Multiplex Detection of Rotavirus A, B, C, Norovirus GI and GII, Adenovirus, Astrovirus and Sapovirus in a Single PCR System in Stool Samples From Children With Acute Diarrhea

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Abstract

We developed a RT-PCR combined with melting curve analysis (RRCMC) method for simultaneous detection of rotavirus A, B, C, norovirus GI and GII, adenovirus, astrovirus and sapovirus. Stool samples were collected from 160 children with acute diarrhea and tested by RRCMC assay. A total of 71 patients were tested positive with norovirus, adenovirus or rotavirus. The RRCMC assay has high specificity. There is no internal cross-reaction through the 8 diarrhea viruses and no cross-reaction of other commonly intestinal pathogens and human genome. The detection limit was ranging from 1×10^2 to 1×10^5 copies/ml for each diarrhea virus. In conclusion, the RRCMC method is a suitable rapid clinical test for infectious viruses, with the advantages of high-throughput, low cost, high sensitivity and specificity.

Background

Virus infection was the most common cause of acute diarrhea in children under 5 years of age. Pathogens of viral diarrhea include rotavirus, norovirus, adenovirus, astrovirus and sapovirus [1]. The main clinical manifestations of viral diarrhea were diarrhea, vomiting, and fever. Furthermore, acute diarrhea can also cause severe dehydration, leading to further complications and hospitalization [2]. Diarrheal diseases lead to a large number of childhood deaths, the rotaviruses account for two fifth [3]. Rotaviruses are double-stranded RNA viruses and belong to family of *Reoviridae* which can be divided into three subtypes: A, B, and C. Rotavirus A is the leading known cause of severe gastroenteritis in infants and young children worldwide[4,5]. Norovirus is a non-enveloped, small RNA virus that contains a single stranded, positive-sense, polyadenylated RNA genome and which was associated with approximately one-fifth of all diarrhea cases [6]. Norovirus can be divided into five groups, of which norovirus GI and norovirus GII groups are mainly related to humans [7]. Human adenovirus is non-enveloped virus which belongs to the family of *Adenoviridae* and have a linear 36-kb dsDNA genome. Human adenovirus is also recognized as an important cause of diarrhea in children [8]. Human astroviruses are small, non-enveloped positive-sense single-stranded RNA viruses in the *Astroviridae* family which are a major cause of diarrhea in children, the elderly, and immunocompromised people [9,10]. Sapovirus is a member of the *Caliciviridae* family, and a single-stranded positive sense RNA virus. Sapovirus infections primarily affect children aged less than 5 years, causing mild to moderate diarrhea and outbreaks in all age groups [11].

In China, the antigen assay is the main tool for rapid screening of some viruses in many hospitals. However, these techniques are insensitive and cross-reactive [12,13]. Serological tests are also widely used as a diagnostic technique in clinical practice. However, due to the time window for antibody production, its early diagnosis value is limited [14]. Moreover, both antigen and antibody detection are not applicable for multiple diarrhea viruses. Nucleic acid detection is widely used because of its high sensitivity, specificity and early diagnosis [7]. At present, clinical nucleic acid diagnosis is mostly based on the detection of DNA / RNA of diarrhea related virus. However, due to the limitation of the number of fluorescent dyes and labeled probes, it is impossible to detect more pathogens in one tube of detection solution. There are multiple PCR tests developed in previous studies [15,16]. Bennett et al developed one-

step multiplex real-time RT-PCR assay simultaneously detecting adenovirus, astrovirus, rotavirus and sapovirus in one tube which has reached the limit detection of fluorescent quantitative PCR in one system [17]. In this study, we developed a Multiplex Probe Melting Curve analysis for detecting rotavirus A, B, C, norovirus GI and GII, adenovirus, astrovirus and sapovirus in one PCR tube.

From March 2019 to May 2019, patients who met the following criteria were recruited in the present study: (1) children under 14 years of age, (2) Patients who visit Children's Hospital of Zhejiang University School of Medicine in the inpatient wards and outpatient departments, (3) Primary diagnosis of acute diarrhea with suspected virus infections. Diarrhea was defined as three or more stools per day of unusual stool shape (liquid, watery, mucous or bloody purulent) within the previous 24 h. This study was approved by the medical ethics committee of the Children's Hospital, Zhejiang University School of Medicine (NO. 2019-IRB-082). Informed consent to participate in the study was obtained from the patients and their parents.

