Human parechovirus infections and child myositis cases associated with genotype 3 in Osaka City, Japan, 2014

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Human parechovirus (HPeV) infects humans early in life and typically causes asymptomatic or mild diseases such as gastrointestinal and respiratory illness but sometimes leads to more serious consequences in neonates and young infants. In 2014, we detected HPeV from 38 patients by real-time reverse transcription-PCR in Osaka City, Japan, and 33 HPeV strains were genotyped based on their VP1 sequences. HPeV genotype 3 (HPeV-3) was the most prevalent and accounted for 22 cases (66.7 %) followed by nine HPeV-1 (27.3 %), one HPeV-2 (3.0 %) and one HPeV-4 (3.0 %). Phylogenetic analysis revealed that detected HPeV-3 strains were divided into three genetically distinct groups. One was characterized by a novel single amino acid deletion mutation at the N terminus of the 2A protein as well as the VP1 sequence, whereas the others were closely related to HPeV-3 strains detected in Japan in either 2008 or 2011. These HPeV-3 groups were detected from patients with various symptoms including three myositis cases. Recent papers have demonstrated that HPeV-3 was the aetiological agent for epidemic myalgia exclusively among adults from Yamagata Prefecture in Japan. Here, we provide clinical details and episodes of three myositis patients including an adult and two children in Osaka City, Japan. Our results suggest that HPeV-3 is a causative agent of myositis not only in adults but also in children.

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INTRODUCTION

Human parechovirus (HPeV) belongs to the genus *Parechovirus*, a member of the family *Picornaviridae*, which is characterized by non-enveloped viral particles harbouring a positive-sense ssRNA genome. The HPeV genome encodes a single polyprotein sandwiched between 5′ and 3′ UTRs. This precursor polyprotein undergoes proteolytic cleavage to give rise to mature viral proteins. The viral capsid proteins (VP0, VP3 and VP1) are encoded near the N terminus of the polyprotein, and non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) are located downstream (Stanway & Hyypia¨, 1999).

HPeV-1 and -2 were formerly called echovirus 22 and 23, respectively, and were later reclassified as members of the genus *Parechovirus* (Wigand & Sabin, 1961; Ghazi *et al*., 1998; Mayo & Pringle, 1998). A third HPeV (HPeV-3) was reported in 2004, and since then, up to 17 genotypes of HPeV have been identified and classified based on VP1 sequences (*Ito et al*., 2004; *Benschop et al*., 2006; *Al-Sunaidi et al*., 2007; *Watanabe et al*., 2007; *Benschop et al*., 2008; *Kim Pham et al*., 2010; *Pham et al*., 2011; *Nix et al*., 2013; *Chuchaona et al*., 2015).

The majority of HPeV infections appear to occur early in life without specific symptoms; however, disease manifestations involved with many of the currently described

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; HPeV, human parechovirus; RT, reverse transcription.
genotypes have been reported, ranging from gastroenteritis and respiratory infections to neurological disease, particularly in neonates (Harvala & Simmonds, 2009). Although HPeV-1 is frequently isolated from healthy, asymptomatic children, it generally causes mild gastrointestinal or respiratory illness, although more serious consequences of infection, such as myocarditis and encephalitis, have been reported (Stanway et al., 2000). Accumulating evidence shows that HPeV-3 seems to be associated with more severe diseases including sepsis-like syndrome, meningitis, encephalitis and hepatitis in neonates and young infants (Boivin et al., 2005; Harvala et al., 2010; Khatami et al., 2015).

In Japan, HPeV-3 epidemics occur every 2–3 years and, following this pattern, were observed in 2008, 2011 and 2014 (Mizuta et al., 2013; Aizawa et al., 2014). Mizuta et al. (2012) first demonstrated that HPeV-3 was associated with symptoms of myositis, also known as epidemic myalgia (Mizuta et al., 2012). Interestingly, the myalgia patients were exclusively adults and were reported only from Yamagata Prefecture, although many HPeV-3-infected children were observed in two prior HPeV-3 epidemics in Japan (Mizuta et al., 2012, 2013).

