Molecular Epidemiology of Norovirus Outbreaks in Norway during 2000 to 2005 and Comparison of Four Norovirus Real-Time Reverse Transcriptase PCR Assays

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During the period from January 2000 to August 2005 a total of 204 outbreaks of norovirus gastroenteritis were diagnosed at the Norwegian Institute of Public Health. A clear increase in the norovirus activity was seen in healthcare institutions during the winter seasons. Polymerase sequence analysis of norovirus strains from 122 outbreaks showed that 112 were caused by GII strains (91.8%). Two norovirus variants seen during the study period—GIIB and GII.4—were predominant between January 2000 and September 2002, whereas GII.4 was predominant from September 2002 onward. The highest norovirus activity was seen during the 2002–2003 and 2004–2005 seasons with the emergence of new GII.4 variants. This study describes the molecular epidemiology of norovirus strains circulating in Norway during the five previous seasons and compares four norovirus real-time reverse transcriptase PCR assays. A suitable assay for routine diagnostics is suggested.

Noroviruses (NoVs) are estimated to be responsible for 60 to 80% of all human gastroenteritis outbreaks worldwide (4, 9, 16, 19, 23, 30, 34). Norovirus infections have become an increasing problem in healthcare institutions such as nursing homes and hospitals because outbreaks often result in closed wards (6, 15, 19, 22). Although the main mode of transmission in health institutions is person to person, NoVs are also efficiently transmitted via food, water, and contaminated surfaces (3, 17, 14, 21). A low infectious dose, short-lived immunity, and high stability in the environment make these viruses especially contagious (5, 7).

Noroviruses are classified into five genogroups (GI to GV), and human NoVs belong to genogroups GI, GII, and GIV (4, 17). Since no unified genotyping system for NoVs exists, genogroups can be divided into various numbers of genotypes (1, 13, 33, 37). The design of a single reverse transcriptase PCR (RT-PCR) assay for the detection of all genotypes has been a challenge due to the genomic diversity of NoVs (36). Earlier RT-PCR assays were based on the polymerase region since this was expected to be the most conserved. In a recent study, however, the highest degree of conservation was demonstrated in the polymerase-capsid junction region (12). Several broadly reactive real-time RT-PCR assays amplifying part of this junction have been developed (11, 12, 20, 26). The sensitivity has varied between the assays; however, an evaluation of the different assays using a common panel of strains has not been performed. Reliable and rapid NoV diagnostics is important for correct interventions in different settings, e.g., in institutions and in food-borne outbreaks.

Due to the predominance of assays amplifying the polymerase region, NoV genotyping has been mainly based on the

polymerase typing system in epidemiological studies. Most of these studies describe a predominant circulation of strains belonging to GII, especially in health institutions (4, 15, 25, 27, 29, 32, 35). GII.4 has been the most common genotype in Europe during recent years, and two new genetic GII.4 variants have emerged since January 2002 (19, 22, 25). Detailed genotype or sequence data are essential for the detection of new emerging variants and for the investigation of epidemiologically linked outbreaks.

The aims of the present study were to describe the molecular epidemiology of NoV strains circulating in Norway between 2000 and 2005, to compare four published NoV real-time RT-PCR assays on a panel of stool samples, and to use the best assay in the retesting of stool samples sent to the Norwegian Institute of Public Health between 1998 and 2004.

MATERIALS AND METHODS

Clinical specimens. (i) **Molecular epidemiology.** The Norwegian Institute of Public Health is the national reference laboratory for NoVs. Stool samples from outbreak cases of gastroenteritis were included in the study. The samples were received between January 2000 and August 2005. The majority of the samples were from outbreaks in hospitals and nursing homes in different areas of Norway. An outbreak of gastroenteritis was defined as two or more cases of acute gastroenteritis occurring in a given setting within a period of 2 weeks. No screening data on other enteric agents were available for these samples.

(ii) **RT-PCR comparison.** Four real-time RT-PCR assays (10, 11, 20, 31) were compared by using a panel of 97 stool samples analyzed in our laboratory between 1998 and 2004. The panel included 76 NoV-positive and 11 NoV-negative samples as classified by a polymerase RT-PCR published by Vinje et al. (34, 36). The remaining 10 samples had previously not been tested by the real-time RT-PCR. Based on the sequence data from the polymerase region of 42 NoV-positive samples, the following genotypes were included in the panel: GI.1, GI.2, GI.3, GI.4, GI.5, GII.1, GII.2, GII.3, GII.4, GII.6, GII.7, GIIB, and GIIC.

