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Real-Time Detection of Noroviruses in Surface Water by Use of a Broadly Reactive Nucleic Acid Sequence-Based Amplification Assay

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Noroviruses are the most common agents causing outbreaks of viral gastroenteritis. Outbreaks originating from contaminated drinking water and from recreational waters have been described. Due to a lack of cell culture systems, noroviruses are detected mostly by molecular methods. Molecular detection assays for viruses in water are often repressed by inhibitory factors present in the environment, like humic acids and heavy metals. To study the effect of environmental inhibitors on the performance of nucleic acid sequence-based amplification (NASBA), we developed a real-time norovirus NASBA targeting part of the RNA-dependent RNA polymerase (RdRp) gene. Specificity of the assay was studied with 33 divergent clones that contained part of the targeted RdRp gene of noroviruses from 15 different genogroups. Viral RNA originated from commercial oysters, surface waters, and sewage treatment plants in The Netherlands. Ninety-seven percent of the clones derived from human noroviruses were detected by real-time NASBA. Two clones containing animal noroviruses were not detected by NASBA. We compared the norovirus detection by real-time NASBA with that by conventional reverse transcriptase PCR (RT-PCR) with large-volume river water samples and found that inhibitory factors of RT-PCR had little or no effect on the performance of the norovirus NASBA. This consequently resulted in a higher sensitivity of the NASBA assay than of the RT-PCR. We show that by combining an efficient RNA extraction method with real-time NASBA the sensitivity of norovirus detection in water samples increased at least 100 times, which consequently has implications for the outcome of the infectious risk assessment.

Noroviruses belong to the family *Caliciviridae* and contain a positive-sense, single-stranded RNA genome of approximately 7.6 kb (14). Based on sequence information of the genes encoding the viral RNA-dependent RNA polymerase (RdRp) and the capsid protein, the norovirus genus is divided into five genogroups (genogroup I [GGI] to GGV) that can be divided further into genetic clusters, each represented by a prototype virus. The diversity of norovirus variants increases continually due to the generation of new variants, with two groups of strains predominating in the past 5 years (22, 29, 33, 34, 39). Noroviruses that cluster within GGI and GGII are mostly human pathogens. They are the most common agents involved in outbreaks of gastroenteritis. Outbreaks originating from contaminated drinking water as well as from recreational water have been described previously (4, 11, 18, 21, 37).

Due to the lack of a cell culture system, noroviruses are currently detected mainly by molecular techniques. With the increase in knowledge of norovirus sequences, many molecular detection assays that detect a wide variety of norovirus strains have been developed, e.g., reverse line blot hybridization (47), reverse transcriptase PCR (RT-PCR) (1, 13, 46), and nucleic acid sequence-based amplification (NASBA) (17, 36). In the last decade, several different regions of the norovirus genome have been analyzed for their potential to detect the large variety of norovirus strains as well as newly occurring norovirus variants. Many primers target the RdRp gene in open reading frame 1 (ORF1) (1, 13, 46) or the capsid protein gene in ORF2 (2, 15, 48). Recently, the conserved ORF1-ORF2 junction of the norovirus genome (26) was explored for real-time detection of a broad range of noroviruses in stool samples and naturally contaminated shellfish (24, 25, 32). However, the RdRp gene and the capsid gene (2) are more appropriate for genotyping, indicating that the highly conserved ORF1-ORF2 junction is more suitable for detection than for genotyping.

Quantitative data on the concentrations of noroviruses present in drinking water or recreational water are indispensable for assessment of the public health risks caused by norovirus infections. Detection of noroviruses by real-time methods facilitates the generation of quantitative data. Several RT-PCR assays have been described for detection of noroviruses in water by endpoint detection, but probe-specific real-time quantitative detection in environmental samples has been described only by Haramoto et al. (20), amplifying the ORF1-ORF2 junction (25). Quantification of viral RNA by RT-PCR is mainly dependent on the efficiency of the copy DNA synthesis by reverse transcriptase, which in real-time assays generally takes only 10 to 20 min. The action of reverse transcriptase in NASBA is not a separate reaction as is the case with RT-PCR but is an integrated part of the entire amplification process, which probably makes NASBA more appropriate for quantitative detection of RNA. Moreover, NASBA does not use *Taq* polymerase enzymes that are sensitive to inhibitory factors present in the environment, such as humic acids and heavy metals (43), which might make NASBA less prone to environmental inhibitors. Norovirus NASBA assays by end-

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TABLE 1. Primers and probes used for conventional RT-PCR and real-time NASBA

