in the other 5 years. In 2004, apart from one B1 strain, we isolated only B2 strains. We isolated B2 strains every year and A2 strains every year between 2005 and 2009. The nucleotide identities among strains within the A2, B1 and B2 subgenogroups in Yamagata were between 94 and 100%, 99 and 100% and 97 and 100%, respectively.

hMPV has its main clinical impact in the late winter and early spring in countries with moderate climates (2, 3, 7, 8). In reality, studies of 5–7 years duration from Sweden and Austria together with our present study support the notion that hMPV infections occur in these seasons every year (10, 11). Data from a yearly longitudinal study from Austria and our present study also suggest that no or only a few hMPV are detected in September and October (10, 11). Thus, the period consisting of September and October might be the low season for hMPV activity in the northern hemisphere. Taken together, data based on longitudinal studies of over 5 years duration in Sweden, Austria and Yamagata suggest that the high season for hMPV is from winter to spring (between January and May) and the low season is in the fall (around September and October) in the northern hemisphere, although hMPV could be present year round in the community (10, 11). Such a seasonality pattern is similar to that of respiratory syncytia virus (RSV), for which it was shown that the virus is rarely isolated during August or September and most outbreaks peak in February to March, although continuous isolation has been found throughout the year (2). Another 4-year study from Brazil indicated that hMPV outbreaks peak in the winter months (July and August) or in spring (October) when hRSV infection rates declined (9). Thus, hMPV has its main clinical impact in winter or in spring months in Southeastern Brazil, as in countries with moderate climates.

The question remains as to whether there is a difference in dominance among the four subgenogroups in a community. Although several papers have described the co-circulation of plural subgenogroups with a dominant subgenogroup in a limited area and for a limited period, this phenomenon has not been well analyzed in a longitudinal study. Even longitudinal studies from Sweden and Austria showed no information regarding this phenomenon (10, 11). Data from a 3-year study from Brazil indicated that B1 and B2 were dominant in 2004 and in 2005 and A2 and B2 were dominant in 2006, although only a few A1 isolates were detected between 2004 and 2006 (9). Our present study suggested that A2 and B2 are present almost every year, whereas other subgenogroups appear less frequently in a community such as Yamagata. Of course, we also have to determine whether the Vero E6 cell line is sensitive to A1 as well as to other subgenogroups or whether A1 rarely circulates in the community. Interestingly, the major subgenogroup changed from one subgenogroup to the other each year from 2007 (B2) to 2008 (A2) to 2009 (B2). This phenomenon interests us in terms of the relationship between the predominant subgenogroup and the subgenogroup-specific herd immunity in Yamagata.

Unlike influenza A virus, which is an epidemic virus that causes diseases that rapidly spread to many people (18), hMPV might be an endemic virus. Yang *et al.* reported that hMPV F protein remains conserved over decades, whereas influenza viruses show progressive drift over time (6). Our data support the notion of hMPV endemicity in a community, as hMPV (especially subgenogroups A2 and B2 strains) were isolated throughout most of the study period. Although we only sequenced 441 bp of the F region for the A2, B1, and B2 subgenogroups of Yamagata strains, their identities were 94–100%, which was almost identical to the data (93.5–97.6%) reported by Yang *et al.* (6).

In conclusion, our study suggested that the high season of hMPV is between January and May and the low season is around September and October, although hMPV could be present year round in a community such as Yamagata. Furthermore, our results indicated that A2 and B2 are endemically present almost every year as major types without progressive drift, whereas other subgenogroups appear less frequently in the community.

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High-dose lorazepam for convulsive status epilepticus in an infant with holoprosencephaly

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Key words lorazepam, refractory seizure, semilobar holoprosencephaly.

Holoprosencephaly (HPE) is characterized by severe brain malformation due to early arrest in brain cleavage and rotation, and the prevalence is only about 1 in 10 000.[1] HPE has various central nervous system (CNS) manifestations, including epilepsy and mental retardation.[2] In particular, approximately half of the children with the semilobar type of HPE have at least one seizure, and about half of these have difficult-to-control seizures.[3] In Western countries, intravenous lorazepam is frequently used as a first-line anti-epileptic drug for the control of status epilepticus due to congenital CNS abnormalities, however this drug is not available in Japan.[4,5] Lorazepam has a longer duration of action than other benzodiazepines such as diazepam and has few side-effects (i.e. respiratory depression).

We encountered a case of refractory status seizures due to semilobar HPE in a 6-month-old Japanese girl. The patient was first treated with conventional anticonvulsants, including phenobarbital, diazepam, clobazam, and valproic acid. However, we failed to control the severe daily seizures with these drugs. The patient also had life-threatening complications, including status epilepticus, hypoxia, apnea and tachycardia. Treatment with high-dose oral lorazepam drastically improved these complications. Here, we report on the patient's clinical course and discuss the beneficial effects of high-dose oral lorazepam when treating intractable seizures.

Case report

The patient was born at 36 weeks and 2 days of gestation by natural vaginal delivery. A prenatal cranial ultrasound study revealed ventriculomegaly. She was small for dates (birthweight 2068 g). The parents were non-consanguineous and had one healthy girl. At birth, features of incomplete midline facial development such as hypotelorism and absence of a nasal septum were found. At 16 h after birth, she had generalized tonic–clonic seizures with some mild abnormal vital signs, such as low-grade fever, slight hypoxia, and tachycardia. On electroencephalography (EEG), frequent convulsive waves were observed. A brain computed tomography (CT) scan suggested semilobar HPE (Fig. 1). In addition, chromosome analysis showed 45, XX, der (15; 18) (q10; q10), and add (19)(p13.3). These present as deletions of 18p and dup(19)(q13.3). HPE is the most severe congenital malformation with deletions of 18p (18p-syndrome).[1] The dup(19)(q13.3) is associated with some congenital anomalies, including cleft lips, cardiac defects, and cerebral atrophy.[6] Thus, we diagnosed the condition as semilobar HPE accompanied by convulsive status epilepticus. First, phenobarbital (loading dose, 20 mg/kg) was given as a suppository. After the loading dose, the patient received maintenance doses (8 mg/kg/24 h in two divided doses). However, even on day 4 after initiation of treatment, the seizures could not be controlled completely by phenobarbital alone. Another anticonvulsant, midazolam (0.25 mg/kg/h), was

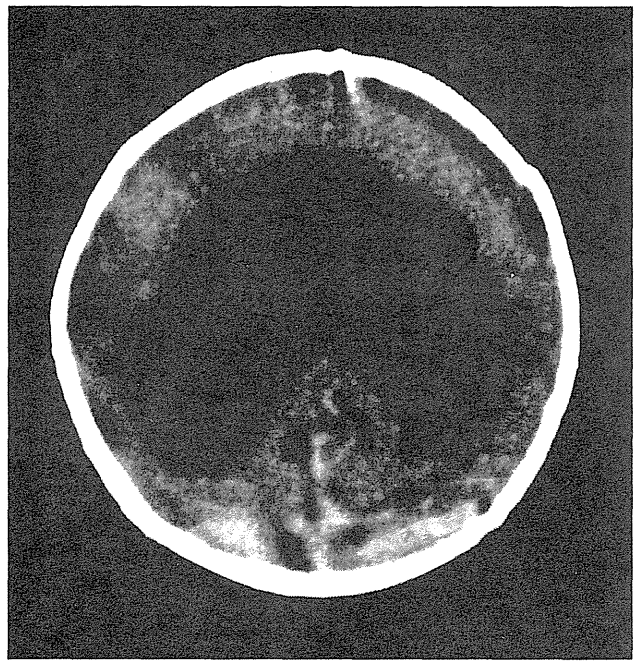

Fig. 1 Computed tomography (CT) study of the brain. An axial CT scan obtained 8 h after birth shows separation of the hemispheres posteriorly but not anteriorly. Anterior horns of the lateral ventricles are absent. There is also an incomplete separation of the basal ganglia.

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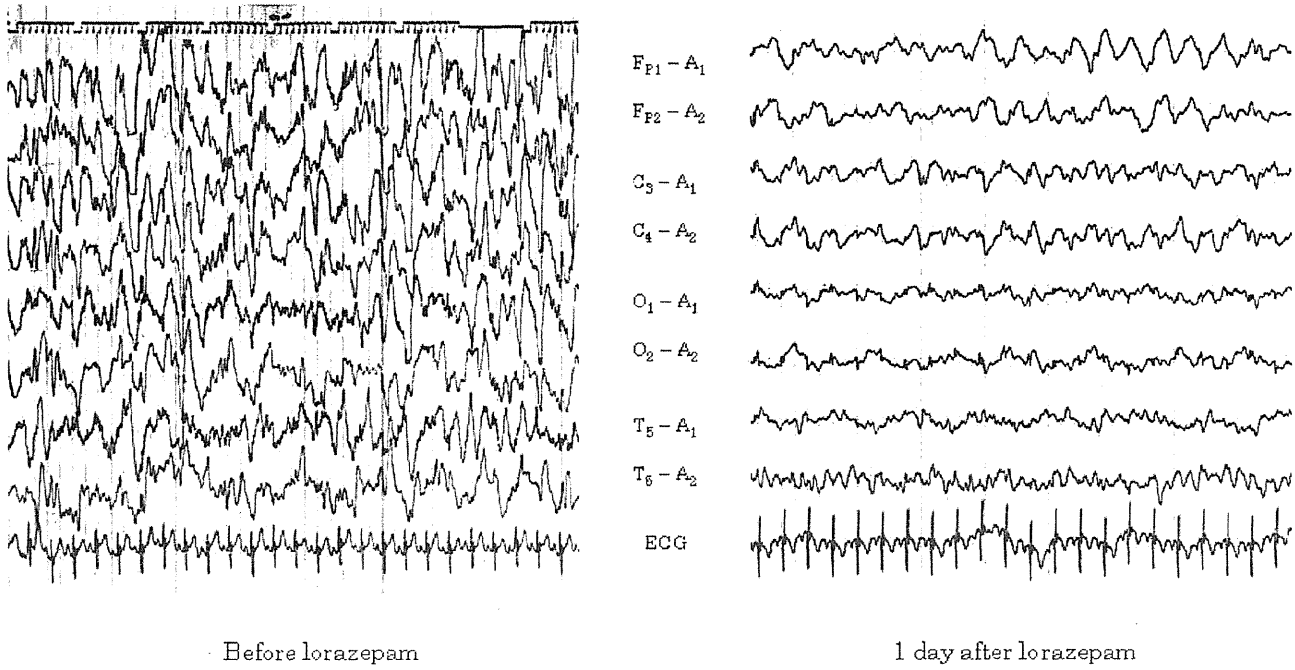

