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Evaluation of a new real-time reverse transcription polymerase chain reaction assay for detection of norovirus in fecal specimens

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ABSTRACT

A new real-time reverse transcription polymerase chain reaction (RT-PCR) assay, the AccuPower Norovirus Real-time RT-PCR Kit, was evaluated in detection of human norovirus in stool specimens. Studies for detection limit, dynamic range, reproducibility, and cross-reactivity were performed. A total of 281 fecal specimens were tested using the AccuPower Norovirus Real-time RT-PCR Kit, and the results were compared with those obtained using another real-time RT-PCR system. Norovirus positivity and genotype were confirmed by direct sequencing. The lowest mean numbers of genome copies of GI and GII that could be detected by the assay were 12.3 and 5.6 RNA copies/reaction, respectively. The positive, negative, and overall percent agreements between the 2 real-time PCR assays were 99.0% (96/97), 95.1% (175/184), and 96.4% (271/281), respectively. The AccuPower Norovirus Real-time RT-PCR system showed good analytical and clinical performance and may be a useful diagnostic tool for norovirus infection.

1. Introduction

Noroviruses are one of the most frequent causes of acute nonbacterial gastroenteritis in all age groups worldwide (Glass et al., 2009; Patel et al., 2009). Previous studies reported that noroviruses were responsible for 93% of the 233 gastroenteritis outbreaks between 1997 and 2000 in the United States and 85% of the 3714 outbreaks between 1995 and 2000 in Europe (Dolin, 2007). Moreover, they are responsible for an estimated 218,000 deaths each year among children younger than 5 years in developing countries and 1.1 million hospitalizations worldwide (Patel et al., 2008).

Noroviruses are a genetically diverse group of single-stranded RNA, non-enveloped viruses in the *Caliciviridae* family (King et al., 2011). Noroviruses are classified into 5 genogroups by phylogenetic analysis of the capsid protein, of which genogroups II (GII), I (GI), and IV (GIV) (rarely), in order of greatest to lowest numbers, are responsible for human outbreak (Zheng et al., 2006).

Various diagnostic methods have been developed, including electron microscopic examination of fecal specimens, reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, enzyme immunoassays, and immunochromatographic assays (Atmar and Estes, 2001). Among these assays, RT-PCR or real-time RT-PCR is currently considered the standard method of detection of noroviruses in clinical, food, and environmental samples (Butot et al., 2010).

This study aimed to evaluate the analytical and clinical performance of a newly developed real-time RT-PCR system, the AccuPower Norovirus Real-time RT-PCR Kit (Bioneer Co., Daejeon, South Korea), for detecting human norovirus GI and GII qualitatively in fecal specimen.

2. Materials and methods

2.1. Clinical samples for comparison study

A total of 281 fecal suspensions (10–20% fecal specimen diluted with saline) collected and stored at −70 °C between August 2010 and April 2011 were used in this study. These comprised 109 norovirus-positive and 172 norovirus-negative stool samples, as determined using a norovirus enzyme-linked immunosorbent assay (ELISA; RIDASCREEN Norovirus, R-Biopharm, Darmstadt, Germany). Of the samples, 166 (59.1%) were collected from male patients, with ages ranging from 3 days to 84 years (median, 1.42 years). The presence of norovirus GI and GII was tested using the AccuPower Norovirus Real-time RT-PCR Kit, and the results were compared with those obtained by another real-time RT-PCR system, the RIDAGENE Norovirus V (R-Biopharm). Norovirus positivity was confirmed by direct sequencing.

2.2. AccuPower Norovirus Real-time RT-PCR assay

RNA extracts from fecal suspensions were prepared using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and QIAcube platform (Qiagen). The viral RNA was extracted from 140 μL of stool suspension and finally eluted in 50 μL of elution buffer. For the AccuPower Norovirus Real-time RT-PCR assay (Bioneer Co.), 5 μL of

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RNA extract was mixed with 45 μL of master mix, and real-time RT-PCR was performed using a PCR thermocycler (Exicycler 96 Real-Time Quantitative Thermal Block; Bioneer Co.), under the following conditions: reverse transcription at 45 °C for 15 min, predenaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 55 °C for 5 s. An internal positive control consisting of RNA sequences unrelated to the norovirus target sequence was co-amplified in each reaction to determine whether PCR was inhibited by the sample. All the procedures were performed according to the manufacturer's instructions.

2.3. RIDA GENE Norovirus Real-Time RT-PCR assay for a comparison study

For the RIDAGENE Norovirus V assay (R-Biopharm), 5 μL of RNA extract was mixed with 20 μL of master mix, and real-time RT-PCR was performed using a PCR thermocycler (Rotor-Gene Q; Qiagen), under the following conditions: reverse transcription at 50 °C for 15 min, 45 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 55 °C for 30 s. In each PCR reaction, the internal positive control is co-amplified and detected to determine possible RT-PCR inhibitions. All the procedures were performed according to the manufacturer's instructions.

2.4. Norovirus genotyping

Norovirus genotyping was performed as described previously (Kim et al., 2008). For 1-step RT-PCR, the specific primers GI-F1M (nt 5342)/GI-R1M (nt 5671) and GII-F1M (nt 5058)/GII-R1M (nt 5401) targeting open reading frame 2–encoding capsid protein (VP1) were used. Nested PCR was also performed using the primers GI-F2 (nt 5357)/GI-R1M (nt 5671) and GII-F3 (nt 5088)/GII-R1M (nt 5401).