Primer or probe	Sequence[a]	Type of primer or probe[h]	Application(s)
JV12Y[b]	5′ ATACCACTATGATGCAGAYTA 3′	+ Primer	RT-PCR/NASBA
JV13i[b]	5′ TCATCATCACCATAGAAIGAG 3′	− Primer	RT-PCR
T7-JV13i[b]	5′ aattctaatacgactcactatagggagaaggTCATCATCACCATAGAAIGAG 3′	− Primer	NASBA
Ni[c,d]	5′ GAATTCCATCGCCCACTGGCT 3′	+ Primer	NASBA
T7-Ni	5′ aattctaatacgactcactatagggagaaggAGCCAGTGGGCGATGGAATTC 3′	− Primer	NASBA
SR48/50/52[e]	5′ GTGAACAGYATAAAYCAYTGG 3′	+ Primer	NASBA
T7-SR48/50/52	5′ aattctaatacgactcactatagggagaaggatcCCARTGRTTTATRCTGTTCAC 3′	− Primer	NASBA
GGIi[f]	5′ ATGGAYGTTGGYGAYTATGT 3′	SB probe	RT-PCR
GGIIi[f]	5′ GAAYTCCATCRCCCAYTG 3′	SB probe	RT-PCR
UK3[d,f]	5′ GTCCCCTGACATCATACAGGCT 3′	SB probe	RT-PCR
JV5[f]	5′ CTCACCAGAGGTTGTCCAAGC 3′	SB probe	RT-PCR
MBNVG2.1[g]	5′ FAM cgatcgGTCCCCTGACATCATACAGGCTcgatcg DABSYL 3′	Mol beacon	NASBA
MBNVG2.2[g]	5′ FAM cgatcgACAGGACTAGGCCCCGACAcgatcg DABSYL 3′	Mol beacon	NASBA
MBNVG2.4[g]	5′ FAM cgatcgTCAGGTCTCTCACCAGATGTTcgatcg DABSYL 3′	Mol beacon	NASBA
MBNVG1.1[g]	5′ FAM cgatcgACAGGCCTATCACCCGAcgatcg DABSYL 3′	Mol beacon	NASBA
MBNVG1.2[g]	5′ FAM cgtcacTGGCTTATCACCTGATgtgacg DABSYL 3′	Mol beacon	NASBA
MBNVG1.3[g]	5′ FAM cgatcgTATCACCTGATGTTATACAATCCcgatcg DABSYL 3′	Mol beacon	NASBA
MBGGIc	5′ FAM gcgATGGAIGTTGGIGACTATGTcatcgc DABSYL 3′	Mol beacon	NASBA
MBGGIIi	5′ FAM cgatcgGAAITCCATCICCCAITGcgatcg DABSYL 3′	Mol beacon	NASBA
MBUK3	5′ FAM cGTCCCCTGACATCATACAGGCTgggacg DABCYL 3′	Mol beacon	NASBA
MBJV5	5′ FAM cgatgCTCACCAGAGGTTGTCCAAGCgcatcg DABSYL 3′	Mol beacon	NASBA

[a] Uppercase letters indicate norovirus-specific sequences. DABSYL, 4-dimethylaminoazobenzene-4′-sulfonyl.
[b] See reference 45.
[c] See reference 36.
[d] See reference 12.
[e] See reference 1.
[f] See reference 44.
[g] See reference 16.
[h] + Primer, forward primer; − Primer, reverse primer; SB probe, specific probe for detection by Southern blotting; Mol beacon, molecular beacon.

point detection have been described previously for detection of noroviruses in clinical samples (17, 36) and in food (23). Recently, we described the successful real-time detection of enteroviruses in large-volume water samples by NASBA (40), indicating that a real-time NASBA assay for the detection of norovirus RNA might be applicable to water samples as well. Furthermore, not only the assay for detection of viruses in water but also the processing of water and the RNA extraction are very important for the estimation of virus concentrations in the original water sample. Generally, direct detection of noroviruses in surface water is not possible due to their low concentrations. We have recently published a method for concentration of enteroviruses and the extraction of enterovirus RNA from large volumes of water, which increased the detected enterovirus concentrations in the original water sample at least 100 times (40). In the present study, this method was analyzed for its capability to isolate norovirus RNA as well. Furthermore, we developed a real-time NASBA assay for the detection of norovirus RNA and compared detection efficiencies and specificities with those obtained by conventional RT-PCR. Differences in specificities were shown by analysis of a collection of norovirus clones containing part of the RdRp gene of noroviruses originating from commercial oysters, surface waters, and sewage treatment plants in The Netherlands (30, 31, 44). Differences in sensitivity between the two methods were studied with large-volume river samples and revealed that RT-PCR inhibitory factors have little or no effect on the performance of the norovirus NASBA.

MATERIALS AND METHODS

Sampling and concentration by UF. Large volumes of river water (approximately 600 liters) were collected and concentrated by a conventional filter adsorption-elution method as was previously described (40). Two-thirds of the eluate was further concentrated by ultrafiltration (UF) using a cellulose-acetate filter (nominal molecular weight limit, 10,000) under high pressure (300,000 Pa). The remaining one-third of the eluate was concentrated by two-phase separation.

Two-phase separation and RNA isolation. Conventional viral RNA isolation from the concentrate following two-phase separation was done by a modified in-house protocol of the Boom method (5) described by Lodder et al. (30, 40).

RNA isolation from ultrafiltered concentrate. RNA was isolated from 12.5 μl or 125 μl ultrafiltered concentrate. After the addition of 500 μl of NucliSens lysis buffer (bioMérieux, Boxtel, The Netherlands) and 50 μl of NucliSens magnetic silica particle suspension (bioMérieux, Boxtel, The Netherlands), RNA was isolated as was described previously (40). A NucliSens miniMAG instrument (bioMérieux, Boxtel, The Netherlands) was used to collect and wash the magnetic silica particles. Nucleic acids were recovered from the particles during a 5-min incubation period at 60°C by use of 50 μl elution buffer.

Norovirus RT-PCR. The conserved RNA-dependent RNA polymerase gene of the norovirus genome was used as the target for amplification. RT-PCR was performed with primer pair JV12Y/JV13i, which was previously described (45). The specificity of the detected noroviruses was confirmed by hybridization of RT-PCR products with a mixture of four specific probes (GGIi, GGIIi, UK3, and JV5) as was described previously (44) (Table 1).

