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Technical advance

Fast detection of Noroviruses using a real-time PCR assay and automated sample preparation

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Abstract

Background: Noroviruses (NoV) have become one of the most commonly reported causative agents of large outbreaks of non-bacterial acute gastroenteritis worldwide as well as sporadic gastroenteritis in the community. Currently, reverse transcriptase polymerase chain reaction (RT-PCR) assays have been implemented in NoV diagnosis, but improvements that simplify and standardize sample preparation, amplification, and detection will be further needed. The combination of automated sample preparation and real-time PCR offers such refinements.

Methods: We have designed a new real-time RT-PCR assay on the LightCycler (LC) with SYBR Green detection and melting curve analysis (T_m) to detect NoV RNA in patient stool samples. The performance of the real-time PCR assay was compared with that obtained in parallel with a commercially available enzyme immunoassay (ELISA) for antigen detection by testing a panel of 52 stool samples. Additionally, in a collaborative study with the Baden-Wuerttemberg State Health office, Stuttgart (Germany) the real-time PCR results were blindly assessed using a previously well-established nested PCR (nPCR) as the reference method, since PCR-based techniques are now considered as the "gold standard" for NoV detection in stool specimens.

Results: Analysis of 52 clinical stool samples by real-time PCR yielded results that were consistent with reference nPCR results, while marked differences between the two PCR-based methods and antigen ELISA were observed. Our results indicate that PCR-based procedures are more sensitive and specific than antigen ELISA for detecting NoV in stool specimens.

Conclusions: The combination of automated sample preparation and real-time PCR provided reliable diagnostic results in less time than conventional RT-PCR assays. These benefits make it a valuable tool for routine laboratory practice especially in terms of rapid and appropriate outbreak-control measures in health-care facilities and other settings.

Background

NoV, members of the family Caliciviridae, are single-stranded RNA, nonenveloped viruses. Based on antigenic and genetic distinctions NoV (formerly called Norwalk-like viruses) can be divided in 5 different genogroups [1]. Since the first identification of this pathogen in 1972 [2],

NoV have become one of the most commonly reported causative agents of large outbreaks of non-bacterial gastro-enteritis worldwide as well as sporadic gastroenteritis in the community [3-6]. NoV usually cause acute self-limited infections in humans of all ages. However, NoV infection can be severe in frail elderly persons, young children, and immunocompromised persons and medical attention may be required to prevent dehydration and nosocomial spread. After an incubation period of 1 to 3 days, the clinical manifestations are characterized by diarrhoea that lasts 12 to 60 hours and is frequently accompanied by a variety of other symptoms such as nausea, vomiting, and abdominal cramps. Headache and low-grade fever are also commonly reported with NoV illness (reviewed in [7]). Viral spread from person-to-person via the faecal-oral route or through vomiting is the major mode [8], but also the importance of food and water in disease transmission is well-recognized [9-11].

Initially, direct and immune electron microscopy (EM) were used to detect the presence of NoV in fecal specimens, however EM diagnosis has a low sensitivity and is dependent upon collection of samples 2–3 days after the onset of illness. Moreover, EM is not routinely implemented in the laboratory because of technical limitations and dependency on trained medical staff for its operation.

With the breakthrough in cloning and sequencing of NoV genome, antigen detection assays based on baculovirus-expressed NoV capsid antigens have been developed (reviewed in [12]). These assays are type-specific, however they are not broadly reactive. Subsequently, RT-PCR assays have become the method of choice both due to their unsurpassed sensitivity and the ability to detect genetically diverse NoV strains [13-20]. Today, there is keen interest for standardization and automation for molecular tests.

The combination of automated sample preparation using the MagNA Pure extraction robot and the LightCycler PCR system (both Roche Applied Science, Mannheim, Germany) now provides such a standardized application format. Unlike the classic RT-PCR techniques, the closed format of real-time PCR avoids carryover contaminations with amplified products. As a result of reduced cycle times and lack of post-amplification processing steps, the turn-around time of the assay is considerably shorter and test results can be generated in a timely manner.

