Short communication

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

Pattara Khamrind, Makiko Okame, Aksara Thongprachum, Nattika Nantachat, Shuichi Nishimura, Shoko Okitsu, Niwat Maneekarn, Hiroshi Ushijima

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.

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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 viral 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
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 BE9 and VP7-1, ADG9-1F and ADG9-1R, G8N1 and G8N2, 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, 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 MgCl2 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 for 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

<table>
<thead>
<tr>
<th>Virus and primer</th>
<th>Sequence 5’–3’</th>
<th>Sense</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
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<td>Astrovirus</td>
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<td>PreCAP1</td>
<td>GGA CTG CAA AAC AGC TAC TGT</td>
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<td>82b</td>
<td>GTG ACC CAC CAG CCA TCC CT</td>
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<td>Group A rotavirus</td>
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<td>VP7 1(F)</td>
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<td>Ad1</td>
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<td>F1</td>
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<td>COG2F</td>
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<td>GCMP-F</td>
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<td>C94b</td>
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<td>SVL5317</td>
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patterns of mixed-infections were a wide variety. Triple infection among group A rotavirus/norovirus GII/enterovirus was found in 1 sample. Mixed-infection of 2 viruses between enterovirus and norovirus GII and between enterovirus and group A rotavirus were detected in 3 and 2 samples, respectively. Each mixed-infection of sapovirus with astrovirus or enterovirus or parechovirus, also was observed. In addition, mixed-infections of parechovirus with group A rotavirus or norovirus GII or adenovirus or enterovirus were found in each sample. One other sample was found to have a mixed-infection between adenovirus and astrovirus. In this panel of specimens tested, norovirus GII, group B rotavirus, Aichi virus, or bocavirus were detected.

To evaluate the efficiency of new multiplex PCR method, 10 known cDNA of the targeted viruses were used also as the template for the novel multiplex PCR method. As shown in Fig. 1, the expected PCR product sizes for the targeted viruses were observed in agarose gel at a 100 bp and between enterovirus and group A rotavirus were detected in 2 samples, respectively. Each mixed-infection of sapovirus with astrovirus or enterovirus or parechovirus, also was observed. In addition, mixed-infections of parechovirus with group A rotavirus or norovirus GII or adenovirus or enterovirus were found in each sample. One other sample was found to have a mixed-infection between adenovirus and astrovirus. In this panel of specimens tested, no norovirus GII, group B rotavirus, Aichi virus, or bocavirus were detected.

Fig. 1. Agarose gel electrophoresis demonstrating the expected PCR product sizes of 10 viruses detected from clinical samples by the novel multiplex PCR method. Lanes 1–10 represent the specific viruses detected: sapovirus (100 bp), Aichi virus (158 bp), group C rotavirus (205 bp), parechovirus (270 bp), norovirus GII (330 bp), norovirus GII (387 bp), enterovirus (440 bp), adenovirus (482 bp), group A rotavirus (569 bp), and astrovirus (719 bp). Lane 10 is the negative control and M represents the standard 100 bp DNA ladder marker.

was found in each sample. Another sample had mixed-infection between adenovirus and parechovirus. It was, however, observed that the results with 51 samples were not concordant between the conventional multiplex PCR and novel multiplex PCR. In this case, a monoplex PCR was carried out as the confirmatory test, and it was interesting to note that the results with 33 samples by the novel multiplex PCR and 18 samples by the conventional multiplex PCR were in agreement with the monoplex PCR.

Viral gastroenteritis is one of the most common illnesses in humans worldwide, and different viral agents have been described to associate with the disease. The clinical presentation of patients with acute gastroenteritis symptoms is not generally indicative of a specific pathogen. Therefore, a rapid, sensitive, and specific diagnostic test would be helpful in the administration of appropriate treatment for the patients. For this reason, a new multiplex PCR method was developed for the detection of 10 pathogenic viruses that are currently known to be associated with diarrheal disease in humans (group A and C rotaviruses, adenovirus, norovirus GII, norovirus GI, sapovirus, astrovirus, Aichi virus, parechovirus, and enterovirus). The advantage of a novel multiplex PCR is that 10 targeted viruses can be detected simultaneously in a single tube. The method could differentiate multiple types of viruses on the basis of different amplicon sizes by combining the primers from conventional multiplex PCR methods (set A, B, and C). However, in a novel multiplex PCR, the detection of group B rotavirus and bocavirus was omitted, because the prevalence rates of these 2 viruses were much lower than those of other viral pathogens. It was observed clearly that the newly designed specific primers for the detection of group A and C rotaviruses, Aichi virus, and sapovirus could be used in combination with the primers reported previously for the detection of these viruses in clinical samples. In comparison between conventional and novel multiplex PCR methods, the overall detection rates for viruses causing diarrhea were not different (46.4% versus 47.2%). However, a novel multiplex PCR requires a shorter time to perform the test. The non-concordant results of the clinical samples between these 2 multiplex PCR methods were confirmed by monoplex PCR, and it was shown clearly that the novel multiplex PCR was in better agreement with mono-
plex PCR results when compared to the conventional multiplex PCR method. It might be possible that newly designed primers incorporated in this novel multiplex PCR were designed based on virus sequences circulating recently and, therefore, could detect recent strains circulating in patients. A problem encountered frequently with the multiplex PCR assay is a reduction in sensitivity, due to competition for reagents when multiple templates are amplified in a single reaction. Therefore, the sensitivity and specificity of this assay should be validated and evaluated further on a larger scale of clinical specimens and compared with the conventional monoplex PCR. The preliminary results showed the potential application of the novel multiplex PCR method as a rapid and cost-effective diagnostic tool for detecting a variety of viruses causing diarrhea.

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References