Cloning, sequencing, and phylogenetic analysis. Hybridization-positive norovirus RT-PCR products were excised from 2% agarose gel and purified with a QIAquick gel extraction kit (QIAGEN, Hilden, Germany). The purified PCR products were cloned into a pGEM T-easy vector system II (Promega, Leiden, The Netherlands) and analyzed for the presence of the correct insertion size by direct PCR amplification with M13 forward and M13 reverse primers supplied by the manufacturer. PCR products hybridizing with a mix containing probes GGIi, GGIIi, UK3, and JV5 (Table 1) were purified with a PCR purification kit and sequenced with a Big Dye Terminator cycle sequencing ready reaction. The

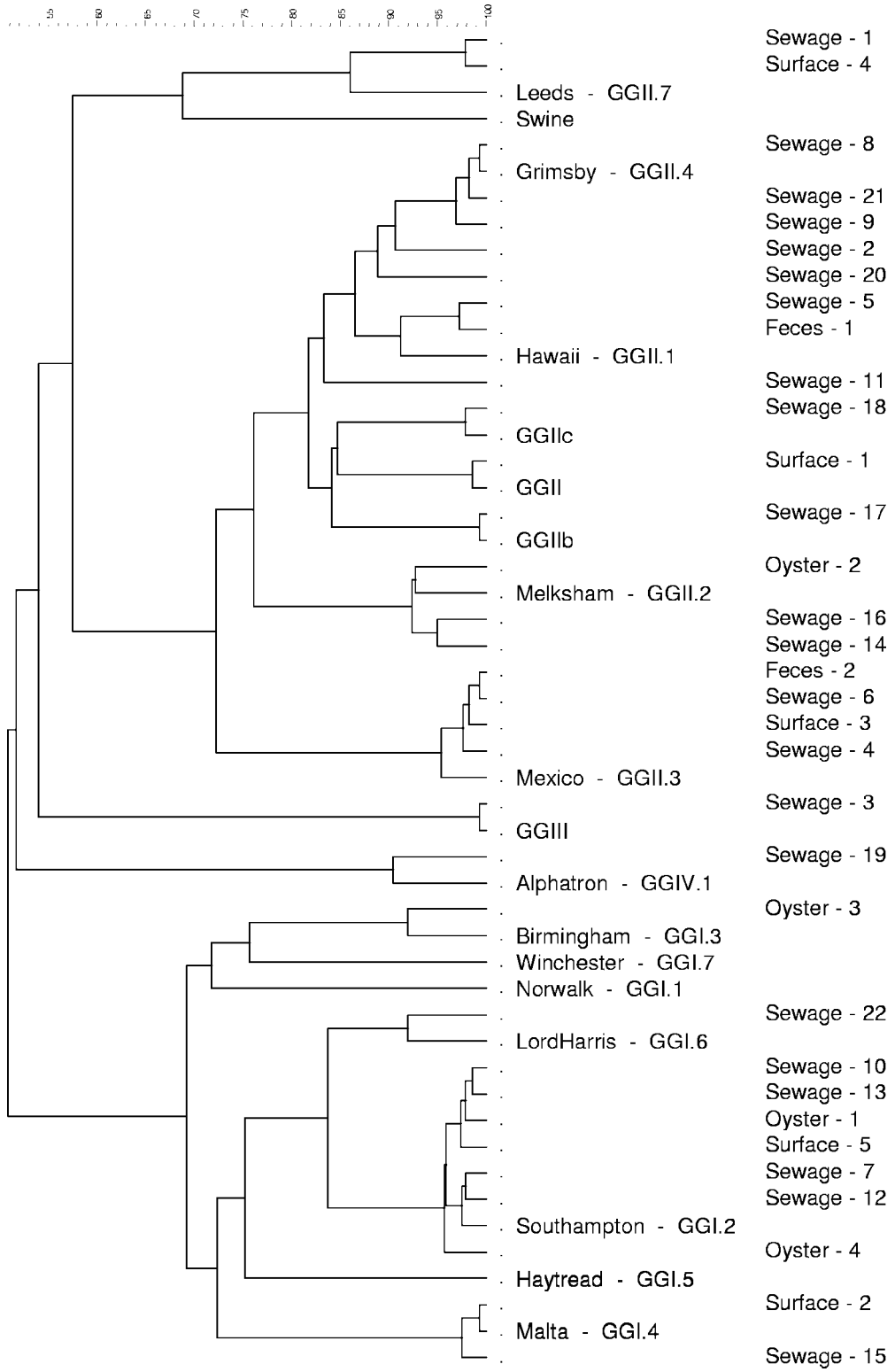


FIG. 1. Phylogenetic comparison of the genetic relationship of norovirus sequences isolated from Dutch commercial oysters, sewage, and surface water samples and the consensus sequences of norovirus prototypes (GG) found in the population (27). The genetic relationship is based on a 145-nucleotide fragment of the RdRp gene. The right column indicates the sources of the norovirus strains and the clone number of each.

