Validation of a norovirus multiplex real-time RT-PCR assay for the detection of norovirus GI and GII from faeces samples

S. JONES*, P. E. DOUARRE†, J. O'LEARY*, D. CORCORAN*, J. O'MAHONY† and B. LUCEY*†

*Department of Medical Microbiology, Cork University Hospital;
†Department of Biological Sciences, Cork Institute of Technology, Cork, Ireland

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ABSTRACT

Norovirus is a leading cause of infectious non-bacterial gastroenteritis. The virus is highly contagious and has multiple modes of transmission, presenting a growing challenge to hospital-based healthcare. In this study, a total of 120 stool samples are tested for the presence of norovirus GI and GII by the Roche two-step Lightcycler 2.0 assay incorporating primers and probes produced by TIB Molbiol, and the results are compared with results from the National Virus Reference Laboratory. The Roche/TIB Molbiol assay produced 51 positive results and 69 negative results. Discrepancy analysis was performed for six conflicting results using a second real-time polymerase chain reaction (PCR) assay (Roche/TIB Molbiol) and this confirmed that four of the five discrepant positive results were true positives. A single discrepant negative result generated by the Roche assay remained negative using the second assay. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated to be 98%, 98.6%, 98.0% and 98.6 %, respectively. Melting curve analysis was used to differentiate genogroups I and II and this showed that 92% of strains belonged to genogroup II.

KEY WORDS: Norovirus.
Polymerase chain reaction.
Reverse transcription.

Introduction

Norovirus has emerged as the most common cause of acute non-bacterial gastroenteritis, causing outbreaks and sporadic cases in children and adults worldwide.[1] Spread is predominantly by the faecal-oral route through ingestion of contaminated food or water. This single-stranded positive sense RNA virus is highly contagious, and as few as 10 virions can be sufficient to infect a healthy adult.

Outbreaks are common in semiclosed conditions such as hospitals, cruise ships, nursing homes, prisons and schools.[2,3] Norovirus epidemics place an enormous burden on the healthcare system. In the United Kingdom, outbreaks have been estimated to cost £1 million per 1000 hospital beds.[4] The prompt diagnosis of infection is vital in reducing both the cost and spread of an outbreak.

The norovirus genus consists of five genogroups (GI–GV). In general, GI, GII and, to a much lesser extent, GIV are associated with human gastroenteritis.[5] Genogroup II/genotype 4 (GII/4, Bristol/Lordsdale group) strains are the most prevalent aetiological agents in Ireland and around the world, accounting for approximately 85% of outbreaks.[6,7]

The aim of the present study is to evaluate the Roche LightCycler two-step real-time reverse transcription polymerase chain reaction (RT-PCR) assay (incorporating primers and probes from TIB Molbiol) for the rapid detection of norovirus GI and GII for use in a diagnostic laboratory, and to compare results with detection by an alternative RT-PCR assay provided by the National Virus Reference Laboratory (NVRL), Dublin, Ireland.

Materials and methods

Clinical samples

Between October 2008 and January 2009, a total of 120 anonymised stool specimens bearing a request for norovirus testing (but of unknown norovirus status) were obtained from the Department of Microbiology at Cork University Hospital (CUH) from patients who presented with acute diarrhoea. All samples were stored at –80°C prior to testing. A sample of each specimen was sent to NVRL for norovirus testing by real-time PCR using the ABI7500 TaqMan platform and an in-house assay based on the methods described by Kageyama *et al.*[8] and Ferns *et al.*,[9] respectively, for GI, GII and an internal control.

Controls

A GI and a GII positive control (TIB Molbiol, Berlin, Germany) and a negative control (cDNA substituted with 5 µL PCR-grade water) were included in each LightCycler run. An internal control (MS2 phage, TIB Molbiol) was incorporated with each test sample and control to be co-amplified with the target sequence.

RNA extraction

All samples were diluted in Stool Transport and Recovery (STAR) buffer (Roche Diagnostics, Mannheim, Germany), as

Correspondence to: Dr. Brigid Lucey
Department of Biological Sciences, Cork Institute of Technology, Cork, Ireland.
Email: brigid.lucey@cit.ie

recommended by Logan *et al.*,[10] to obtain a 20% (v/v) suspension, which was then vortex-mixed to homogenise the sample. The stool suspension was clarified, as recommended by Witlox *et al.*,[11] by centrifugation at 3500 x*g* for 15 min, and the resultant supernatant was centrifuged at 7000 x*g* for 30 min.

