Molecular epidemiological study of human rhinovirus species A, B and C from patients with acute respiratory illnesses in Japan

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Recent studies suggest that human rhinovirus species A, B and C (HRV-ABCs) may be associated with both the common cold and severe acute respiratory illnesses (ARIs) such as bronchiolitis, wheezy bronchiolitis and pneumonia. However, the state and molecular epidemiology of these viruses in Japan is not fully understood. This study detected the genomes of HRV-ABCs from Japanese patients (92 cases, 0–36 years old, mean ± SD 3.5 ± 5.0 years) with various ARIs including upper respiratory infection, bronchiolitis, wheezy bronchiolitis, croup and pneumonia between January and December 2010. HRV-ABCs were provisionally type assigned from the pairwise distances among the strains. On phylogenetic trees based on the nucleotide sequences of the VP4/VP2 coding region, HRV-A, -B and -C were provisionally assigned to 14, 2 and 12 types, respectively. The present HRV-A and -C strains had a wide genetic diversity (>30% divergence). The interspecies distances were 0.230 ± 0.063 (mean ± SD, HRV-A), 0.218 ± 0.048 (HRV-B) and 0.281 ± 0.105 (HRV-C), based on nucleotide sequences, and 0.075 ± 0.036 (HRV-A), 0.049 ± 0.022 (HRV-B) and 0.141 ± 0.064 (HRV-C) at the deduced amino acid level. Furthermore, HRV-A and -C were the predominant species and were detected throughout the seasons. The results suggested that HRV-A and -C strains have a wide genetic divergence and are associated with various ARIs in Japan.

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Abbreviations: ARI, acute respiratory illness; HRV-ABCs, human rhinovirus species A, B and C; NPS, nasopharyngeal swab; URI, upper respiratory infection.

The GenBank/EMBL/DDBJ accession numbers for the HRV sequences determined in this study are AB628096–AB628187.
INTRODUCTION

Human rhinoviruses (HRVs) are a group of positive-sense ssRNA viruses belonging to the genus *Enterovirus* in the family *Picornaviridae*. Previous reports have suggested that HRVs are responsible for various acute respiratory illnesses (ARIs) including the common cold, bronchiolitis and pneumonia (Turner & Couch, 2007). In addition, recent reports strongly suggest that HRVs may induce exacerbation of wheezing and/or asthma (virus-induced asthma) (Busse et al., 2010; Chung et al., 2007; Khadadah et al., 2010). Thus, these viruses may be associated with ARIs and other severe respiratory illnesses, such as wheezy bronchiolitis and asthma (Busse et al., 2010; Chung et al., 2007; Gern, 2009; Khadadah et al., 2010).

HRVs were previously classified into two species, species A (HRV-A) and B (HRV-B), containing over 100 serotypes (Turner & Couch, 2007). However, a genetically heterogeneous third species, HRV species C (HRV-C), was discovered recently (Lamson et al., 2006; McErlean et al., 2007). Among the species, HRV-A and -C appear to be associated mainly with ARIs and virus-induced asthma, whilst HRV-B has been detected in a relatively small number of patients with ARIs (Linsuwanon et al., 2009; Smuts et al., 2011). Notably, HRV-C species can be detected in most countries and may be associated with various ARIs including upper respiratory infection (URI), bronchiolitis, wheezy bronchiolitis and pneumonia (Smuts et al., 2011; Watanabe et al., 2010), although the epidemiology is not exactly known. However, it is not known whether HRV-A, -B and -C (HRV-ABCs) are associated with severe ARIs such as bronchiolitis, wheezy bronchiolitis and pneumonia. In addition, the epidemiology of HRV-ABCs detected from patients with ARIs is unclear in Asian areas, including Japan.