Here, we describe the application of a SYBR Green based real-time PCR assay to directly detect the presence of NoV RNA in stool specimens using the LC instrument. The results of the new assay were evaluated against antigen ELISA and nPCR results. The objectives of this study were to determine the test characteristics such as reliability and reproducibility, as well as to assess the clinical value of the new assay. A particular goal was to combine a real-time PCR-based approach with an automated sample preparation system, which is especially beneficial for clinical laboratories handling large numbers of specimens.

Methods
Samples
A total of 52 routine diagnostic samples from different patients were used in this study. The samples were collected from January 2003 through April 2003. Although we have collected the samples within a short period of time, the samples originate from various geographical areas in Germany. Of the 52 samples, 38 were obtained from patients implicated in several outbreaks of supposed gastroenteritis in different settings, including nursing and residential homes, and hospitals. Unfortunately, only data of age and gender are available (13.1% were less than 10 years of age, 39.5% between 10 and 60 years, and 47.4% more than 60 years old). The remaining 14 samples were cases of single sporadic gastroenteritis obtained from children <5 years of age, which have been already tested for rotavirus and adenovirus by antigen ELISA. These patients presented clinical symptoms of acute diarrhoea (loose or watery stools, diarrhoea with vomiting, nausea, and associated symptoms including fever or signs of dehydration).

Fecal specimens were collected each in sterile containers and immediately processed upon receipt for real-time PCR and antigen ELISA and stored at -20°C until further processing by nPCR.

Sample processing
For real-time PCR testing, a 10% (wt/vol) stool suspension was prepared in 500 μl PCR-grade water, and centrifuged at 16.000 × g for 5 min at room temperature. 200 μl of the clarified supernatant was used for extraction of RNA on the MagNA Pure instrument.

Automated nucleic acid isolation
Viral RNA preparation was performed on the automated MagNA Pure instrument by using the MagNA Pure total nucleic acid isolation kit (Roche Applied Science, Mannheim, Germany). The MagNA Pure is a benchtop instrument that can extract nucleic acids from 32 samples in parallel. The instrument software guides the user through the set up procedure and automatically calculates the amount of reagents needed for processing. All remaining pipetting steps of the procedure were automatically performed with aerosol-preventive, disposable reaction tips. After incubation with a chaotropic lysis buffer and proteinase K, nucleic acids were released and bound to magnetic glass particles. Unbound molecules are removed by

Table 1: Summary of NoV primers used

| Real-time PCR (polymerase gene) | | | |
Primer	Location	Oligonucleotide sequence (5' - 3')	Reference
NV p110[a]	4865 - 4883	ACDATYTCATCATCACCATA	18
NV p36[a]	4487 - 4505	ATAAAAGTTGGCATGAACA	15
NV NI[b]	4495 - 4515	GAATTCCATCGCCCACTGGCT	17

| Nested PCR (polymerase gene) | | | |
Primer	Location	Oligonucleotide sequence (5' - 3')	Reference
Primer 32[b]	4226 - 4245	ATGAATATGAATGAAGATGG	23
Primer 36[a]	4980 - 4961	ATTGGTCCTTCTGTTTTGTC	23
Primer 35[a]	4890 - 4871	GTTGACACAATCTCATCATC	23
Primer 33[b]	4280 - 4299	TACCACTATGATGCAGATTA	23

Annealing site refer to [a]GenBank accession no. M87661 and [b]GenBank no X86557

several washing steps and RNA eluted in 60 μl elution buffer. Purified RNA was stored at -20°C.

Primer
Primer sets from previously published assays which amplify a conserved region within the RNA-dependent RNA polymerase gene were used. Sequences were compared against those listed in GenBank by using the basic local alignment search tool (BLAST) to ensure a high level of specificity against NoV. Oligonucleotide primers used in this study are listed in Table 1.