Here, we detected a high number of HPeV-3- and HPeV-1-infected patients with various symptoms in Osaka City, Japan, in 2014, and we describe not only HPeV-3-positive adult but also child myositis cases. Thus, our findings indicate that HPeV-3 infection may lead to myositis in both children and adults.

**METHODS**

**Sample collection.** During 2014, clinical specimens were obtained from patients in a virus surveillance system supported by 14 sentinel hospitals in Osaka City, Japan (Kaida et al., 2010).

**Myositis cases.** Clinical tests were performed only for routine diagnosis, and these data were obtained retrospectively after HPeV-3 detection. Informed consent was obtained from each patient or from the parents if the patient was a child.

**RNA extraction and cDNA synthesis.** Viral RNA was extracted from specimens using a QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription (RT) was performed using random hexamers and either Reverse Transcriptase XL (from avian myeloblastosis virus) (TaKaRa Bio) or SuperScript III (Thermo Fisher Scientific), as described previously (Iritani et al., 2014; Kaida et al., 2014).

**HPeV detection.** HPeV was detected by real-time RT-PCR targeting the 5’ UTR using a QuantiTect Multiplex PCR kit (Qiagen) and primer/probe set described previously (Nix et al., 2008). To conduct multiplex real-time RT-PCR, Cy5-labelled HPeV probe (AN257-Cy5: 5’-CCTRYGGGTACCTYCWGGGCATCCTTC) was constructed and used as described below.

**Testing for other viral pathogens.** Faecal specimens from patients showing gastrointestinal symptoms were tested for other gastroenteritis viruses. Rotavirus A and adenovirus 40/41 were tested by ELISA as described previously (Iritani et al., 2014). Norovirus was tested by real-time RT-PCR as described previously (Kageyama et al., 2003). Sapovirus, human astrovirus and HPeV were tested by multiplex real-time RT-PCR using a QuantiTect Multiplex PCR kit (Qiagen) and primer/probe sets for sapovirus (labelled with 6-carboxyfluorescein) (Oka et al., 2006), human astrovirus (HAstV-F: 5’CTACAGAAGAGCAACCTCCATCG; HAstV-R: TTGGTAGCCATTCTCACCTTGTG; HAstV-probe: VIC-CATTTGAGGCGAGGAC) and HPeV (labelled with Cy5) (Nix et al., 2008). Specimens from patients with respiratory symptoms and exanthematous disease were tested for other respiratory viruses and rash-associated viruses, respectively, by multiplex real-time PCR as described previously (Kaida et al., 2012, 2014). Specimens from patients exhibiting other symptoms were checked by virus isolation tests with Vero and RD-185 cells as described previously (Kaida et al., 2011).

**RT-PCR and sequencing.** HPeV-positive samples by real-time RT-PCR were used for further analysis. The VP1 region of HPeV was amplified by nested RT-PCR using Ex Taq DNA polymerase (TaKaRa Bio) as described previously (Pham et al., 2010). The PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and used for direct sequencing with a BigDye Terminator Cycle Sequencing kit (Thermo Fisher Scientific) on a 3130 Genetic Analyzer (Thermo Fisher Scientific). Samples in which the VP1 region failed to be amplified were confirmed by sequencing of the 5’ UTR with the primers used in real-time RT-PCR.

**HPeV genotyping and phylogenetic analysis.** The determined nucleotide sequence of detected HPeV was used as the query in a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the genotype of the top-scoring HPeV reference strain was determined as the genotype of a query sequence. Nucleotide alignment was confirmed by CLUSTALW in MEGA 6.0 software (Tamura et al., 2013). On the basis of Akaika information criteria with a correction for finite sample sizes, the optimal evolutionary model that best fitted each sequence dataset was identified, and a maximum-likelihood tree with 1000 bootstrap replications was constructed using MEGA 6.0 software (Tamura et al., 2013). GENETYX v.11 (GENETYX) was used for amino acid alignment of the VP1/2A protein.

**Statistical analysis.** χ² tests were conducted to determine whether the frequencies of symptoms were significantly different between HPeV genotypes. A value of P<0.05 was considered significantly different.