Retesting of stool samples. A total of 372 stool samples randomly collected from selected periods between November 1998 and April 2004 were subsequently retested by the real-time RT-PCR assay that performed best on the

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TABLE 1. Comparison of results of real-time RT-PCR assays used in this study

Assay	Genogroup	Technology	Oligonucleotide	Sequence (5'-3')	Location ^a	Reference
A	GI	TaqMan	JJV1F (+)	GCCATGTTCCGTTGGATG	5282–5299	11
			JJV1R (–)	TCCTTAGACGCCATCATCAT	5377–5358	
			JJV1P probe	FAM-TGTGGACAGGAGATC GCAATCTC-EDQ ^b	5319–5341	
	GII	TaqMan	JJV2F (+)	CAAGAGTCAATGTTTAGGTGGATGAG	5003–5028	12
			COG2R (–)	TCGACGCCATCTTCATTACACA	5100–5080	
			RING2-TP probe	TET-TGGGAGGGCGATCGCAATCT-EDQ	5048–5067	
B	GI	TaqMan	NV65a (+)	TGGACAGGRGATCGCRATCT	5321–5340	10
			NV120 (–)	AYATCACCGGGGTATTRTT	5593–5574	
			TM6 probe	FAM-GCGTCCTTAGACGCCATCATCATTTAC-TAMRA	5354–5380	
	GII	TaqMan	NV107a (+)	AGCCAATGTTTCAGATGGATG	5007–5026	
			NV117 (–)	TCGACGCCATCTTCATTAC	5100–5081	
			TM3 probe	FAM-TGGGAGGGCGATCGCAAT CTGGC-TAMRA	5048–5070	
C	GII	TaqMan	QNIF2d (+)	ATGTTTCAGRTGGATGAGRTTCTCWGA	5012–5037	20
			COG2R (–)	TCGACGCCATCTTCATTACACA	5100–5080	12
			QNIFS probe	TET-AGCACGTGGGAGGGCGATCG-EDQ	5042–5061	20
D	GI	SYBR green	MON 432 (+)	TGGACICGYGGICCYAAYCA	5093–5112	31
			MON 434 (–)	GAASCGCATCCARCGGAACAT	5308–5288	
	GII	SYBR green	MON 431 (+)	TGGACIAGRGGICCYAAYCA	4820–4839	
			MON 433 (–)	GGAYCTCATCCAYCTGAACAT	5033–5013	

^a Nucleotide positions are based on the Norwalk (GI) (accession no. M87661) and Lordsdale (GII) (accession no. X86557) sequences.

^b EDQ, eclipse dark quencher.

panel. A total of 56 (15%) of these samples had previously tested positive and 316 (85%) had tested negative with the in-house diagnostic RT-PCR assay used at the time (the nested RT-PCR or the conventional one-step RT-PCR).

Sample preparation and RNA extraction. Stool extracts (10%) were prepared in phosphate-buffered saline (pH 7.2 to 7.4) by standard methods immediately after arrival at the laboratory (8). RNA was isolated from 140-μl unfrozen stool extracts by QIAmp microspin columns (viral RNA minikit; QIAGEN) according to the manufacturer's protocol. The RNA was eluted in 60 μl of elution buffer and aliquots were stored at –70°C until testing.

Diagnostic RT-PCRs. Three different diagnostic RT-PCR assays were used during the study period. An in-house nested RT-PCR was used between January 1996 and November 2003 (32), a conventional one-step RT-PCR assay was used between November 2003 and December 2004 (34), and a real-time GI-GII duplex TaqMan assay has been used since January 2005 (11). All kits were used for amplification according to manufacturer's protocol.

Real-time RT-PCRs included in the comparison. The primers and probes in the four published real-time RT-PCR assays (10, 11, 20, 31) are listed in Table 1. Assays A, B, and C were based on the TaqMan technology, whereas assay D was based on the SYBR green technology. The QuantiTect probe RT-PCR kit (QIAGEN) and the QuantiTect SYBR green RT-PCR Kit (QIAGEN) were used for amplification.

Some of the published reaction and cycling conditions were slightly modified to optimize the reactions and to adapt the performance to our real-time PCR machine. Optimization was performed on four fecal samples containing different NoVs (two GI and two GII). Assay A included primers (250 nM), GI probe (250 nM), and GII probe (100 nM) under the following amplification conditions: (i) RT for 30 min at 50°C, (ii) 15 min at 95°C to activate the *Taq* polymerase, and (iii) 40 cycles of 15 s at 95°C and 60 s at 55°C. The same cycling conditions were used in all TaqMan assays, except for the annealing temperature that was increased to 56°C in assay B and to 60°C in assay C. Assay B included primers (600 nM), GI probe (400 nM), and GII probe (200 nM), while assay C used 200 nM concentrations of each of the primers and probe (GII). The SYBR green assay D included 500 nM concentrations of each primer, and the reverse transcription was run at 50°C for 30 min, followed by 15 min at 95°C and 40 cycles of 20 s at 95°C, 60 s at 50°C, and 6 s at 77°C (optical read). An optical read at 77°C was chosen because this was 2°C below the melting temperature of the specific amplicons, and detection of spurious products was eliminated. A melting-curve analysis completed the run in order to verify the specificity of the amplification products. Six PCR products with different melting points were also run in a gel

to visualize the size of the amplicons. In all assays an increased ramp time was included between annealing and denaturation to optimize polymerization.

The real-time reactions were run on a SmartCycler (Cepheid) with a final reaction volume of 25 μl. All samples yielding discordant results were retested. Samples that were negative in the duplex assays (A and D) were retested in a monoplex version of the same assays. A reduced concentration of the GI probe (100 nM) was used in the monoplex version of the assay A.