Fig. 2 Electroencephalograms (ECG) before (left panel) and after (right panel) lorazepam administration.

given intravenously. This treatment was effective and the seizures transiently disappeared. Oral administration of clobazam, the same benzodiazepine as midazolam, was started, and the dose of midazolam was tapered slowly over a period of 1 month. After stopping treatment with midazolam, seizures (such as myoclonic seizures) sometimes appeared, but they could be controlled by many anticonvulsants, including phenobarbital, clobazam, diazepam, and valproic acid.

However, at 6 months of age, the severe generalized tonic–clonic seizures recurred with various severe symptoms such as tachycardia (200 b.p.m.), high fever, rolling of the eyes, and hypoxic attacks. All anticonvulsants were maintained at therapeutic serum levels, including phenobarbital (serum concentration 53.4 μg/mL) and clobazam (serum concentration 50 ng/mL). Treatment with midazolam was also resumed at a maximum dose of 0.3 mg/kg/h but was not effective. The patient was then treated with intravenous thiopental sodium (maximum dose: 2.0 mg/kg/h). However, she continued to have repeated, severe seizures and was in a life-threatening condition. Accordingly, as an ultimate treatment, we started high-dose lorazepam therapy (2 mg/kg/24 h) by oral administration, after approval by the local ethics committee of the Tokyo Metropolitan Hachioji Children's Hospital and with written informed consent from the patient's parents. However, lorazepam is available only as a tablet form for anxiety disorders in Japan. Thus, we made a finely milled powder from the tablets, mixed this with distilled water, and administered it through a gastric tube. Thirty minutes after lorazepam administration, seizure activity immediately ceased. The heart rate decreased to 120 b.p.m. and the fever reduced. On EEG, diffuse bilateral spike-wave complexes (hypsarrhythmia-like) were observed during status seizures before lorazepam administration. After lorazepam administration, the spike-wave complexes completely disappeared (Fig. 2). No status seizure was seen, but tonic seizures occurred a few times a day. Thus, the dose of lorazepam was gradually increased to a maximum dose of 5 mg/kg/24 h at 7 months of age. Finally, we continued lorazepam for 2 months for the treatment of myoclonus.

The patient is now 2 years old. Her seizures are controlled by phenobarbital (8 mg/kg/24 h) and clobazam (0.6 mg/kg/24 h). On an as-needed basis, lorazepam (0.5 mg/kg/dose) is used about 3 times per month only for refractory myoclonus. No clinically significant side-effects of lorazepam, such as excessive sedation or respiratory depression, have been observed. Thus, high-dose oral lorazepam was far more effective and safe for controlling seizures than both intravenous midazolam and thiopental.

In Japan, lorazepam is not officially approved for treatment of seizures in infants, children and adults. To avoid adverse events, we measured serum concentrations of lorazepam by using high-performance liquid chromatography (SCG, Saitama, Japan). We checked lorazepam serum concentrations at 0, 30, 60, 120 and 180 min after administration of each dose (0.2 mg/kg) (Fig. 3). The peak value was at 30 min. The elimination rate constant and distribution volume were 0.18/h and 0.67 L/body, respectively.

Discussion

HPE cases generally show various CNS signs. About 50% of HPE cases with convulsions manifest with refractory seizures resulting in multiple drug resistance to anticonvulsants.[3] We showed that a case of refractory seizures due to semilobar HPE was controlled with lorazepam, while various other anticonvul-

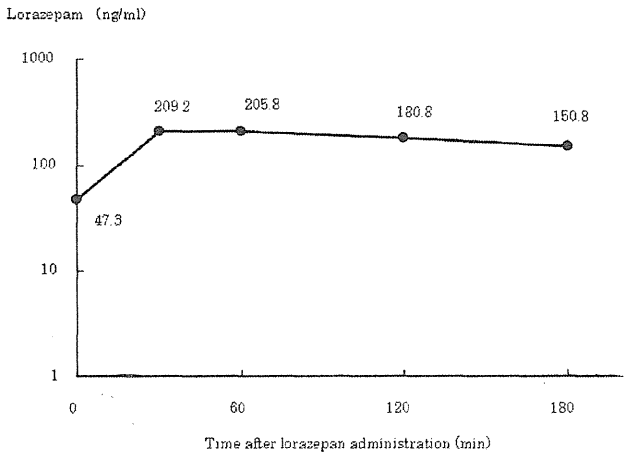

Fig. 3 Change in serum concentration after administration of lorazepam. Lorazepam was not administrated for 24 h before measuring concentrations.

sant drugs were not effective. Lorazepam is a benzodiazepine and used as an anti-anxiety drug in general. This medication is also frequently used in Europe and the USA to control status epilepticus due to various CNS diseases, including congenital CNS abnormalities.[4,7] Although lorazepam is not generally used to control status epilepticus caused by CNS diseases in children and adults in Japan,[5] this drug may be a first-line anti-epileptic drug when other conventional anticonvulsants are not effective for refractory seizures in semilobar HPE.

We first used phenobarbital, diazepam and valproic acid to control the myoclonic seizures in our patient. However, these were not effective. At the beginning phase of the refractory seizures, we also used another benzodiazepine drug, midazolam. Midazolam was effective for controlling status epilepticus and was administered for 1 month. At 6 months of age, we resumed administration of midazolam to control the refractory seizures; however the treatment was not effective. We did not know why midazolam was no longer effective. Our patient also had life-threatening complications, including status epilepticus, apnea, and tachycardia. Therefore, we used a relatively high dose of lorazepam. She was successfully treated with this anticonvulsant and had no side-effects. Previous reports demonstrated that lorazepam is effective in status epilepticus and refractory seizures.[4] Lorazepam has a longer duration of action than diazepam and readily crosses the blood–brain barrier after rapid absorption. These pharmaceutical properties might be associated with the efficacy of lorazepam. In the present case, we assumed the suitable oral dose of lorazepam for status epilepticus as previously described.[8] We thought that the oral equivalent (1.2 mg/kg/dose) of the intravenous dose used elsewhere (0.1 mg/kg/dose) was too high compared with the recommended intravenous dose (0.1 mg/kg/dose). Thus, we administrated 2 mg/kg/24 h (1.0 mg/kg/dose every 12 h). This dose was very effective for the suppression of status epilepticus and did not produce any side-effects, such as respiratory depression or hypotension. At 30 min and at 7 h after administration of this dose, the serum concentrations of lorazepam were 715.4 and 415.6 ng/mL, respectively. This concentration may be high (therapeutic range: 50–240 ng/mL). However, relatively high doses of various anticonvulsants may be used to suppress the status epilepticus.[9,10], Next, the ordinary therapeutic dose of lorazepam must be determined. We measured serum concentration of the drug after oral administration. Thirty minutes after oral administration of 0.2 mg/kg/dose, the serum drug concentration was 209.2 ng/mL, suggesting that this was a suitable oral dose (Fig. 3). Few reports on high-dose lorazepam have been published.[11] Additional large studies regarding oral lorazepam may be needed, although our dose was effective in the present case.

The recommended treatment for convulsive status epilepticus in children in Japan includes intravenous diazepam as a first-line treatment, followed by intranasal or intravenous midazolam, intravenous phenytoin, and intravenous barbiturates.[5] Previous experiences have shown that intravenous diazepam was ineffective in these cases. In accordance with these regulations and findings, we administered midazolam initially but found it to be ineffective. In addition, in the present case, we could only administer the drugs with a small digital vein, because it was difficult to prick other peripheral veins with a needle. Induced vasculitis may be necessary to administer phenytoin via a small vein route. Thus, we did not use this drug. Other anticonvulsants, such as paraldehyde and lidocaine (xylocaine) may be applicable. However, paraldehyde is not available in Japan. In addition, lidocaine is not definitively recommended, although it is included in the guidelines for the medical treatment of pediatric status epilepticus.[5]

It has been suggested that HPE involves midline structural abnormalities that may include endocrinological disturbance. In such cases, it is possible that lorazepam works against intractable seizures through a unique mechanism. However, the detailed mechanisms of anticonvulsants are not yet known. A large number of case studies involving the administration of lorazepam in refractory seizures with cerebral dysgenesis, including HPE, may be needed to address these issues.

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Intestinal amebiasis with Henoch–Schönlein purpura

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Key words Henoch–Schönlein purpura, intestinal amebiasis.