Stools were collected and added approximately 250μl to 1ml normal saline. The mixtures were centrifuged at 6,000 rpm at 20℃ for 30s. A total of 200μl supernatant was separated and DNA/RNA was extracted by SSNP-2000A nucleic acid automatic extraction instrument and Nucleic acid extraction or purification kits (Art.No：SDK60105) abovementioned instrument and kit be from Jiangsu Bioperfectus Biotechnology Technologies Co, (Jiangsu, China).

The principles of RRCMC are reported in previous study and as follows[18]: Firstly, Taqman probes labeled with the same fluorescence dyes and primers was designed to target four viruses, and each probe is designed with a not fully complementary 3' phosphorylation closed melting curve oligonucleotide. Taqman probes with sequences of varying length can be distinguished if the probes themselves, independent of hybridising to 3' phosphorylation closed melting curve oligonucleotide, have different melting temperatures. Secondly, if a virus target is present, its corresponding probe is consumed during PCR amplification; the amplification curve appeared which indicated this channel viruses positive. Comparing melting profiles of the probes with negative control after the reaction reveals which probes have been consumed and melting curve decreased; this in turn indicates which targets are present in a sample. The primers and probes were as shown in table.1.

The reagents were supported by Jiangsu Bioperfectus Biotechnology Technologies Co, China. PCR reactions system in a final volume of 30μl consist of 4 components: 10μl of nucleic acid amplification reaction solution (dNTPs、Tris、KCl、$MgCl_2$), 10μl primer/probe mix (primer: 200-300 nM/μL, Taqman probe: 100-150 nM/μL, 3' phosphorylation closed melting curve oligonucleotide: 100-300 nM/μL), 5μl Enzyme mixture(M-MLV and *Taq*), and 5μl template. Amplification reactions and melting profiles were performed in a real-time PCR SLAN-96P system (HONGSHI, China). The thermal profile was: 50℃ for 20 min; 95℃ for 5 min; 8 cycles of 95℃ for 10 sec and 60℃ for 40 sec; 40 cycles of 95℃ for 10 sec, 55℃ for 30 sec, and 58℃ for 15 sec. Fluorescence measurements were recorded during the read steps at 58℃. Post-amplification melting profile had the following conditions: after the last cycle of PCR, heat at 95℃ for 15 sec, cool to 20℃ and slowly increase the temperature at speed of 0.03℃/s. The

fluorescence emission data is continually collected during the rising temperatures. The negative derivative of the emission reading, with respect to temperature, is plotted against the temperature to form melting curves, and the peak of the curve corresponds to the Tm of the probe. Rotavirus C, astrovirus, rotavirus B, and norovirus GI were detected by FAM channel, and rotavirus A, sapovirus, norovirus GII, and adenovirus were detected by VIC channel.

Clinical strains of rotavirus A, rotavirus B, rotavirus C, GI, norovirus GII adenovirus, sapovirus, and astrovirus were tested by RRCMC assay. As shown in Fig.1, comparing to negative control, and according to the melting curve temperature, the order of detection for virus were rotavirus C, astrovirus, rotavirus B, and norovirus GI in FAM channel. In VIC channel of RRCMC assay, the order of detection for virus were rotavirus A, sapovirus, norovirus GII, and adenovirus with melting curve temperature rising.