A negative control containing water and a positive RT-PCR control containing RNA from one GI- and one GII-positive stool sample were included in each run.

DNA sequencing and phylogenetic analysis. Genetic characterization was performed by direct sequencing of the PCR fragment. At least one positive sample per outbreak was processed for sequencing of the RNA polymerase region (240 nucleotides). PCR products were sequenced in both directions using the ABI Prism BigDye terminator cycle sequencing kit and the ABI Prism 310 genetic analyzer (Applied Biosystems). Sequences were edited by using Sequencher 3.0.1 software (Gene Codes Corp.). Multiple alignments and phylogenetic trees were constructed by using MEGA version 3.0 (18).

The sequences obtained in the present study were aligned to the following EMBL/GenBank NoV genotype strains and to strains from the sequence database of European Network (14, 36): Lordsdale/1993/UK (X86557), Farmington Hill (AY502023), Hokkaido2004 (AB231362), Wortley/90/UK, Den Haag/99/NL, Berlin2000 (AF312524), Spania2001 (AJ487474), Gotenberg2001 (AF365989), Melksham/94/UK (X81879), Sundvall (AJ626563), Leeds/90/UK, Alphantron (AY185554), Birmingham/93/UK, Desert Shield 1990 (U04469), Chiba/87/JP, Norwalk/1968/US, Norwalk (M87661), Southampton/1991/UK (L07418), and Sindlesham/95/UK (Fig. 1). The NoV genogroups and genotypes were assigned according to a recently proposed classification system (1, 7, 16).

Nucleotide sequence accession numbers. The sequences obtained in this study have been submitted to GenBank under the following accession numbers (isolate numbers are given in parentheses): AM397872 (776-04), AM397873 (2681-02), AM397874 (4398-02), AM397875 (4117-03), AM397876 (4266-03), AM397877 (4200-02), AM397878 (886-01), AM397879 (1024-00), AM397880 (4469-03), AM397881 (314-04), AM397882 (6498-00), AM397883 (148-05), AM397884 (352-01), AM397885 (1299-01), AM397886 (164-04), AM397887 (2851-02), AM397888 (4167-03), AM397889 (1793-05), AM397890 (477-05), AM397891 (5577-02), AM397892 (785-05), AM397893 (5587-02), AM397894 (4448-00), AM397895 (114-01), AM397896 (754-04), AM397897 (2202-01), AM397898 (1504-02), AM397899 (749-04), and AM397900 (7105-02).

FIG. 1. NoV activity in Norway between January 2001 and August 2005. The figure shows the number of positive outbreaks (pos OB) analyzed in our laboratory and the total number of positive samples diagnosed in Norway. The food- or waterborne outbreaks are marked “FW OB.”

RESULTS

Outbreaks of NoV gastroenteritis in Norway from 2000 to 2005. During the five seasons studied, 204 of 310 gastroenteritis outbreaks were diagnosed as NoV positive in our laboratory. The majority of the outbreaks were from hospitals and nursing homes (171 of 204 [84%]), and the number of NoV outbreaks ranged between 9 and 67 per year (Table 2). Several other laboratories in Norway perform RT-PCR for NoVs, and the number of positive samples is routinely reported to our laboratory. These reports, however, do not allow us to determine whether the NoV-positive samples were from outbreaks or from sporadic cases. Figure 1 shows the monthly distribution of the NoV outbreaks diagnosed in our laboratory and the NoV-positive samples reported from all diagnostic laboratories. The positive samples diagnosed in our laboratory represent ca. 25% of all NoV-positive samples diagnosed in Norway. A clear increase was seen during winter and spring, with a peak

between December and March. Between June and October, only a few NoV-positive outbreaks were observed. A total of 29 food- or waterborne (FW) outbreaks were detected during the study period, and all but one of these outbreaks were noninstitutional. The usual seasonality was not observed with the FW outbreaks since 11 of 29 occurred between May and September. A marked increase in NoV activity was observed in September 2002, along with a change in the usual seasonal pattern and the emergence of the new GII.4-2002 variant. The new variant dominated the remainder of the extended 2002-2003 season. The NoV activity decreased during the next season, whereas an increase was again observed in November 2004 (Fig. 1). This rise in NoV activity in November 2004 concurrent with the emergence of a new GII.4 variant was similar to that observed in 2002.

