Identification and Characterization of Two Strains of Human Parechovirus 4 Isolated from Two Clinical Cases in Fukuoka City, Japan

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Reverse transcription-PCR targeting the VP0 gene of human parechoviruses (HPeVs) was used to identify two isolates from two Japanese children’s stool specimens. Molecular analysis revealed that these isolates belonged to HPeV type 4, and their nucleotide identity in the P1 region was 85.0%.

Human parechoviruses (HPeVs), members of the Parechovirus genus of the Picornaviridae family, are genetically classified into six types. HPeVs have been associated with gastrointestinal and respiratory symptoms and also with severe symptoms such as transient paralysis and neonatal sepsis in young children (3, 5). HPeV type 1 (HPeV1) and HPeV3 infections are common worldwide (5, 6); however, other HPeV infections, especially HPeV4 infections, are less common. HPeV4 strains have been isolated from young children with fever, TORCH syndrome, and lymphadenitis in a few instances (2, 10, 11).

This paper describes the identification and characterization of HPeV4 strains using molecular and immunological techniques. In 2001 and 2005, we isolated viruses from stool specimens from a 1-year-old girl with herpangina and from a 3-year-old boy with acute gastroenteritis, respectively, in Fukuoka City, Japan. The stool specimens were cultured weekly at 37°C for three passages on Caco-2, RD-18S, VeroE6, and HEp-2 cells. Picornaviridae-like cytopathic effects were observed in the Caco-2, RD-18S, and VeroE6 cell lines. To identify these isolates, a molecular typing method based on reverse transcription-PCR (RT-PCR) and direct sequencing was carried out. Viral RNAs were extracted from cell culture supernatants by using a QIAamp viral RNA mini kit (Qiagen, Germany). RT-PCRs were performed with specific primers (187/011, 188/011, and 189/011 for the enterovirus VP1 gene and E23P1/HPV-N1 for the HPeV VP0 gene) (5, 9).

Table 1 shows the molecular typing results from the cell culture samples. In case 1, the Caco-2 and RD-18S cell culture samples were positive by RT-PCR for enterovirus, and human coxsackievirus group A type 2 was identified by direct sequencing of the VP1 amplicon. In both case 1 and 2, all of the Caco-2, RD-18S, and VeroE6 culture samples were positive for HPeV by RT-PCR. The VeroE6 culture sample from case 1 and the Caco-2 culture sample from case 2 were designated Fuk2001-282 and Fuk2005-123, respectively. These amplicon sequences were compared with sequences from HPeV reference strains HPeV1 Harris, HPeV2 Williamson, HPeV3 A308-99, HPeV4 K251176-02, HPeV4 T75-4077, HPeV5 T92-15, and HPeV6 NIH561-2000.

Both Fuk2001-282 and Fuk2005-123 showed high similarities to the VP0 gene of the HPeV4 reference strains (positions 710 to 1,339 on HPeV1 Harris); the nucleotide identities of Fuk2001-282 to HPeV4 K251176-02 and HPeV4 T75-4077 were 84.9% and 89.5%, respectively, and those of Fuk2005-123 to K251176-02 and T75-4077 were 85.4% and 85.2%, respec-

**Table 1.** Results of molecular typing based on RT-PCR and direct sequencing*

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Date of onset</th>
<th>Clinical diagnosis</th>
<th>Specimen</th>
<th>Cell culture</th>
<th>Molecular typing result</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>F</td>
<td>September 2001</td>
<td>Herpangina</td>
<td>Stool</td>
<td>Caco-2, RD-18S, VeroE6</td>
<td>CA2, HPeV4</td>
<td>Fuk2001-282</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>M</td>
<td>December 2005</td>
<td>Acute gastroenteritis</td>
<td>Stool</td>
<td>Caco-2, RD-18S, VeroE6</td>
<td>CA2, HPeV4</td>
<td>Fuk2005-123</td>
</tr>
</tbody>
</table>

* F, female; M, male; CA2, human coxsackievirus group A type 2.

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The identity between Fuk2001-282 and Fuk2005-123 was 85.4%; therefore, these strains were not genetically identical (data not shown).

The full-length nucleotide sequence of the Fuk2005-123 strain was determined by using a primer-walking strategy. The complete genome of Fuk2005-123 was 7,349 nucleotides long, containing an open reading frame of 6,549 nucleotides. HPeV1, -2, -4, -5, and -6 contain RGD (arginine-glycine-aspartic acid) motifs, which are known to associate with cell attachment (4, 5). Fuk2005-123 also contained an RGD motif at the C terminus of the VP1 gene.

A SimPlot analysis of complete sequences of the reference HPeV strains against the sequence of the Fuk2005-123 strain was performed to analyze genetic relationships and recombination events between HPeVs (Fig. 1) (8). Nucleotide sequence alignments were generated with MEGA software, version 3.1 (7), and the plots were created with SimPlot software, version 3.5.1. The plots for the P1 region show that Fuk2005-123 is relatively closer to the HPeV4 reference strains than to the HPeV1, -2, -3, -5, and -6 reference strains, whereas in the P2, P3, and untranslated regions, the identities between Fuk2005-123 and the reference strains vary. Therefore, the P1 region is suitable for comparing nucleotide identities among HPeVs. Recent studies reveal that recombination plays a role in the evolution of HPeVs (1); however, the recombination breakpoint of Fuk2005-123 was not detected.

A phylogenetic tree based on the P1 region nucleotide sequences of Fuk2001-282, Fuk2005-123, and the HPeV reference strains was constructed by the neighbor-joining method using MEGA (Fig. 2). These strains cluster into six groups; Fuk2001-282 and Fuk2005-123 belong to the HPeV4 group. Fuk2001-282 is closely related to the HPeV4 NII370-93 reference.
ence strain, which was isolated from a 5-year-old Japanese boy with lymphadenitis in 1993 (11). The P1 region nucleotide and amino acid identities between the Fuk2001-282 strain, the Fuk2005-123 strain, and the HPeV4 reference strains were 84.2 to 88.3% and 97.4 to 97.9%, respectively (Table 2); these results indicate that Fuk2001-282 and Fuk2005-123 are HPeV4 strains. The nucleotide identity between Fuk2001-282 and Fuk2005-123 was only 85.0%, although these strains were isolated in the same region within only four years of each other.

To identify HPeV4 by immunological assay, neutralization tests were performed using a specific antiserum against the Fuk2005-123 strain (12). The antiserum was obtained from a rabbit that had been injected subcutaneously with purified strain Fuk2005-123 at the Nippon Biotest Laboratories (Japan) (11). The antiserum against Fuk2005-123 did not cross-react with HPeV1, -2, -3, and -6 strains and neutralized both the Fuk2001-282 and Fuk2005-123 strains (data not shown).

In our infectious disease surveillance, two HPeV4 strains, Fuk2001-282 and Fuk2005-123, were isolated in Fukuoka City. HPeV4 strains have rarely been isolated in Japan; the first isolation was described by Watanabe et al. (11), and this report is the second. The results of our molecular study demonstrate that the Fuk2001-282 and Fuk2005-123 strains are genetically distinct; however, it is uncertain whether these strains are indigenous or nonindigenous to Fukuoka City. In the future, a seroepidemiological study should be undertaken to understand the epidemiology of HPeV4 infection in Japan.

**Nucleotide sequence accession numbers.** The full-length sequence of Fuk2005-123 and the partial sequence of the P1 region of Fuk2001-282 have been deposited in GenBank under accession numbers AB433629 and AB433630, respectively.

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### REFERENCES