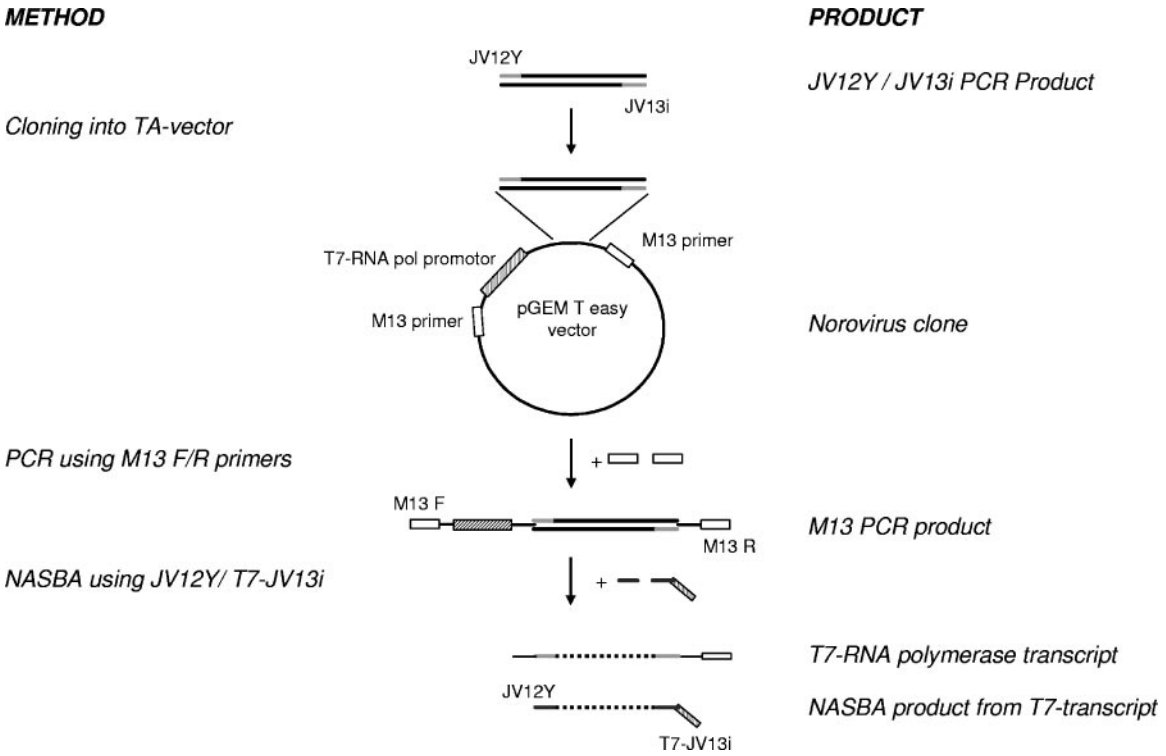


FIG. 2. Schematic representation of successive steps performed for the analysis of norovirus clones by NASBA. The starting material was the PCR product generated by conventional RT-PCR using the primers JV12Y and JV13i. The PCR product was cloned into a pGEM T-easy vector, amplified by PCR using M13 forward (M13 F) and reverse (M13 R) primers, and sequenced to determine the orientation of the insert. A dilution of the correctly oriented PCR product was analyzed by NASBA using primers JV12Y and T7-JV13i. The T7 RNA polymerase (pol) enzyme present in the NASBA reaction mixture transcribed the M13 PCR product, generating a sense RNA fragment of the RdRp gene of norovirus, which enabled the JV12Y/T7-JV13i-specific NASBA reaction. A positive NASBA signal indicated that the clone, and thus the corresponding norovirus genotype, was detected. In this way, 33 different clones were analyzed using three different primer and probe mixes.

computer program Bionumerics (v2.0; Applied Maths, Kortrijk, Belgium) was used to align 145 nucleotides of the generated norovirus sequences with the unweighted-pair group method using average linkages after multiple sequence alignments of a 145-nucleotide segment of the RNA polymerase gene.

Norovirus NASBA. Noroviruses were detected using primers Ni, JV12Y, and JV13i, the last of which was labeled with a T7 RNA polymerase promoter to increase specificity (18, 36). Different combinations of molecular beacons were used for detection: mix A, containing MBNVG2.1, MBNVG2.2, and MBNVG2.4; mix B, containing MBNVG1.1, MBNVG1.2, and MBNVG1.3; and mix C, containing MBGGIc, MBGGIIi, MBUK3, and MBJV5 (Table 1). All molecular beacons contained a 6-carboxyfluorescein (FAM) fluorophore at the 5′ end. NASBA reactions were done by using a NucliSens Basic kit (bioMérieux, Boxtel, The Netherlands) according to the instructions of the manufacturer. Briefly, 5 μl RNA or dilutions of this RNA, 80 mM KCl, final primer concentrations of 0.2 μM, and final molecular beacon concentrations of 0.1 μM for each beacon in a total volume of 15 μl were incubated at 65°C for 5 min followed by an incubation of 5 min at 41°C. Five microliters of enzyme mix supplied in the basic kit was added, followed by an incubation of 2 h at 41°C with a NucliSens EasyQ analyzer for real-time detection of NASBA amplicons (bioMérieux, Boxtel, The Netherlands).

Estimation of norovirus concentrations in water. Estimation of the numbers of viral genomes by RT-PCR (PCR-detectable units [PDU]) and NASBA (NASBA-detectable units [NDU]) was performed with 10-fold serially diluted RNA samples (endpoint dilution). Virus concentrations in the undiluted samples were estimated as most probable numbers by determining the presence or absence of virus genomes in the 10-fold RNA dilutions, under the assumption that negative samples do not contain viral RNA. Application of the Poisson distribution was justified by the assumption that the infectious virus particles or viral RNA was dispersed randomly in the sample. The maximum-likelihood method was used to estimate the number of virus particles in the undiluted sample (35). A negative binomial model gives the best fit for the distribution of virus particles in the original and diluted samples. The 95% confidence interval was estimated for each virus concentration.

RESULTS

Characterization of the specificity of the norovirus NASBA. A NASBA assay uses different enzymes and conditions for amplification than RT-PCR and might therefore be less prone to environmental inhibitors. To study this, several matrices (oysters, sewage, and surface waters) were analyzed for the presence of norovirus RNA by RT-PCR using an assay that had been developed for specific detection of norovirus in stool specimens from humans by use of primers JV12Y and JV13i (45). To be able to compare the specificity of RT-PCR to that of real-time NASBA, the identities of the detected strains had to be determined. Therefore, RT-PCR products derived from norovirus-positive oysters, sewage, and surface water samples were cloned in a TA cloning vector downstream of a T7 RNA polymerase promoter. Part of their sequences, 145 nucleotides, was subjected to phylogenetic analysis and was compared to sequences of consensus strains (30, 31, 44). Two fecal samples were included as positive controls. We selected 33 constructs that were divergent and contained the norovirus RT-PCR product in the correct orientation for transcription of positive-stranded norovirus RNA. The 33 selected clones belonged to 15 genogroups, of which 13 are known to be infectious to

humans; GGI.2, GGI.3, GGI.4, GGI.6, GGII, GGIIb, GGIIc, GGIId, GGII.1, GGII.2, GGII.3, GGII.4, and GGII.7 (Fig. 1). Two strains that are known to be infectious to animals were found: GGIV.1 and GGIII.