**RESULTS**

**HPeV genotypes in Osaka City**

The HPeV screening was conducted with 364 specimens derived from 297 patients with various symptoms including fevers (11.8 %), neurological symptoms (17.5 %), respiratory symptoms (18.2 %), exanthematous disease (6.4 %), gastroenteritis symptoms (40.1 %) and other symptoms (arthralgia, myositis, sepsis, myocarditis, pericarditis, heart arrest, apnoeic events and shock with peripheral circulation failure) (6.1 %). We detected HPeV in blood, cerebrospinal fluid, faeces, nasal mucus and throat swab specimens of 38 patients by real-time RT-PCR targeting a well-conserved 5’ UTR region (Table 1). Twenty-four patients (63.2 %) were infants younger than 1 year old including eight neonates (<1 month old) (Table 1). HPeV was the only pathogen present in all specimens except for a throat swab of patient no. 12, in which human coronavirus OC43 was also detected (see Methods).
### Table 1. Characteristics of the 38 patients in whom HPeV was detected in Osaka City, Japan, 2014, and the HPeV genotypes

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Sex</th>
<th>Strain ID</th>
<th>Month sampled</th>
<th>HPeV-positive clinical samples</th>
<th>Symptoms or signs</th>
<th>HPeV genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 days</td>
<td>M</td>
<td>Po14-01</td>
<td>May</td>
<td>Cerebrospinal fluid, faeces, throat swab</td>
<td>Fever (40 °C)</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>2</td>
<td>21 days</td>
<td>M</td>
<td>Po14-02</td>
<td>May</td>
<td>Cerebrospinal fluid</td>
<td>Fever (39 °C), papules</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>3</td>
<td>1 year</td>
<td>F</td>
<td>Po14-03</td>
<td>June</td>
<td>Faeces</td>
<td>Fever (38.5 °C), gastroenteritis, diarrhoea</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>4</td>
<td>18 days</td>
<td>M</td>
<td>Po14-04</td>
<td>June</td>
<td>Faeces, nasal mucus</td>
<td>Fever (39 °C), rash, erythema, sepsis, apnoea</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>5</td>
<td>0 months</td>
<td>M</td>
<td>Po14-05</td>
<td>June</td>
<td>Cerebrospinal fluid</td>
<td>Fever (39 °C)</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>6</td>
<td>6 months</td>
<td>M</td>
<td>Po14-06</td>
<td>June</td>
<td>Blood</td>
<td>Fever (39.5 °C), papules</td>
<td>HPeV+</td>
</tr>
<tr>
<td>7</td>
<td>1 month</td>
<td>F</td>
<td>Po14-07</td>
<td>June</td>
<td>Cerebrospinal fluid</td>
<td>Fever (38.5 °C)</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>8</td>
<td>0 months</td>
<td>M</td>
<td>Po14-08</td>
<td>June</td>
<td>Cerebrospinal fluid</td>
<td>Fever (38.6 °C)</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>9</td>
<td>1 year</td>
<td>F</td>
<td>Po14-09</td>
<td>July</td>
<td>Throat swab</td>
<td>Rash</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>10</td>
<td>3 years  9 months</td>
<td>M</td>
<td>Po14-10</td>
<td>July</td>
<td>Faeces</td>
<td>Abdominal pain</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>11</td>
<td>2 months</td>
<td>M</td>
<td>Po14-11</td>
<td>July</td>
<td>Cerebrospinal fluid</td>
<td>Fever (38.8 °C), vomiting</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>12</td>
<td>6 years  7 months</td>
<td>M</td>
<td>Po14-12</td>
<td>July</td>
<td>Faeces, throat swab</td>
<td>Fever (40 °C), myositis, pharyngitis, conjunctivitis</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>13</td>
<td>2 months</td>
<td>M</td>
<td>Po14-13</td>
<td>July</td>
<td>Blood, cerebrospinal fluid, faeces, nasal mucus</td>
<td>Fever (39.1 °C), papules, symptoms of shock, peripheral circulatory failure</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>14</td>
<td>1 month</td>
<td>F</td>
<td>Po14-14</td>
<td>July</td>
<td>Faeces, throat swab</td>
<td>Fever (39 °C)</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>15</td>
<td>1 month</td>
<td>F</td>
<td>Po14-15</td>
<td>July</td>
<td>Faeces, throat swab</td>
<td>Fever (38.9 °C)</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>16</td>
<td>8 months</td>
<td>M</td>
<td>Po14-16</td>
<td>July</td>
<td>Faeces</td>
