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Simultaneous Detection of Waterborne Viruses by Multiplex Real-Time PCR

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Norovirus, Rotavirus group A, the Hepatitis A virus, and Coxsackievirus are all common causes of gastroenteritis. Conventional diagnoses of these causative agents are based on antigen detection and electron microscopy. To improve the diagnostic potential for viral gastroenteritis, internally controlled multiplex real-time polymerase chain reaction (PCR) methods have been recently developed. In this study, individual real-time PCRs were developed and optimized for specific detections of Norovirus genogroup I, Norovirus genogroup II, Rotavirus group A, the Hepatitis A virus, and Coxsackievirus group B1. Subsequently, individual PCRs were combined with multiplex PCR reactions. In general, multiplex real-time PCR assays showed comparable sensitivities and specificities with individual assays. A retrospective clinical evaluation showed increased pathogen detection in 29% of samples using conventional PCR methods. Prospective clinical evaluations were detected in 123 of the 227 (54%) total samples used in the multiplex real-time PCR analysis. The Norovirus genogroup II was found most frequently (23%), followed by Rotavirus (20%), the Hepatitis A virus (4.5%), Coxsackievirus (3.5%), and Norovirus genogroup I (2.6%). Internally controlled multiplex real-time PCR assays for the simultaneous detection of Rotavirus, Coxsackievirus group B, the Hepatitis A virus, and Norovirus genogroups I and II showed significant improvement in the diagnosis of viral gastroenteritis.

Keywords: viral gastroenteritis, multiplex, Real-time PCR, RT-PCR, PCR

Introduction

Viral gastroenteritis is a major contributor to health problems in developing countries. Although mortality due to viral gastroenteritis is low in developed countries, its related morbidity and economic consequences are nonetheless significant (Maarseveen *et al*., 2010). A number of viruses, including Noroviruses (NV), Rotaviruses (HRV), the Hepatitis A virus (HAV), and the Coxsackie virus B1 (CVB1), can result in human viral gastroenteritis. NV is members of the family Caliciviridae (Maarseveen *et al*., 2010). NV has significant global public health impacts, being the most common etiological agent for gastroenteritis outbreaks and a common cause of acute gastroenteritis in children (Pang *et al*., 1999; Fankhauser *et al*., 2002). These genetically diverse RNA viruses are divided into 5 genogroups (GI–GV), of which genogroups GI, GII, and occasionally GIV, have been associated with infections in humans (Maarseveen *et al*., 2010). Another frequent cause of viral gastroenteritis in young children is HRV. HRV belong to the family Reoviridae, and are categorized into 7 different serogroups (A–G) based on the antigenic specificity of the middle-layer protein of the virus and the electrophoretic mobility pattern of the 11 segments of the viral genome double-stranded RNA (Estes, 2001). Of these 7 groups, groups A–C are known to infect humans, and group A HRVs have been most commonly associated with severe and life-threatening diseases in children worldwide (Estes, 2001).

Genotyping of HRV can be accomplished by targeting the genes encoding the 2 outer capsid proteins, VP7 andVP4. The G and P serotypes are determined by antigenic specificities of the VP7 and VP4 proteins, respectively. Group A HRVs have been classified into 14 G serotypes, with G1–G4 and G9 considered to be the most globally common types with clinical importance (Hoshino *et al*., 2004). The HAV is an enterically transmitted pathogen that is a global public health concern, causing substantial morbidity, primarily in developing countries (Tanaka, 2000). The HAV genome is a positive-strand RNA of approximately 7.5 kb with a single open reading frame (ORF) encoding a large polyprotein containing approximately 2230 amino acids (Koff, 1998). CVBs are small, icosahedral, non-enveloped viruses containing a single-stranded, positive-sense RNA molecule that is approximately 7500 nucleotides long. The CVB genome, which consists of a long uncapped 5′ nontranslated region (NTR), a single ORF, and a polyadenylated 3′ NTR, concomitantly serves as a messenger RNA for the synthesis of viral proteins and as a template for virus replication (Selinka *et al*., 1998).