Henoch–Schönlein purpura (HSP), which is characterized by non-thrombocytopenic, purpuric and systemic vasculitis of the small vessels of the skin, joints, gastrointestinal tract, and kidney, is often reported to be associated with various infections such as β-hemolytic streptococcus, varicella, rubella, rubeola, hepatitis B, mycoplasma, mumps, measles, human parvovirus B19, coxsackie virus, adenovirus, salmonella, clostridium, tuberculosis, and HIV.[1–4] HSP, however, is rarely reported in association with amebiasis.[3–6]

Only three reports have been found in a MEDLINE search: two in the English-language literature[4,5] and one in the Japanese-language literature.[6] The two in the English-language literature were both from Turkey.[4,5] Here, we report a very rare case of intestinal amebiasis associated with HSP; the patient had abdominal pain and bloody diarrhea preceding the skin lesions, leading to diagnostic difficulties.

Case report

An 8-year-old girl, who was previously healthy, presented with a 3 day history of abdominal pain with watery diarrhea. The abdominal pain was colicky and diffuse, but more severe in the epigastric and periumbilical areas. Nausea, vomiting and fever, however, did not develop. Initial physical examination indicated a lethargic and irritable child, who had tenderness but no rebound tenderness on palpation of the abdomen. The medical and dietary history was unremarkable.

Laboratory studies of blood and urine included a white blood cell count, platelet count, coagulation profiles, serum electrolytes, acute phase reactants, serum transaminases, renal function tests, total bilirubin, amylase, the Widal test, and urinary analysis; all of the test results were normal but the stool was positive for occult blood and pus cells. Additional laboratory studies indicated positive ameba antibodies in the serum, and the wet mount confirmed amebic cysts in the stool. Other serology, however, such as serum anti-nuclear antibody, IgG, IgA, IgM, IgE, and gastrin were normal. The tests for other infectious agents were negative and included a tuberculin skin test; stool cultures, antigens (on enzyme-linked immunosorbent assay) or toxins for salmonella species, shigella species, pathogenic *Escherichia coli*, Vibrio species, *Yersinia enterocolitica*, *Clostridium difficile*, *C. perfringens*, rotavirus, norovirus, adenovirus, and astrovirus; non-amoebic ova or parasites in stool; blood or urine culture.

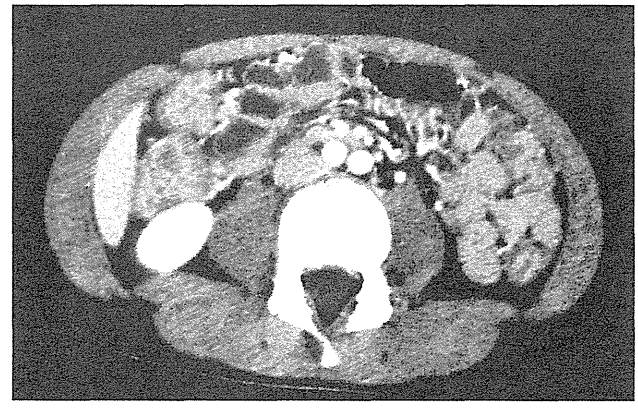

Fig. 1 Abdominal computed tomography on admission showing diffuse circumferential wall thickening of the colon with enhanced mucosa and edematous submucosa, which was suggestive of colitis.

Abdominal computed tomography on admission indicated diffuse circumferential wall thickening of the colon with enhanced mucosa, edematous submucosa and a small amount of ascites, which was suggestive of colitis (Fig. 1). Even though an inflammatory etiology was suspected, empirical antibiotics

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Detection and Phylogenetic Analysis of Human Rhinoviruses in Okinawa, Japan

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Human rhinoviruses (HRVs) are the cause of common colds and asthmatic exacerbation (1). Phylogenetic analysis of the *VP4/VP2* sequences of HRVs has revealed that all HRV serotypes except serotype 87 belong to 2 different species, HRV-A and HRV-B (2). Recently, several groups have reported the presence of a new HRV species, HRV-C (3,4). Although HRV-C cannot be cultured, it is distributed worldwide and is found in association with community outbreaks of acute respiratory infections (ARIs) (4,5). In Japan, HRV-A isolated from patients with ARIs in Yamagata Prefecture has been phylogenetically analyzed (6). However, the molecular epidemiology of HRVs from Okinawa Prefecture is not well known. Therefore, we performed phylogenetic analysis of the *VP4/VP2* sequences of HRVs detected in patients with ARIs and other viral infections in Okinawa Prefecture from June 2008 to January 2010.

Viral RNA was extracted from the nasopharyngeal swabs by using a QIAamp Viral RNA Mini kit (Qiagen, Valencia, Calif., USA) and suspended in DNase/RNase-free water. After RNA extraction, cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif., USA) and random hexamer primers (Takara, Shiga, Japan), and PCR was performed using the primers E2 and OL68-1 as described previously (7,8). Amplicons were purified using a QIAquick PCR Purification kit (Qiagen) and the nucleotide sequences were determined by direct sequencing. Partial nucleotide sequences (393 nt) of the *VP4/VP2* region of HRV were phylogenetically analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (9). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbor-joining (NJ) method (10). The reliability of the tree was estimated using 1,000 bootstrap replications.

In the present study, 13 HRV strains were detected by RT-PCR in patients with ARIs and other viral infections. Figure 1 shows a phylogenetic tree based on the *VP4/VP2* sequences including the present strains and reference strains. Of the 13 new strains, 4 (31%) were classified into HRV-A, 3 (23%) into HRV-B, and 6 (46%) into HRV-C.

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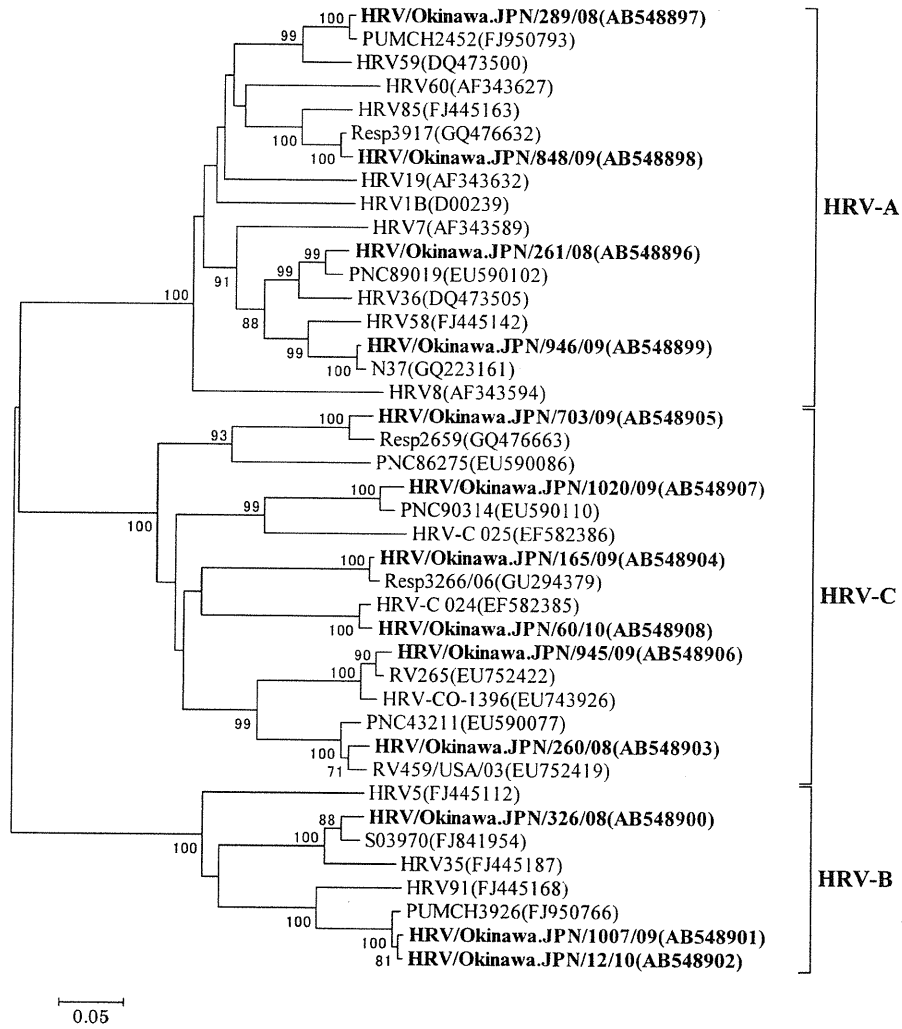

Fig. 1. Phylogenetic tree based on the *VP4/VP2* coding region sequences (393 nt) of the 41 human rhinoviruses (HRVs) including the present strains and reference strains. The present strains are shown as bold letters. Numbers in parentheses indicate the Genbank accession number. The numbers at each branch indicate the bootstrap value for the clusters.

The 4 present strains belonging to HRV-A were located in 4 distinct subclusters formed by the serotype known reference strains (HRV 59, HRV 85, HRV 36, and HRV58). The 3 present strains belonging to HRV-B were located in 2 distinct subclusters formed by the serotype known reference strains (HRV 35 and HRV 91). The 6 present strains belonging to HRV-C also segregated into 6 distinct subclusters formed by the reference strains (HRV-C 025, PNC86275, Resp3266/06, HRV-C 024, HRV-CO-1396, and PNC43211). These Okinawa strains analyzed in this study were also similar to other strains (PUMCH2452, N37, and PUMCH3926 from China, Resp3917 and Resp2659 from the United Kingdom, PNC89019 and PNC90314 from Finland, RV265 and RV459 from the USA, and S03970 from Spain). The nucleotide sequences of the present strains belonging to HRV-C were 59.3-64.6%, 56.4-64.8%, and 69.1-99% identical to HRV-A, HRV-B, and HRV-C reference strains, respectively. These results suggest that HRVs from Okinawa have diverse genetic variations.

