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Short communication

A single-tube multiplex PCR for rapid detection in feces of 10 viruses causing diarrhea

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A novel multiplex polymerase chain reaction assay was developed to identify 10 viruses in a single tube. The assay was targeted to detect group A and C rotaviruses, adenovirus, norovirus GI, norovirus GII, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus. A total of 235 stool samples were collected from infants and children with acute gastroenteritis in Kyoto, Japan, from 2008 to 2009, then tested by this novel multiplex PCR and compared with a multiplex PCR described previously, which used 3 primer sets. The novel multiplex PCR could detect the targeted viruses in 111 of the 235 (47.2%) stool samples. Of these, 9 out of 10 types of viruses were identified, including group A rotavirus, norovirus GII, enterovirus, sapovirus, adenovirus, parechovirus, group C rotavirus, astrovirus, and norovirus GI. In contrast, the multiplex PCR that used 3 sets of primers could detect the targeted viruses in 109 of the 235 (46.4%) stool samples. Among these, 8 types of viruses were identified, including group A rotavirus, norovirus GII, enterovirus, adenovirus, parechovirus, group C rotavirus, sapovirus, and astrovirus. The results suggested that the new multiplex PCR is useful as a rapid and cost effective diagnostic tool for the detection of major pathogenic viruses causing diarrhea.

Acute gastroenteritis is one of the most common illnesses in humans worldwide. An estimated 25–30% of all deaths among children younger than 5 years of age are caused by virus infections. Different types of viruses such as rotavirus, norovirus, sapovirus, astrovirus, and adenovirus have been known to associate with these diseases (Clark and McKendrick, 2004). In recent years, several novel viruses have been discovered, mostly by advanced molecular screening methods (Tang and Chiu, 2010; Svraka et al., 2010). Recently, Aichi virus, parechovirus, enterovirus, and human bocavirus have been considered as agents associated with diarrhea in humans (Stanway et al., 2000; Phan et al., 2005; Pham et al., 2007, 2010; Reuter et al., 2009; Chow et al., 2010). The standard laboratory methods for diagnosing viral infections are based mainly on viral isolation in cell culture. However, in those cases, some viruses cannot be isolated by the cell culture system. For epidemiological study, application of reverse transcription-polymerase chain reaction (RT-PCR) and sequencing techniques have become the standard methods for the detection and characterization of those viral pathogens (Yan et al., 2003, 2004).

RT-multiplex PCR methods had been developed previously for the detection of 8 viruses causing diarrhea using 2 sets of specific primers, set A and B (Yan et al., 2003, 2004). Set A was used to identify group A, B, and C rotaviruses and adenovirus, while set B could detect norovirus (genogroup GI and GII), sapovirus, and astrovirus. Most recently, another RT-multiplex PCR for the detection of 4 additional viruses (Aichi virus, parechovirus, enterovirus, and bocavirus) has been reported and a new set (set C) of specific primers was described (Pham et al., 2010). Although these 3 sets of primers showed good results for detecting several types of viruses causing diarrhea, each one had to be used in a separate reaction. In order to develop a simple, rapid, and cost-effective diagnostic tool for screening clinical specimens, a novel multiplex PCR for simultaneous detection of 10 viruses causing diarrhea (group A and C rotaviruses, adenovirus, norovirus GI, norovirus GII, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus) in a single reaction tube has been developed.

A total of 235 stool samples were collected from infants and children with acute gastroenteritis in Kyoto, Japan, from 2008 to 2009. Only patients with clinical diagnosis of acute gastroenteritis with watery diarrhea were included in this study. The ages of the

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Table 1
Oligonucleotide primers used for the amplification of 10 viruses causing diarrhea.

Virus and primer	Sequence 5′–3′	Sense	Amplicon size (bp)	Reference
Astrovirus				
PreCAP1	GGA CTG CAA AGC AGC TTC GTG	+	719	Yan et al. (2003)
82b	GTG AGC CAC CAG CCA TCC CT	–		Yan et al. (2003)
Group A rotavirus				
VP7 1′(F)	AAA GGA TGG CCA ACA GGA TCA GT	+	569	Yan et al. (2004)
End9(s)	GTA TAR AAH ACT TGC CAC CAT	–		This study
Adenovirus				
Ad1	TTC CCC ATG GCI CAY AAC AC	+	482	Yan et al. (2004)
Ad2	CCC TGG TAK CCR ATR TTG TA	–		Yan et al. (2004)
Enterovirus				
F1	CAA GCA CTT CTG TTT CCC CGG	+	440	Zoll et al. (1992)
R1	ATT GTC ACC ATA AGC AGC CA	–		Zoll et al. (1992)
Norovirus GII				
COG2F	CAR GAR BCN ATG TTY AGR TGG ATG AG	+	387	Yan et al. (2003)
G2SKR	CCR CCN GCA TRH CCR TTR TAC AT	–		Yan et al. (2003)
Norovirus GI				
G1SKF	CTG CCC GAA TTY GTA AAT GA	+	330	Yan et al. (2003)
G1SKR	CCA ACC CAR CCA TTR TAC A	–		Yan et al. (2003)
Parechovirus				
Ev22(+)	CYC ACA CAG CCA TCC TC	+	270	Joki-Korpela and Hyypia (1998)
Ev22(-)	TRC GGG TAC CTT CTG GG	–		Joki-Korpela and Hyypia (1998)
Group C rotavirus				
GCMP-F	CAA ATG ATT CAG AAT CTA TTG	+	205	This study
G8NA2	GTT TCT GTA CTA GCT GGT GAA	–		Yan et al. (2004)
Aichi virus				
C94b	GACTTCCCCGGAGTCGTCGTCT	+	158	Yamashita et al. (2000)
AiMP-R	GCR GAG AAT CCR CTC GTR CC	–		This study
Sapovirus				
SLV5317	CTC GCC ACC TAC RAW GCB TGG TT	+	100	Yan et al. (2003)
SMP-R	CMW WCC CCT CCA TYT CAA ACA C	–		This study