Molecular epidemiology of NoVs. A total of 122 of 204 NoV outbreaks were randomly selected for sequencing analysis. Eight of the sequenced outbreaks occurred in 2000, 12 occurred in 2001, 29 occurred in 2002, 30 occurred in 2003, 24 occurred in 2004 and 19 occurred in 2005 (Table 2). A total of 112 of 122 outbreaks (91.8%) were caused by NoVs belonging to GII, and 94 of the GII isolates (84%) clustered within the GII.4 genotype. All but 2 of the 93 institutional outbreaks were caused by NoVs belonging to GII. Only 10 outbreaks were caused by GI strains, and 8 of these were associated with FW transmission. The remaining 17 sequenced FW outbreaks were caused by GII strains (17 of 29 [57%]). Seven GI outbreaks occurred between May and September. Polymerase sequence analysis revealed a variety of NoV genotypes circulating in Norway between January 2000 and August 2005 (Fig. 2): four GI genotypes (GI.1, GI.3b, GI.4, and GI.6) and seven GII genotypes (GII.1, GIIC, GIIB, GII.2, GII.3, GII.4, and GII.7). Data for 10 GI outbreaks, the 25 sequenced FW outbreaks, the GII outbreaks not belonging to GII.4, and some of the panel

TABLE 2. Annual occurrence of the predominant NoV genotypes causing outbreaks of gastroenteritis in Norway between January 2000 and August 2005^a

Yr	No. of outbreaks							
	NoV	Sequenced	GII.4	GII.4-2002	GII.4-2004	GIIB	GI	FW
2000	9	8	4	0	0	3	1	3
2001	24	12	3	0	0	8	1	5
2002	67	29	26	22	0	0	4	12
2003	59	30	26	26	0	1	1	8
2004	25	24	19	5	14	0	3	0
2005	20	19	16	0	16	0	0	1
Total	204	122	94	53	30	12	10	29

^a Results for food- or waterborne outbreaks (FW) are shown in the last column.

samples are presented Fig. 2. The remaining institutional GII.4 outbreaks not included in the phylogenetic tree are described in Table 2. The NoV strains detected during the 2000-2001 and 2001-2002 seasons grouped in two major clusters: one cluster being closely related to similar GIIB strains detected in Spain and Sweden (2, 28) during the same period and the other cluster being closely related to the Farmington Hill strain. The nucleotide sequence homology between our GIIB sequences and the GIIB sequences from Sweden and Spain was between 97 and 99%. The nucleotide sequence homologies between our GII.4 sequences and the Farmington Hill strain was between 98 and 100% and between our GII.4 sequences and the Lordsdale prototype strain was ca. 90%. A complete dominance of the GII.4 strains was observed from September 2002 (Table 2). The nucleotide sequence homology between the GII.4 sequences detected since August 2004 and the new GII.4-2004 variant was between 98 and 100% (data not shown). The emergence of the new GII.4-2002 variant, with a shift in a specific motif from AACTTG to AATCTG (22), was registered for the first time in September 2002. This new variant dominated the two following seasons but was replaced by the other new GII.4-2004 variant in August 2004. The GII.4-2004 variant was detected in all but five outbreaks between August 2004 and August 2005. Apart from the GII.4 and GIIB clusters, several outbreaks were caused by GII.2, GII.7, and GI.3b strains (Fig. 2).

Comparison of real-time RT-PCRs. Changes made to the reaction and cycling conditions yielded a higher analytical sensitivity (a lower cycle threshold [C_T] value). Assays A, B, and D detected the same four GI genotypes included in the panel, while none of the assays detected the GI.2 genotype (Table 3). The panel contained 12 GI-positive samples, and 9 were detected by assay A, 10 were detected by assay B, and 11 were detected by assay D. The average C_T values for the nine GI samples detected by each assay were 25.7 for assay A, 30.7 for assay B, and 25.0 for assay D. The mean difference between C_T values in the two TaqMan assays A and B was 4.9 ± 2.6 . The TaqMan assays A, B, and C showed identical results regarding the detection of the genotyped GII samples, whereas SYBR green assay D missed three of the samples. The average C_T values for the 25 GII-positive samples detected by all of the assays were 23.8 for assay A, 22.2 for assay B, and 22.6 for assays C and D. The mean difference between C_T values in the assays with highest and lowest average was 1.5 ± 1.2 , which indicates that the assays have similar analytical sensitivity for detecting GII strains included in the panel. The samples that tested negative in assays A and D were also negative in the monoplex versions of these two assays. The three TaqMan assays (A, B, and C) showed a high specificity regarding identification of correct NoV genogroup (Table 3). The melting-curve analysis in the assay D showed melting points

TABLE 3. Results from the comparison of four NoV real-time assays with a panel of 97 stool samples^a

Sample		No. of positive samples ^b					
Type	No.	Assay A		Assay B		Assay C	Assay D
		GI	GII	GI	GII	(GII)	(GI/GII)
GI.1	2	1	0	2	0	0	2
GI.2	1	0	0	0	0	0	0
GI.3	7	6	0	6	0	0	7
GI.4	1	1	0	1	0	0	1
GI.5	1	1	0	1	0	0	1
Total	12	9	0	10	0	0	11
GII.1	1	0	1	0	1	1	1
GII.2	1	0	1	0	1	1	1
GII.3	1	0	1	0	1	1	0
GII.4	18	0	16	0	16	16	15
GII.6	1	0	1	0	1	1	1
GII.7	3	0	3	0	3	3	3
GIIB	2	0	2	0	2	2	2
GIIC	3	0	3	0	3	3	2
Total	30	0	28	0	28	28	25
Positive	34	4	25	4	25	26	22 ^c
Negative	11	0	3	0	4	4	3
Not tested	10	0	7	0	6	6	3

^a A total of 87 samples had previously been tested by a conventional NoV RT-PCR and classified as NoV positive ($n = 76$) or negative ($n = 11$). Ten stool samples had not previously been tested. Genotyping of 42 strains was performed in the polymerase region. The table gives the number of samples with different GI and GII genotypes. All samples were tested by each of the assays.

