Simultaneous Detection of Five Enteric Viruses Associated with Gastroenteritis by Use of a PCR Assay: a Single Real-Time Multiplex Reaction and Its Clinical Application

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We developed a highly sensitive reverse transcription and multiplex real-time PCR (rtPCR) assay that can identify five viruses, including six genogroups, in a single reaction: norovirus genogroups I and II; sapovirus genogroups I, II, IV, and V; human rotavirus A; adenovirus serotypes 40 and 41; and human astrovirus. In comparison to monoplex rtPCR assays, the sensitivities and specificities of the multiplex rtPCR ranged from 75% to 100% and from 99% to 100%, respectively, evaluated on 812 clinical stool specimens.

Norovirus genogroup I (NV GI) and NV GII (1, 2), sapovirus (SaV) genogroups I, II, IV, and V (3), human rotavirus A (HRV) (4), adenovirus serotypes 40 and 41 (AdV) (5), and human astrovirus (AstV) (6) are the most common causes of viral gastroenteritis and result in large outbreaks of viral diarrhea (7–11). Rapid and accurate detection methods are essential for response to viral diarrhea outbreaks and management of the patients (12, 13). Current detection methods include electron microscopy (14), enzyme immunoassay (EIA) (15), conventional or real-time PCR (rtPCR) (16–19), and Luminex xMAP technology (12, 13). These methods are laborious (14) or need post-gel electrophoresis (18) or allow simultaneous detection of three or four viral pathogens in one reaction (16, 17, 19) or need opening of reaction tubes for hybridization (12, 13). Here, based on the principle of multicolor combinatorial probe coding (MCPC) (20, 21), we developed a more effective multiplex rtPCR assay for the simultaneous detection of five major enteric viruses in a single reaction.

All gene sequences retrieved from GenBank were aligned using Clustal W software. The primers and modified molecular beacon probes based on the conserved regions were designed using Primer Premier (version 5.0), Oligo (version 6.31), and mfold/nucleic acid folding software (http://mfold.rna.albany.edu/?q=mfold/dna-folding-form). The sequences of the primers for NV and HRV were adapted from the literature (19, 22). The primers had a common tag sequence at the 5’ ends to keep the formation of primer dimers to a minimum. Equine arteritis virus (EAV) was employed as an internal control to monitor the inhibition of amplification (23).

All primers and probes were commercially synthesized, labeled, and purified by the company Sangon Biotech (Shanghai, China) (see Table S1 in the supplemental material).

Ten positive samples in which SaV, NV GI, NV GII, HRV, AdV, and AstV were identified by PCR with sequencing were used for preparation of the plasmid DNA standards and initial multiplex rtPCR assay development. Viral DNA and RNA of clinical stool samples were extracted using a Roche High Pure Viral Nucleic Acid kit (Roche Diagnostics, Penzberg, Germany). For the reverse transcription, 5 μl of extracted RNA was transcribed into cDNA using a Primer Script 1st strand cDNA synthesis kit (TaKaRa Biotechnology Company, Dalian, China).

Multiplex rtPCR was conducted using a Bio-Rad CFX96 real-time PCR system. The final volume was 25 μl, which included 1X PCR buffer, 3 mM MgCl₂, 2 μl of deoxynucleoside triphosphate (2.5 mM), 1 U of Taq polymerase, 0.1 μM to 0.9 μM concentrations of specific primers and probes (see Table S1 in the supplemental material), and 5 μl of cDNA or DNA template. Optimal cycling conditions included a first stage initiated by heating at 95°C for 3 min followed by a second stage of five cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 15 s and finally by a third stage of 40 cycles of 95°C for 10 s, 55°C for 32 s, and 72°C for 15 s. FAM (6-carboxyfluorescein), HEX (hexachloro-6-carboxyfluorescein), 6-carboxy-X-rhodamine (ROX), Cy5, and Quasar 705 fluorescence was detected and recorded at each step of primer annealing during the third stage.