NASBA amplification utilizes three enzymes for the amplification of RNA, including T7 RNA polymerase. Because the selected clones contained an upstream T7 RNA polymerase promoter, the T7 RNA polymerase enzyme present in the NASBA reaction was able to transcribe those clones, resulting in the production of norovirus RNA fragments that were bordered by JV12Y/JV13i sequences (Fig. 2). RT-PCR primer JV13i was modified for inclusion into a real-time NASBA assay by the addition of a T7 RNA polymerase promoter, resulting in primer T7-JV13i (Table 1). Two forward primers were included in the NASBA reaction, JV12Y and Ni (36), and amplification products were detected by hybridization using three different molecular beacon probes: MBNVG2.1, MBNVG2.2, and MBNVG2.4 (mix A) (Table 1). Twenty-one of 33 clones (63.6%) were detected by real-time NASBA (Table 2). Of the 12 clones that were not detected, 4 of them belonged to GGII (GGII.d and GGII.2) and 6 to GGI (GGI.2, GGI.3, and GGI.6). Neither of the two clones derived from animal strains (GGIV.1 and GGIII) was detected.

To improve the detection efficiency, we analyzed whether a second probe mix, containing beacon probes MBNVG1.1, MBNVG1.2, and MBNVG1.3 (mix B) (16), reduced the number of undetected clones. As is shown in Table 2, in total, 25 of 33 clones were detected. Clones belonging to GGIIb and GGII.2 were detected when mix B was used for hybridization, indicating that only one clone belonging to GGII (GGIId) was not detected by NASBA. Of the GGI clones, four clones of GGI.2, one GGI.6 clone, and the two clones derived from animal strains (GGIV.1 and GGIII) remained undetected. Thus, the additional hybridization with probe mix B increased the percentage of detected clones derived from human noroviruses from 67.7% to 80.6%.

The analyzed clones were originally detected by RT-PCR using JV12Y and JV13i primers (Table 1), indicating that all clones contain the JV12Y/JV13i primer binding sites and thus should be able to be amplified by NASBA. This suggests that clones might be missed by NASBA detection due to the use of different probes. For hybridization following RT-PCR, the probes GGIi, GGIIi, UK3, and JV5 were used, where especially GGIi is highly specific for detection of GGI clones. We therefore designed molecular beacons containing the sequences of the RT-PCR probes (MBGGIc, MBGGIIi, MBUK3, and MBJV5 [mix C]) by using the Mfold web server (51) and analyzed whether these beacons were able to detect any of the missing clones. Although a GGIIb clone that was also detected by mix A gave a positive result, none of the missing clones were reactive with any of the mix C beacons. Further optimization of the assay by using the more-GGI-specific primer SR48/50/52 in antisense (T7-SR48/50/52) orientation (Table 1) in combination with JV12Y and detection with the GGI-specific probe MBGGIc resulted in detection of the five missing GGI.2 clones as well as the missing GGI.6 clone. This increased the percentage of detected clones derived from human noroviruses from 80.6% to 96.8%. Neither the addition of primer Ni in the antisense orientation (T7-Ni) nor the addition of primer SR48/50/52 in the sense orientation resulted in detection of the clone belonging to GGIId or the two missing clones derived from animals. This indicates that molecular beacons MBGGIIi, MBUK3, and MBJV5 did not detect any additional clones. Overall, optimal results were reached when three separate norovirus NASBA assays were run, one using primers JV12Y, Ni, and T7-JV13i in combination with probe mix A, a second using the same primers in combination with probe mix B, and a third using primers JV12Y and T7-SR48/50/52 in combination with probe MBGGIc. The second and third runs were also analyzed combined. This slightly reduced the sensitivity of the assay.