^b Assay A, duplex with TaqMan technology (11); assay B, monoplex (GI and GII) with TaqMan technology (10); assay C, monoplex (GII) with TaqMan technology (20); assay D, duplex with SYBR green technology (31).

^c The positive samples include the four GI samples detected by assays A and B.

in the range of 79 to 83°C. However, it was not possible to differentiate between GI and GII strains based on melting point (data not shown). In NoV-containing samples the melting-curve analysis showed no interference from unspecific polymerization. Gel visualization of six PCR products obtained in assay D verified the amplicon size and the absence of spurious products. Regarding the remaining 55 untyped stool samples, similar results were obtained with assays A, B, and C, whereas several samples were not detected by assay D. According to assays A and B, 4 of the 55 samples contained GI strains. These four strains were also detected by assay D (Table 3).

Based on the results from the conventional RT-PCR on 87 stool samples in the panel, 76 were NoV positive and 11 were NoV negative. Altogether, assay A detected 69 samples (13 GI and 56 GII), assay B detected 71 samples (14 GI and 57 GII), and assay D detected 61 samples. Assay C gave the highest score, with the detection of 58 GII-positive samples. The conventional RT-PCR detected the highest number of positive

FIG. 2. Phylogenetic tree derived from 12 NoV GI and 39 NoV GII strains detected in Norway during the study period. The phylogenetic tree was based on 240 bp from the polymerase region and was constructed by the neighbor-joining method using MEGA3 software. Branch lengths are related to the degree of divergence between the strains. The major branches are marked with GI and GII. Bootstrap values for branches of >50% are given. The food- or waterborne outbreaks are marked "FW," and the institutional outbreaks are marked "INSTITUTION." Data for GI outbreaks, FW outbreaks, GII outbreaks not belonging to GII.4, and some of the panel samples are presented. Two of the GI samples are only included in the stool panel.

samples ($n = 76$); however, four samples were only detected by the real-time assays.

Retesting of stool samples collected from 1999 to 2004. Of the 372 stool samples, 56 (15%) included in the retesting were previously classified as NoV positive based on the in-house diagnostic RT-PCR. In total, 96 samples (26%) tested as NoV positive when retested with assay A (91 GII and 5 GI). The 56 samples previously classified as NoV positive were also positive with assay A, whereas the 40 samples classified as NoV negative were all GII positive with assay A.

DISCUSSION

The clear increase in NoV activity seen in many European countries during autumn 2002 was due to the emergence of a new GII.4 variant. A similar trend was seen in Norway, as indicated by the significant increase in the number of positive samples analyzed in or reported to our laboratory. Figure 1 shows the variation in NoV activity observed during the five seasons studied. In accordance with other studies, the highest NoV activity was seen during the 2002-2003 season. Based on the number of positive samples reported to our institute, a high NoV activity was also recognized during the 2004-2005 season. This was similar to the high NoV activity observed in Finland, The Netherlands, and England during the same season (DIVINE-NET). The increased NoV activity seen in Norway during these two seasons is most likely due to the emergence of the new GII.4 variants reported to the European Network in 2002 and 2004. Although the real number of NoV outbreaks occurring during the study period is unknown, the present study shows that the NoV activity and seasonality seen in Norway has been similar to that reported from other European countries.

The complete picture of different NoV genotypes circulating in Norway during the study period remains unknown. Nevertheless, the present study shows that there have been two dominant NoV clusters: GIIB and GII.4. Both of these genotypes were predominant during the 2000-2001 and 2001-2002 seasons, and the strains within the GIIB cluster showed 98 to 100% sequence homology to the GIIB strains from Spain, Sweden, and Finland in 2001 (2, 25, 28). The next season started with the emergence of the new GII.4-2002 variant, and the strains clustering with the GII.4 genotype have been predominant since 2002. This genotype has been shown to persist from year to year. A <5% nucleotide sequence difference was detected between the GII.4 sequences, and the different GII.4 variants clustered together in the phylogenetic tree (data not shown). Although the GII.4 genotype seems to undergo continuous genetic changes, only a few point mutations were seen in the polymerase region between the different GII.4 sequences. One of these mutations was detected in a specific motif, and the change in this motif reflected the GII.4 variant detected. However, to gain more insight into the pathogenic potential of the different variants, other regions, e.g., the capsid region, must be analyzed. The new GII.4-2002 variant appeared in Norway in September 2002, whereas the GII.4-2004 variant appeared in August 2004. The delayed detection of the new variants in Norway compared to other European countries was probably due to the limited number of outbreaks selected for sequencing. The new variants were detected in 83 of 94

GII.4 outbreaks between September 2002 and August 2005 (Table 2).

