Pediatrics & Therapeutics
ISSN: 2161-0665

Chen et al., Pediat Therapeut 2014, 4:3
DOI: 10.4172/2161-0665.1000208

Research Article Open Access

Development of Two Multiplex Real-Time PCR Assays for the Rapid Detection of RNA and DNA Viruses Associated with Gastroenteritis in Pediatric Patients

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Rec date: Mar 12, 2014, **Acc date:** Jul 18, 2014, **Pub date:** Jul 21, 2014

Abstract

Background: Viral gastroenteritis is one of the most common causes of morbidity and mortality in infants and young children in China.

Objectives: To develop a rapid and sensitive real time PCR method capable of detecting several viruses associated with gastroenteritis in pediatric patients simultaneously.

Methods: Two multiplex real-time PCR assays, the first targeting the RNA viruses: rotavirus, norovirus and parachovirus and the second the DNA viruses: human adenovirus and human bocavirus 2, were designed and assessed for their specificity and sensitivity. The multiplex assays were evaluated using clinical samples and compared to conventional RT-PCR/PCR. Results The multiplex assays developed in the study were successful in detecting the five target viruses. No cross-reactions with a panel of other human viruses were presented. The assays were sensitive enough to detect as little as one copy of *in vitro* transcribed target RNA or plasmid DNA in a single reaction. Compared to conventional RT-PCR or PCR, the multiplex assays showed acceptable sensitivity from 86.2% to 95.7% together with high specificity (97.2% to 99.5%) in detecting rotavirus, norovirus and adenovirus.

Conclusion: The development of these two multiplex assays should result in significant improvement in the screening of viral pathogens associated with pediatric gastroenteritis in China.

Keywords: Multiplex real-time PCR assays; Rotavirus; Norovirus; Parachovirus; Adenovirus; Bocavirus; Gastroenteritis

Introduction

Gastroenteritis is a major, but a substantially neglected, public health problem in developing countries. In China, it is second only to respiratory tract infections as one of the most common causes of morbidity and mortality in infants and young children [1]. Gastroenteritis can be caused by different bacterial, viral and parasitic pathogens. The predominant viral pathogens are rotavirus, norovirus, enteric adenovirus (ADV), astrovirus, coronavirus, Coxsackie virus, and ECHO virus [2]. Studies have shown that the prevalence of these viruses has increased in recent years, with some of them, previously believed to be rare, causing a wider range of serious diseases [3-6]. In addition, due to the development of new and highly sensitive diagnostic techniques, a variety of novel human viruses have been identified. For example, human bocavirus (HBoV) which is associated with acute respiratory infections was only first reported in 2005 and is now considered as the second most pathogenic parvovirus in humans [7,8]. Likewise, HBoV2 was reported to be associated with gastroenteritis only as recently as 2009 [9]. Human parechovirus (HPeV) discovered in 1956 but overlooked for many years has been recently associated with infant deaths in Wisconsin, USA, [10,11]. Therefore, the spectrum of viral pathogens associated with gastroenteritis is constantly changing, underlining the importance of accumulating knowledge on the prevalence of these viruses in order to implement more effective prevention and control measures in China.

For the identification of these viruses in the clinical setting, rapid, sensitive and high throughput detection methods are required. Molecular based detection methods have become the front-line diagnostic procedures for many laboratories [12] with real-time PCR being a preferred approach due to its many advantages over other diagnostic procedures, including quantification of the target RNA/DNA, lower risk of contamination, decreased hands-on time, and better specificity of detection. Considering the limited amounts of stool samples available for analysis in laboratory and the cost of each test (about RMB ¥80 per test), this study reports the development and evaluation of two multiplex real-time PCR assays for the detection of RNA viruses (e.g. rotavirus, norovirus and parachovirus) and DNA viruses (ADV and HBoV2) associated with pediatric acute gastroenteritis.

Material and Methods

Clinical samples

This study was approved by the Ethical Committee of the Capital Institute of Pediatrics.

Stool specimens were obtained from 207 infants and children who attended the affiliated Children's Hospital to the Capital Institute of Pediatrics (CIP) with acute gastroenteritis. These included 150 from the outpatient units and 57 hospitalized children during the period of November 2010 to October 2011. The male to female ratio was 134:73 and ages ranged from 12 days to 12 years old with an average age of 14 months and 15 days. Acute gastroenteritis was defined as having three or more loose or liquid bowel movements per day.