RNA was extracted from 200 µL of the aqueous phase from the pretreated stool using the High Pure Viral RNA kit (Roche Diagnostics), according to the manufacturer's instructions.

RNA quantification

The manually extracted nucleic acid was quantified using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific). Samples with an extracted total nucleic acid concentration <50 ng/µL were re-extracted prior to reverse transcription (RT).

Generation of complementary DNA

The RT reaction was performed on a 2720 Thermal Cycler (Applied Biosystems, Warrington, UK) using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics), according to the manufacturer's instructions. All reagents were maintained on ice prior to RT, and the resulting cDNA samples were stored at –20°C for single use.

Real-time PCR

Real-time PCR was performed on the LightCycler 2.0 using software version 4.05. Primers (specific for a 94 bp fragment of the junction between ORF1 and ORF2), probes and a 110 bp internal control from the LightMix Norovirus Kit (TIB Molbiol) were used in conjunction with the LightCycler FastStart DNA Master HybProbe Kit (Roche Diagnostics), according to the manufacturers' instructions. Primers and probe sequences were not obtained commercially. The thermal cycling conditions were as follows: denaturation for 10 min at 95°C followed by 45 cycles of 95°C for 10 sec, 56°C for 10 sec and 72°C for 5 sec. Amplification at 530 nm was indicative of a positive norovirus result; a signal at 610 nm was indicative of amplification of the internal control and a melting curve at 670 nm denoted norovirus GII. A second assay was provided by TIB Molbiol which incorporated primers with the sequences shown in Table 1, and this generated a 90 bp product specific for norovirus GII.

Interpretation of results

The following criteria were established to assign positive and negative results by retrospective analysis. Samples with a cycle threshold (Ct) value <40, which displayed a sigmoidal amplification curve, were considered to be positive for norovirus. A run was considered invalid if the negative control did not show an internal control signal at 610 nm. Negative results which did not display amplification of the internal control at 610 nm were considered invalid and were repeated.

The second derivative maximum method was used for crossing point calculation (as recommended by TIB Molbiol), rather than the fit points method (which is more error prone due to operator influence). The traffic light system, which assigns positive or negative results based on software calculations, was not employed as it was found to give a high level of false-positive results that were not consistent with observations.

Table 1. Norovirus GII-specific assay (TIB Molbiol) for discrepancy analysis of results generated by NVRL and Cork University Hospital.

Primer name	Sequence (5'–3')	Tm (°C)
NV107 GG2	AGAGCCAATGTTCAGATGGAT	54.1
NV119	TCGACGCCATCTTCATTCAC	56.8
NV-II-TM3	FAM-TGGGAGGGCGATCGCAATCTGGC-BHQ1	

Melting curve analysis

Homogeneity of the PCR product and hence the specificity of the amplification reactions was assessed by melting curve analysis. Following the 45 cycles, the product was heated to 95°C for 20 sec, cooled to 40°C for 20 sec and then heated to 85°C at a ramp rate of 0.2°C/sec. Sample fluorescence was monitored continually while the temperature increased, and this resulted in the production of melting curves. These were converted automatically into melting peaks by plotting the negative derivative of fluorescence versus temperature (–dF/dT versus T).

Agarose gel electrophoresis

To facilitate further analysis, the RT-PCR product (20 µL) from the LightCycler capillary tube was recovered by centrifugation of the inverted capillary in a 1.5 mL Eppendorf tube (Sarstedt, Ireland). A 10 µL volume of PCR product was applied to a 1.5% agarose gel (Sigma-Aldrich, USA) and visualised under ultraviolet (UV) light following ethidium bromide staining.

Results

Of the 120 samples tested, 51 were positive for norovirus (GI or GII) and 69 were negative at CUH (Table 2). In all cases, the negative control gave a negative result for that run, and positive controls showed Ct values of <27 cycles. A minority of positive norovirus test results (n=10) with Ct values of 19–26 cycles showed no internal control (IC) signal at 610 nm, suggesting that the IC was out-competed for reagents in these cases. Ct values were 26–32 cycles when the IC was not out-competed.