Of the 4 patients with HRV-A infection, 3 were clinically diagnosed with an upper respiratory tract infection (URTI) and 1 was diagnosed with pneumonia. The 3 patients with HRV-B infection were separately diagnosed with a lower respiratory tract infection (LRTI), pneumonia, and viral myocarditis. Finally, of the 6 patients with HRV-C infection, 2 had URTI, 3 had LRTI, and 1 had viral meningitis. However, we could not estimate the relevance of pathogenicity with HRV species or strains because of the small number of samples in this study.

In conclusion, our results suggest that genetically diverse HRVs, including those belonging to HRV-C (a new species), are distributed in Okinawa. However, additional epidemiological and molecular epidemiological studies may be needed to better understand HRV infection in Okinawa Prefecture.

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LETTER TO THE EDITOR

Sequence and phylogenetic analyses of Saffold cardiovirus from children with exudative tonsillitis in Yamagata, Japan

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To the Editor,

Saffold cardiovirus (SAFV) of the genus Cardiovirus and family Picornaviridae was recently recovered from faecal specimens of an infant with fever of unknown origin [1]. SAFV has also been detected in children with diseases such as gastroenteritis, respiratory tract infection, and non-polio acute flaccid paralysis [2–5]. However, the epidemiology and pathogenicity of SAFV is not exactly known. In this study, we detected SAFV in children with exudative tonsillitis and conducted sequence and phylogenetic analyses.

We obtained nasopharyngeal swabs from 37 patients with typically exudative tonsillitis between August and December 2009. Informed consent was obtained from the parents of all subjects for the donation of the nasopharyngeal samples used in this analysis. Initially, we sought to isolate or detect pathogens from these samples using cell culture methods, quick immunochromatography (as used to detect Streptococcus), and polymerase chain reaction (PCR; as used to detect Epstein–Barr virus [6]). To isolate various viruses, we used 7 different cell lines (Vero E6, HEp-2, HEL, MDCK, GMK, HMV-II, and RD18S cells) [7,8]. These cells may be sensitive to the various agents of exudative tonsillitis – parainfluenza viruses, influenza viruses, herpes simplex viruses, adenovirus, and respiratory syncytial virus [7,8]. However, these pathogens were not isolated or detected from the samples provided.

Next, we attempted to detect SAFV using a nested reverse transcriptase PCR (RT-PCR). We extracted RNA from the samples and amplified the *VP1* coding region of SAFV by nested RT-PCR. Primer sets were newly designed by Primer Express ® version 1.5 software (Applied Biosystems LLC, Foster City, CA, USA) [9]. Primer sequences were as follows: 5′-HAA RCA RGR YTG GAR YTT YNT NAT GTT-3′ (primer 315F) and 5′- DGG BCK DGG RCA RWA VAC YCT CAT-3′ (primer 738R) as outer primers, and 5′-AAR CAR GRY TGG ARY TTY DTH ATG TTY TC-3′ (primer 316F) and 5′-RTT RKK RAA RTY NGM RDA NCY RTT RAA CCA-3′ (primer 621R) as inner primers. Reverse transcription was performed for 10 min at 30°C, 45 min at 37°C, and 5 min at 95°C using random hexamers (TAKARA BIO Inc., Otsu, Japan). First and nested PCR conditions were as follows: 5 min at 94°C, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, ending with elongation for an additional 10 min at 72°C. To prevent carryover contamination of nested-PCR, we took general precautions as previously described [10,11]. As a result, we obtained amplicons from 9 children who showed typical exudative tonsillitis symptoms, including fever (>38°C). They lived in Yamagata Prefecture, Japan and were aged between 2 and 7 y (mean ± standard deviation, 3.8 ± 1.6 y). Fever lasted for 1 to 3 days (1.8 ± 0.7 days). Amplicons were sequenced and aligned (453 bp) [3,5,12]. Next, we performed phylogenetic

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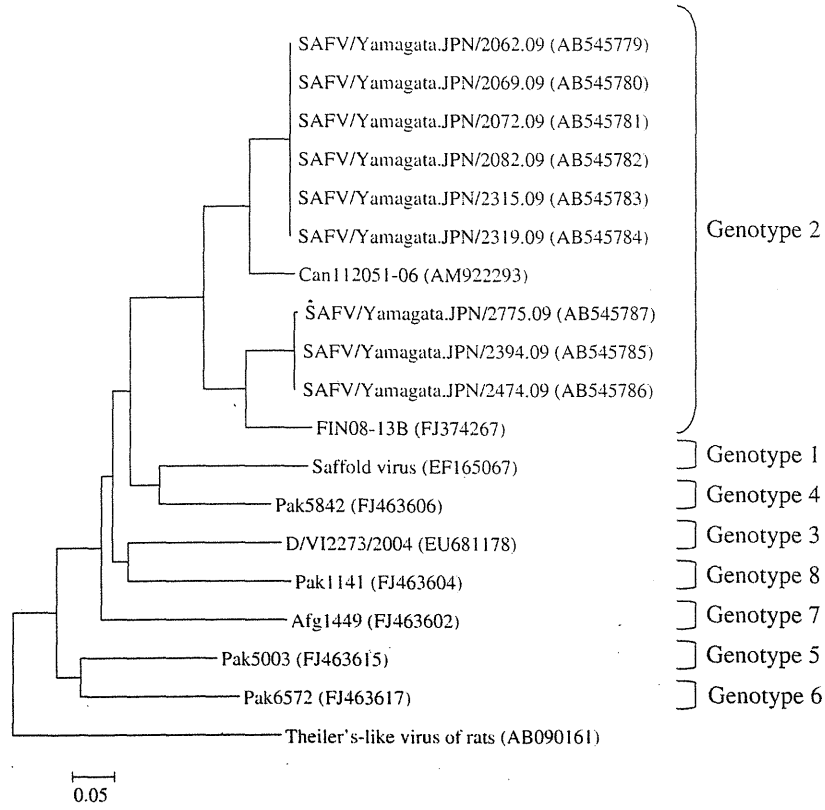

Figure 1. A phylogenetic tree of Saffold cardiovirus (SAFV) based on *VP1* gene (327 nt), constructed using *VP1* gene (GenBank accession numbers are given in parentheses).

analysis. Evolutionary distances were estimated using Kimura's 2-parameter method and phylogenetic trees were constructed using the neighbour-joining method. The reliability of each tree was estimated using 1000 bootstrap replications [9].

The present strains belonged to genotype 2 (see Figure 1) and were further divided into 2 distinct clusters [3,5]. Nucleotide identity among the present strains was 82.2–100%. Thus, we consider that the causative agent of exudative tonsillitis in these children was associated with SAFV. Recent reports have shown the detection of SAFV in children with diarrhoea [12,13], and other viruses, such as rotavirus and norovirus, have been found to co-infect with SAFV [14]. As a SAFV infection may be asymptomatic, the full clinical and/or biological spectrum remains to be established [15] and, to the best of our knowledge, no association between tonsillitis and SAFV infection is known. Together, our results suggest that, alongside other viruses and bacteria, SAFV is a causative agent of tonsillitis.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Laboratory and Epidemiology Communications

Phylogenetic Analysis of Human Metapneumovirus from Children with Acute Respiratory Infection in Yamaguchi, Japan, during Summer 2009

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Human metapneumovirus (hMPV) belongs to the family *Paramyxoviridae*, genus *Metapneumovirus*. It is an important causative agent of acute respiratory infections (ARIs) in humans (1) whose epidemiology in Japan is not known. To better understand the molecular epidemiology of domestic strains of hMPV, we performed phylogenetic analysis of hMPV detected from children with ARIs in Yamaguchi Prefecture during July and August 2009.

Table 1 shows patient and virus data. Virus RNA was extracted from throat swabs using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA) and suspended in DNase/RNase-free water. After RNA extraction, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as previously described (2). Amplicons were purified using a MinElute PCR Purification Kit (Qiagen), and the nucleotide sequences were determined by direct sequencing (2). We performed phylogenetic analysis based on the F gene of hMPV strains using Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (3). Evolutionary distances were estimated using Kimura's two-parameter method, and a phylogenetic tree was constructed using the neighbor-joining method (4). The reliability of the tree was estimated using 1,000 bootstrap replications.