Specimens were prepared as 10% (w/v) suspensions in sterile phosphate-buffered saline (PBS) and centrifuged at 4,000 rpm for 15 min. A total of 150 µl of the supernatant was used for nucleic acid extraction. The remaining specimens were kept frozen at -70°C for further analysis.

		Target	Refs.	Length
Multiplex assay #1				
Rotavirus				
Rota F1	CATGACGCCAGCCGTAAAT	VP6	FJ998275	100 bp
Rota R1	TTCACAAACTGCAGACTCAATACGT			
Rota P1-FAM	FAM-CATTATTTCCGCAAGCACAACCTTTTCAAC-BHQ1			
Rota F2	AAACAGAAGACAAM(A/C)GAACR(G/A)GGTTTTACA	VP6	EF583020	119 bp
Rota R2	GCCACATCGTACCCATCAAGT			
Rota P2-FAM	FAM-TCAGCK(T/G)TCATTCACACTGAAY(C/T)AGATCACA-BHQ1			
Norovirus				
Noro F1	CCAATGTTY(T/C)AGR(G/A)TGGATGAGR(A/G)TTY(C/T)T	RNA-dependent	AB231348	78bp
Noro R1	TTCACAAAACTGGGAGCM(C/A)AGAT	RNA polymerase		
Noro P-Cy5	Cy5-CGATCGCCCTCCCACGTGCT-BHQ2			
Parachovirus				
HPeV F	CAGCGGAW(T/A)CM(A/C)ACACCTGGTA	5' end	AJ889918	206 bp
HPeV R	CCAGATCAGATCCAY(T/C)AGTGTCACTTG	Non-coding region		
HPeV P-HEX	HEX-ACGGGTACCTTCTGGGCATCCTTCG-BHQ2			
Multiplex assay #2				
Adenovirus				
ADV F1	TCGATGATGCCGCAATG	Hexon	AB610527	71bp
ADV R1	TCGATGATGCCGCAGTG			
ADV F2	AGGCCCGGGCTCAGATACT			
ADV R2	AGGCCCGGGCTCAGGTACT			
ADV P-Cy5	Cy5-ATGCACATCGCCGGGCAGGACG-BHQ2			
HBoV 2				
HBoV2F	TTGCTCCTGGGACTGAACGT	NS1	GU301645	77bp
HBoV2R	TTCCCTGACAGGATCATCTTC			
HBoV2P-FAM	FAM-TCATGATCCAACTAAGAAACTGCGCACCA-BHQ1			

Table 1: Primers and Probes for Multiplex 1 and Multiplex 2 real-time PCR assays.

Nucleic acid extraction

Nucleic acids, both DNA and RNA, were extracted from all specimens using Trizol (Invitrogen, USA) according to the manufacturers' instructions. The extracted RNA and DNA were dissolved in 20 µl DEPC (diethylpyrocarbonate) treated water and 25 µl 8 mM NaOH, respectively.

Primers and taqman probes

The primers and the TaqMan probes were designed using the Primer Express version 2.0 (Applied Biosystems) software around the conserved regions of sequences of rotavirus group A, norovirus GI and GII, HPeV 1-3, ADV, and HBoV2 deposited in GenBank (Table 1).

Multiplex real-time PCR assays

Two multiplex assays were designed using different commercial real-time assay kits. Multiplex assay #1 targeted the RNA viruses (e.g. rotavirus, norovirus and HPeV) while Multiplex assay #2 was designed for the DNA viruses ADV and HBoV2.

For Multiplex assay #1, a one-step Real-Time PCR assay was developed using the primers and probes as listed in Table 1 and the TaqMan® One-step PCR master reagent kit (Applied Biosystems). Extracted RNA (5μl) of each sample combined with primers (800nM) and probes (200 nM) were denatured by heating at 95°C for 5 min and then snap-chilled on ice for 5 min. Then 2×Master mix and 40×RT-mix were added to the reaction in a final volume of 25μl. The thermal cycling program consisted of a 30 min cycle at 48°C, a 10 min cycle at 95°C and 45 cycles consisting of 95°C for 15 s followed by 60°C for 1 min.