Figure 1 shows results at 530 nm and 670 nm for two positive tests and one negative test, along with the negative control and positive GI and GII controls. Sample 109, showing a positive result and a Ct value of 24.5, out-

Table 2. Comparison between NVRL results and real-time RT-PCR LightCycler assay results using primers (specific for a 94 bp fragment of the junction between ORF1 and ORF2), probes and an 110 bp internal control from the LightMix Norovirus Kit (TIB Molbiol) in combination with the LightCycler FastStart DNA Master HybProbe Kit (Roche Diagnostics).

	No. of samples tested by NVRL real-time PCR	
CUH result	Positive	Negative
Positive	46	5
Negative	1	68

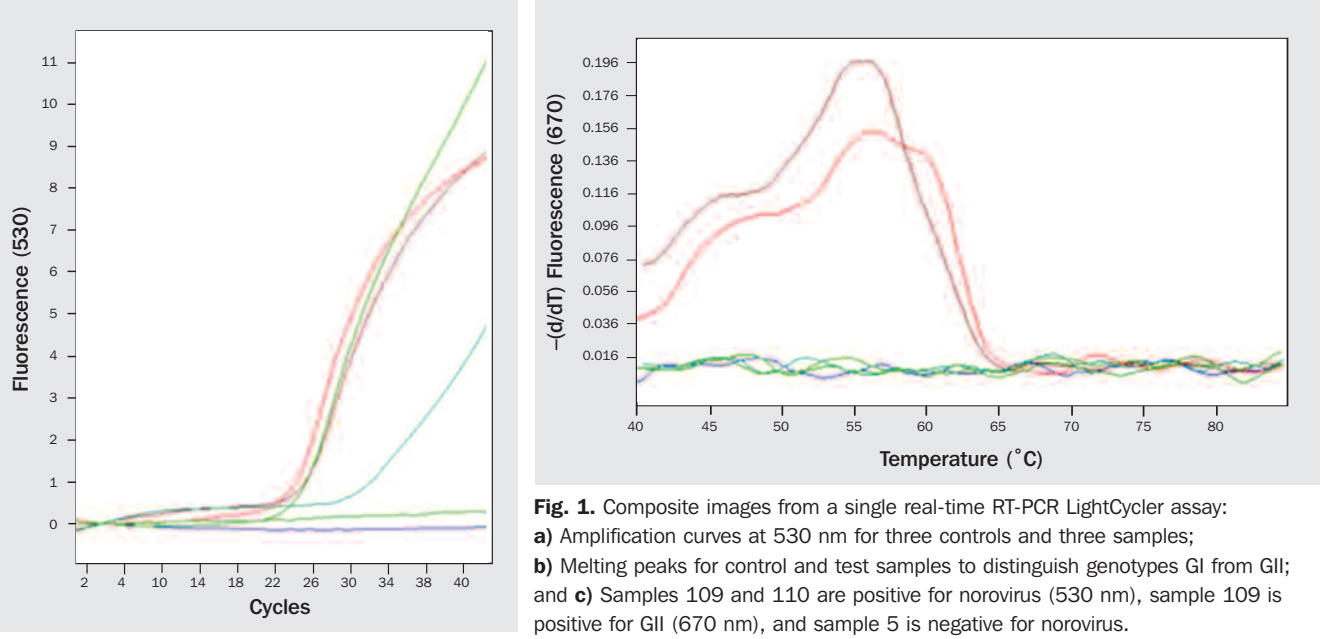

Fig. 1. Composite images from a single real-time RT-PCR LightCycler assay: **a)** Amplification curves at 530 nm for three controls and three samples; **b)** Melting peaks for control and test samples to distinguish genotypes GI from GII; and **c)** Samples 109 and 110 are positive for norovirus (530 nm), sample 109 is positive for GII (670 nm), and sample 5 is negative for norovirus.

competed the IC for reaction components at 610 nm (data not shown).

There were six consistent discrepant results (after repeat testing at CUH) between the NVRL results and the results of the norovirus real-time RT-PCR LightCycler assay (Table 2).

A sample reported as positive by NVRL was repeatedly negative at CUH. Re-extraction, reverse transcription and re-examination of the specimen by real-time PCR failed to produce an amplicon, and the internal control phage RNA was detected within defined limits. This was recorded as a false-negative result.

