matson, D. O. 1998b Calicivirus emergence from ocean reservoirs: zoonotic and interspecies movement. *Emerg. Infect. Dis.* **4**, 13–20.

Sobsey, M. D. 1999 Progress Report: Detection and Occurrence of Human Caliciviruses in Drinking Water, http://es.epa.gov/ncer/progress/grants/98/drinking/sobsey99.html.

Sugieda, M., Nagaoka, H., Kakishima, Y., Ohshita, T., Nakamura, S. & Nakajima, S. 1998 Detection of Norwalk-like virus genes in the caecum contents of pigs. *Arch. Virol.* **143**, 1215–1221.

Sunen, E. & Sobsey, M. 1999 Recovery and detection of enterovirus, hepatitis A virus and Norwalk virus in hardshell clams (*Mercenaria mercenaria*) by RT-PCR methods. *J. Virol. Methods* **77**, 179–187.

Tamura, M., Natori, K., Kobayashi, M., Miyamura, T. & Takeda, N. 2000 Interaction of recombinant Norwalk virus particles with the 105-kilodalton cellular binding protein, a candidate receptor molecule for virus attachment. *J. Virol.* **74**, 11589–11597.

Taylor, J. W., Gary, G. W. Jr & Greenberg, H. B. 1981 Norwalk-related viral gastroenteritis due to contaminated water. *Am. J. Epidemiol.* **114**(4), 584–592.

Thruston-Enriquez, C., Hass, N., Jacangelo, J. & Gerba, C. P. 2003 Chlorine inactivation of adenovirus type 40 and feline calicivirus. *Appl. Environ. Microbiol.* **69**(7), 3979–3985.

USEPA (United States Environmental Protection Agency) 1998 *Drinking Water Contaminant Candidate List*. EPA 815-F-98-002. Washington, DC.

van der Poel, W. H. M., Vinje, J., van der Heide, R., Herrera, M., Vivo, A. & Koopmans, M. P. 2000 Norwalk-like calicivirus genes in farm animals. *Emerg. Infect. Dis.* **6**, 36–41.

Vinjé, J. & Koopmans, M. P. G. 1996 Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. *J. Infect. Dis.* **174**, 610–615.

Vinjé, J. & Koopmans, M. P. G. 2000 Simultaneous detection and genotyping of 'Norwalk-like viruses' by oligonucleotide array in a reverse line blot hybridization format. *J. Clin. Microbiol.* **38**, 2595–2601.

Vinjé, J., Altena, S. A. & Koopmans, M. 1997 The incidence and genetic variability of small round-structured viruses in outbreaks of gastroenteritis in The Netherlands. *J. Infect. Dis.* **176**, 1374–1378.

Vinjé, J., Deijl, H., van der Heide, R., Lewis, D., Hedlund, K., Svensson, L. & Koopmans, M. P. G. 2000 Molecular detection and epidemiology of Sapporo-like viruses. *J. Clin. Microbiol.* **38**, 530–536.

White, L. J., Ball, J. M., Hardy, M. E., Tanaka, T. N., Kitamoto, N. & Estes, M. K. 1996 Attachment and entry of recombinant Norwalk virus capsids to culture human and animal cell lines. *J. Virol.* **70**, 6589–6597.

Wilson, I. G. 1997 Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **63**, 3741–3751.

Wyn-Jones, A. P., Pallin, R., Dedoussis, C., Shore, J. & Sellwood, J. 2000 The detection of small round-structured viruses in water and environmental materials. *J. Virol. Meth.* **87**, 99–107.

Yuen, L. K. W., Catton, M. G., Cox, B. J., Wright, P. J. & Marshall, J. A. 2001 Heminested multiples reverse transcription-PCR for detection and differentiation of Norwalk-like virus genogroups 1 and 2 in fecal samples. *J. Clin. Microbiol.* **39**, 2690–2694.

First received 21 October 2003; accepted in revised form 4 March 2004