For Multiplex assay #2 a multiplex real-time PCR assay was developed using primers and probes for ADV and HBoV2 (Table 1) and the TaqMan® universal PCR master reagent kit (Applied Biosystems). The total reaction volume was 25μl containing 800 nM of each primer, 200 nM of each probe, the TaqMan universal PCR Master Mix Reagent components, and 2μl of DNA extracted. The thermal cycling parameters consisted of a 2 min cycle at 50°C, a 10 min cycle at 95°C, and 40 cycles consisting of 95°C for 15 s followed by 60°C for 1 min.

All of the amplification reactions were conducted using the ABI Prism 7500 Sequence Detection System (Applied Biosystems). Each reaction included a negative extraction control, a DEPC-treated water control, and positive controls (e.g. *in vitro*-transcribed RNA or plasmid DNA as described below). An acceptable run was one with the expected results from each control. The samples were considered as positive if the amplification plots showed definite exponential increases in the fluorescent signal.

Sensitivity and Specificity of the Multiplex Real-time PCR Assays

The suitability and specificity of the primers and probes shown in Table 1 were examined using the NCBI/ Primer-BLAST (http://www.ncbi. nlm.nih.gov /BLAST).

Targeted genes for each virus were amplified from positive stool samples by PCR using primers shown in Table 1, and then purified and cloned into pGEM-T vector (Invitrogen) to obtain positive control plasmid DNA. Subsequently, plasmid DNAs from RNA viruses were transcribed into RNA by an in-vitro transcription system T7 RiboMAX™ Express Large Scale RNA Production System (Promega) by following the manufacturer's instructions. *In vitro*-transcribed RNAs and plasmid DNAs were quantified using the Quanti-iT™ ssRNA or dsDNA BR Assay kit and the Qubit™ fluorometer (2-100 ng) (Invitrogen, Lot: 55318A). They were then used to evaluate the sensitivity of the two multiplex real-time PCR assays compared to individual real-time PCRs by analyzing serial tenfold dilutions in triplicate.

A panel of 17 total nucleic acid extracts derived from isolates or prototype strains including Coxsackievirus types A16 (Cox A16) and B3 (strain Nancy, Cox B3 Nancy), enterovirus type 71 (EV71), cytomegalovirus (strain AD169, CMV AD169), herpes simplex virus types 1 and 2 (HSV1, HSV2), human metapneumovirus (HMPV), influenza virus H1N1, H3N2, and B (InfA1, InfA3, InfB), parainfluenza virus types 1, 2, 3, and 4 (PIV 1-4), respiratory syncytial virus subtype A (strain Long, RSVA Long) and B (strain 18537, RSV B 18537), and human rhinovirus 14 (strain 1059, HRV14 1059) was used to assess the specificity of the multiplex assays.

The two multiplex assays were compared with conventional RT-PCR or PCRs for the detection of rotavirus, norovirus and ADV previously developed in our laboratory [13-16]. No conventional RT-PCR or PCR methods were available in our laboratory for HBoV2 and HPeV. Therefore, the specificity of the multiplex assays in detecting HBoV2 and HPeV was confirmed by sequencing of HBoV2 and HPeV positive samples.

Statistics

Data analyses were performed using the SPSS 16.0 statistical package (SPSS Inc., Chicago, Illinois, USA).

Results

Specificity and sensitivity of the multiplex real-time PCR assays

An NCBI/Primer-BLAST analysis of the forward and reverse primers for each candidate virus confirmed their specificity and did not reveal any cross-reactivity with human genes or other viruses.

In addition, from the panel of 17 total nucleic acid RNA or DNA extracts derived from viral isolates or reference strains (e.g. Cox A16, Cox B3 Nancy, EV71, CMV AD169, HSV1, HSV2, HMPV, InfA1, InfA3, InfB, PIV 1-4, RSVA Long, RSV B 18537, and HRV14 1059), no amplifications were observed confirming the specificity of these multiplex assays.

The sensitivity of the two multiplex assays compared to the monoplex assays was evaluated by testing serial dilutions (from 10^3 to 10^0 copies) of *in vitro*-transcribed RNA or plasmid DNA of the target viruses in triplicate. For rotavirus, norovirus, HPeV, ADV and HBoV2, comparable sensitivities were obtained in multiplex and monoplex assays, although the mean Ct values were higher in multiplex assays (Table 2).