Five samples reported as negative by NVRL were consistently positive after repeat isolation of the viral RNA from the stool sample, reverse transcription and re-examination of the specimen by RT-PCR. Agarose gel electrophoresis showed two bands, one at approximately 110 bp (indicative of the IC) and one at approximately 100 bp (indicative of a norovirus product). The second confirmatory assay using the primers and probe shown in Table 1 generated amplicons of the expected size for four of these samples, and one false-positive result was therefore recorded.

Melting curve analysis

A total of 46 out of the 50 samples confirmed to be positive for norovirus by the LightCycler real-time RT-PCR assay displayed melting peaks between 50˚C and 60˚C at 670 nm (Fig. 1), categorising these samples as GII-positive results. GI-positive samples accounted for 8% (four samples) of all the positive results, showing an amplification curve at 530 nm only.

Assay performance

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated to be 98%, 98.6%, 98.0% and 98.6%, respectively, on the basis of an expanded gold standard where any positive result generated by this study that did not match the NVRL result was investigated further using the alternative norovirus real-time PCR assay.

c

Sample	530 nm	670 nm
Negative control	Negative	Negative
Positive control GI	Positive	Negative
Positive control GII	Positive	Positive
Sample 109	Positive	Positive
Sample 110	Positive	Negative
Sample 5	Negative	Negative

Discussion

Norovirus infection is recognised as a primary cause of viral gastroenteritis worldwide. It is highly contagious and causes epidemics that place an enormous burden on the healthcare system.[12] Studies of hospital outbreaks have shown that prompt implementation of strict infection control procedures can prevent further intrahospital spread.[13] Therefore, a rapid, sensitive and specific assay is required for norovirus detection in stool samples, with workload varying from single cases of presumptive viral gastroenteritis to the large numbers generated by mass outbreaks that occur mainly in winter.

The purpose of the present study was to evaluate a method for use in the microbiology department at CUH that would allow testing to be conducted on site, rather than by referral to NVRL. The cost per test of the method under evaluation was approximately €15, compared to approximately €45 per test when outsourced. On-site testing produced results within 24 hours, which facilitated outbreak management by the hospital's infection control team.

Of the 120 samples tested in this study, only one false-positive result was recorded, where the CUH assay was not matched either by the confirmatory assay or NVRL testing. The single positive result from NVRL that was unmatched by either assay undertaken at CUH was recorded as a false negative. It is possible that the samples used for these assays at CUH did not contain the target sequence and that

increasing the number of replications of this assay might have generated a positive result. Alternatively, however, unidentified sequence variations within the conserved ORF1/ORF2 junction region of the norovirus genome targeted by this assay may have led to this negative result, which could only have been confirmed by sequencing the amplicon for this sample generated by the NVRL assay.

Melting curve analysis at 670 nm produced melting peaks for 46 of the 50 true positive results for norovirus by the LightCycler real-time PCR assay, categorising these samples as GII strains. Four samples (8%) which tested positive for norovirus by the LightCycler real-time PCR assay did not produce melting peaks, categorising these samples as GI strains. A molecular epidemiological study of norovirus strains circulating in Ireland from 2003 to 2004 by Waters *et al*.[14] found GI to be responsible for 5.2% of cases while GII was responsible for 94.8% of cases.

A common problem with real-time PCR is the presence of PCR inhibitors that may cause false-negative results.[15] The LightCycler norovirus assay addresses this problem in two ways. First, STAR buffer (Roche Diagnostics), which was chosen at the beginning of this study, binds directly to inhibitory substances, enhancing their removal during RNA extraction. Second, the inclusion of an MS2 phage internal control in each sample capillary, including in negative and positive controls, permits effective identification of PCR inhibition in samples (4.2% of samples tested in this investigation) which could be responsible for false-negative results.

It is important that the sensitivity of this assay remains stable over time. Therefore, tests that target the most conserved area of the genome are preferred. The junction sequence between ORF1 and ORF2 used in this study is currently regarded as the most conserved region of the norovirus genome; however, awareness of circulating strains within the population at any given time is vital in ensuring the sensitivity of the detection method. The literature should be monitored regularly to maintain the high standard of this assay.

In conclusion, the LightCycler norovirus real-time RT-PCR assay described in this study is an accurate, sensitive, specific and rapid method for the detection of norovirus genogroups I and II, and is at least as good as the reference method. □

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