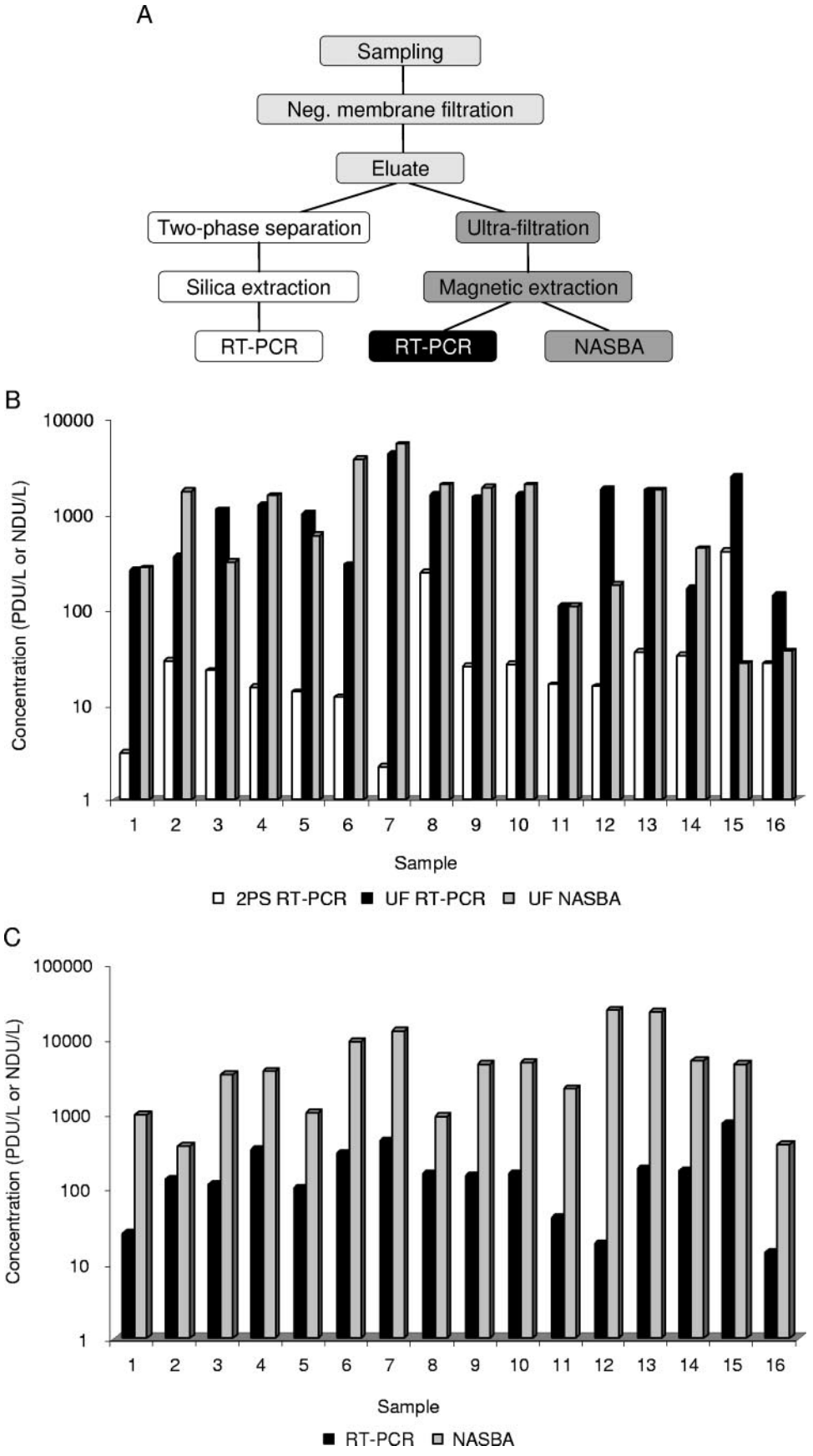
TABLE 2. Detection of norovirus clones of different genogroups by probe mixes A, B, and C

Genogroup	Source of strain (clone no.)[a]	Detection result with probe mix[b]:		
		A	B	C
GGII.7	Sewage (1)	+	−	ND
	Surface water (4)	+	−	ND
GGII.4	Sewage (8)	+	−	ND
	Sewage (21)	+	−	ND
	Sewage (9)	+	−	ND
	Sewage (2)	+	−	ND
	Sewage (20)	+	−	ND
GGII.1	Sewage (5)	+	+	ND
	Feces (1)	+	+	ND
GGIId	Sewage (11)	−	−	−*
GGIIc	Sewage (18)	+	−	ND
GGII	Surface water (1)	+	−	ND
GGIIb	Sewage (17)	+	−	+
GGII.2	Oyster (2)	−	+	−*
	Sewage (16)	−	+	−*
	Sewage (14)	−	+	−*
GGII.3	Feces (2)	+	−	ND
	Sewage (6)	+	−	ND
	Surface water (3)	+	−	ND
	Sewage (4)	+	−	ND
GGIII	Sewage (3)	−	−	−*
GGIV.1	Sewage (19)	−	−	−*
GGI.3	Oyster (3)	−	+	−*
GGI.6	Sewage (22)	−	−	+*
GGI.2	Sewage (10)	+	−	+*
	Sewage (13)	−	−	+*
	Oyster (1)	−	−	+*
	Surface water (5)	−	−	+*
	Sewage (7)	+	−	ND
	Sewage (12)	+	−	ND
	Oyster (4)	−	−	+*
GGI.4	Surface water (2)	+	+	ND
	Sewage (15)	+	+	−*

[a] Clones are listed according to Fig. 1.
[b] +, clone was detected; −, clone was not detected; ND, not determined. Results marked with an asterisk (*) also include results obtained with primers T7-SR48/50/52, JV12Y, and MBGGIc.

In addition, specificity of the molecular beacons was determined by analysis of several other waterborne RNA viruses, such as poliovirus types 1 and 2 (Sabin), rotavirus WA, coxsackie B4 virus, reovirus 3, and hepatitis A virus. None of these viruses were detected by either mix of molecular beacons, confirming the specificity of norovirus detection by NASBA.

Analysis of water samples: comparison of concentration by a two-phase separation method and concentration by ultrafiltration. Noroviruses in surface waters were concentrated by filtration of water samples on a negatively charged membrane.