HRV-A and -C may have a wide genetic diversity (Mizuta et al., 2010; Wisdom et al., 2009). Indeed, our previous report indicated that HRV-A strains isolated from Japanese people with various ARIs showed >30% divergence based on sequences of the VP4/VP2 coding region and were classified into many clusters by phylogenetic analysis (Mizuta et al., 2010). It has been suggested recently that HRV-ABCs have a unique genetic diversity (McIntyre et al., 2010; Simmonds et al., 2010). In addition, HRV-ABCs may be type assigned using pairwise distances (p-distances) (McIntyre et al., 2010; Simmonds et al., 2010). Thus, we applied this method to provisionally type assign the detected strains of HRV-ABCs in the present study. On this basis, we performed a molecular epidemiological study of HRV-ABCs detected in Japanese patients with various ARIs including URI, bronchiolitis, wheezy bronchiolitis, croup and pneumonia.

METHODS

**Study samples.** A total of 501 nasopharyngeal swab (NPS) samples were collected from patients with ARI. Culture methods and RT-PCR were applied to all samples to detect the various respiratory viruses such as influenza viruses (subtypes A, B and C), parainfluenza viruses (types 1–4), adenoviruses, respiratory syncytial virus, human metapneumovirus and enteroviruses, as well as respiratory bacteria (Echevarria et al., 1998; Miura-Ochiai et al., 2007; Nakauchi et al., 2011; Parveen et al., 2006; Takao et al., 2004). HRV-A, -B or -C alone was detected in 92 of the NPS samples (18.4%) and genetic analysis of the strains was performed.

**Subjects.** The 92 Japanese patients in whom HRV-A, -B or -C was detected were enrolled in the present study. The samples were obtained by the local health authorities of Tochigi and Yamaguchi prefectures for the surveillance of viral diseases in Japan between January and December 2010. Informed consent was obtained from the subjects, or from the parents of underage subjects, for the donations of samples used in this study. Patients were diagnosed with URI and severe ARIs such as bronchiolitis, wheezy bronchiolitis, croup or pneumonia (Table 1). The diagnosis of ARIs was conducted as described previously (Cherry, 2003; Robert, 2003). All patients were aged 0–36 years (mean ± 3.5 ± 5.0 years).

**RNA extraction, RT-PCR and sequencing.** All procedures were performed as described previously (Mizuta et al., 2010). Briefly, for the extraction of viral RNA, RT-PCR and sequence analysis, NPS samples were centrifuged at 3000 g at 4 °C for 15 min and the supernatants were used for RT-PCR and sequence analysis, as described previously (Mizuta et al., 2010). Viral nucleic acid was extracted from the samples using a QIAamp Viral RNA Mini kit (Qiagen). The reverse transcription reaction mixture was incubated with random hexamers at 42 °C for 90 min, followed by incubation at 99 °C for 5 min and amplification by thermal cycling. Using this cDNA, part of the VP4/VP2 coding region was amplified by PCR with cycling parameters of 3 min at 94 °C for denaturation, followed by 40 cycles of 94 °C for 30 s, 60 °C for 1 min and 68 °C for 2 min, with a final elongation for 7 min at 68 °C. Purification of DNA fragments and nucleotide sequence determination were performed as described previously (Mizuta et al., 2010). We analysed the nucleotide sequences (nt 623–1012; 390 bp) and the deduced amino acid sequences (130 aa) of the VP4/VP2 coding region. We took general precautions to prevent any carry-over contamination of PCR, as described previously (Lam et al., 2007).

**Phylogenetic analysis and calculation of p-distances.** Phylogenetic analysis of the nucleotide sequences of the partial VP4/VP2 coding region of HRVs (nt 623–1012) was conducted using the CLUSTAL W program available on the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/top-j.html) and Tree Explorer (http://www.megasoftware.net/). Evolutionary distances were estimated according to Kimura’s two-parameter method and a phylogenetic tree was reconstructed using the neighbour-joining method (Kimura 1980; Saitou & Nei, 1987). The reliability of the tree was estimated with 1000 bootstrap replications. In addition, to assess interspecies frequency distributions of HRVs, we calculated p-distances for all of the strains, including the present strains and reference strains, as described previously (Mizuta et al., 2010).