Real-time PCR
Real-time PCR was done using the LightCycler system with SYBR Green detection and T_m analysis. QuantiTect™ SYBR® Green RT-PCR kit was purchased from Qiagen, (Qiagen, Hilden, Germany). Amplification is performed in a total volume of 20 μl containing 10 μl of kit-supplied QuantiTect™ SYBR® Green RT-PCR Master mix (including HotStar Taq DNA polymerase, reaction buffer, dNTP mix, and SYBR dye), 0.5 μM of each primer, 1 μl RNase-free water, 0.2 μl kit-supplied QuantiTect RT mix (reverse transcriptase), and 6.8 μl of probe. Prior to cycling, the glass capillaries are sealed, placed into the LC sample carousel, and centrifuged at 700 × g for 5 s in the LC carousel centrifuge (Roche). Amplification conditions consisted of four consecutive phases: (i) a reverse transcription step (50°C for 20 min), (ii) an initial denaturation step in order to activate the HotStarTaq DNA Polymerase (95°C for 15 min), (iii) a touch-down profile for 10 cycles (94°C for 5 sec, 65°C for 15 sec and 72°C for 25 sec) with a 1°C step size temperature ramp per cycle from 65°C to 55°, (iv) an amplification step for 26 cycles (94°C for 15 sec, 55°C for 15 sec, and 72°C for 25 sec). To identify PCR products generated in the presence of SYBR Green, a T_m analysis is performed by increasing the temperature from 60°C to 90°C at a linear transition rate of 0.1°C/sec. Fluorescence of the samples is monitored continuously while the temperature is increased. SYBR Green is released upon denaturation, which results in a decreasing fluorescence of the signal. The LC software calculates the T_m, i.e. the rate of change in fluorescence (-dF/dT) is displayed as a function of temperature. All samples were analyzed once. Recommended optimisation by titration of appropriate primer concentrations was performed.

Controls
To monitor crossover contaminations 200 μl of PCR-grade water was included in the RNA extraction procedure and used as a negative control. To ensure quality and reproducibility of data, both a low-run control (i.e. purified NoV RNA) and a negative control were always applied in each run. In order to prevent PCR carryover contamination extraction of nucleic acids and amplification were performed in different rooms. Barrier-filtered pipette were used in all manipulations with the samples.

Interpretation
A sample was only considered positive if both the sample quantification curve became exponential, and the PCR product was correctly differentiated by the T_m analysis step.

NoV detection by nested RT-PCR
NoV RNA was extracted from coded patients's specimens using a method based on the lysing and nuclease-inactivating properties of guanidinium thiocyanate with the nucleic acid-binding properties of silica particles [21]. A 20% (wt/vol) stool suspension was prepared in 1 ml PCR-grade water, vortexed, and centrifuged at 16.000 × g for 3 min. Reaction vessels were prepared with 900 μl lysis buffer (5.25 M GuSCN, 50 mM Tris HCl, pH 6.4, 20 mM

EDTA, 1.3% wt/vol Triton X-100) and 40 µl of silica matrix to a 2 ml Eppendorf reaction tube. 300 µl of the clarified supernatant was added to the reaction vessels, and immediately vortexed. After 10 min at room temperature, the vessel was vortexed again and centrifuged at 16.000 × g for 15 s. The supernatant was disposed of by suction. The silica pellet was subsequently washed twice with 1 ml washing buffer (5.25 M GuSCN, 50 mM Tris HCl, pH 6.4), twice with 1 ml ethanol 70% (vol/vol), and once with 1 ml acetone. After centrifugation (3 min at approximately 16.000 × g), the supernatant was disposed of by suction. The vessels were dried at 56°C with open lids in a heat block for 10 min. For elution, 75 µl nuclease free water and 1 µl rRNasin (Promega, Mannheim, Germany) were added, and the closed vessel was vortexed briefly, and incubated for 10 min at 56°C. The vessel was vortexed again and centrifuged for 3 min at 16.000 × g. For reverse transcription (RT) 5 µl of the DNA and RNA containing supernatant was used. RT was carried out with the AMV-RT (Roche Applied Science, Mannheim, Germany) and the antisense primer 36. RT was performed for one cycle (25°C, 10 min; 42°C, 60 min; 98°C, 5 min). Part of the cDNA was amplified by nested PCR using primer 32 and primer 36 for the first round, and primer 33 and primer 35 for the second round. Amplifications were performed for 35 cycles (94°C, 30 s; 42°C, 30 s; 72°C, 45 s). The cycles were proceeded by a 1 min denaturation and followed by a 3 min extension at 72°C. PCR products were visualized on agarose gels after electrophoresis coupled with ethidium bromide staining and viewed on a UV transilluminator. Nested PCR was performed in a second laboratory without knowledge of LC-PCR test results. The sensitivity of the nPCR using serial dilutions of spiked clinical specimens was comparable to real-time PCR analysis. Primer sequence and nested RT-PCR set up have been previously described [22].