Recently, real-time polymerase chain reaction (PCR) assays have demonstrated greater sensitivity and specificity for the detection of viruses associated with gastroenteritis compared to antigen-detection assays (Maarseveen *et al*., 2010). In addition, diagnostic use of conventional multiplex PCR assays

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Table 1. Primer and probe sequences of the multiplex assays

Virus	Primer	Sequence (5′→3′)	Region	Reference
NV	NV F	CAA GAG TCA ATG TTT AGG TGG ATG AG	ORF2	This study
	NV R	TCG ACG CCA TCT TCA TTC ACA		
	NV P	FAM-TGG GAG GGC GAT CGC AAT CT-BHQ1		
HRV	HRV F	ACA GGT TGG TGG CTC AGA TGT ACT	NSP3	This study
	HRV R	GCC ACC ATT TCT TCC AAT TCA CTC GC		
	HRV P	ROX-ACA GCT GAT CCA ACG ACA ATG CCA CA-BHQ2		
HAV	HAV F	TCTTGCCGTTGATACTCCTTGGGT	VP1/2A	This study
	HAV R	ATCCAGTGCTCCAGACACAGCATA		
	HAV P	TYE705-GCT CTT GGA ACT GTC AGA TTT AAC ACA AGG-IABkFQ		
CVB	CVB F	AAACCCAAACATGTGAAGGCGTGG	5' end	This study
	CVB R	TGGTAATGTTTGAGCGCGTTGTGG		
	CVB P	HEX-ACC GCC GAG GCT ATG TCA ATA TGA GA-BHQ1		

for the detection of various agents of viral gastroenteritis has been described (Nguyen *et al*., 2007; Li *et al*., 2009).

In the present study, we aimed to improve the diagnostic potential of viral gastroenteritis by molecular methods. Internally controlled multiplex real-time PCR assays were developed for the simultaneous detection of HAV, HRV, NV, and CVB in samples from patients with gastroenteritis. Thereafter, patient outcomes were determined upon implementation in a diagnostic laboratory.

Materials and Methods

Clinical samples

One hundred eighteen fecal samples from 118 patients obtained between November 2006 and May 2012 were received at the Gyeonggi-do Institute of Health & Environment. In addition, clinical stool samples were isolated from 109 one-year-old patients with acute gastroenteritis in Seoul, South Korea in February 2006. Samples were obtained from the Waterborne Virus Bank (Seoul, Korea). The stool samples were stored at -70°C until use.

Viral RNA isolation and cDNA synthesis

Viral genomic RNA was extracted from 140 ml of a 10% fecal suspension with the QIAamp Viral RNA Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. cDNA synthesis was performed using 10 μl of extracted RNA with the iScript^{TM} cDNA Synthesis Kit (Bio-Rad, USA).

Primers and probes

Primers and probes used in this study (Table 1) were designed from conserved regions of viral genomes using Beacon Designer software version 5.1 (Premier Biosoft International, USA) and the PrimerQuest website (Integrated DNA Technologies, USA). BLAST analyses using the Nucleotide collection database (National Center for Biotechnology Information, USA), were performed to ensure specificity of the primer and probe sequences.

Optimization of PCR conditions

Multiplex real-time PCR assays were developed for the simultaneous detection of NV GI, NV GII, HRV, HAV, and CVB. The melting temperature (Tm) value was calculated using modified DNA sequences of the primers and probes that were designed for multiplex real-time PCR. Probes for NV, HRV, HAV, and CVB were regulated with FAM, ROX, TYE705, and HEX dyes, respectively, to achieve simultaneous detection of all viruses. Next, forward and reverse primers at various concentrations (50, 500, and 750 nM) were cross-reacted based on each primer and probe set. The probes were cross-reacted using the same procedure, but with concentrations of 50, 100, 150, 200, and 250 nM. Because the detection efficiencies of forward and reverse primers were both excellent at a concentration of 750 nM, this was the final concentration of primers used and the multiplex real-time PCR was optimized at 750 nM. With respect to probes, the ideal concentration of each virus was chosen to be greater than 100 nM, as the detection efficiency was best in this range.

Table 2. Primer sequences for conventional RT-PCR analyses

Virus	Primer	Sequence (5′→3′)	Location	Region
NoV GI	GI-FIM	CTGCCCGAATTYGTAAATGATGAT	5342-5365[a]	Capsid
	GI-RIM	CCAACCCARCCATTRTACATYTG	5649-5671[a]	
NoV GII	GII-FIM	GGGAGGGCGATCGCAATCT	5049-5067[b]	
	GII-RIM	CCRCCIGCATRICCRTTRTACAT	5367-5389[b]	
HRV	ddrv-1	GGCGCCGCTCYTTTTRATGTATGGTATTGAATTACCAC	6-38[c]	VP7
	ddrv-2	GGCGCCCTTTAAAATANAYDGADCCWRTYGGCCA	346-373[c]	

[a] GenBank accession no. M87661
[b] GenBank accession no. X86557
[c] GenBank accession no. HQ392461

Table 3. Comparison of the sensitivities of the monoplex and multiplex real-time PCR assays using serial dilutions of cDNA