The GIIB and GII.4 genotypes have been associated with the majority of hospital outbreaks (4, 25), and this seems to be the case also in Norway. This was not surprising since the majority of outbreaks included in the present study were institutional. Due to the high hygienic level in hospitals, it has been speculated that only the most resistant NoV strains are capable of causing institutional outbreaks (25). This may be one explanation for the global distribution of GII.4 strains in hospital outbreaks. However, little is known about the environmental stability, the dose needed to produce an active infection, or the pathogenic potential of different NoV genotypes. Different strains may also induce different degrees of immunity. To our knowledge, different virulence factors have not yet been linked to different genotypes. Figure 2 shows that the majority of the FW outbreaks were also caused by GII strains. This is probably due to the dominating circulation of NoV GII strains in the population. On the other hand, GI strains caused 8 of 25 FW outbreaks (32%) and 2 of 93 institutional outbreaks (2%), which indicates that GI strains may be more resistant to environmental conditions that favor FW transmission. This assumption is supported by a Finnish study in which the detection of GI strains in waterborne outbreaks was more frequent than expected (24). However, due to the limited number of GI outbreaks detected in the present study, further investigation is needed.

The comparison of four published NoV real-time RT-PCRs showed that assay A satisfied the combined requirements for sensitivity and simplicity. Real-time RT-PCR is less cumbersome and safer with regard to cross-contamination than conventional RT-PCR. To optimize our NoV diagnostics, a published conventional polymerase RT-PCR was compared to a SYBR green assay (assay D) amplifying a different part of the polymerase region and to three TaqMan assays amplifying the polymerase-capsid junction (assays A, B, and C). Assay A, which detects the most prevalent genotypes, was included in the comparison for the convenience of running a GI and GII duplex reaction. Jothikumar et al. had previously demonstrated that assay A was more sensitive than the assay described by Kageyama et al. (11, 12). These two assays contain identical GII reverse primer and probe. Loisy et al. also demonstrated that the GI assay described by Kageyama et al. performed poorly on NoV-positive shellfish (20). Their modified version of the Kageyama GII primers and probe performed well and was therefore included in the present study (assay C). Assay B amplified a different part of the polymerase-capsid junction in GI strains compared to assay A, whereas the GII primers and probe were located in the same area as in assays A and C. SYBR green assay D had been shown to detect a broad spectrum of NoV genotypes (GI 1 to 5 and GII 1 to 8) and was included due to a different location in the polymerase region and the duplex form.

Conclusions regarding the specificity and sensitivity of the real-time assays included in the present study were based on the RT-PCR results on the panel (Table 3). This panel represents the different genotypes that circulated in Norway between 1998 and 2005. The panel was originally classified by the broadly reactive polymerase primers and the sequence of their amplicons (34). These primers were improved variants of pub-

lished primers which performed well in an international study on NoV RT-PCR assays (36). The three TaqMan assays (A, B, and C) detected a similar number of NoV-positive samples. Although SYBR green assay D performed well on GI samples, the total number of positive samples detected was lower than with the TaqMan assays. These results are probably not due to a lower sensitivity of the SYBR green technology compared to the TaqMan technology. Some of the samples missed by the SYBR green assay D yielded low C_T values in the TaqMan assays. This indicates a high NoV copy number, and these samples should therefore be NoV positive even in a less sensitive assay. The observed C_T values for the assay D were also similar to the C_T values in assays A, B, and C. The assay D primers were located in a different part of the genome, and this may have resulted in a lower detection rate of GII strains (Table 3). The TaqMan assays showed equal NoV detection rates, and the main difference in performance between assays A and B is displayed in the C_T values from the genotyped GI samples. The mean difference in the C_T values was 4.9 ± 2.5 , with assay A yielding the best result. This indicates that assay A has a higher analytical sensitivity than assay B, but this may only partly explain why Jothikumar et al. failed to detect the GI samples with assay B in their comparison of assays A and B (11). Regarding the clinical sensitivity, both assay A and assay B performed well in the present study. The lower analytical sensitivity observed in the detection of GI samples with assay B may be due to the location of primers and probes in a less conserved area of the polymerase-capsid junction (12). The conventional RT-PCR detected the highest number of positive samples. This may be due to a bias in the panel because it was classified by the same conventional RT-PCR. In addition, the real-time PCRs were run on RNA that may have been degraded during storage. On the other hand, the real-time assays detected NoV in four stool samples previously classified as NoV negative.

Although assays A and B showed equal clinical sensitivity in the present study, assay A showed a higher analytical sensitivity for GI strains compared to assay B. Therefore, assay A probably has an increased clinical sensitivity on fecal samples with a low viral load. The practical and economical advantages of a duplex assay were also considered important when assay A was established as the new NoV-diagnostic RT-PCR. Although assay D is a duplex assay and SYBR green is a less expensive technology, the lower detection rate for GII samples and the lack of differentiation between GI and GII strains make it less suitable as a diagnostic tool. Assay C gave a slightly higher detection rate compared to the other assays and could be used as a first-line GII screening method.