A
Sampling
Neg. membrane filtration
Eluate
Two-phase separation
Ultra-filtration
Silica extraction
Magnetic extraction
RT-PCR
RT-PCR
NASBA
B
Concentration (PDU/L or NDU/L)
10000
1000
100
10
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sample
2PS RT-PCR
UF RT-PCR
UF NASBA
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Concentration (PDU/L or NDU/L)
100000
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1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sample
RT-PCR
NASBA

Two secondary concentration methods were compared. One method was based on a two-phase separation using dextran T40 and polyethylene glycol 6000 (30, 40). After additional purification steps, RNA was extracted using guanidinium (iso)thiocyanate-silica according to the method of Boom et al. (5) followed by detection of norovirus RNA by RT-PCR (Fig. 3A). The second method was based on ultrafiltration and was followed by RNA isolation with magnetic silica beads (Fig. 3A). We analyzed 16 samples of 600 liters of surface water from the river Maas by both concentration/extraction procedures. Norovirus concentrations were estimated semiquantitatively on 10-fold serially diluted RNA samples by calculating the mean concentration of norovirus RNA in the initial water samples (Fig. 3B). Analysis of RNA that was extracted after two-phase separation showed that norovirus RNA was present in all samples, with detected virus concentrations ranging from 2 (0.1 to 20) PDU per liter of water to 414 (60 to 2,236) PDU/liter. Ultrafiltration prior to RNA isolation with magnetic silica beads resulted in norovirus concentrations ranging from 108 (6 to 500) to 4,303 (271 to 38,570) PDU/liter (Fig. 3B). Differences in virus concentrations in a single sample with either of the concentration methods varied from 1 to 3 $\log_{10}$ units, demonstrating the superiority of the ultrafiltration assay. Therefore, this method was used in the following experiments.

Comparing sensitivities of norovirus NASBA and RT-PCR in river water samples. To compare detection of norovirus RNA by RT-PCR and NASBA, the 16 samples derived from the river Maas were subjected to real-time NASBA analyses. Norovirus concentrations were estimated on 10-fold serially diluted RNA samples as was done for RT-PCR. Amplification products were detected by hybridization using probe mix A, rendering 15 norovirus-positive samples. Dilutions that gave negative results with probe mix A were additionally analyzed with probe mix B and with primer T7-SR48/50/52 and MBGGIc, because positive results in these dilutions effect the estimation of the virus concentration. As shown in Fig. 3B, all samples gave positive results, with concentrations ranging from 27 (2 to 122) NDU/liter to 5,498 (714 to 39,810) NDU/liter. The mean virus concentration was comparable to the mean concentration found by RT-PCR: 1,405 NDU/liter versus 1,241 PDU/liter, respectively. No norovirus RNA was detected in sample 15 upon use of probe mix A, which confirmed that additional detection with probe mix B and MBGGIc increased the sensitivity of the assay.

To analyze whether the NASBA enzymes are less affected by inhibitory factors in the RNA samples than enzymes used in RT-PCR, detection by NASBA and RT-PCR on RNA isolated from 12.5 μl of concentrated sample (40) was compared with detection from a 125-μl sample. Therefore, we isolated RNA from the 16 river Maas samples by using sample volumes of 125 μl for RNA extraction. RNA samples were analyzed for the presence of noroviruses by RT-PCR and NASBA, followed by estimation of the virus concentrations in the initial water sample. Comparing detection of norovirus RNA by RT-PCR in 12.5-μl and 125-μl samples revealed that estimated virus concentrations were higher in all of the RNA samples isolated from the 12.5-μl concentrate, with the exception of samples 6 and 14, which gave comparable virus concentrations (Fig. 3B and C). The mean virus concentration found in sample volumes of 12.5 μl was 1,241 PDU/liter, whereas 193 PDU/liter was detected when volumes of 125 μl were analyzed. This suggests that the increased concentration of inhibitory factors present in the larger sample volume reduced the efficiency of detection by RT-PCR, leading to a decreased sensitivity of the assay.

Analysis of RNA samples derived from 12.5 μl and 125 μl concentrate by NASBA showed that in 14 of 16 samples of 125 μl virus, concentrations were higher than concentrations found in the 12.5-μl samples. This suggests that the RT-PCR inhibitory factors have little or no effect on the performance of the norovirus NASBA. Detection of RNA isolated from 12.5-μl samples by RT-PCR and NASBA resulted in comparable mean virus concentrations. Analysis of 125-μl samples by NASBA resulted in a much higher mean virus concentration than that determined by RT-PCR analysis (6,345 NDU/liter versus 193 PDU/liter, respectively), indicating that the NASBA reaction was more sensitive than the RT-PCR.

DISCUSSION

To compare the specificities and sensitivities of conventional RT-PCR (45, 46) and real-time NASBA, we adjusted the RT-PCR primers JV12Y and JV13i for NASBA application by adding a T7 RNA polymerase promoter sequence downstream of the reverse RT-PCR primer JV13i. Furthermore, a second forward primer, Ni, was included in the reaction as was previously described (36). Amplification by primers Ni/JV13i generates a product of 113 nucleotides, whereas primers JV12Y/JV13i generate a product of 327 nucleotides, which is rather long for optimal real-time detection. This was demonstrated by analysis of a number of norovirus clones derived from RT-PCR products of oysters, sewage, and surface water samples with either one of the forward primers JV12Y or Ni in combination with JV13i. Only a GGI.4 clone was detected by combining the primers JV12Y and T7-JV13i. The combination of Ni and T7-JV13i detected most of the clones; some were

FIG. 3. Norovirus detection in 16 samples derived from the river Maas. (A) Schematic representation of virus concentration, RNA extraction, and detection of viral RNA by either RT-PCR or NASBA from water samples. Viruses were concentrated by filtration on a negatively charged (Neg.) membrane followed by a second concentration step based on either two-phase separation (2PS) or UF. In the 2PS method, RNA was extracted using guanidinium (iso)thiocyanate-silica according to the method of Boom et al. (5), whereas UF-concentrated samples were extracted using magnetic silica particles. (B) Detection of norovirus RNA following concentration by either 2PS or UF and detection by conventional RT-PCR. UF-concentrated water samples were also analyzed by real-time NASBA. Virus concentrations were estimated semiquantitatively on 10-fold serially diluted RNA samples by calculating the mean concentration of norovirus RNA in the initial water sample in PDU/liter or NDU/liter. (C) Detection of norovirus RNA on UF-concentrated water samples. RNA was extracted with magnetic silica particles from 10 times more UF concentrate (125 μl) than that used for the experiment shown in panel B. Virus concentrations were determined either by conventional RT-PCR (PDU/liter) or by real-time NASBA (NDU/liter).