<td>Fever (38.5 °C), rash, erosion of tongue, non-pitting oedema</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>17</td>
<td>3 years  2 months</td>
<td>M</td>
<td>Po14-17</td>
<td>August</td>
<td>Faeces</td>
<td>Upper airway inflammation, diarrhoea</td>
<td>HPeV-4</td>
</tr>
<tr>
<td>18</td>
<td>1 month</td>
<td>F</td>
<td>Po14-18</td>
<td>July</td>
<td>Cerebrospinal fluid, nasal mucus</td>
<td>Fever (40 °C)</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>19</td>
<td>1 month</td>
<td>M</td>
<td>Po14-19</td>
<td>August</td>
<td>Cerebrospinal fluid, faeces, throat swab</td>
<td>Fever</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>20</td>
<td>1 month</td>
<td>F</td>
<td>Po14-20</td>
<td>August</td>
<td>Faeces, throat swab</td>
<td>Fever (39 °C), hepatic dysfunction</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>21</td>
<td>0 months</td>
<td>M</td>
<td>Po14-21</td>
<td>August</td>
<td>Faeces</td>
<td>Fever (38.9 °C), upper airway inflammation, rash, erythema</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>22</td>
<td>0 months</td>
<td>M</td>
<td>Po14-22</td>
<td>August</td>
<td>Blood, cerebrospinal fluid, faeces, nasal mucus</td>
<td>Fever (39 °C), encephalitis, convulsion</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>23</td>
<td>0 months</td>
<td>F</td>
<td>Po14-23</td>
<td>August</td>
<td>Cerebrospinal fluid, throat swab</td>
<td>Fever (38 °C)</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>24</td>
<td>8 months</td>
<td>F</td>
<td>Po14-24</td>
<td>September</td>
<td>Faeces</td>
<td>Diarrhoea</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>25</td>
<td>22 years</td>
<td>M</td>
<td>Po14-25</td>
<td>September</td>
<td>Faeces, throat swab</td>
<td>Fever (40 °C), headache, arthralgia, myositis, hypotension, conjunctivitis, hepatic dysfunction, pharyngitis, sepsis</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>26</td>
<td>7 months</td>
<td>M</td>
<td>Po14-26</td>
<td>September</td>
<td>Faeces, throat swab</td>
<td>Fever (38.4 °C), pharyngitis, herpangina</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>27</td>
<td>1 year   2 months</td>
<td>M</td>
<td>Po14-27</td>
<td>September</td>
<td>Faeces</td>
<td>Fever (38.8 °C), pneumonia, diarrhoea</td>
<td>HPeV-2</td>
</tr>
<tr>
<td>28</td>
<td>9 months</td>
<td>F</td>
<td>Po14-28</td>
<td>September</td>
<td>Faeces</td>
<td>Fever (38.3 °C), papules</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>29</td>
<td>6 years  11 months</td>
<td>M</td>
<td>Po14-29</td>
<td>September</td>
<td>Faeces</td>
<td>Vomiting, abdominal pain</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>30</td>
<td>6 months</td>
<td>M</td>
<td>Po14-30</td>
<td>October</td>
<td>Faeces</td>
<td>Diarrhoea, vomiting</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>31</td>
<td>1 year</td>
<td>F</td>
<td>Po14-31</td>
<td>October</td>
<td>Faeces</td>
<td>Diarrhoea</td>
<td>HPeV+</td>
</tr>
</tbody>
</table>
The VP1 region of 33 HPeV strains was amplified by RT-PCR and classified into nine HPeV-1, one HPeV-2, 22 HPeV-3 and one HPeV-4 genotype by sequencing and BLAST search analysis (Table 1). HPeV-1 was detected mainly in patients with gastroenteritis symptoms including diarrhoea, vomiting and/or abdominal pain (6/9, 66.7 %). HPeV-2 and -4 were detected from faecal specimens of patients showing respiratory tract symptoms and diarrhoea. Although HPeV-3 was detected from patients showing various symptoms, most cases (20/22, 90.9 %) were accompanied by fever. More severe symptoms such as sepsis-like syndrome (patient nos 4 and 25), shock with peripheral circulation failure (patient no. 13) and encephalitis (patient no. 22) were seen in four cases (18.2 %) (Table 1). HPeV-3 was also detected from three myositis cases (patient nos 12, 25 and 32; 13.6 %) and four patients who developed gastroenteritis symptoms (patient nos 3, 10, 11 and 32; 18.2 %) (Table 1). When compared with HPeV-1 patients, HPeV-3 patients had significantly more fevers ($\chi^2 = 5.1; P=0.024$) and fewer gastroenteritis symptoms ($\chi^2 = 8.7; P=0.003$).