Retesting of 372 randomly chosen stool samples previously analyzed in our laboratory between 1998 and 2004 was performed with assay A. The goal was to achieve an extended comparison of assay A and the conventional RT-PCRs and to reveal whether false negatives had contributed to the low prevalence of NoV-positive samples in Norway. The number of positive samples increased from 56 (15%) to 96 (26%). The results demonstrate the increased clinical sensitivity of assay A compared to the former diagnostic RT-PCRs. However, the difficulty of designing an RT-PCR assay that detects all NoV strains is demonstrated by our results since assay A did not detect all of the positive stool samples in our panel. It is,

however, difficult to estimate the true false-negative rate of the RT-PCR methods used in the present study. Electron microscopy could probably have detected some samples missed by RT-PCR, i.e., new genetic variants, but was not available in our laboratory.

In summary, the NoV activity observed in Norway has been similar to that seen in other European countries during the same period. The present study also indicates that the strains circulating in Norway are similar to the strains detected in other countries. In general, DIVINE-NET has demonstrated the importance of common sequence databases in the detection of new NoV variants. It is also important to further enhance the capacity for national and international surveillance across Europe of NoV outbreaks.

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REFERENCES

1. Ando, T., J. S. Noel, and R. L. Fankhauser. 2000. Genetic classification of "Norwalk-like" viruses. *J. Infect. Dis.* **181**(Suppl. 2):S336-S348.
2. Buesa, J., B. Collado, P. Lopez-Andujar, R. Abu-Mallouh, D. J. Rodriguez, D. A. Garcia, J. Prat, S. Guix, T. Llovet, G. Prats, and A. Bosch. 2002. Molecular epidemiology of caliciviruses causing outbreaks and sporadic cases of acute gastroenteritis in Spain. *J. Clin. Microbiol.* **40**:2854-2859.
3. de Wit, M. A., M. P. Koopmans, and Y. T. van Duynhoven. 2003. Risk factors for norovirus, Sapporo-like virus, and group A rotavirus gastroenteritis. *Emerg. Infect. Dis.* **9**:1563-1570.
4. Fankhauser, R. L., S. S. Monroe, J. S. Noel, C. D. Humphrey, J. S. Bresee, U. D. Parashar, T. Ando, and R. I. Glass. 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* **186**:1-7.
5. Fankhauser, R. L., J. S. Noel, S. S. Monroe, T. Ando, and R. I. Glass. 1998. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* **178**:1571-1578.
6. Glass, R. I., J. Noel, T. Ando, R. Fankhauser, G. Belliot, A. Mounts, U. D. Parashar, J. S. Bresee, and S. S. Monroe. 2000. The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics. *J. Infect. Dis.* **181**(Suppl. 2):S254-S261.
7. Green, K., M. Chanock, and A. Kapikian. 2001. Human caliciviruses, p. 841-874. *In* D. M. Knipe and P. M. Howley (ed.), *Fields virology*, 4th ed. Lippincott-Raven Publishers, Philadelphia, Pa.
8. Grist, N., E. Bell, E. Follett, and G. Urquhart. 1979. Diagnostic methods in clinical virology. Blackwell Scientific Publications, Oxford, England.
9. Hedlund, K. O., E. Rubilar-Abreu, and L. Svensson. 2000. Epidemiology of calicivirus infections in Sweden, 1994-1998. *J. Infect. Dis.* **181**(Suppl. 2):S275-S280.
10. Hohne, M., and E. Schreier. 2004. Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. *J. Med. Virol.* **72**:312-319.
11. Jothikumar, N., J. A. Lowther, K. Henshilwood, D. N. Lees, V. R. Hill, and J. Vinje. 2005. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl. Environ. Microbiol.* **71**:1870-1875.
12. Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama. 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J. Clin. Microbiol.* **41**:1548-1557.
13. Kageyama, T., M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, S. Kojima, R. Takai, T. Oka, N. Takeda, and K. Katayama. 2004. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to norovirus in Japan. *J. Clin. Microbiol.* **42**:2988-2995.
14. Koopmans, M., H. Vennema, H. Heersma, E. van Strien, Y. Van Duynhoven, D. Brown, M. Reacher, and B. Lopman. 2003. Early identification of common-source foodborne virus outbreaks in Europe. *Emerg. Infect. Dis.* **9**:1136-1142.
15. Koopmans, M., M. J. Vinje, M. De Wit, I. Leenen, W. van der Poel, and Y. Van Duynhoven. 2000. Molecular epidemiology of human enteric caliciviruses in The Netherlands. *J. Infect. Dis.* **181**(Suppl. 2):S262-S269.
16. Koopmans, M., J. Vinje, E. Duizer, M. De Wit, and Y. van Duynhoven. 2001.