detected by JV12Y as well as by primer Ni in combination with T7-JV13i (GGII.b and GGII.2). Thus, molecular beacons targeting the norovirus RNA in between primers JV12Y and Ni target the inefficiently produced large amplicons generated by JV12Y–T7-JV13i. This might explain the inefficient detection of GGI, because GGI-specific probes target this region. Indeed, use of primer T7-SR48/50/52 (1) combined with JV12Y, generating a 236-nucleotide fragment, resulted in the detection of the missing GGI clones. The animal-derived clones were not detected using primer Ni in the forward or reverse (T7-Ni) orientation or primer SR48/50/52. Although primers and probes used for RT-PCR and NASBA are identical, some strains are not detected by NASBA but are detected by RT-PCR. This might be explained by the fact that DNA is denatured during Southern blot hybridization, which eliminates secondary and tertiary structures, which is not the case with NASBA, possibly obstructing hybridization of probes and subsequent real-time detection.

Although one human norovirus strain (GGIId) and two animal strains were not detected by real-time NASBA, detected virus concentrations in small sample volumes were similar to those found by RT-PCR. This might indicate either that the undetected strains were not present in the analyzed water samples or that additional norovirus strains were detected by NASBA but missed by RT-PCR. Moreover, NASBA appeared to be more sensitive when increased sample volumes were analyzed. Increasing the input volume 10 times also increased the amount of RT-PCR inhibitory factors present in the RNA to be analyzed, as was demonstrated by the fact that the efficiency of the RT-PCR decreased. The 10-fold increase in sample volume did not have a negative effect on NASBA. Common PCR inhibitors found in environmental samples include humic acids and heavy metals (49). Humic acids have been reported to inhibit *Taq* polymerase activity during PCR, but DNA-DNA hybridization (43) and the sequestration of Mg^{2+} ions by various compounds may inhibit amplification as well. Either the NASBA enzymes are less prone to inhibition or reaction conditions are more optimal to facilitate amplification, such as the presence of 15% dimethyl sulfoxide in the reaction mixture (41).

Although diversity and frequency of norovirus strains have changed over time, the occurrence of GGII strains has been reported predominantly with two groups of strains, GGIIb and GGII.4, in the past 5 years (10, 22, 28, 29, 33, 34, 38, 39). The human norovirus strain that was not detected by NASBA, GGIId, has not been reported to occur in patients for several years now. The distributions of genotypes in water and oysters appear to be different from those among humans, which is demonstrated by the much higher contribution of GGI genotypes in water and oysters (6, 19, 24). It is possible that those GGI viruses represent animal norovirus strains which can be introduced into the environment by excretion in feces. Those GGI viruses may be less pathogenic to humans or spread less efficiently from person to person. This idea is supported by the finding that GGI strains are detected more frequently in sporadic cases of acute gastroenteritis than in outbreak cases (28). Also, differences in inactivation or stability in the environment might explain the differences in strains present in the population or in the environment. To study the stability of genogroup I and II noroviruses, a cell culture method is required but not yet available (9).

By use of conventional RT-PCR, many primers that target the polymerase gene (RdRp) in ORF1 (1, 13, 46) or the capsid protein gene in ORF2 (2, 15, 48) have been described previously. However, several groups have described the conserved ORF1-ORF2 junction of the norovirus genome (26) as the amplification target for real-time detection of a broad range of noroviruses in stool samples and naturally contaminated shellfish (22, 24, 25, 32). Because of the conserved characteristics of the junction region, it is less suitable for genotyping of detected norovirus strains. With real-time NASBA, we target the RdRp gene, which is a commonly used region for typing by sequence analysis (2), indicating that the NASBA amplification product can be genotyped directly. Sequence analyses of NASBA products in which amplification products were cloned prior to sequencing have been described previously (42), but dependent on the nature of the analyzed sample, direct RNA sequencing might be possible as well. Fecal samples containing high concentrations of one virus can be sequenced directly, but environmental samples or food samples containing low concentrations of many different viruses have to be cloned prior to sequencing.

Estimated norovirus concentrations determined by NASBA on RNA extracted from the 125 μl ultrafiltered concentrate appeared to increase by 1 to 3 $\log_{10}$ units compared to concentrations determined by the two-phase separation method and RT-PCR (Fig. 3B and C). These increased virus concentrations consequently affect the estimation of the public health risk via consumption of drinking water and exposure to surface water by recreation. More extensive drinking water treatment and sewage treatment processes will be required to comply with the Dutch legislation for drinking water, which imposes a maximum infection risk by waterborne pathogens of 1 in 10,000 persons per year. Norovirus concentrations determined by either RT-PCR or NASBA might overestimate the infectious risk, because molecular methods detect genomic RNA derived from infectious as well as defective virus particles. However, in the absence of a cell culture system, information on the presence of viral genomes obtained by detection with molecular methods is the best alternative to assess potential health risks. Although knowledge of replication and packaging of noroviruses in somatic cells increases rapidly (3) and assays are available to culture feline and canine caliciviruses (7) as well as murine noroviruses (50), determination of the concentration of infectious human noroviruses is not yet feasible (8). To characterize the risks associated with norovirus infection, infectivity data are required. For enteroviruses, the ratio between infectious virus particles and viral genomes has been studied (A. M. De Roda Husman et al., unpublished data). These data may be extrapolated to noroviruses until a norovirus culture system is available.

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