The phylogenetic tree revealed that 14 out of the 18 strains detected during the 2009 summer season were clustered in the A2 subgroup, 3 strains were in the B1 subgroup, and 1 strain was in the B2 subgroup (Fig. 1). The 14 strains clustered in subgroup A2 were further classified into 2 subclusters. The nucleotide identities among the strains within the subgroups were 98.4 to 100% for subgroup A2 and 100% for subgroup B1. The nucleotide identities among the strains within the two subclusters of the A2 subgroup were 99.7 and 100%, respectively. In one of the subclusters of the A2 subgroup to which 11 strains belonged, an epidemiological rela-

Table 1. Patient and human metapneumovirus data

Patient	Age (y)	Sex	Diagnosis	Onset date	Sampling date	Strain	Subgroup	GenBank accession no.
1	1	F	Bronchiolitis	28 Jul. 2009	29 Jul. 2009	Yamaguchi 09-01	A2	AB533235
2	1	F	Pneumonia	26 Jul. 2009	29 Jul. 2009	Yamaguchi 09-03	A2	AB533239
3	2	F	Pneumonia	27 Jul. 2009	29 Jul. 2009	Yamaguchi 09-04	A2	AB533236
4	5	F	Bronchiolitis	30 Jul. 2009	31 Jul. 2009	Yamaguchi 09-07	A2	AB533237
5	2	M	Bronchiolitis	31 Jul. 2009	1 Aug. 2009	Yamaguchi 09-09	A2	AB533238
6	3	M	Bronchiolitis	31 Jul. 2009	3 Aug. 2009	Yamaguchi 09-10	A2	AB533240
7	8	M	Pneumonia	4 Aug. 2009	6 Aug. 2009	Yamaguchi 09-13	A2	AB533241
8	4	M	Bronchiolitis	4 Aug. 2009	6 Aug. 2009	Yamaguchi 09-14	A2	AB533242
9	9	F	Bronchiolitis	2 Aug. 2009	3 Aug. 2009	Yamaguchi 09-15	B2	AB533243
10	2	F	Pneumonia	7 Aug. 2009	8 Aug. 2009	Yamaguchi 09-17	B1	AB533244
11	4	M	Pneumonia	9 Aug. 2009	10 Aug. 2009	Yamaguchi 09-20	A2	AB533245
12	3	F	Bronchiolitis	8 Aug. 2009	10 Aug. 2009	Yamaguchi 09-22	A2	AB533246
13	4	M	Bronchiolitis	8 Aug. 2009	11 Aug. 2009	Yamaguchi 09-26	A2	AB533247
14	1	F	Bronchiolitis	12 Aug. 2009	13 Aug. 2009	Yamaguchi 09-29	A2	AB533248
15	6	F	Pneumonia	11 Aug. 2009	13 Aug. 2009	Yamaguchi 09-30	B1	AB533249
16	3	M	Bronchiolitis	11 Aug. 2009	13 Aug. 2009	Yamaguchi 09-31	B1	AB533250
17	4	M	Pneumonia	19 Aug. 2009	21 Aug. 2009	Yamaguchi 09-37	A2	AB533251
18	0 (3 mo)	F	Bronchiolitis	19 Aug. 2009	21 Aug. 2009	Yamaguchi 09-38	A2	AB533252

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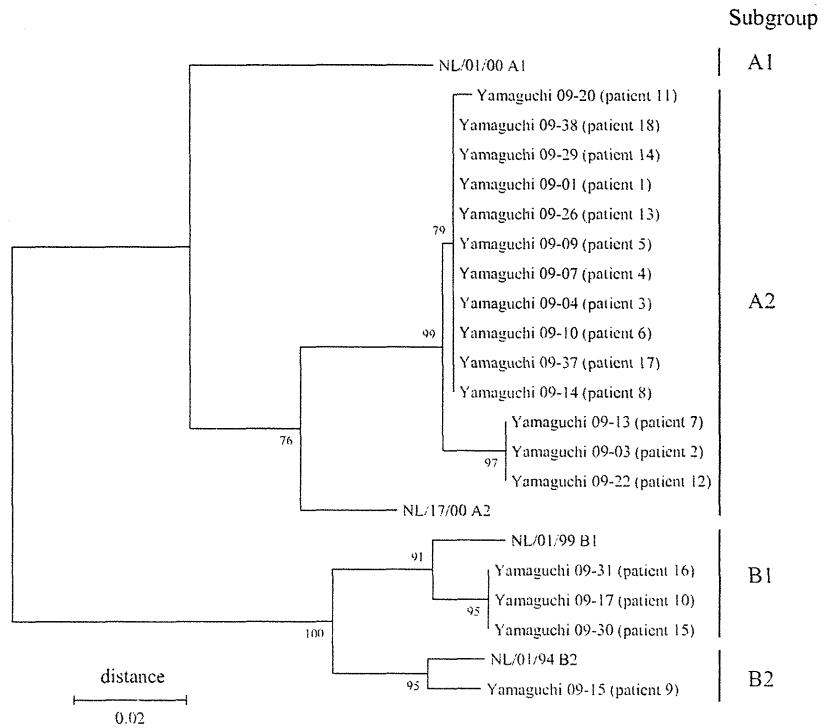

Fig. 1. Phylogenetic tree constructed on the basis of partial sequences of the human metapneumovirus F gene. Distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers above the branches are bootstrap probabilities (%). Reference strains were NL/1/00, NL/17/00, NL/1/99, and NL/1/94.

tion was found. Five out of the 11 patients (patients 1, 4, 5, 6, and 13) and 2 patients (patients 8 and 14) attended the same nursery schools, respectively. In addition, patients 4 and 8 were from the same family. In the other subcluster, patients 2 and 12 attended the same nursery school, and patients 7 and 12 were from the same family.

In summary, hMPV was detected from a total of 18 children with ARIs during July and August 2009 in Yamaguchi Prefecture. All of these patients resided in the same city, indicating that a regional outbreak of hMPV occurred among the children. Phylogenetic analysis revealed that subgroup A2 was predominant in the outbreak, and that subgroups B1 and B2 cocirculated. Our findings suggested that this outbreak had at least four different infection sources. Although there have been few reports of hMPV being detected in the summer season (5–9), the results of the present study suggest that hMPV infections should be taken into consideration in children with ARIs throughout the year.

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Phylogenetic and cluster analysis of human rhinovirus species A (HRV-A) isolated from children with acute respiratory infections in Yamagata, Japan

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ABSTRACT

We performed phylogenetic and cluster analysis of human rhinovirus species A (HRV-A) isolated from 76 children with acute respiratory infection in Yamagata prefecture, Japan during the period 2003–2007. Phylogenetic trees based on the nucleotide and amino acid sequences of the *VP4/VP2* coding region showed that the present strains could be classified into 11 and 8 clusters, respectively. The homology among the present strains ranged from 66.6% to 100% at the nucleotide level and 84.7% to 100% at the amino acid level. The interspecies distance (mean ± standard deviation) was calculated to be 0.235 ± 0.048 at the nucleotide level and 0.076 ± 0.033 at the amino acid level. In addition, the phylogenetic trees created based on the nucleotide and amino acid sequences showed that HRV-A strains belonging to some clusters were associated with both upper respiratory infection and wheezy bronchiolitis, while other strains were associated with upper respiratory infection alone. These results suggest that the present HRV-A isolates had a wide nucleotide divergence and were associated with acute respiratory infection, including upper respiratory infection and wheezy bronchiolitis, in Yamagata prefecture, Japan during the investigation period.

1. Introduction

Human rhinoviruses (HRVs), which are positive sense-stranded RNA viruses belonging to the *Picornaviridae* family, cause acute respiratory infection (ARI) in humans and are the major pathogen for the common cold (Turner and Couch, 2007). HRV, the major pathogen for the common cold, is responsible for around 50% of asthma exacerbations and is one of the factors that can direct the infant immune system towards an asthmatic phenotype (Nicholson et al., 1993; Johnston et al., 1996; Jackson et al., 2008). In addition, accumulating evidence suggests that some patients with asthma or exacerbation of asthma may be associated with HRV infection, although the pathophysiology of this association is not clear at present (Wos et al., 2008). Thus, HRV is being reevaluated as an important agent of ARI in humans (Papadopoulos et al., 2002; Imakita et al., 2000; Wos et al., 2008).

More than 100 serotypes of HRV have been confirmed, and these viruses have been classified into many clusters on the basis of genetic analysis (Savolainen et al., 2002a,b). Recently, it was reported that HRV can be classified into 3 species (HRV-A, -B, and -C) on the basis of phylogenetic analysis of the *VP4* gene (Lau et al., 2007). Strains belonging to HRV-A and -B species are culturable and have been implicated as the most prevalent viruses associated with ARI (Savolainen et al., 2002a), whereas those of the HRV-C species are not culturable. However, the genetic characteristics and epidemiology of domestic HRV-A infection are not known. Therefore, as a general indicator of domestic infection, we performed phylogenetic and cluster analysis of HRV-A isolated from patients

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with ARI in Yamagata prefecture, Japan during the period 2003–2007.

2. Materials and methods

2.1. Isolates and patients

We analyzed 76 HRV-A isolates obtained from patients with upper respiratory infection (URI) (70 patients) or wheezy bronchiolitis (6 patients) referred to pediatric clinics in Yamagata prefecture during the period 2003–2007. The samples were obtained by the local health authority of Yamagata prefecture for the surveillance of viral diseases in Japan. We diagnosed URI, also known as the common cold, and URI that typically affects the upper airway which includes the nose (sinusitis), throat (pharyngitis), and voice box (laryngitis) (Cherry, 2003). Wheezy bronchiolitis was diagnosed due to the presence of wheezing alone or chest retractions in association with a URI (Robert, 2003). A detailed listing of patients and isolates is provided in Table 1. All patients were under 16 years of age (5.8 ± 3.3 years; mean ± standard deviation [SD]). No significant seasonal variation of HRV-A isolation was seen in this study.

2.2. Cell culture and virus isolation

To isolate various respiratory viruses, we used HEF, HEp-2, Vero E6, MDCK, RD-18S and GMK cells in this study. Cells were grown in Eagle's minimal essential medium (Nissui No. 3; Nissui Pharmaceutical Co., Tokyo, Japan) containing 5–10% fetal bovine serum or calf serum at 37 °C in a humidified atmosphere of 5% CO_2 (Mizuta et al., 2008). Cells were grown in 96 well microplates (Greiner Bio-One, Frickenhausen, Germany).