In order to confirm sensitivity of RRCMC assay, the target fragment of each virus was cloned into pGH plasmid vector. And for this, we prepared a certain-fold dilution series from 10^7 copies/mL to 10^2 copies/mL. The results showed that the detection limitation of the RRCMC method was at least as follows: rotavirus A: 1.0×10^2, rotavirus B: 2.0×10^3, rotavirus C: 1.3×10^4, adenovirus: 1.0×10^4, GI: 1.0×10^5, GII: 1.0×10^5, astrovirus: 4.0×10^3, sapovirus: 4.0×10^3. To determine the specificity of RRCMC assay, clinical strains of rotavirus A, rotavirus B, rotavirus C, GI, GII adenovirus, sapovirus, and astrovirus were used as controls and all clinical strains were confirmed by commercial real-time PCR assay (Jiangsu Bioperfectus Biotechnology Technologies Co, LTD). All were detected positive and there were no false positive or false negative results. There is no internal cross-reaction between 8 viruses by using RRCMC assay while each virus was positive. No fluorescence was detected and no cross-reaction was found in DNAs extracted from the human genome, *Salmonella*, *Shigella*, diarrhea causing *Escherichia coli*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus* in this test.

A total of 160 cases clinically diagnosed as acute diarrhea were enrolled into this study from March 2019 to May 2019, and stool samples were collected and tested by RRCMC assay. There were 108 samples from boys while 52 samples from girls, which yielded a male-to-female ratio of 2.08:1. A total of 71 samples were tested positive by RRCMC assay with positive rate of 44.38%. Of these, 48(44.44%, 48/108) were from boys and 23(44.23%, 23/52) were from girls. As shown in Fig.2, among 71 positive samples, norovirus has the highest positive rate of 10.00% (16/160) comprised 15 cases of norovirus GII (9.38%, 15/160) and 1 case of GII (0.63%, 1/160), successively followed by adenovirus (9.38%, 15/160), rotavirus A (8.75%, 14/160) and astrovirus (8.13%, 13/160), sapovirus (3.13%, 5/160), norovirus (GII) and astrovirus co-infection (1.88%, 3/160), rotavirus A and norovirus (GII) co-infection (1.25%, 2/160), rotavirus A and astrovirus co-infection (0.63%, 1/160), rotavirus A and sapovirus co-infection (0.63%, 1/160), rotavirus C and norovirus (GII) co-infection (0.63%, 1/160). We also analyzed the age distribution of virus positive children. The median age of children with rotavirus A infection was 1.33 years old (0.53-7.90), norovirus (GII) was 1.25 years old (0.15-13.58), adenovirus was 1.74 years old (0.50-11.74), astrovirus was 1.33 years old (0.15-8.82) and sapovirus was 2.58 years old (1.08-5.33).

Acute diarrhea is still a major problem for children especially in developing countries, and it is associated with a large number of morbidity, mortality and costs. [19]Accurate diagnosis of viral diarrhea can avoid the abuse of antibiotics after misdiagnosis as bacterial diarrhea, and we can carry out targeted treatment as early as possible. At the same time, the clinical symptoms of these viruses are similar in the early stage of infection, but there are great differences in the clinical severity in the later stage. Rotavirus A can cause severe clinical symptoms such as dehydration and electrolyte turbulence, while Enteroadenovirus can cause mild to moderate dehydration in most children [20]. Norovirus and sapovirus can cause significantly less clinical symptoms in children than rotavirus A [21-22]. Our results also revealed that the median age of children infected with each diarrhea virus was very close (1-2 years), except for astrovirus. Therefore, the accurate identification of viral pathogens at early infectious period can individualize the treatment and management of children to avoid the occurrence of serious clinical symptoms. Many diagnostic thechonology have been used for the detection of diarrhea viruses, including Serological method immune electron microscopy, ,and enzyme-linked immunosorbent assays (ELISA). However, these techniques are time-consuming and are low in sensitivity and specificity [2]. In order to detect common viruses in children with diarrhea diseases, the high throughput, multiplex real-time RT-PCR was developed for the detection of adenovirus, astrovirus, rotavirus or sapovirus from stool samples in previous study [15-17]. However, the detection capacity of this method is not large enough.