Detection of clinical specimens

Among the 207 stool samples used for a retrospective clinical evaluation, 60 samples (29.0%, 60/207) were positive for rotavirus, 23 (11.1%, 23/207) positive for norovirus and 16 (7.7%, 16/207) positive for HPeV as determined by Multiplex assay #1. Using Multiplex assay #2, 28 (13.5%, 28/207) of the samples were positive for ADV and 12 (5.8%, 12/207) for HBoV2. Of these positive samples determined by the two multiplex assays, 20 (9.7%, 20/207) were positive for 2 or 3 viruses, including 3 (1.4%, 3/207) positive for rotavirus and HBoV2, 9 (4.3%, 9/207) positive for rotavirus and ADV, 4 (1.9%, 4/207) for rotavirus and norovirus, 1 (0.5%, 1/207) for norovirus and HPeV , 1

(0.5%, 1/207) for norovirus, rotavirus and HBoV2, 1 (0.5%, 1/207) for norovirus, rotavirus and HPeV, and 1 (0.5%, 1/207) for rotavirus, ADV and HBoV2.

Targeted virus	RNA/DNA	Ct value of multiplex		Ct value of monoplex	
	copies/reaction	Mean	SD	Mean	SD
Rotavirus	**Multiplex assay #1**				
	1×10^0	31.5	0.5	31.5	0.6
	1×10^1	28	0.15	28.1	0.4
	1×10^2	24.8	0.3	24.9	0.2
	1×10^3	21.3	0.1	21.5	0.1
Norovirus	**Multiplex assay #1**				
	1×10^0	36.5	0.42	34.9	0.35
	1×10^1	29.5	0.1	28.4	0.4
	1×10^2	25.9	0.1	23.9	0.25
	1×10^3	22.3	0.06	20.3	0.2
HPev	**Multiplex assay #1**				
	1×10^0	27	0.29	27.3	0.3
	1×10^1	23.6	0.5	24.2	0.5
	1×10^2	21.6	0.56	19.8	0.6
	1×10^3	17.7	0.26	16.3	0.4
ADV	**Multiplex assay #2**				
	1×10^0	30.5	0.47	29.4	0.2
	1×10^1	28	0.06	26.4	0.3
	1×10^2	24.8	0.12	23.2	0.3
	1×10^3	20.4	0.1	18.8	0.1
HBoV2	**Multiplex assay #2**				
	1×10^0	34.2	0.38	33.9	0.2
	1×10^1	32.9	0.17	31.7	0.1
	1×10^2	28.7	0.31	28.1	0.2
	1×10^3	24.6	0.57	23.7	0.2
*Mean Ct value from triplicate experiments SD: standard deviation					

Table 2: Sensitivities of multiplex Real-time PCR assays compared with monoplex Real-time PCR assays using serial dilutions of *in vitro*-transcribed RNA/DNA

Compared to the conventional RT-PCR/PCR, Multiplex assay #1 had a sensitivity of 86.2% and 95.7%, and specificity of 97.2%, and 99.5% with Kappa values of 0.85 and 0.95 for rotavirus and norovirus, respectively (A kappa value of over 0.75 indicates good correlation between the two methods). Multiplex assay #2 had a sensitivity of 89.3%, and specificity of 98.9% (Kappa value 0.90) in the detection of ADV (Table 3). Among these 56 positive samples for rotavirus (Table 3), 39 were classified as genotype P [8] a and 2 as genotype P [8] b when these samples were genotyped by hybridization with Digoxin-labelled probes of the group A VP4 gene [14]. On the other hand, when these samples were genotyped by hybridization with Digoxin-labelled probes of the group A VP7 gene [13], 14 were identified as genotype G3, 21 as genotype G1, 2 as genotype G9 and 1 as both genotype G1 and G3. Others among the 56 positive samples were

undetermined because of the low quality of the RT-PCR amplification product. These results show that Multiplex assay #1 is efficient in detecting rotavirus group A (serotypes A to G) known to cause the majority of infections in humans.

Multiplex real-time PCR assays	Samples (N)	Conventional RT- PCR/PCR		Sensitivity (%)	Specificity (%)	Kappa Value
		+	-			
Rotavirus	60	56	97.2	86.2	97.2	0.85
Norovirus	23	22	99.5	95.7	99.5	0.95
Adenovirus	28	25	98.9	89.3	98.9	0.90

Table 3: Results of the Multiplex Real-time PCR assays in detecting rotavirus, norovirus and adenovirus compared to conventional RT-PCR/ PCR.