To confirm whether the target genes selected were specific for each virus, nucleic acids of Giardia lamblia, Entamoeba histolytica, Cryptosporidium parvum, Staphylococcus aureus, Shigella sp., Yersinia enterocolitica, Campylobacter jejuni, and human enterovirus 71 and 10 enteric virus-positive samples were tested by multiplex rtPCR. No cross-reaction was observed, and all five of the targeted genes were identified correctly by the multiplex rtPCR (see Fig. S1 in the supplemental material), indicating that the developed assay had good specificity. The detection limits of the multiplex rtPCR...
The assay were assessed twice using seven serial 10-fold dilutions (10⁷ to 10¹ copies/μl) of the plasmid DNA standards, and four serial 10-fold dilutions (10⁶ to 10³ copies/μl) of the plasmid DNA standards were prepared for the analysis of interreproducibility in triplicate experiments. The detection limit for each of the five viruses was 10 copies/μl. Amplification efficiency ranged from 87.6% to 99.2% with strong correlation coefficients (R² ≥ 0.99) (see Table S2 and Fig. S2 in the supplemental material). The difference in the threshold cycle (C_T) values among the triplicate experiments was less than one cycle, and the coefficient of variation (CV) for each target virus ranged from 0.06% to 2.3%, which indicated that the assay was reproducible (see Table S3 in the supplemental material).

Due to the clinical samples often containing mixed infections (24–27), HRV and AdV were used in conjunction as representatives to simulate coinfection. The 2.5-μl plasmid DNA standards were combined in equal amounts ranging from 10² to 10⁶ copies/μl for multiplex rtPCR. HRV and AdV could be simultaneously detected apart from when the number of HRV copies was 10,000 times higher than the number of AdV copies, which prevented the AdV from being detected (Fig. 1).

To further validate the multiplex rtPCR assay’s specificity and sensitivity, 812 clinical stool samples were collected from 10 hospitals in four cities in Guangdong province and were detected using the newly developed assay with direct comparison with commercial monoplex rtPCR kits (ZJ Bio-Tech, Shanghai, China). All monoplex rtPCR-positive products of clinical samples were sequenced for the further confirmation. Among the 812 stool samples, 372 were virus positive. Of those, 29 stool specimens were found to be coinfected with two viruses, including 11 coinfected with NV GII and AdV, 10 coinfected with HRV and AdV, and 5 coinfected with NV GII and HRV, as well as 3 coinfected with NV GI and NV GII (Table 1).

Compared with monoplex rtPCR, the sensitivity of the multiplex rtPCR assay ranged from 75% to 100% and the specificity ranged from 99% to 100%. The agreement ranged from 97.8% to 100%, and the kappa correlation ranged from 0.85 to 1.0 (Table 1).

This study had a limitation that requires further improvement. Two NV GI samples, 10 HRV samples, 1 AdV sample, and 12 NV GII samples were positive by monoplex rtPCR but negative by multiplex rtPCR, which was due to nucleic acid degradation. More clinical samples will be collected for further validation.

![FIG 1 Detection results of simulation for mixed infection according to C_T value. Panels A and B indicate the mixed infections between rotavirus group A (HRV) and adenovirus (AdV). Different copy numbers (0, 10², 10⁴, and 10⁶ copies) of HRV were combined with 10² (columns with small squares), 10⁴ (columns with large squares), and 10⁶ (columns with horizontal lines) copies of AdV. All C_T values are means of the results of duplicate experiments.](http://jcm.asm.org/)

| TABLE 1 Performance of multiplex real-time PCR assay compared to monoplex real-time PCR |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Species and multiplex rtPCR result | No. of samples with monoplex rtPCR result | Sensitivity (%) | Specificity (%) | Agreement (%) | Kappa value |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Sapovirus Positive | 4 | 0 | 100.0 | 100.0 | 100.0 | 1.00 |
| Negative | 0 | 808 | | | | |
| Norovirus GI Positive | 6 | 0 | 75.0 | 100.0 | 99.7 | 0.85 |
| Negative | 2 | 804 | | | | |
| Rotavirus group A Positive | 116 | 5 | 92.0 | 99.2 | 98.2 | 0.92 |
| Negative | 10 | 681 | | | | |
| Adenovirus Positive | 22 | 1 | 95.7 | 99.9 | 99.8 | 0.98 |
| Negative | 1 | 788 | | | | |
| Norovirus GII Positive | 190 | 6 | 94.0 | 99.0 | 97.8 | 0.94 |
| Negative | 12 | 604 | | | | |
| Astrovirus Positive | 8 | 1 | 88.9 | 99.9 | 99.8 | 0.88 |
| Negative | 1 | 802 | | | | |
ease outbreaks and provide the laboratory data for epidemiological research as well as clinical care of patients and then strengthen general public health.

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