The seasonal transition of HPeV genotypes is shown in Fig. 1. HPeV-3 detection peaked in July (summer), while HPeV-1 was mainly detected during September and November (autumn) after HPeV-3 levels declined (Fig. 1).

### Phylogenetic analysis of HPeVs

HPeV strains detected during 2014 in Osaka City were named using a combination of ‘Po14-’ and the case number (strain ID in Table 1). Among the HPeV-1 strains detected in this study, VP1 sequences shared 86.4–100 % nucleotide identity and 92.2–100 % amino acid identity. Five strains (Po14-16, Po14-29, Po14-30, Po14-34 and Po14-35) were closely related to one another with $\geq 97.6$ % nucleotide identity, whereas the nucleotide identity of the other combinations was $< 95$ %, even between Po14-24 and Po14-28 (94.1 %) (Fig. 2b).

HPeV-2 is rare worldwide, and the VP1 gene sequences of only seven HPeV-2 strains are accessible in GenBank. HPeV-2 strain Po14-27, detected from patient no. 27, showed a sequence identity of 77.8–84.1 % and 95.1–99.1 % at the nucleotide and amino acid levels, respectively, with the other HPeV-2 reference strains (Fig. 2a).

The detected HPeV-3 strains shared 94.1–100 % nucleotide identity and 98.1–100 % amino acid identity with one another. These strains were divided into three genetically distinct groups. One was more closely related to strains isolated in Yamagata Prefecture in 2008 (lineage A), and another was more closely related to strains isolated in Yamagata Prefecture in 2011 (lineage B) (Fig. 2c). A previous study showed that these strains were distinct from one another but were detected from myositis patients (Mizuta et al., 2013). The third group, comprising seven strains (Po14-02, Po14-08, Po14-09, Po14-10, Po14-11, Po14-14 and Po14-32), was distinct from the other detected strains in Osaka City with 94.1–96.9 % nucleotide identity (lineage C) (Fig. 2c). In addition, lineage C was characterized by its unique 2A sequence. The PCR method for amplification of the HPeV VP1 region reported by Pham et al. (2010) allowed us to sequence part of the 2A region. We found that the detected HPeV-3 strains in lineage C possessed one amino acid deletion in the N terminus of the 2A protein. As shown in Fig. 3, the deletion mutation