NoV detection by antigen ELISA
The RIDASCREEN® Norwalk-like Virus kit was purchased from R-Biopharm (R-Biopharm, Darmstadt, Germany) and used for the detection of Norwalk-like viruses (NLVs) of the genogroup I and II in stool specimens. This enzyme immunoassay is based on monoclonal antibodies bound on the surface of the microwell strips to capture the antigen in stool specimens. All reagents were kit-supplied. For sample preparation, stool samples were diluted 1:6 (v/v) in the diluent buffer, mixed thoroughly, allowed 5–10 min to settle, and further processed according to the protocol recommended by the manufacturer. Briefly, 100 µl supernatant of the prepared stool suspension are transferred into separate wells and incubated at room temperature for 60 min. After complete removing of the liquid, the wells are washed 5 times with 300 µl wash buffer. Two drops of enzyme conjugate are added to each well, incubated at room temperature for 30 min. After 5 times wash-

ing (see above), the bound conjugate was determined by the addition of a chromogen and enzyme substrate. The absorbance is measured at 450 nm. The kit cut-off used is determined by addition of 0.15 absorbance units to the measured absorption (450 nm) of the negative control.

Statistical analysis
Comparisons involving C_t values of PCR-positive ELISA-positive and PCR-positive but ELISA-negative samples were analysed by the Mann-Whitney U test using statistical software SPSS, version 11.5.1 (SPSS inc., Chicago, USA). For comparison of proportion the chi square test was used.

Results
Optimization strategies
Real-time PCR affords continuous monitoring of the PCR reaction cycle by cycle by using fluorescence. The detection in our assay is based on the binding of the fluorescent dye SYBR Green, which binds all double stranded DNA molecules. Since this process is sequence independent, the characteristic T_m of the target is determined by performing a melting curve analysis on the PCR products. To reduce the formation of primer-dimers and non-specific products, which compete with formation of specific PCR products, leading to reduced amplification efficiency, we include a touch-down step in the thermal cycling program. By using restricted annealing temperature, formation of unexpected double-stranded DNA products and primer-dimers were not observed (Fig. 1). In addition, both purity and concentration of primers are essential for optimal working conditions.

Detection range, sensitivity, specificity, reproducibility
To validate the real-time assay prior to application, we determined the detection range using tenfold serial dilutions of a patient's stool sample. The assay was able to detect NoV RNA within a range of up to 5 logs. The resulting curve with a slope of -3.251, and a coefficient of correlation r = -0.99 indicates a high efficiency of RT-PCR and a strong linear relationship (results not shown). T_m analysis of the PCR product revealed a distinct temperature at which the double stranded amplicon is denatured (mean T_m 78.9°C; range 78.2 to 81.2). No increase in fluorescence signal from primer-dimers or other non-specific amplification products (distinguished from the specific product through their lower T_m) was monitored (Fig. 1). To assess specificity, we tested the assay with RNA or DNA (high run controls as templates) from several viral pathogens usually associated with gastroenteritis diseases. No cross-amplification was observed when a panel of enteric viruses, including hepatitis A, hepatitis E, enterovirus, rotavirus and adenovirus, was tested as described above for NoV (results not shown). Reproducibility of the assay was evaluated by measuring the threshold cycle (C_t), i.e.