	Mean C_T value monoplex[a] (SD)	Mean C_T value multiplex[b] (SD)
Norovirus		
10^{-1} dilution	5.51(0.78)	6.06(0.49)
10^{-2} dilution	6.48(0.32)	7.86(0.11)
10^{-3} dilution	11.67(0.40)	12.48(0.41)
10^{-4} dilution	15.30(0.11)	16.40(0.23)
Rotavirus		
10^{-1} dilution	6.11 (0.50)	7.78(0.25)
10^{-2} dilution	10.70(0.10)	12.27 (0.38)
10^{-3} dilution	14.50(0.18)	15.77(0.47)
10^{-4} dilution	18.26(0.18)	19.34(0.45)
Coxsackievirus		
10^{-1} dilution	6.86 (0.26)	6.93(0.04)
10^{-2} dilution	8.77(0.32)	9.17(0.63)
10^{-3} dilution	13.73(0.11)	13.80(0.32)
10^{-4} dilution	17.57(0.77)	17.95(0.78)
Hepatitis A Virus		
10^{-1} dilution	3.32 (0.35)	4.77(0.50)
10^{-2} dilution	7.74(0.64)	8.01(0.36)
10^{-3} dilution	11.42(0.26)	11.47(0.49)
10^{-4} dilution	15.07(2.10)	15.88(0.19)

[a] Real-time RT-PCR; [b] Multiplex real-time PCR

Multiplex real-time PCR

After optimization of each primer and probe set in monoplex PCR reactions, multiplex PCR analysis was performed for simultaneous virus detection. Multiplex PCR reactions were composed in such a way to minimize any decrease in sensitivity due to the multiplexing procedure.

The assay, which targeted NV, HRV, HAV, and CVB, was performed in a 50 µl reaction mixture, consisting of 25 µl iQ™ Multiplex Powermix (Bio-Rad), 0.5 mM $MgCl_2$, 0.75 µM of each primer, or 0.1 µM in the case of NV, 0.1 µM HRV, 0.1 µM HAV, and CVB, 0.1 µM probe, and 5 µl of cDNA. The reaction conditions involved incubation at 95°C for 3 min followed by 39 cycles of denaturation (95°C for 10 sec), and annealing/extension (60°C for 30 sec), and was performed using MyiQ and CFX96 Real-Time Detection Systems (Bio-Rad).

Sensitivities of monoplex and multiplex real-time PCR assays

For each target, viral RNA was isolated from positive fecal samples, and its cDNA was synthesized. Subsequently, the sensitivities of multiplex assays and individual PCR reactions were compared by quadruplicate analyses of serial tenfold dilutions of cDNA.

Furthermore, similar experiments were performed for the simultaneous detection of multiple targets. This was accomplished by spiking serial dilutions of cDNA of 1 target with a constant strong-positive concentration [cycle threshold (C_T)<32] of a second target. Each target was systematically spiked with all other targets present in the multiplex assay (e.g., serial HAV cDNA dilutions were spiked with a constant concentration of either HRV cDNA or NV cDNA).

Specificities of monoplex and multiplex real-time PCR assays

A panel of parasitic, bacterial, and viral pathogens that could have been potentially present in the fecal samples was used to evaluate the specificity of multiplex real-time PCR assays. This panel included *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Vibrio parahaemolyticus*, *Clostridium difficile*, *Clostridium perfringens*, *Salmonella enteritidis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and enterovirus 71.

Retrospective clinical evaluation

In total, 227 fecal samples from 227 patients were used for retrospective clinical evaluations of the conventional RT-PCR assays. For detection of NV GI, NV GII, and HRV, reverse transcription PCR (RT-PCR) was performed using the One-step RT-PCR Kit (Qiagen) with the primers (GIFIM, GIRIM), (GIIFIM, GIIRIM), and (ddrv-1, ddrv-2), which were based on sequences of the NV ORF2 and HRV VP7 region (Table 2). 5 ml of viral RNA was used as the template, which was combined with 20 ml of the premixed kit solution. The PCR was carried out in a PCR System S1000™ thermal cycler (Bio-Rad) according to the following protocol: 1 initial RT step at 50°C for 30 min, followed by PCR activation at 95°C for 15 min; 35 cycles of amplification at 30 sec at 94°C, 30 sec at 56°C, and 30 sec at 72°C; with a final extension step of 5 min at 72°C. The PCR products were then electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