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Sex</th>
<th>Strain ID</th>
<th>Month sampled</th>
<th>HPeV-positive clinical samples</th>
<th>Symptoms or signs</th>
<th>HPeV genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>6 years</td>
<td>M</td>
<td>Po14-32</td>
<td>October</td>
<td>Throat swab</td>
<td>Fever (37.5 °C), erythematous rash, myositis, pharyngitis, stomatitis, diarrhoea, upper airway inflammation</td>
<td>HPeV-3</td>
</tr>
<tr>
<td>33</td>
<td>1 year</td>
<td>M</td>
<td>Po14-33</td>
<td>October</td>
<td>Throat swab</td>
<td>Fever (38.9 °C), convulsion, upper airway inflammation</td>
<td>HPeV*</td>
</tr>
<tr>
<td>34</td>
<td>2 months</td>
<td>F</td>
<td>Po14-34</td>
<td>October</td>
<td>Faeces, nasal mucus</td>
<td>Fever (38 °C), gastroenteritis</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>35</td>
<td>3 months</td>
<td>F</td>
<td>Po14-35</td>
<td>November</td>
<td>Faeces</td>
<td>Fever (39 °C), vomiting</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>36</td>
<td>1 year</td>
<td>M</td>
<td>Po14-36</td>
<td>November</td>
<td>Faeces</td>
<td>Convulsion, gastroenteritis, impaired consciousness</td>
<td>HPeV-1</td>
</tr>
<tr>
<td>37</td>
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<td>M</td>
<td>Po14-37</td>
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<td>Gastroenteritis</td>
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</tr>
<tr>
<td>38</td>
<td>1 year</td>
<td>M</td>
<td>Po14-38</td>
<td>December</td>
<td>Faeces</td>
<td>Fever (40.5 °C), diarrhoea, pneumonia, wheezing</td>
<td>HPeV*</td>
</tr>
</tbody>
</table>

M, male; F, female.

*HPeV* was confirmed by sequencing of the 5' UTR due to failure to amplify the VP1 region by RT-PCR.

Table 1. cont.
of one Asn residue (AAT or AAC codon) at position 779 was seen in all seven HPeV-3 strains in lineage C. This mutation was not found in any of the HPeV-3 strains accessible in GenBank. Glu, Arg and Ala residues at positions 764, 769 and 775, respectively, were also unique residues near the region in between VP1 and 2A in lineage C (Fig. 3).

HPeV-4 strain Po14-17 showed up to 97.0% nucleotide identity with the other HPeV-4 strains by BLAST search analysis (maximum with HPeV-4/652580, GenBank accession no. FJ373167, isolated in the Netherlands) (Fig. 2a).

**HPeV-3 from two children and an adult with myositis**

Clinical myositis is manifested by fever, chills, weakness, hypotonia, tenderness and oedema of the involved muscle groups. Myoglobinemia, myoglobinuria and an elevated creatine kinase (CK) level are often found (Modlin, 2010). The clinical details of individual HPeV-3-associated myositis cases in two children and an adult are described below.

Patient no. 12 (Po14-12), a 6-year-old boy, had fever, and subsequent pain in the gastrocnemius muscle of both legs 3 days later, resulting in hospitalization due to difficulties in standing and sitting. At this point, in addition to the symptoms shown in Table 1, his serum CK and myoglobin levels were 3101 IU l\(^{-1}\) and 1603 ng ml\(^{-1}\), respectively. On day 2 of hospitalization, his muscular pain was somewhat reduced, although the CK values were elevated up to 4807 IU l\(^{-1}\). His aldolase and aspartate aminotransferase (AST) levels were also beyond the normal range. Faecal and throat swab specimens, but not a urine sample, were positive for HPeV-3 (lineage A) (Fig. 2c). On day 3 of hospitalization, he recovered from the pain and was able to walk without the help of others. Myoglobinuria was not observed. On day 7 of hospitalization, his CK and AST levels returned to normal. He was discharged on day 9 following hospitalization.

Patient no. 32 (Po14-32), also a 6-year-old boy, had fever, coughing and diarrhoea at the disease onset. The next day, he felt discomfort in his extremities. Two days later, he had pain in the gastrocnemius muscle of both legs and had difficulty walking alone. An erythematous rash appeared on his extremities and face, and he was hospitalized 3 days after the disease onset. CK and myoglobin levels in his serum were 5613 IU l\(^{-1}\) and 1603 ng ml\(^{-1}\), respectively, at this point. AST and alanine aminotransferase (ALT) levels were also beyond the normal range. On day 2 of hospitalization, a throat swab specimen was collected and was positive for HPeV-3 (lineage C) (Fig. 2c). On day 4 of hospitalization, his muscular pain dissipated completely, although the CK level was elevated to 9294 IU l\(^{-1}\), but this then decreased to 1448 IU l\(^{-1}\) on day 6 of hospitalization. Myoglobinuria was not observed. His C-reactive protein value was normal throughout his hospitalization. He was discharged on day 8 of hospitalization.