This paper describes the development and validation of a real-time RT-PCR plus melting profile analysis (RRCMC) assay, which will allow rapid and simultaneous detection of rotavirus A, B, C, norovirus GI and GII, adenovirus, astrovirus and sapovirus in stool samples, it only takes about 3-5 hours to finish the testing process. The cost of RRCMC assay is about 10 dollars each sample, so it is an inexpensive novel diagnostic method. Because of the conservative characteristics of primers and probes and the control of melting curve, RRCMC assay has high specificity. There is no internal cross-reaction between 8 diarrhea viruses. The specificity of the assay was also confirmed by testing a panel of other commonly intestinal pathogens and no-template controls and no false positive results were encountered. Previous study suggested that there was a significant correlation between severity and the virus copies, and high sensitivity of PCR assay is helpful for early diagnosis [16]. Our study showed that the detection limit of the RRCMC method was ranged from 1×10^2 to 1×10^5 copies/ml for each diarrhea associated virus. Among them, the sensitivity of rotavirus detection is the highest (1×10^2 copies/ml) and the sensitivity of norovirus (GI and GII) detection is the lowest (1×10^5 copies/ml).

In 160 patients with acute diarrhea, a total of 71 patients were tested positive by RRCMC. In our study, rotavirus A, C, norovirus GI and II, adenovirus, astrovirus and sapovirus were detected by RRCMC, and norovirus, adenovirus and rotovirus were the most common viruses caused acute diarrhea disease in children in precious study. Rotavirus B was not detected in this study. In further studies, we will enroll more clinical samples from children with acute diarrhea disease to evaluate the effect of RRCMC method.

In this study, we developed a double probes plus multiple melting curve technique for detecting rotavirus A, B, C, norovirus GI and GII, adenovirus, astrovirus and sapovirus in one PCR tube. This novel method has the advantages of low cost because of single tube reaction, high sensitivity and specificity, and is

suitable for general fluorescent quantitative PCR instrument, which is suitable for rapid clinical application. So the RRCMC method would be a valuable supplementary test in clinical practice.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors declare that they had no confict of interest.

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Table

Table 1

Sequence of probes and primers

Virus	primer/probe	seqence（5’-3’）
rotavirus A	F	ATTGAAGCAGAWTMTGATTCTG
	R	ACAAATCYTYRATCAATTGCAT
	P	VIC-ATTCAGATAGTGATGAYGGWAAATGTA-BHQ1
	Ph	TACATTTCCCTTCATCACTATTTGAAT-Phosphorylation
rotavirus B	F	CTCAGTTCTTGTAGTATATTAGCTATCG
	R	CTCCACGAGGCAATTTGATA
	P	FAM-AACGCTTCTATGGATTTTAATGTTTTTCTTCAG-BHQ1
	Ph	CTGAATAAAAACTTTAATATCCATATAAGTGTT-Phosphorylation
rotavirus C	F	CCATTCTCTTCATTCTTTTCATTT
	R	TGGAAAATAAATACATAAAGATTCAAC
	P	FAM-TAATTGTGTAGAGTGGTCACAAGGTCAGATG-BHQ1
	Ph	CATCTGATCTTGTTACCACTTTACATAATTA-Phosphorylation
norovirus GI	F	TTCCATGATTTGAGTTTGTGG
	R	GGTGCCATCCATGTTTGTT
	P	FAM-CAGGAGACCGCGATCTCCTGC-BHQ1
	Ph	GCATGAGATCTCGTTTTCCTG-Phosphorylation
norovirus GII	F	AAAGACTGTCTGAAGCTACTCCT
	R	GACCAGATTTAGTGATAAACACAAT
	P	VIC-CTCCCCTGCAAGAGGCCATTT-BHQ1
	Ph	AAATGTCCTTTTGTAGGGTAG-Phosphorylation
astrovirus	F	AATTGCTCAAAGAGGAAATAGA
	R	GCAAGTATVCCATTGATTTCAT
	P	FAM-AGAACTGCAATGGAACGTGAGATGAAG-BHQ1
	Ph	CTTCTTCTCATGTTCTATTGTAGTTCT-Phosphorylation
sapovirus	F	CCAATGTCAATTACGACCAG
	R	TCCATTTCAAACACTAATTTGG
	P	VIC-CCACCTACGAATCTTGGTTCATAGGCG-BHQ1