After the plates were washed with phosphate-buffered saline without calcium and magnesium (PBS), 100 μL of maintenance medium was added to each well of the plates. Each specimen was then centrifuged at 3000 rpm for 15 min and 75 μL of the supernatant was inoculated into 2 wells of each cell line. The inoculated plates were incubated at 33 °C in a CO_2 incubator. We observed the plates two or three times per week for cytopathic effects (CPEs) over 14 days for all cell lines, without passage or medium change (Mizuta et al., 2008). When a suspected HRV CPE was observed, we passaged and stored the cells at −80 °C until analysis. Viral identification was carried out by reverse-transcription polymerase chain reaction (RT-PCR) and sequence analysis (Savolainen et al., 2002a).

2.3. RNA extraction, RT-PCR, and sequencing

For RNA extraction, RT-PCR, and sequence analysis, infected culture fluids 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 (Savolainen et al., 2002a; Iwai et al., 2006). Briefly, viral RNA was extracted from 140 μL supernatant with a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). The RT-PCR procedure was performed according to the manufacturer's instructions (Access RT-PCR System; Promega, Madison, WI). The primers for RT-PCR were as follows: 5′ GGG ACC AAC TAC TTT GGG TGT CCG TGT 3′ (9895 forward; sense [534–560 nt]), and 5′ GCA TCI GGY ARY TTC CAC CAC CAN CC 3′ (9565 reverse; antisense [1083–1058 nt]). The following protocol was used: 45 min at 48 °C (reverse transcription), 2 min at 94 °C (denaturation), and 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min followed by 7 min at 68 °C in the last cycle for elongation. Purification of DNA fragments and nucleotide sequence determination were performed as described previously (Iwai et al., 2006).

2.4. Phylogenetic analysis and calculation of pairwise genetic distances

For phylogenetic analysis, the nucleotide sequences (positions 623–1012; 390 bp) and deduced amino acid sequences (130 aa) of the partial HRV *VP4/VP2* coding region were analyzed phylogenetically with the CLUSTAL W program available on the DNA Data Bank of Japan homepage (http://www.ddbj.nig.ac.jp/index-j.html) and TreeView (version 1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Evolutionary distances were estimated according to the Kimura's 2-parameter method, and the phylogenetic tree was constructed with the neighbor-joining method (Kimura, 1980; Saitou and Nei, 1987). The reliability of the tree was estimated with 1000 bootstrap replications. Genetic distances among reference (representative) strains may be important in phylogenetic analysis. Thus, to construct the phylogenetic tree, we preferably selected the reference strains having constant genetic distances (interspecies distances, 0.237 ± 0.030), and used the following as references strains in this study: HRV1B, HRV7, HRV8, HRV9, HRV12, HRV15, HRV16, HRV19, HRV28, HRV29, HRV47, HRV49, HRV55, HRV56, HRV60, and HRV63. In addition, some human rhinovirus species B (HRV-B), human rhinovirus species C (HRV-C) and human enterovirus species D (HEV-D) were used as reference strains as previously described (Piralla et al., 2009). Echovirus 11(Echo-11), which belongs to the human enterovirus species B was used as the outgroup (Piralla et al., 2009). In order to assess interspecies frequency distributions and the frequency distribution of each intercluster of HRV-A, we calculated pairwise genetic distances for all of the strains, including the present isolates and reference strains, as previously described (Katayama et al., 2002).

3. Results

3.1. Phylogenetic and cluster analysis

Phylogenetic trees based on the nucleotide sequences (390 nt) and the deduced amino acid sequences (130 aa) of the *VP4/VP2* coding region, including the present strains ($n = 76$) and representative reference strains ($n = 16$) belonging to HRV-A, are shown in Fig. 1a and b, respectively. In addition, we show the corresponding phylogenetic trees for the representative reference strains alone (Fig. 1c and d, respectively). Phylogenetic trees based on the nucleotide and deduced amino acid sequences for the present and reference strains of HRV-A species could be classified into 11 and 8 clusters, respectively. At the nucleotide level, the homology among the present strains ranged from 66.6% to 100%, while at the deduced amino acid level the homology ranged from 84.7% to 100%. On the phylogenetic tree based on the nucleotide sequences, the numbers of the present strains in each cluster were as follows: Cluster 1, 6 strains; Cluster 2, 8 strains; Cluster 3, 7 strains; Cluster 4, 8 strains; Cluster 5, 10 strains; Cluster 6, 7 strains; Cluster 7, 4 strains; Cluster 8, 4 strains; Cluster 9, 6 strains; Cluster 10, 10 strain; and Cluster 11, 6 strains. On the phylogenetic tree based on the deduced amino acid sequences, the numbers of the present strains in each cluster were as follows: Cluster 1, 20 strains; Cluster 2, 9 strains; Cluster 3, 10 strains; Cluster 4, 1 strain; Cluster 5, 11 strains; Cluster 6, 4 strains; Cluster 7, 19 strains; and Cluster 8, 2 strains. Notably, on the phylogenetic tree based on nucleotide sequences, Clusters 2, 3, 5 and 9 were associated with both URI and wheezy bronchiolitis, whereas other strains were associated with URI alone. As well, on the phylogenetic tree based on amino acid sequences, Clusters 3, 7, and 8 were associated with both URI and wheezy bronchiolitis. These results suggest that the present HRV-A strains which had a wide nucleotide divergence (66.6–100% homology) were circulating in the Yamagata area during the investigation period.

Table 1
Patients' data, HRV strain, and cluster.