- Molecular epidemiology of human enteric caliciviruses in The Netherlands. Novartis Found. Symp. **238**:197–214.
17. Koopmans, M., C. H. von Bonsdorff, J. Vinje, D. De Medici, and S. Monroe. 2002. Foodborne viruses. *FEMS Microbiol. Rev.* **26**:187–205.
 18. Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.* **5**:150–163.
 19. Lindell, A. T., L. Grillner, L. Svensson, and B. Z. Wirgart. 2005. Molecular epidemiology of norovirus infections in Stockholm, Sweden, during the years 2000 to 2003: association of the GGIb genetic cluster with infection in children. *J. Clin. Microbiol.* **43**:1086–1092.
 20. Loisy, F., R. L. Atmar, P. Guillon, P. Le Cann, M. Pommepuy, and F. S. Le Guyader. 2005. Real-time RT-PCR for norovirus screening in shellfish. *J. Virol. Methods* **123**:1–7.
 21. Lopman, B., Y. Van Duynhoven, F. X. Hanon, M. Reacher, M. Koopmans, and D. Brown. 2002. Laboratory capability in Europe for food-borne viruses. *Euro. Surveill.* **7**:61–65.
 22. Lopman, B., H. Vennema, E. Kohli, P. Pothier, A. Sanchez, A. Negredo, J. Buesa, E. Schreier, M. Reacher, D. Brown, J. Gray, M. Iturriza, C. Gallimore, B. Bottiger, K. O. Hedlund, M. Torven, C. H. von Bonsdorff, L. Maunula, M. Poljsak-Prijatelj, J. Zimsek, G. Reuter, G. Szucs, B. Melegh, L. Svensson, Y. van Duynhoven, and M. Koopmans. 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. *Lancet* **363**:682–688.
 23. Lopman, B. A., M. H. Reacher, Y. van Duynhoven, F. X. Hanon, D. Brown, and M. Koopmans. 2003. Viral gastroenteritis outbreaks in Europe, 1995–2000. *Emerg. Infect. Dis.* **9**:90–96.
 24. Maunula, L., I. T. Miettinen, and C. H. von Bonsdorff. 2005. Norovirus outbreaks from drinking water. *Emerg. Infect. Dis.* **11**:1716–1721.
 25. Maunula, L., and C. H. von Bonsdorff. 2005. Norovirus genotypes causing gastroenteritis outbreaks in Finland 1998–2002. *J. Clin. Virol.* **34**:186–194.
 26. Mohamed, N., S. Belak, K. O. Hedlund, and J. Blomberg. 2005. Experience from the development of a diagnostic single tube real-time PCR for human caliciviruses, norovirus genogroups I and II. *J. Virol. Methods* **132**:69–76.
 27. Noel, J. S., R. L. Fankhauser, T. Ando, S. S. Monroe, and R. I. Glass. 1999. Identification of a distinct common strain of “Norwalk-like viruses” having a global distribution. *J. Infect. Dis.* **179**:1334–1344.
 28. Nygard, K., M. Torven, C. Ancker, S. B. Knauth, K. O. Hedlund, J. Giesecke, Y. Andersson, and L. Svensson. 2003. Emerging genotype (GGIb) of norovirus in drinking water, Sweden. *Emerg. Infect. Dis.* **9**:1548–1552.
 29. Reuter, G., T. Farkas, T. Berke, X. Jiang, D. O. Matson, and G. Szucs. 2002. Molecular epidemiology of human calicivirus gastroenteritis outbreaks in Hungary, 1998 to 2000. *J. Med. Virol.* **68**:390–398.
 30. Reuter, G., H. Vennema, M. Koopmans, and G. Szucs. 2006. Epidemic spread of recombinant noroviruses with four capsid types in Hungary. *J. Clin. Virol.* **35**:84–88.
 31. Richards, G. P., M. A. Watson, R. L. Fankhauser, and S. S. Monroe. 2004. Genogroup I and II noroviruses detected in stool samples by real-time reverse transcription-PCR using highly degenerate universal primers. *Appl. Environ. Microbiol.* **70**:7179–7184.
 32. Vainio, K., K. Stene-Johansen, J. T. Oystein, A. L. Bruu, and B. Grinde. 2001. Molecular epidemiology of calicivirus infections in Norway. *J. Med. Virol.* **65**:309–314.
 33. Vinje, J., J. Green, D. C. Lewis, C. I. Gallimore, D. W. Brown, and M. P. Koopmans. 2000. Genetic polymorphism across regions of the three open reading frames of “Norwalk-like viruses.” *Arch. Virol.* **145**:223–241.
 34. Vinje, J., R. A. Hamidjaja, and M. D. Sobsey. 2004. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. *J. Virol. Methods* **116**:109–117.
 35. Vinje, J., and M. P. Koopmans. 1996. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in The Netherlands. *J. Infect. Dis.* **174**:610–615.
 36. Vinje, J., H. Vennema, L. Maunula, C. H. von Bonsdorff, M. Hoehne, E. Schreier, A. Richards, J. Green, D. Brown, S. S. Beard, S. S. Monroe, E. de Bruin, L. Svensson, and M. P. Koopmans. 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. *J. Clin. Microbiol.* **41**:1423–1433.
 37. Zheng, D. P., T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass, and S. S. Monroe. 2005. Norovirus classification and proposed strain nomenclature. *Virology* **346**:312–323.