** Provisional type assignment of HRV-ABCs in the present strains.** It has been suggested that HRV-C strains can be type assigned (Simmonds et al., 2010). In addition, previous reports suggest that, if the correct species p-distance values are used, this method may be applicable to the type assignment of HRV-A and -B, as well as HRV-C, on the phylogenetic trees (McIntyre et al., 2010; Simmonds et al., 2010). Thus, we thought that it would be possible to conduct type assignment of the present strains (HRV-ABCs). In this study, VP4/VP2 sequences that were available and which showed >10% divergence from other species in the sequences in this region were provisionally type assigned.
RESULTS

Seasonal variation of HRV-ABCs

HRV-ABCs were detected in 92 of 501 NPS samples from patients with ARIs (18.4%). HRV-A and -C were detected throughout the investigation period (Fig. 1). However, the monthly distribution differed among the species: HRV-A and -C were predominantly detected in April–June and September–December, respectively, whilst HRV-B was detected sporadically. HRV-A strains were the most prevalent (47/92; 51.1 %), followed by HRV-C (43/92; 46.7 %) and HRV-B (2/92; 2.2 %).

Phylogenetic analysis, provisional type assignment and homology analysis

First, we reconstructed phylogenetic trees based on the nucleotide (390 nt) and deduced amino acid (130 aa) sequences of HRV-A and -C with regard to the reference strains (Fig. 2a–d). Using these reference strains and the present strains, we then reconstructed phylogenetic trees based on the nucleotide sequences and deduced amino acid sequences of the VP4/VP2 coding region (Fig. 2e, f). Based on the nucleotide sequences, HRV-A, -B and -C were provisionally assigned to 14, 2 and 12 types, respectively, in the phylogenetic trees. The number of HRV-A strains in each type in the phylogenetic tree based on the nucleotide sequences was as follows: HRV45, three strains; HRV7, five strains; HRV46, one strain; HRV53, one strain; HRV20, four strains; HRV12, seven strains; HRV23, two strains; HRV40, six strains; HRV59, three strains; HRV76, two strains; HRV11, two strains; HRV19, one strain; HRV10, three strains and HRV16, seven strains. Two HRV-B strains were provisionally assigned to HRV3 and HRV17 based on the nucleotide sequences. The number of HRV-C strains in each type, based on nucleotide sequences, was as follows: g2-11 (HRV-C39), five strains; SA365412 (pat14), six strains; tu304 (HRV-C38), one strain; IN-36 (HRV-C49), one strain; g2-25 (HRV-C40), two strains; RV546 (pat18), two strains; Cd08-1009 U (pat28), one strain and g2-23 (HRV-C41), eight strains. In addition, four possibly unique types of HRV-C strains were found. The number of HRV-C strains assigned to each of these types was as follows: not typed (A) (HRV/Yamaguchi/2010/YU64 type), five strains; not typed (B) (HRV/Yamaguchi/2010/YU42 type), one strain; not typed (C) (HRV/Tochigi/2010/353), one strain; and not typed (D) (HRV/Yamaguchi/2010/YU74 type), 10 strains. The HRV-A and -C strains detected in most types were associated with bronchiolitis and/or wheezy bronchiolitis.

Interspecies p-distances of HRVs

We calculated the interspecies distances of HRVs by the distribution of p-distances (Figs 3 and 4). Among the present and reference strains, the interspecies distances based on the nucleotide sequences were 0.230 ± 0.063 (mean ± s.d., HRV-A), 0.218 ± 0.048 (HRV-B) and 0.075 ± 0.036 (HRV-C), 0.049 ± 0.022 (HRV-B) and 0.141 ± 0.064 (HRV-C) at the deduced amino acid level. HRV-C strains had the longest interspecies p-distances based on the nucleotide and amino acid sequences.