Patient	Age (year)	Sampling date	Disease	Strain	Cluster	GenBank accession no.
1	16	23 January, 2003	URI*	HRVi/Yamagata.JPN/4.03	8**, 1***	AB474093
2	3	25 March, 2003	URI	HRVi/Yamagata.JPN/13.03	3, 5	AB474094
3	15	18 April, 2003	URI	HRVi/Yamagata.JPN/16.03	5, 3	AB474095
4	7	18 April, 2003	URI	HRVi/Yamagata.JPN/16-2.03	1, 7	AB474096
5	11	30 April, 2003	URI	HRVi/Yamagata.JPN/18.03	9, 2	AB474100
6	9	30 April, 2003	URI	HRVi/Yamagata.JPN/18-2.03	3, 5	AB474101
7	5	7 May, 2003	URI	HRVi/Yamagata.JPN/19.03	11, 6	AB474102
8	7	12 May, 2003	URI	HRVi/Yamagata.JPN/20.03	10, 6	AB474103
9	1	10 May, 2003	URI	HRVi/Yamagata.JPN/19-2.03	6, 1	AB474104
10	1	12 May, 2003	URI	HRVi/Yamagata.JPN/20-2.03	11, 2	AB474105
11	0	9 September, 2003	URI	HRVi/Yamagata.JPN/37.03	3, 5	AB474106
12	4	20 September, 2003	Wheezy bronchiolitis	HRVi/Yamagata.JPN/38.03	9, 8	AB474107
13	2	22 September, 2003	Wheezy bronchiolitis	HRVi/Yamagata.JPN/39.03	2, 7	AB474108
14	2	30 September, 2003	URI	HRVi/Yamagata.JPN/40.03	10, 1	AB474109
15	6	6 October, 2003	URI	HRVi/Yamagata.JPN/41.03	3, 7	AB475008
16	0	10 October, 2003	URI	HRVi/Yamagata.JPN/41-2.03	3, 7	AB474110
17	0	10 October, 2003	LRI	HRVi/Yamagata.JPN/41-3.03	8, 1	AB474111
18	9	20 October, 2003	URI	HRVi/Yamagata.JPN/43.03	3, 7	AB474112
19	0	12 November, 2003	Wheezy bronchiolitis	HRVi/Yamagata.JPN/46.03	3, 7	AB475009
20	6	17 November, 2003	URI	HRVi/Yamagata.JPN/47.03	10, 2	AB474113
21	2	19 November, 2003	URI	HRVi/Yamagata.JPN/47-2.03	9, 8	AB474114
22	11	20 November, 2003	URI	HRVi/Yamagata.JPN/47-3.03	10, 2	AB474115
23	8	21 November, 2003	URI	HRVi/Yamagata.JPN/47-4.03	4, 5	AB474116
24	1	16 December, 2003	Wheezy bronchiolitis	HRVi/Yamagata.JPN/51.03	5, 3	AB474117
25	2	17 December, 2003	URI	HRVi/Yamagata.JPN/51-2.03	10, 1	AB474118
26	7	21 January, 2004	URI	HRVi/Yamagata.JPN/4.04	5, 3	AB474119
27	13	30 March, 2004	URI	HRVi/Yamagata.JPN/14.04	2, 7	AB474120
28	3	26 April, 2004	URI	HRVi/Yamagata.JPN/18.04	2, 7	AB474121
29	7	26 April, 2004	URI	HRVi/Yamagata.JPN/18-2.04	5, 3	AB474122
30	2	7 May, 2004	URI	HRVi/Yamagata.JPN/19.04	10, 1	AB474123
31	10	2 June, 2004	URI	HRVi/Yamagata.JPN/23.04	2, 7	AB474124
32	0	28 July, 2004	URI	HRVi/Yamagata.JPN/31.04	6, 1	AB474125
33	0	16 August, 2004	URI	HRVi/Yamagata.JPN/34.04	4, 5	AB474126
34	1	14 September, 2004	URI	HRVi/Yamagata.JPN/38.04	4, 5	AB474127
35	2	2 November, 2004	URI	HRVi/Yamagata.JPN/45.04	6, 1	AB474128
36	4	20 April, 2005	URI	HRVi/Yamagata.JPN/16.05	6, 1	AB474129
37	1	12 May, 2005	URI	HRVi/Yamagata.JPN/19.05	6, 1	AB474130
38	2	16 May, 2005	URI	HRVi/Yamagata.JPN/20.05	9, 1	AB474131
39	3	24 May, 2005	URI	HRVi/Yamagata.JPN/21.05	9, 1	AB474132
40	6	13 October, 2005	URI	HRVi/Yamagata.JPN/41.05	11, 2	AB474133
41	11	18 October, 2005	URI	HRVi/Yamagata.JPN/42.05	11, 2	AB474134
42	4	28 February, 2006	URI	HRVi/Yamagata.JPN/9.06	5, 3	AB474135
43	10	18 March, 2006	URI	HRVi/Yamagata.JPN/11.06	10, 1	AB474136
44	9	24 April, 2006	URI	HRVi/Yamagata.JPN/17.06	5, 3	AB474137
45	1	1 May, 2006	URI	HRVi/Yamagata.JPN/18.06	10, 6	AB474138
46	3	19 May, 2006	URI	HRVi/Yamagata.JPN/20.06	2, 7	AB474139
47	0	27 May, 2006	Wheezy bronchiolitis	HRVi/Yamagata.JPN/21.06	2, 7	AB474140
48	1	26 May, 2006	URI	HRVi/Yamagata.JPN/21-2.06	5, 3	AB474141
49	8	31 May, 2006	URI	HRVi/Yamagata.JPN/22.06	5, 3	AB474142
50	0	6 June, 2006	URI	HRVi/Yamagata.JPN/23.06	4, 7	AB474143
51	1	6 June, 2006	Wheezy bronchiolitis	HRVi/Yamagata.JPN/23-2.06	2, 7	AB474144
52	1	21 June, 2006	URI	HRVi/Yamagata.JPN/25.06	7, 1	AB474145
53	1	21 June, 2006	URI	HRVi/Yamagata.JPN/25-2.06	8, 2	AB474146
54	2	21 July, 2006	URI	HRVi/Yamagata.JPN/29.06	2, 7	AB474148
55	0	1 December, 2006	URI	HRVi/Yamagata.JPN/48-2.06	8, 1	AB474150
56	1	30 March, 2007	URI	HRVi/Yamagata.JPN/13.07	7, 1	AB474151
57	1	23 May, 2007	URI	HRVi/Yamagata.JPN/21.07	4, 5	AB474152
58	3	2 June, 2007	URI	HRVi/Yamagata.JPN/22.07	1, 8	AB474153
59	3	6 June, 2007	URI	HRVi/Yamagata.JPN/23.07	10, 6	AB474154
60	1	27 June, 2007	URI	HRVi/Yamagata.JPN/26.07	1, 7	AB474155
61	6	22 June, 2007	URI	HRVi/Yamagata.JPN/25.07	1, 7	AB474156
62	2	22 June, 2007	URI	HRVi/Yamagata.JPN/25-2.07	1, 7	AB474157
63	2	3 July, 2007	URI	HRVi/Yamagata.JPN/27.07	4, 5	AB474158
64	10	13 July, 2007	URI	HRVi/Yamagata.JPN/28.07	1, 7	AB474159
65	5	21 August, 2007	URI	HRVi/Yamagata.JPN/34.07	5, 3	AB474160
66	8	25 September, 2007	URI	HRVi/Yamagata.JPN/39.07	9, 4	AB474161
67	0	29 September, 2007	URI	HRVi/Yamagata.JPN/35.07	6, 1	AB474163
68	1	3 October, 2007	URI	HRVi/Yamagata.JPN/40-2.07	5, 3	AB474164
69	2	9 October, 2007	URI	HRVi/Yamagata.JPN/41.07	4, 5	AB474165
70	1	15 October, 2007	URI	HRVi/Yamagata.JPN/42.07	4, 5	AB474166
71	2	18 October, 2007	URI	HRVi/Yamagata.JPN/42-2.07	7, 1	AB474167
72	0	24 October, 2007	URI	HRVi/Yamagata.JPN/43.07	11, 2	AB474168
73	1	1 November, 2007	URI	HRVi/Yamagata.JPN/44.07	6, 1	AB474169
74	0	6 November, 2007	URI	HRVi/Yamagata.JPN/45.07	7, 1	AB474170
75	1	6 November, 2007	URI	HRVi/Yamagata.JPN/45-2.07	11, 2	AB474171
76	2	22 November, 2007	URI	HRVi/Yamagata.JPN/47.07	10, 5	AB474172

* URI: upper respiratory infection.
** Left number: cluster numbers as phylogenetic tree based on the nucleotide sequences.
*** Right number: cluster numbers as phylogenetic tree based on the amino acid sequences.