Specimens positive for HPeV were confirmed to be HPeV-3 by sequence analysis. Sequence analysis of 7 of the 28 samples positive for ADV indicated that they were of serotypes 2, 3, 40, and 41, respectively. Three samples selected randomly from 12 samples positive for HBoV2 were sequenced and confirmed to be HBoV2.

Discussion

Real-time PCR for detecting viruses associated with gastroenteritis has been described as a convenient, useful, and powerful diagnostic tool [17], while multiplex assays of more than one viral gene target in a single tube have the advantage of rapid screening of a large number of potential pathogens in a short time [18]. There are several reports on multiplex real-time PCR assays for detecting viruses associated with gastroenteritis. In 2004, Beuret et al. [19] developed a simple method: a multiplex real-time RT-PCR protocol for the simultaneous detection of norovirus GI and GII, human astroviruses and enteroviruses using SYBR Green reagents [20]. Then, van Marrseveen et al. in 2010 and Feeney et al. in 2011 [20,21] developed multiplex Taqman® assays for the rapid diagnosis of viral gastroenteritis, which allowed for highly sensitive and timely diagnosis of the most prominent causes of viral gastroenteritis, i.e. rotaviruses, noroviruses, astroviruses, group F adenoviruses, and sapoviruses. In order to improve the specificity of these methods, minor groove binder (MGB) assays were used by the authors. Unfortunately, in China, at present such MGB technology is not readily available and so in this study, two multiplex real-time PCR assays without MGB probes were developed in order to detect both RNA and DNA viruses predominantly associated with gastroenteritis, such as rotavirus, norovirus and ADV. In addition to these common viruses, the assays targeted the novel viruses HBoV2 and HPeV that are being increasingly recognized associated with severe gastroenteritis.

The results demonstrated that the multiplex real-time PCR assays were highly specific in detecting the target viruses and showed no cross-reaction with a panel of non-target viruses observed. The assays were also highly sensitive in the detection of 1 copy of *in vitro*-transcribed RNA or plasmid DNA per reaction.

Compared to conventional RT-PCR and PCR, acceptable sensitivity and high specificity values in the detection of rotavirus (86.2%, 97.2%), norovirus (95.7%, 99.5%) and ADV (89.3%, 98.9%) were generated with a good correlation between these methods (e.g. Kappa values of 0.85 for rotavirus, 0.95 for norovirus and 0.90 for ADV).

During the retrospective clinical evaluation of the multiplex PCR assays, a high percentage of samples (71%, 40/56) positive for rotavirus were further genotyped as P[8]a, P[8]b and G1, G3, G9. This result is in concordance with other studies showing that rotavirus group A is a significant cause of gastroenteritis in children [22].

Adenovirus group F consisting of serotypes 40 and 41 have been associated with gastroenteritis. The multiplex real-time PCR developed in this study can detect not only serotypes 40 and 41, but also other serotypes, such as 2 and 3. This will allow for more data to be generated and, ultimately, a better understanding of the relationship between non-enteric adenovirus and gastroenteritis.

In this study, 12 out of 207 samples were positive for HBoV2, and 16 for HPeV. The investigation of new viral agents, such as HBoV2, and viruses playing more important roles in gastroenteritis than previously thought, such as HPeV, may be helpful in understanding the role of viruses other than rotavirus and norovirus in gastroenteritis.

There is one important shortcoming in this study. A limitation of the ABI Prism Sequence Detection System is that only four probe types can be used simultaneously, and for this reason, other viral pathogens associated with gastroenteritis, such as astrovirus, coronavirus, Coxsackie virus, and ECHO virus, were not included in this study.

In summary, two multiplex real-time PCR assays for the simultaneous detection of the RNA viruses rotavirus, norovirus and HPeV, and DNA viruses ADV and HBoV2 with acceptable sensitivity and specificity were developed in this study. It is hoped that these assays will result in a significant improvement in the screening of viral pathogens associated with gastroenteritis.

Conflict of Interest

None declared.

Acknowledgements

The authors would like to thank the doctors and nurses from the Affiliated Children's Hospital to Capital Institute of Pediatrics who devoted their time to collect stool samples and clinical information.

Funding: Beijing Municipal Natural Science Foundation (7112021) and Beijing Municipal Health Bureau "Advanced Personnel Training Program" (No. 2011-3-068).

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