Table 1. Subject data in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>No. strains</th>
<th>Sex (male/female)</th>
<th>Age (year)*</th>
<th>Clinical symptoms</th>
<th>No. patients</th>
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<tr>
<td>HRV-A</td>
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<td>27/20</td>
<td>3.0 ± 5.3</td>
<td>URI</td>
<td>20</td>
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<td></td>
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<td></td>
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<td>19</td>
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<td></td>
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<td>Wheezy bronchiolitis</td>
<td>5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Croup</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pneumonia</td>
<td>2</td>
</tr>
<tr>
<td>HRV-B</td>
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<td>1/1</td>
<td>13.5 ± 13.4</td>
<td>URI</td>
<td>1</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>Bronchiolitis</td>
<td>1</td>
</tr>
<tr>
<td>HRV-C</td>
<td>43</td>
<td>25/18</td>
<td>3.6 ± 3.0</td>
<td>URI</td>
<td>9</td>
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<td></td>
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<td>Bronchiolitis</td>
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<td></td>
<td></td>
<td>Pneumonia</td>
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<tr>
<td>Total</td>
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<td>53/39</td>
<td>3.5 ± 5.0</td>
<td>URI</td>
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<td></td>
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<td>Pneumonia</td>
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</table>

*Data are expressed as mean ± s.d.
HRV-ABCs from patients with various ARIs in Japan

DISCUSSION

In this study, we detected HRV-ABCs from 92 Japanese patients with various ARIs such as URI, bronchiolitis, wheezy bronchiolitis, croup and pneumonia between January and December 2010. Based on the nucleotide sequences of the VP4/VP2 coding region, HRV-A and -C strains showed a wide genetic diversity (>30% divergence) and were classified into many types in the phylogenetic tree (Fig. 2). However, we detected HRV-B from only two cases of ARI. These results suggested that there are various types of HRV-A and -C strains, which may be associated with various ARIs in Japan.

Wisdom et al. (2009) suggested that HRV-A and -C are the predominant strains detected in patients with ARIs in the UK. Moreover, these strains showed a wide genetic diversity and were classified into many types in the phylogenetic tree (Wisdom et al., 2009). Smuts et al. (2011) demonstrated that HRV-ABCs were the predominant strains detected in African children with acute wheezing. In addition, our previous report suggested that HRV-A isolates from children with ARIs (in Yamagata prefecture, Japan) were classified into 11 clusters in the phylogenetic tree with >30% nucleotide divergence of the VP4/VP2 coding region (Mizuta et al., 2010). These results suggest that, in some countries, HRV-A and -C detected in ARI cases are the predominant strains and have varied genetic properties (Mizuta et al., 2010; Wisdom et al., 2009). We then calculated the interspecies distances and divergence of the strains and compared the results with previous reports. HRV-A had similar interspecies distances and divergence (Mizuta et al., 2010). In addition, the p-distances between HRV-C strains (0.281±0.105) were greater than those between HRV-A strains (0.230±0.063). Our present results thus appeared to be compatible with these earlier reports (McIntyre et al., 2010; Mizuta et al., 2010; Simmonds et al., 2010).

It has been suggested recently that the correct type assignment of HRV-C strains is possible if based on the p-distances within HRV-C (McIntyre et al., 2010; Simmonds et al., 2010). In these reports, VP4/VP2 coding regions that are available and that show <10% divergence from other HRV-C species sequences in the region may be provisionally type assigned (Simmonds et al., 2010). Another report suggested that divergence of the VP4/VP2 coding regions may not differ significantly among HRV-ABCs, although the divergence of HRV-C may be greater than that of HRV-A and -B (Simmonds et al., 2010). To the best of our knowledge, it is not known whether this method is applicable to the type assignment of other HRV species, such as HRV-A and -B. However, previous reports suggest that, if the correct species p-distance values are used, this method may also be applicable to the type assignment of HRV-A and -B in the phylogenetic trees (McIntyre et al., 2010; Simmonds et al., 2010). From these findings and our data, we carefully selected suitable reference strains for the type assignment of HRV-ABCs. Finally, through these processes, the present strains were provisionally assigned to various types in the phylogenetic trees. As a result, HRV-A, -B and -C in the phylogenetic trees in this study were classified into 14, 2 and 12 types, respectively. Notably, four unique types of HRV-C strain were found. Further studies may be needed to assign HRV-A and -B strains with certainty using more detailed genetic data.