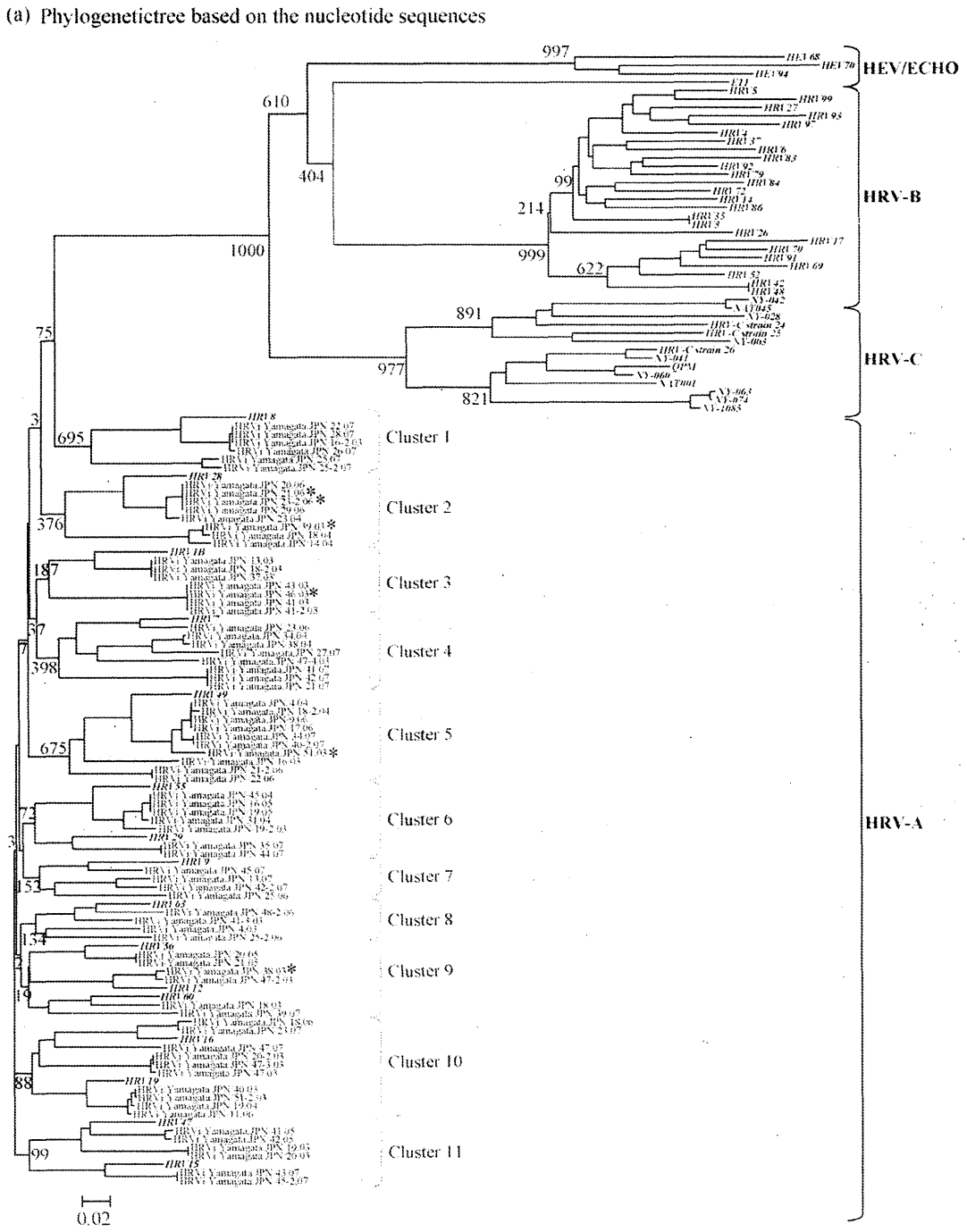


Fig. 1. Phylogenetic tree based on the *VP4/VP2* coding region of human rhinovirus species A (HRV-A), human rhinovirus species B (HRV-B), human rhinovirus species C (HRV-C), and human enterovirus species D (HEV-D). Echovirus 11(Echo-11), which belongs to the human enterovirus species B, was used as an outgroup. (a) Phylogenetic tree based on nucleotide sequences of the *VP4/VP2* coding region (390 nt) including the present strains (76 strains) and representative reference strains (16 strains). (b) Phylogenetic tree based on deduced amino acid sequences of the *VP4/VP2* coding region (130 aa) including the present strains and representative reference strains. Distance was calculated according to Kimura's 2-parameter method, and the tree was plotted with the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Reference strains are shown in bold type. (c and d) Phylogenetic trees based nucleotide sequences and amino acid sequences of the corresponding region for representative reference strains. GenBank accession numbers of references strains are as follows: HRV1B (GenBank accession no. D00239), HRV7 (AF343589), HRV8 (D00239), HRV9 (AF343605), HRV12 (AY016405), HRV15 (AF343630), HRV16 (L24917), HRV19 (AF343632) HRV28 (DQ473508), HRV29 (AF343615), HRV47 (FJ445133), HRV49 (AF343598), HRV55 (AF343621), HRV56 (FJ445140), HRV60 (AF343627), HRV63 (FJ445146), HRV3 (EF173422), HRV4 (AF343655), HRV5 (AF343651), HRV6 (DQ473486), HRV14 (K02121), HRV17 (AF343645), HRV26 (AF343653), HRV27 (AF343654), HRV35 (FJ445187), HRV37 (EF173423), HRV42 (FJ445130), HRV48 (DQ473488), HRV52 (FJ445188), HRV69 (FJ445151), HRV70 (AF343646), HRV72 (AF343650), HRV79 (FJ445155), HRV83 (AF343647), HRV84 (FJ445162), HRV86 (AF343648), HRV91 (FJ445168), HRV92 (FJ445169), HRV93 (EF173425), HRV97 (FJ445172), HRV99 (AF343652), HRV-C strain 24 (EF582385), HRV-C strain 25 (EF582386), HRV-C strain 26 (EF582387), QPM (EF186077), NY-003 (DQ875929), NY-028 (DQ875931), NY-041 (DQ875921), NY-042 (DQ875926), NY-060 (DQ875928), NY-063 (DQ875924), NY-074 (DQ875932), NY-1085 (DQ875925), NAT001 (FF077279), NAT045 (EF077280), HEV68 (EF107098), HEV70 (EV70CG), HEV94 (DQ916376), and Echo-11 (EU167522). *Strains from wheezy bronchiolitis.

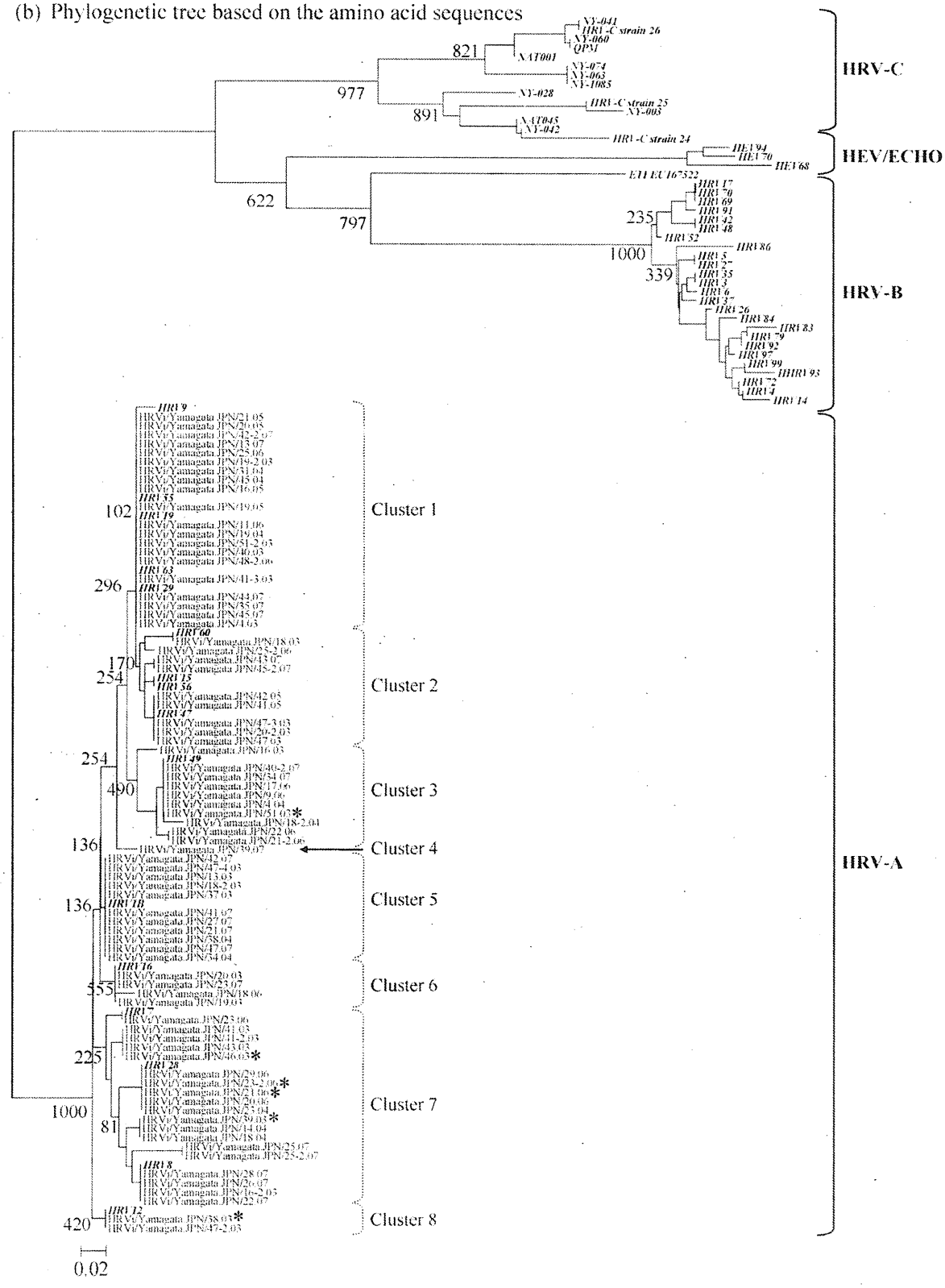
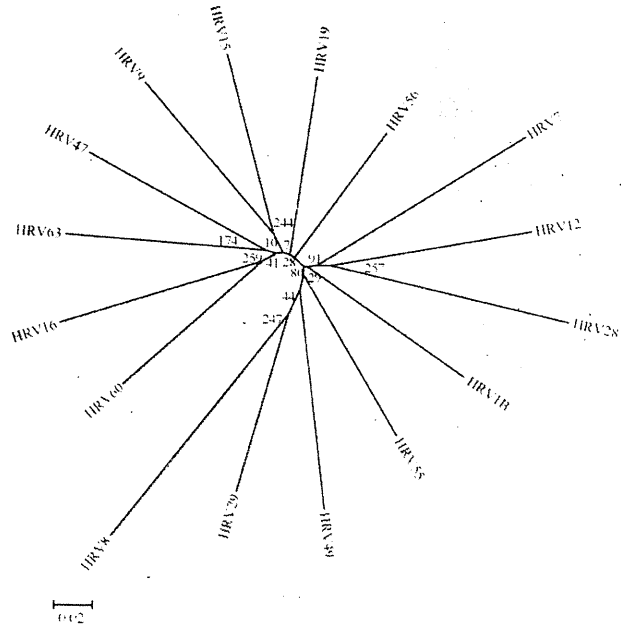


Fig. 1. (*Continued*)

(c) Phylogenetic tree based on the nucleotide sequences (reference strains)



(d) Phylogenetic tree based on the amino acid sequences (reference strains)

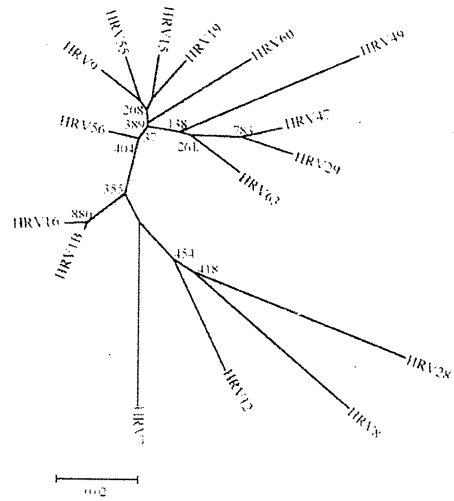


Fig. 1. (*Continued*).

3.2. Distribution of pairwise distances of interspecies of HRV-A based on the nucleotide and amino acid sequences

We calculated interspecies distances of HRV-A by the distribution of pairwise distances (Figs. 2a–k and 3a–i). Among the present and reference strains, the interspecies distances based on the nucleotide and amino acid sequences were 0.235 ± 0.048 and 0.076 ± 0.033 (mean ± SD), respectively (Figs. 2a, 3a). The intercluster distances based on the nucleotide sequences were as follows: Cluster 1, 0.136 ± 0.101 (mean ± SD, Fig. 2b); Cluster 2, 0.115 ± 0.086 (c); Cluster 3, 0.152 ± 0.115 (d); Cluster 4, 0.142 ± 0.062 (e); Cluster 5, 0.085 ± 0.058 (f); Cluster 6, 0.130 ± 0.093 (g); Cluster 7, 0.159 ± 0.053 (h); Cluster 8, 0.167 ± 0.053 (i); Cluster 9, 0.184 ± 0.064 (j); Cluster 10, 0.169 ± 0.072 (k) and Cluster 11, 0.153 ± 0.072 (l). Next, the intercluster distances based on the amino acid sequences were as follows: Cluster 1, 0.031 ± 0.020 (mean ± SD, Fig. 3b); Cluster 2, 0.040 ± 0.016 (c); Cluster 3, 0.012 ± 0.012 (d); Cluster 5, 0.030 ± 0.025 (e); Cluster 6, 0.027 ± 0.020 (f); Cluster 7, 0.079 ± 0.041 (g); and Cluster 8, 0 (h). The longest pairwise intercluster distances based on the nucleotide and amino acid sequences were Cluster 9 and Cluster 7, respectively, whereas the shortest pairwise distances were Cluster 5 and Cluster 8, respectively.