Melting curve analysis
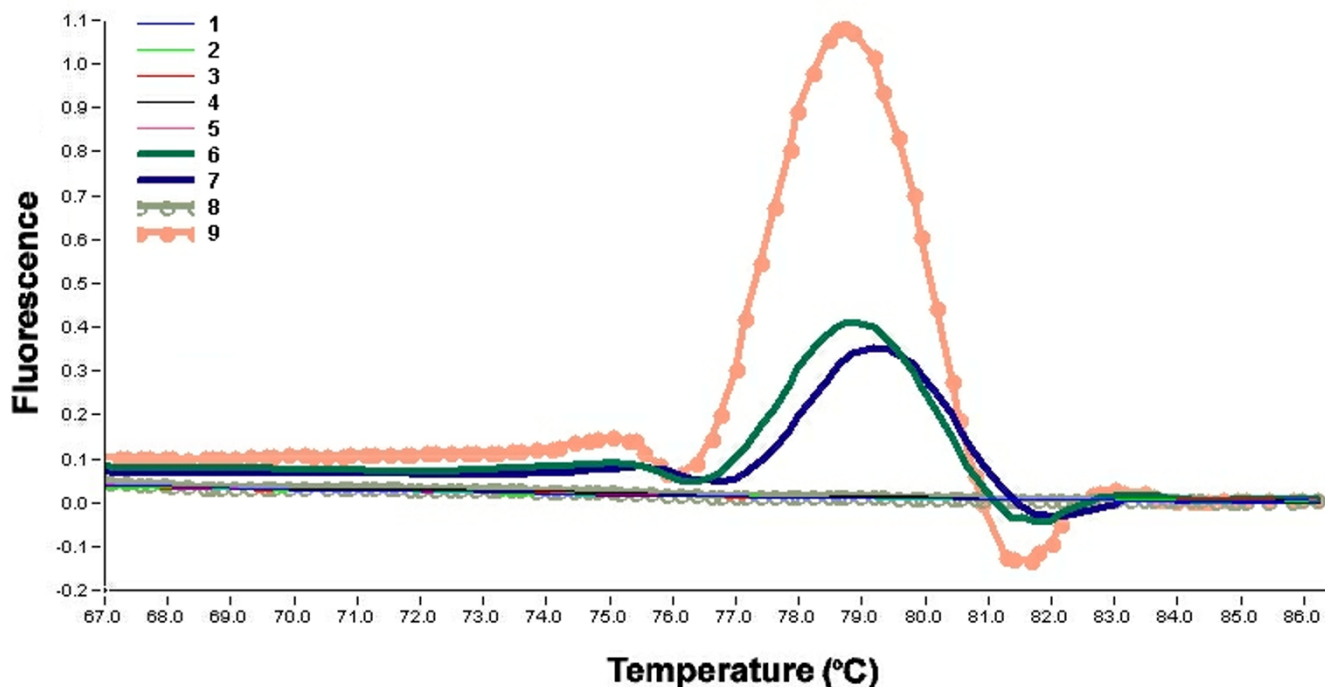
Figure 1
T_m analysis of real-time PCR with SYBR Green as fluorescent dye. T_m analysis allows the differentiation of specific PCR product (amplicon) from non-specific amplification products, such as primer-dimers. Samples: 1 to 7, patient samples (stool); 8, negative control (water); 9, positive control (NoV positive stool). Amplified products from NoV template RNA can be reliably distinguished from non-specific products by different melting points. The melting temperature for the positive samples was 79.0 +/- 0.5°C. Non-specific products melt at lower temperatures.

the cycle with the first detectable increase in fluorescence, of a low run control on three consecutive days six times. To determine the intra-assay precision, three stool specimen were simultaneously tested three times. The assay showed a between-run coefficient of variation (CV) of <3%, while the within-run coefficient was <2%, indicating that the assay was efficiently reproducible.

Comparison of results obtained by real-time PCR, antigen ELISA, and nPCR
Results of real-time PCR were compared with those of antigen ELISA and reference nPCR. We found a 100% correlation between the results of real-time PCR and those obtained by nPCR. By antigen ELISA 18 of 52 (34.6%) samples were positive for NoV, whereas both real-time PCR and nPCR detected 26 (50%), respectively. Of the antigen ELISA-positive samples, only 50% (9/18) were confirmed by the two PCR-based methods. In contrast, real-time PCR and nPCR detected 17 samples that were not identified by antigen ELISA. An identical result in all

three tests was obtained in 50% (26/52) of the samples. Seventeen specimens were negative by all tests. The mean C_t value for real-time PCR was 28.9 [95% confidence interval (CI), 27.3 to 30.5; range 18.5 to 34] and the mean T_m was 78.9°C (95% CI 78.7 to 79.1; range 78.2 to 81.2). All our negative controls showed no fluorescence signal. The mean optical density (OD) value for antigen ELISA was 1.0 (95% CI 0.5 to 1.5; range 0.2 to 3.6). Table 2 shows the combined results of antigen ELISA, real-time PCR, and nPCR for the total of 52 specimens.