The products from the nested PCR were purified using a MEGA quick-spin PCR kit (INTRON Biotechnology, Seongnam, South Korea) and then cloned into the pGEM T-easy vector and analyzed by DNA sequencing. The nucleotide sequences were analyzed using ABI Prism BigDye Terminator version 3.1 (Applied Cosmo Genetech, Seoul, South Korea), and an automated norovirus genotype tool was used to identify norovirus genotypes (available at http://www.rivm.nl/mpf/norovirus/typingtool) (Kroneman et al., 2001).

2.5. Lower limit of detection (LLOD) and dynamic range (linearity)

To quantify the copy number of the norovirus RNA, in vitro transcribed RNA calibrators were prepared as follows: The amplicon of the target region in the norovirus genome was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), and HIT-DH5α competent cells (RBC Bioscience, New Taipei City, Taiwan) were then used for transformation. The cloned plasmid was purified using the AccuPower plasmid mini kit (Bioneer Co.), and in vitro transcription of the target region was performed using the MAXIscript SP6 Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The mass of the in vitro transcribed RNA was determined using the NanoDrop 2000 system (Thermo Fisher Scientific, Wilmington, DE, USA) and converted to copy number. The RNA copy number in the samples was calculated based on the threshold cycle (Ct) value (the number of cycles at which the fluorescence exceeds the threshold), with the corresponding RNA copy number determined from calibration curves prepared using RNA calibrators (Yang et al., 2002).

To evaluate the LLOD, 3-fold serial dilutions from 1000 copies/reaction of GI- and GII-positive controls (cloned plasmids) prepared in distilled water were used. Each diluted sample was tested 20 times. The norovirus concentration in each sample was measured using the AccuPower Norovirus Real-time RT-PCR assay, and the number of norovirus RNA copies per milliliter in each sample was recorded. The LLOD was determined as 95% probability of obtaining a norovirus-positive result using probit regression.

To evaluate linearity, 10-fold serial dilutions from 10^1 to 10^{10} copies/reaction of the GI- and GII-positive controls (cloned plasmids) prepared in distilled water were used. Each diluted sample was tested in 6 replicates in a single run, and linearity analysis was performed using the Analyse-it software version 2.22 (Analyse-it Software Ltd., Leeds, United Kingdom).

2.6. Repeatability/reproducibility

Three norovirus positive controls (cloned plasmids) with different viral loads (10^3, 10^5, 10^7 copies/reaction) for GI and GII, and 1 norovirus-negative control were tested in triplicate at 1 run per day over a period of 20 days. The within-run variation, between-day variation, and total variation (coefficient of variation [CV]) were calculated.

2.7. Cross-reactivity with common enteric viruses and bacteria

Cross-reactivity with the following viruses and bacteria was examined using RNA/DNA extracted from viruses and bacteria. For the viruses, virus culture supernatants or virus-positive fecal samples were used.

Viruses: rotavirus (ATCC Number VR-2018 from American Type Culture Collection; ATCC; http://www.atcc.org), adenovirus type 40 (ATCC VR-931), type 41 (ATCC VR-930), type 31 (ATCC VR-1109), enterovirus type 71 (ATCC VR-784), astrovirus (fecal sample from patient).

Bacteria: *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Salmonella* group B (clinical isolate from patient), *Salmonella* group C (clinical isolate from patient), *Salmonella* group D (clinical isolate from patient), *Salmonella* group E (clinical isolate from patient), *Shigella* group D (clinical isolate from patient), *Yersinia enterocolitica* (clinical isolate from patient), *Campylobacter jejuni* (clinical isolate from patient), *Salmonella typhi* (clinical isolate from patient), *Clostridium difficile* (clinical isolate from patient).

3. Results

3.1. Comparison of results among ELISA and 2 real-time PCR assays

Of the 109 norovirus-positive samples by ELISA, 91 (83.5%) were positive by both PCR kits, and 2 (1.8%) were positive by only the AccuPower kit, whereas 16 cases (14.7%) were negative by both PCR kits. Of the 172 norovirus-negative samples by ELISA, 159 (92.4%) were negative, and 5 (2.9%) were positive by both PCR kits, 1 (0.6%) was positive by the RIDAGENE kit, and 7 (4.1%) were positive by the AccuPower kit (Table 1). Of the 281 samples, 250 (89.0%) showed the same positive or negative results among the 3 methods, whereas 31 (11.0%) showed discrepant results. The total agreement rate was higher between the 2 real-time PCR assays (271/281, 96.4%) than between the ELISA and RIDAGENE real-time PCR assay (257/281, 91.5%) or between the ELISA and AccuPower Real-Time PCR assay (253/281, 90.0%). The percent positive and percent negative agreements between the AccuPower and RIDAGENE PCR kits were 99.0% (96/97) and 95.1% (175/184), respectively.

Ten specimens (3.6%) with discordant results between the 2 real-time PCR are described in Table 2. Nine samples that tested positive by the AccuPower kit but negative by the RIDAGENE kit had genotypes of the norovirus GII, of which 7 were confirmed by direct sequencing. One sample tested negative by the AccuPower kit but positive by the RIDAGENE kit; however, these results could not be confirmed by direct sequencing because of an inadequate amount of the sample.

Detectability of the norovirus genotypes by the AccuPower Real-Time PCR Kit is presented in Table 3. The AccuPower kit detected all of the following genotypes: GI.1 (1/1), GI.3 (1/1), GII.3 (14/14), GII.4 (72/72), GII.6 (1/1), and GII.12 (3/3). Thirteen samples were GII-