Patient no. 25 (Po14-25), a 22-year-old male, had lower back pain at the disease onset. The next day, he had fever (38 °C), headache, and systemic joint and muscular pain. Two days after the onset, he was in a state of impaired consciousness with an increased body temperature (40 °C) and chills, and was subsequently hospitalized. His other vital signs (heart rate 130 beats min\(^{-1}\), 22 breaths min\(^{-1}\)) indicated that he had developed a sepsis-like illness. At this time, the CK value in his blood test was 72 IU l\(^{-1}\). On day 2 of hospitalization, his muscular pain and weakness worsened, his platelet counts decreased (58 000 µl\(^{-1}\)) and his ALT and AST levels were also beyond the normal range. Myelitis and Guillain–Barré syndrome were interpreted as negative by a neurologist. Faecal and throat swab specimens were collected and were positive for HPeV-3 (lineage B) (Fig. 2c). The CK value increased to 253 and 396 IU l\(^{-1}\) on days 3 and 7, respectively, of hospitalization. His muscular pain and weakness improved from day 4 of hospitalization and were absent by day 6 of hospitalization. His white blood cell counts and C-reactive protein level were normal throughout his hospitalization. No symptomatic improvement was recognized following antibiotic use. Notably, his 2-year-old daughter had symptoms of upper airway inflammation prior to his illness (a specimen was not collected from her). This situation is typical for epidemic myalgia and is similar to a previously reported case (Mizuta et al., 2013).

Taken together, all three myositis patients experienced pain in both legs, which prevented them from standing and walking, and exhibited the maximum CK value after the peak pain measurement. It is worth noting that all three HPeV-3 groups (i.e. lineages A–C) were detected in
DISCUSSION

Here, we have described HPeV genotypes detected from patients exhibiting various symptoms in Osaka City, Japan, in 2014. HPeV-1 and -3, the common HPeV genotypes, accounted for the majority of HPeV cases. The differences between HPeV-1 and -3 infections were observed in epidemic seasons and patient profiles (age and clinical manifestations) as described previously (Ito et al., 2010). Although HPeV-3 had not been detected during 2012–2013 in Osaka City (data not shown), the number of HPeV-3 cases increased from May 2014 and peaked in the summer, as observed previously in 2008 and 2011 in Japan (Yamamoto et al., 2009; Mizuta et al., 2013). In contrast, the detection of HPeV-2 is quite rare worldwide, even though it was first isolated in 1956 (Wigand & Sabin, 1961). HPeV-2 infection appears to be associated with gastrointestinal symptoms, but sometimes it is detected in asymptomatic patients as well (van der Sanden et al., 2008;...
Pham et al., 2010). In Japan, detection of HPeV-2 has not been recorded since 2003 according to an Infectious Agents Surveillance Report (http://www.nih.go.jp/niid/en/iars-e.html), and none of the Japanese HPeV-2 sequences have been deposited in online sequence databases. A detected HPeV-2 strain, Po14-27, from a gastroenteritis patient in this study was distinct from other known HPeV-2 strains with low (<85%) nucleotide identity; however, the amino acid identity was higher (>95%). In the case of HPeV-1 strains detected in Osaka City, these showed a higher nucleotide identity but lower amino acid identity (>86% and >92%, respectively) when compared with HPeV-2, indicating that the VP1 protein of HPeV-2 is more conserved than that of HPeV-1, despite a lower general time reversible plus gamma plus invariable sites model including 16 HPeV genotypes. Collapsed branches of HPeV-1 and -3 are expanded in (b) and (c), respectively. Solid squares indicate the strains detected in this study. Arrows indicate the strains detected from myositis patients. The percentage bootstrap support is indicated by the values at each node (values of <80% are omitted). GenBank accession numbers are indicated. Bars indicate genetic distances (nucleotide substitutions per site).