Table 2: Pattern of NoV test results for 52 stool specimens

Elisa	Results of: Real-time PCR	nPCR	No. of specimens (N = 52)
+	+	+	9
-	+	+	17
+	-	-	9
-	-	-	17

Table 3: Comparison of real-time PCR and Elisa test performance to nPCR

Laboratory Test	No. of specimens with test result/no. specimens with result by nPCR as reference (%)	
	Sensitivity	Specificity
Elisa	9/26 (34.6)	17/26 (65.3)
Real-time PCR	26/26 (100)	26/26 (100)

Relative sensitivity

The choice of a reference test is crucial for evaluation of any new assay. In order to ensure specificity of the new real-time PCR assay, we tested all samples by an additional confirmatory assay (nPCR) in a blinded fashion. Comparison of real-time PCR and nPCR as a reference test in 52 clinical specimens resulted in an excellent agreement. Conversely, the results of reference nPCR and antigen ELISA disagreed. The clinical performance of antigen ELISA and real-time PCR after additional testing by nPCR are listed in Table 3.

Viral loads

To explain the substantial difference of the results by real-time PCR and antigen ELISA, we compared the viral load of PCR-positive ELISA-positive specimens with those of PCR-positive but ELISA-negative specimens. This comparison is based on the assumption that high C_t values are representative of low viral load, whereas low C_t values are representative for high viral load. The mean C_t values obtained by real-time PCR for ELISA-positive and ELISA-negative samples were 31.2 (range 26.7 to 34) and 27.7 (range 18.5 to 34), respectively. Statistical significance between the mean C_t values of ELISA-positive and ELISA-negative samples was tested by the Mann-Whitney U test ($p = 0.039$). The actually higher C_t values of ELISA-positive samples indicate that NoV RNA was even present at lower levels in these samples. On the basis of this finding, the failure to detect virus antigen in some real-time PCR positive samples cannot be explained by low virus titer in the samples.

Discussion

It is generally assumed that ELISA antigen techniques are less sensitive than PCR-based assays and therefore we choose the latter as the routine diagnostic assay for detection of NoV RNA in patient specimens. To validate the performance of our new real-time assay we collaborated with another diagnostic laboratory performing nPCR analysis for NoV detection. This approach ensures that primer annealing temperatures are stringent enough and that T_m analysis reliably distinguishes the specific PCR

products and possible PCR by-products. We consider nPCR as a reliable test that can be used as a reference method for the following reasons: (i) the efficiency of the nPCR has been previously shown to work well in outbreak investigations [22-24], and (ii) the nPCR primer set has been proven recently in an international collaborative study [25], adding further confidence to the specificity of the reference test used.

All real-time PCR-positive results were confirmed by nPCR. This congruence offers a firm background for the clinical utility and performance of the new real-time assay. Furthermore, compared to the nPCR protocol, the routine work flow reduced hands on-time and the turna-round time to the final result allowed same-day reporting to the clinician.

Consistent with previous studies [26,27], we found antigen ELISA to be significantly less sensitive than real-time PCR (34.6% vs. 100%; P < 0.001%). We regard the positive results by real-time PCR as reliable on the strength of following reasons. First, the combined use of a stringent hot start PCR and touch-down cycling conditions reduces the formation of non-specific amplification products and primer-dimers. Second, the characteristic T_m of the target was determined by melting curve analysis to prevent false-positive results. Third, and most important, all results were corroborated by the reference nPCR. By use of these compelling criteria, it can be considered that false-positive results are unlikely.