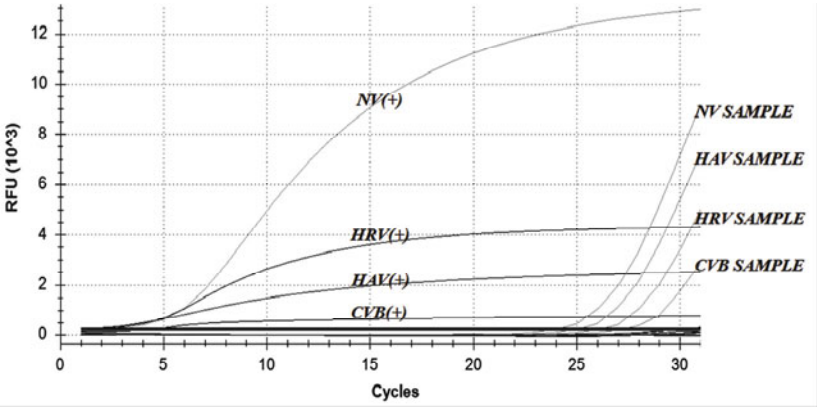

Fig. 1. Analytical and diagnostic specificities of multiplex real-time PCR. The FAM, TYE705, ROX, and HEX fluorescences target NV, HAV, HRV, and CVB genes, respectively. The x-axis represents the cycle number. The y-axis shows the amount of fluorescent signal detected. (+); positive control.

Table 4. Positive results from retrospective and prospective applications of the multiplex PCR assays

Target	Positives (%)	Mixed infections	C_T range
Retrospective application[a]			
NV GI	4 (1.8)	-	-
NV GII	36 (16)	-	-
HRV	26 (11)	-	-
Total	66 (29)	-	-
Prospective application[b]			
NV GI	6 (2.6)	HRV-3	15–29
NV GII	53 (23)	HRV-41	14–28
HRV	46 (20)	NV-44	18–27
HAV	10 (4.5)	NV-2	14–28
CVB	8 (3.5)	-	-
Total	123 (54)	90	

[a] Conventional RT-PCR only
[b] Multiplex real-time PCR only

Results

Sensitivity and specificity of the multiplex real-time PCR assays

Optimized monoplex real-time PCR assays were combined to create multiplex real-time PCR assays. The sensitivity of the multiplex assays was compared to that of the monoplex assays by testing serial dilutions of cDNAs of each target virus in quadruplicate. For HRV, HAV, CVB, and NV, comparable sensitivities were obtained in monoplex and multiplex PCRs, although C_T values were consistently higher in multiplex assays (Table 3).

Similar experiments were performed for the simultaneous detection of multiple targets. The specificity of the multiplex real-time PCR assays was confirmed, because no specific amplification was observed when nucleic acids from the parasitic, bacterial, and viral pathogens were used (see 'Materials and Methods') (Fig. 1).

Prospective applications of the multiplex PCR

In total, 227 fecal samples from 227 patients were used for the retrospective clinical evaluation of the multiplex real-time PCR assays. As shown in Table 4, the RT-PCR analysis detected, in total, 66 (29%) positive samples. NV GI, NVGII, HRV, HAV, and CVB were detected in 6 (2.6%), 53, (23%), 46 (20%), 10 (4.5%), and 8 (3.5%) samples, respectively.

Discussion

In the present study, multiplex real-time PCR assays were developed for the simultaneous detection of NV GI, NV GII, HRV, HAV, and CVB. This proved to be a rapid and highly specific technique for the detection of waterborne viruses. Therefore, multiplex real-time PCR enables more rapid identification of infections in clinical samples compared to a sequencing assay.

Furthermore, a retrospective clinical evaluation showed that multiplex real-time PCR enabled an increase in pathogen detection. While conventional PCR methods detected infection in 29% of samples, prospective clinical evaluations were detected in 123 of 227 (54%) samples with multiplex real-time PCR. Norovirus genogroup II was found the most frequently (23%), followed by rotavirus (20%), the hepatitis A virus (4.5%), Coxsackievirus (3.5%), and norovirus genogroup I (2.6%). Detection efficiencies of monoplex and multiplex real-time PCRs were almost equal, with C_T values between the 2 methods of approximately 1–2. Introduction of more sensitive techniques for the diagnosis of viral gastroenteritis has resulted in a significant increase in the detection rates of enteric viruses. The clinical relevance of positive results with high C_T values remains to be established. Some studies have shown the presence of viral pathogens in samples from asymptomatic individuals using PCR, but viral loads were higher in symptomatic patients (De Wit *et al.*, 2001; Kang *et al.*, 2004; Amar *et al.*, 2007).

These multiplex real-time PCR assays have improved turnaround time significantly and high throughput diagnostic setting this rapid, quality-assured testing enables efficient and economic service delivery.

The multiplex real-time PCR developed and optimized in our laboratory using their primers improved efficiency by requiring only one PCR reaction to detect viral gastroenteritis and reduced procedure time and test volume by 50% as well as saved two-third of the reagent cost.

In summary, the internally controlled multiplex real-time PCR assays for the simultaneous detection of HAV, HRV, NV and CVB resulted in a significant improvement of the diagnosis of viral gastroenteritis in the diagnostic laboratory.

These results strongly demonstrate that multiplex real-time PCR assays could be used for rapid source tracking and monitoring of food-borne viruses.

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