patients ranged from neonate to 5 years old. The study was conducted with approval from the ethical committee in human rights related to human experimentation, Aino University.

The viral genomes were extracted from a supernatant of 10% fecal suspension using a QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). The reverse transcription (RT) was performed using random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan). The RT was carried out at 50 °C for 1 h, followed by 95 °C for 5 min and then rapidly cooled on ice.

For the conventional multiplex PCR, the presence of group A, B, and C rotaviruses and the adenovirus were detected by RT multiplex PCR using the primer set A as a protocol, described previously by Yan et al. (2004). Primers Beg9 and VP7-1′, ADG9-1F and ADG9-1R, G8NS1 and G8NA2, and Ad1 and Ad2 were used for the amplification of group A, B, C rotaviruses and the adenovirus, respectively. For the detection of norovirus GI, norovirus GII, sapovirus, and astrovirus, primer set B; G1SKF and G1SKR, COG2F and G2SKR, SLV5317 and SLV5749, 82b and PreCAP1 was used to amplify these viruses, respectively (Yan et al., 2003). For set C, primers 6261 and 6779, EV22 (+) and EV22 (−), 188F and 542R, F1 and F2 were used to detect Aichi virus, parechovirus, bocavirus, and enterovirus, respectively (Pham et al., 2010; Yamashita et al., 2000; Joki-Korpela and Hyypia, 1998; Zoll et al., 1992).

The cDNA was amplified further for detection of the targeted viruses. The polymerase chain reaction (PCR) components contained 10.9 μl of MilliQ water, 5.0 μl of 5× Colorless GoTaq PCR buffer containing $MgCl_2$ at a final concentration of 1.5 mM in the 1× reaction (Promega, Madison, WI), 2.0 μl of 2.5 mM dNTP Mix (Roche, Germany), 0.5 μl of each 20 pmol/μl primer pairs of mixed-primers (for set A, B, or C), 0.1 μl of 5 units/μl GoTaq DNA polymerase (Promega, Madison, WI), and 3.0 μl of cDNA template. The total PCR mixture was 25.0 μl. The amplification was performed for 30 cycles under the following thermal cycling conditions: 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were detected by electrophoresis through 1.5% agarose gel. Positive results of targeted viruses were assigned based on the expected size of PCR products corresponding to reference viruses. These PCR results were used for comparison with a novel multiplex PCR.

For the novel multiplex PCR, 10 pairs of specific primers for the detection of group A and C rotaviruses, adenovirus, norovirus GI, norovirus GII, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus were mixed in a single reaction (Table 1). The PCR amplification components contained 10.9 μl of MilliQ water, 5.0 μl of 5× Colorless GoTaq PCR buffer (containing $MgCl_2$ at a final concentration of 1.5 mM in the 1× reaction) (Promega, Madison, WI, USA), 2.0 μl of 2.5 mM dNTP Mix (Roche, Mannheim,Germany), 0.2 μl of each 20 pmol/μl of 10 primer pairs, 0.1 μl of 5 units/μl GoTaq DNA polymerase (Promega, Madison, WI, USA), and 3.0 μl of cDNA template. Then, the amplification was performed for 35 cycles under the following thermal cycling conditions: 94 °C for 1 min, 48 °C for 1 min, 72 °C 1 min 15 s and a final extension at 72 °C for 10 min. The PCR product sizes were determined by electrophoresis through 2.5% agarose gel. The gel was stained with SYBR Safe (Invitrogen, CA, USA) and then visualized under ultraviolet light source. All 10 reference targeted viruses which were identified previously in the laboratory were used for standardizing the specificity of this novel multiplex PCR. The primer sequences and expected PCR product sizes are shown in Table 1. When samples from the PCR results were not concordant by conventional and novel methods, monoplex PCR was performed again as a confirmation test.

A total of 235 stool samples were screened by the conventional multiplex PCR method using 3 sets of primers, and it was observed that 109 (46.4%) of them were positive for 8 types of the target viruses. Group A rotavirus was seen to be the most prevalent virus detected in this study (28.5%, 31 out of 109), followed by norovirus GII (22.9%, 25 out of 109), enterovirus (12.9%, 14 out of 109), adenovirus (7.3%, 8 out of 109), parechovirus (6.4%, 7 out of 109), group C rotavirus (4.6%, 5 out of 109), sapovirus (2.8%, 3 out of 109), and astrovirus (1.8%, 2 out of 109). In addition, mixed-infection among 2 or 3 viruses were observed as well (12.8%, 14 out of 109). The