Different sample volume analyzed could account for the differing results. To proof this, we compared the viral load of PCR-positive ELISA-positive specimens with those of PCR-positive but ELISA-negative samples. The first cycle at which the fluorescence passes from background levels to clearly detectable is called the threshold cycle (Ct). This value serves as a tool for calculating semiquantitatively the amount of starting template in the clinical sample, i.e. the higher the C_t, the lower the amount of starting templates. Surprisingly, the mean C_t values were significantly higher in PCR-positive ELISA-positive samples, indicating the presence of even lower levels of viral RNA. In this regard, it is particularly relevant that in stool NoV antigens are present in higher concentrations than intact virus particles [28] suggesting that stool antigen concentrations could not have affected the performance of antigen ELISA. Although, the C_t values depend greatly on the homology of primer and target sequences, it is unlikely that the results are biased by a special NoV genotype, since the samples originate from different geographical regions in Germany. Sequence determination of these strains was not done. Alternatively, the failure to detect viral antigen in some of the real-time PCR positive samples could be due to the wide genetic diversity of NoV. Although anti-

gen ELISA are broadly reactive, the monoclonal antibodies used in the test may detect only antigenically similar or the most closely related NoV strains.

Only 9 of 18 ELISA-positive results could be confirmed by real-time PCR and nPCR, respectively, Factors that may adversely affect the sensitivity of the amplification assays include inefficient RNA extraction procedure, PCR inhibitors in faecal samples, and RNA degradation before testing. To rule out PCR inhibitors, we spiked all 9 ELISA-only positive samples with a NoV low-run control, but no PCR inhibitiors were detected.

Other reasons for the inability to detect NoV RNA could be low level of intact viral particles, or sequence variation (e.g., mismatches at critical positions in the primer binding site) in the viral RNA polymerase region. This is consistent with previous reports of genomic diversity of NoV strains even in the conserved region of open reading frame 1 (ORF1) coding for the viral RNA polymerase [29,30]. To rule out that we have missed these strains due to genetic variability, we have tested in addition all nine ELISA-only positive samples with a PCR-protocol using the primer pair JV12-JV13 targeting the RNA-dependent RNA polymerase gene [25]. All 9 ELISA-only positive samples were also negative by using this protocol. Thus, it is very unlikely that the negative result by real-time PCR was related to genetic variability.

On the other hand, false positive results of the antigen ELISA cannot be excluded. Two of the nine ELISA-only-positive samples had OD values that were in the equivocal range of the assay, i.e. marginally above the cut-off level of the test. These results were not resolved upon retesting. Notably, three of the samples with the high OD values of 2.5, 3.3, and 3.6 in the antigen ELISA were negative by real-time PCR.

Clearly the design of primer and probes capable for detecting a broad range of NoV strains is an ongoing challenge given the genetic diversity of the NoV genus. Recently, a real-time RT-PCR assay was established with sequence-specific probes for detection of NoV [31]. The use of SYBR Green offers the advantage of real-time detection even in the case of template sequence variation, which may abolish detection using hybridization probes.

There are two limitations to this study: (i) no internal control is added to the specimens prior to nucleic acid extraction as provided in part by commercially developed assays, and (ii) since our assay is based on SYBR Green, the assay cannot distinguish between NoV of GI and GII.

In conclusion, we describe a real-time PCR assay which detects the presence of NoV RNA in stool samples. The new assay provides accurate diagnostic results in less time than currently employed conventional PCR-assays. The automated sample preparation offers high throughput without cross-contamination of specimens during processing as well as standardization of operation steps. There is a keen interest in rapid and reliable assay in clinical situations to contain the spread of the virus in hospitals or other settings such as geriatric care facilities.

Competing interests
None declared.

Authors' contributions
MS conceived and designed the study, carried out the assay development, data analysis, and wrote first draft of manuscript. RO carried out nPCR analysis, participated in the design of the study and critical review of the manuscript. GS conceived and designed the study and participated in the writing of the manuscript. SB performed antigen ELISA testing and critically reviewed the manuscript. PK provided expert input for writing. GE provided expert input for writing and supervised the study. All authors have read and approved the final manuscript.

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