Fig. 2. Maximum-likelihood phylogram based on partial nucleotide sequences of the VP1 gene (HPeV-1, 678 nt; HPeV-2, 675 nt; HPeV-3, 663 nt; HPeV-4, 681 nt) of HPeV strains detected in Osaka City, Japan, in 2014. (a) Phylogenetic tree with the general time reversible plus gamma plus invariable sites model including 16 HPeV genotypes. Collapsed branches of HPeV-1 and -3 are expanded in (b) and (c), respectively. Solid squares indicate the strains detected in this study. Arrows indicate the strains detected from myositis patients. The percentage bootstrap support is indicated by the values at each node (values of <80% are omitted). GenBank accession numbers are indicated. Bars indicate genetic distances (nucleotide substitutions per site).
nucleotide identity. Reclusive HPeV-2 may mildly infect humans, probably asymptptomatically in most cases, and circulate while accumulating silent mutations in its genome.

Detected HPeV-3 strains were classified phylogenetically into three groups, one of which, not detected in past HPeV-3 epidemics in Japan, was characterized by not only its VP1 sequence but also a unique single amino acid deletion mutation positioned in the N terminus region of the 2A protein that has not been reported previously, to the best of our knowledge. However, this deletion does not seem to affect viral pathogenesis because no prominent differences were observed in clinical manifestations between patients infected with these three HPeV-3 groups.

In 2008 and 2011, HPeV-3-associated epidemic myalgia occurred in Yamagata Prefecture, Japan, exclusively in adults, although HPeV-3 outbreaks occurred among children in both years (Mizuta et al., 2012, 2013). While HPeV-3-associated myalgia in adults has hitherto been reported only from Yamagata Prefecture, here we found an adult patient in Osaka City, thus demonstrating that it is no longer an endemic disease in Yamagata Prefecture.

We also found two 6-year-old children infected with HPeV-3, exhibiting myositis symptoms. In another report, HPeV-3 isolated from a throat swab of an 8-year-old boy showing myositis was recorded, although neither the extent of the disease nor the aetiological link between HPeV-3 and myositis was mentioned (Watanabe et al., 2007). Taken together, our data provide further evidence that HPeV-3 infections may cause myositis not only in adults but also in children.

Pleurodynia (Bornholm disease), probably the most well-known viral myositis, is an acute enteroviral infection of skeletal muscle characterized by fever and sharp, spasmodic pain in the chest or upper part of the abdomen and has usually been observed during infrequent outbreaks (Modlin, 2010). Coxsackievirus B is the most important aetiological agent of epidemic pleurodynia, although other viruses including echovirus and coxsackievirus A are also implicated in this disease (Modlin, 2010). From our data and previous reports, HPeV-3 and myositis was mentioned (Watanabe et al., 2012, 2013). Instead, the myositis cases in this report, HPeV-3 isolated from a throat swab of an 8-year-old boy showing myositis was recorded, although neither the extent of the disease nor the aetiological link between HPeV-3 and myositis was mentioned (Watanabe et al., 2007). Taken together, our data provide further evidence that HPeV-3 infections may cause myositis not only in adults but also in children.

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Fig. 3. Alignment of amino acid sequences of VP1/2A region of HPeV-3 strains detected in Osaka City, Japan, during 2014. A typical HPeV-3, strain A308/99 (GenBank accession no. AB084913; Ito et al., 2004), was used for comparison and encodes a polyprotein composed of 2177 aa and VP1 protein located at aa 546–771 and followed by the 2A protein. Amino acid residues that differed from HPeV-3 reference strain A308/99 are shown. Amino acid deletions are shown by grey shading.
members may be stricken almost simultaneously or in rapid succession, separated by several days, with epidemic pleurodynia (Modlin, 2010). HPeV-3 caused different degrees of clinical manifestations among family members (Mizuta et al., 2013). However, as HPeV-3 is likely to cause myositis in both adults and children, multiple family members may develop symptoms of myositis derived from HPeV-3 infection similar to epidemic pleurodynia.

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REFERENCES