	Ph	CGCTTATGAATCAAGATTCGTATGTGG-Phosphorylation
adenovirus	F	CTTACAAAGTGCGCTTTACG
	R	AGGGTTTAAAGCTGGGGC
	P	VIC-CATGGCCAGCACCTACTTTGACATC-BHQ1
	Ph	GATTTCAAATTAGTTGCTGTCCTTG-Phosphorylation

Figures

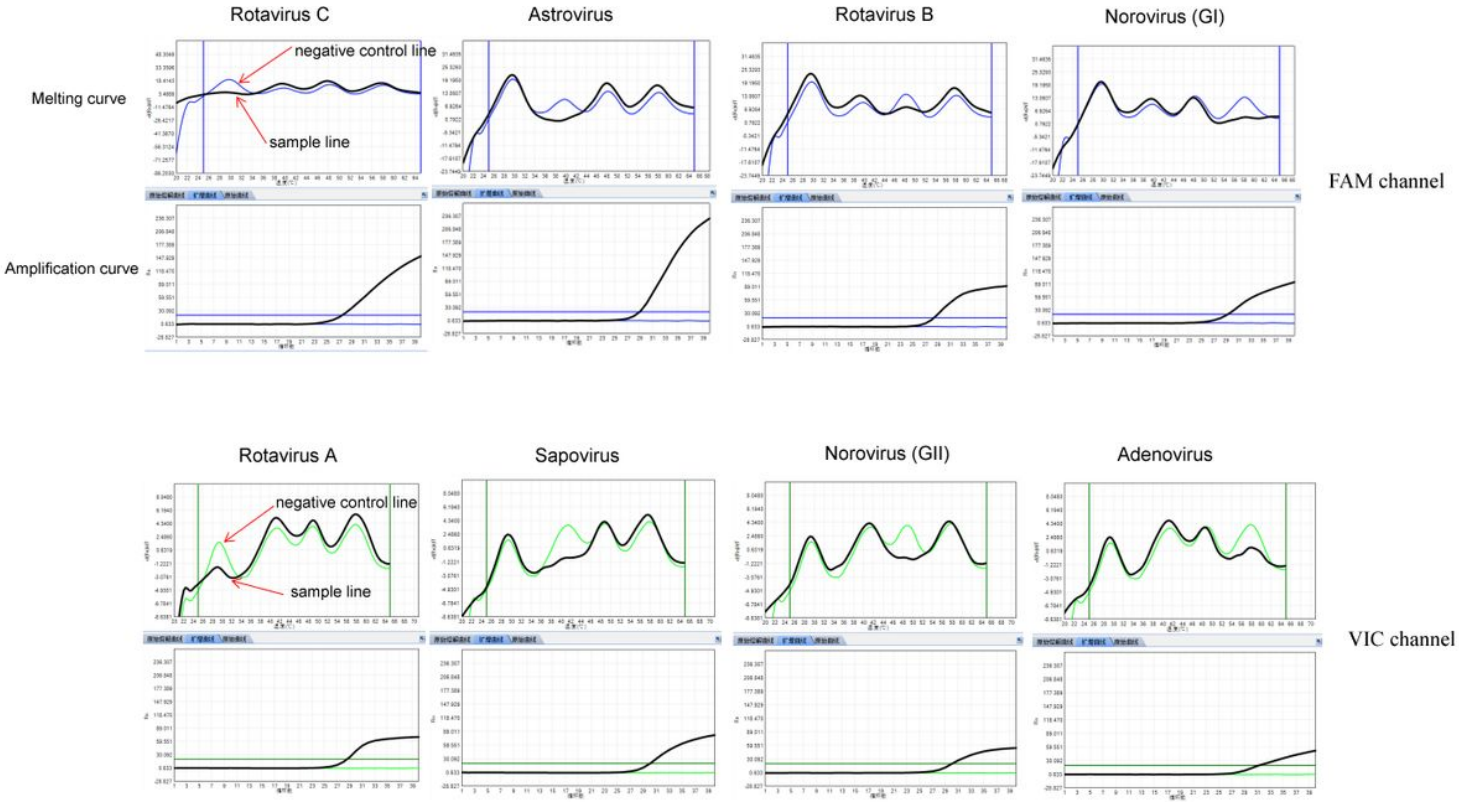

Figure 1

Amplification and melting curve of RRCMC assay

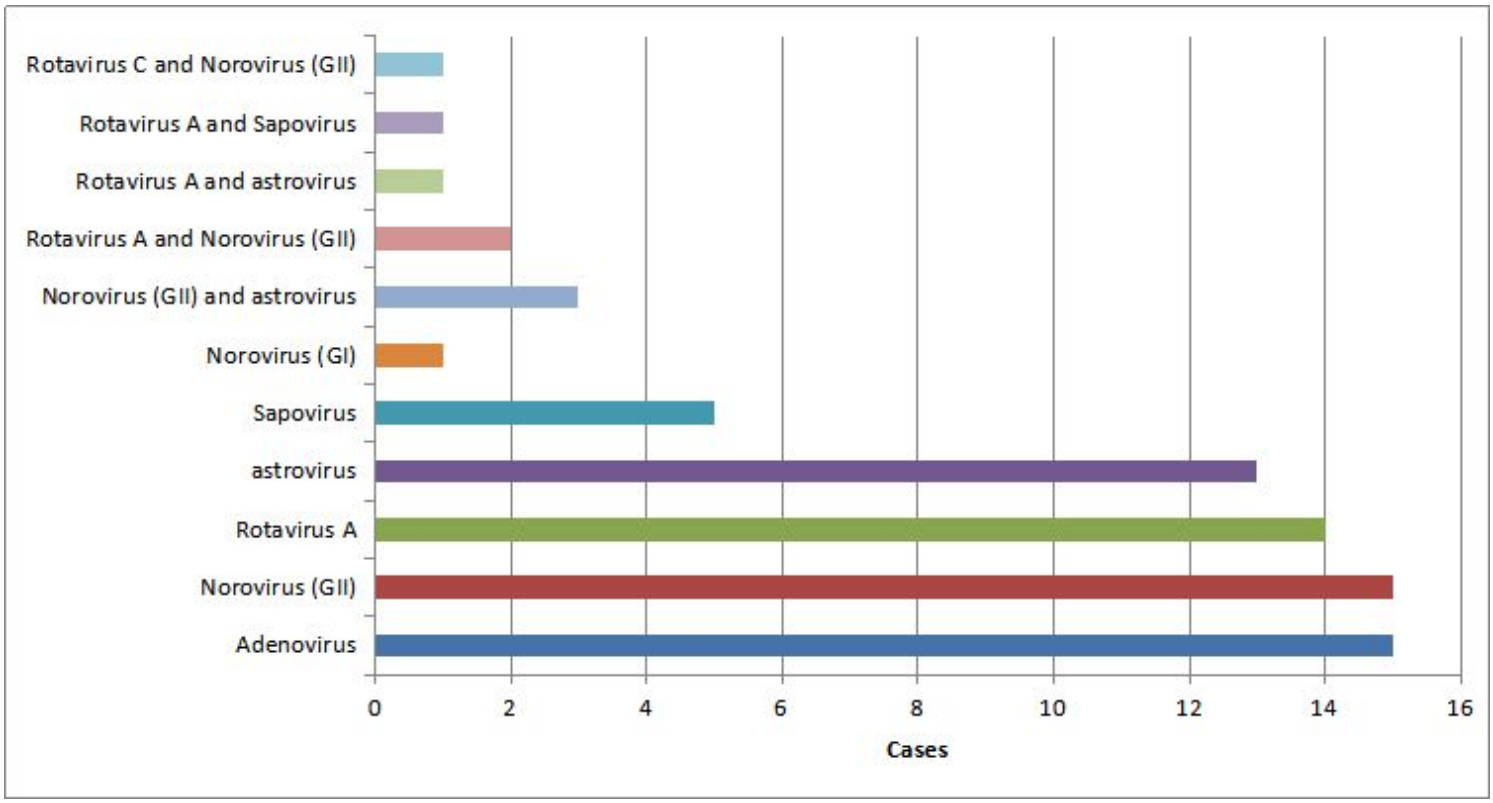

Figure 2

Results of clinical samples by using RRCMC assay