HRVs were previously thought to be associated mainly with the common cold, producing mild respiratory symptoms (Gern, 2010; Turner & Couch, 2007). For this reason, the optimum propagation temperature of HRVs may be 32–35 °C in vitro (Papadopoulos et al., 1999; Schroth et al., 1999). However, a recent study suggested that HRVs can propagate in lower airway tissues and this may be an important factor in the development of airway obstruction, coughing and wheezing that can lead to bronchiolitis and pneumonia (Mosser et al., 2005). HRVs are being re-evaluated as an important agent of ARIs in humans (Imakita et al., 2000; Papadopoulos et al., 2002; Wos et al., 2008). A very recent study suggested that HRVs are a major agent in the induction of wheezing and exacerbation of asthma (Khadadah et al., 2010). In the present cases, HRV-A and -C were detected from patients with severe ARIs such as bronchiolitis, wheezy bronchiolitis, croup and pneumonia. In addition, another recent study has suggested a novel organ culture method to enable the propagation of HRV-C (Bochkov et al., 2011), although this species could not be isolated by previous cell-culture methods. Thus, details of the pathogenesis and epidemiology of HRV-ABCs may be elucidated in the future.

The viruses detected during the investigation period, as illustrated in Fig. 1, showed the seasonal variations of HRVs in Japan. HRV-B strains were detected twice, in February and November, whilst HRV-A and -C were the predominant species and were detected throughout the period. A recent study suggested that HRV-C has a stronger link to severe respiratory illness, such as virus-induced asthma, than HRV-A and -B strains (Miller et al., 2009). Indeed, our results revealed a similar significance (P<0.05). Thus, HRV-ABCs might be associated with
Fig. 2. See figure legend on p. 417.
severe ARIs such as bronchiolitis, wheezy bronchiolitis, croup and pneumonia throughout the seasons in Japan. This study used samples obtained from ARI patients who tested negative for other respiratory viruses, but they were not tested for any additional viruses and therefore dual infections were not excluded. Fig. 2. Phylogenetic trees based on the VP4/VP2 coding region (390 bp) of HRV-ABCs. (a–e) Phylogenetic trees based on the nucleotide sequences (a) and deduced amino acid sequences (b) of HRV-A reference strains, the nucleotide sequences (c) and deduced amino acid sequences (d) of HRV-C reference strains, and the nucleotide sequences of the VP4/VP2 coding region of HRV-ABCs and human enterovirus species D (HEV-D) (e). The reference strains used for type assignment were obtained from Picornaviridae.com (http://www.picornaviridae.com/). The following reference strains were used: HRV3, HRV7, HRV10, HRV11, HRV12, HRV16, HRV17, HRV19, HRV20, HRV23, HRV35, HRV40, HRV45, HRV46, HRV53, HRV59, HRV76, g2-11 (HRV-C39), tu304 (HRV-C38), IN-36 (HRV-C49), g2-25 (HRV-C40), g2-23 (HRV-C41), SA365412 (pat14), RV546 (pat18) and Cd08-1009 U (pat28). Echovirus 11 (E11), which belongs to the human enterovirus species B, was used as an outgroup. Bars, substitutions per nucleotide position. (f) Phylogenetic tree of the deduced amino acid sequences of the VP4/VP2 coding region (130 aa), including the present strains and representative reference strains. The tree was reconstructed by neighbour-joining analysis with labelling of the branches showing ≥70% bootstrap support. Bar, substitutions per amino acid position.

Fig. 3. Distributions of pairwise interspecies distances for HRV-(a) HRV-B(e) and HRV-C(c) based on the nucleotide sequences of the VP4/VP2 coding region.

(a) Number of subjects

(c) Number of subjects

(e) Number of subjects

 pairwise distance 0.281±0.106 (mean ± SD)

 pairwise distance 0.218±0.048 (mean ± SD)

 pairwise distance 0.230±0.063 (mean ± SD)
infection cannot be excluded. In addition, we cannot exclude the possibility that HRVs are present asymptotically in humans, because samples from healthy persons were not tested.

In conclusion, various genetic types of HRV-ABCs appear to be associated with ARIs, including URI, wheezy bronchiolitis, croup and pneumonia, in the Japanese population. Further and larger studies are warranted to address the detailed genetic properties of HRVs.

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