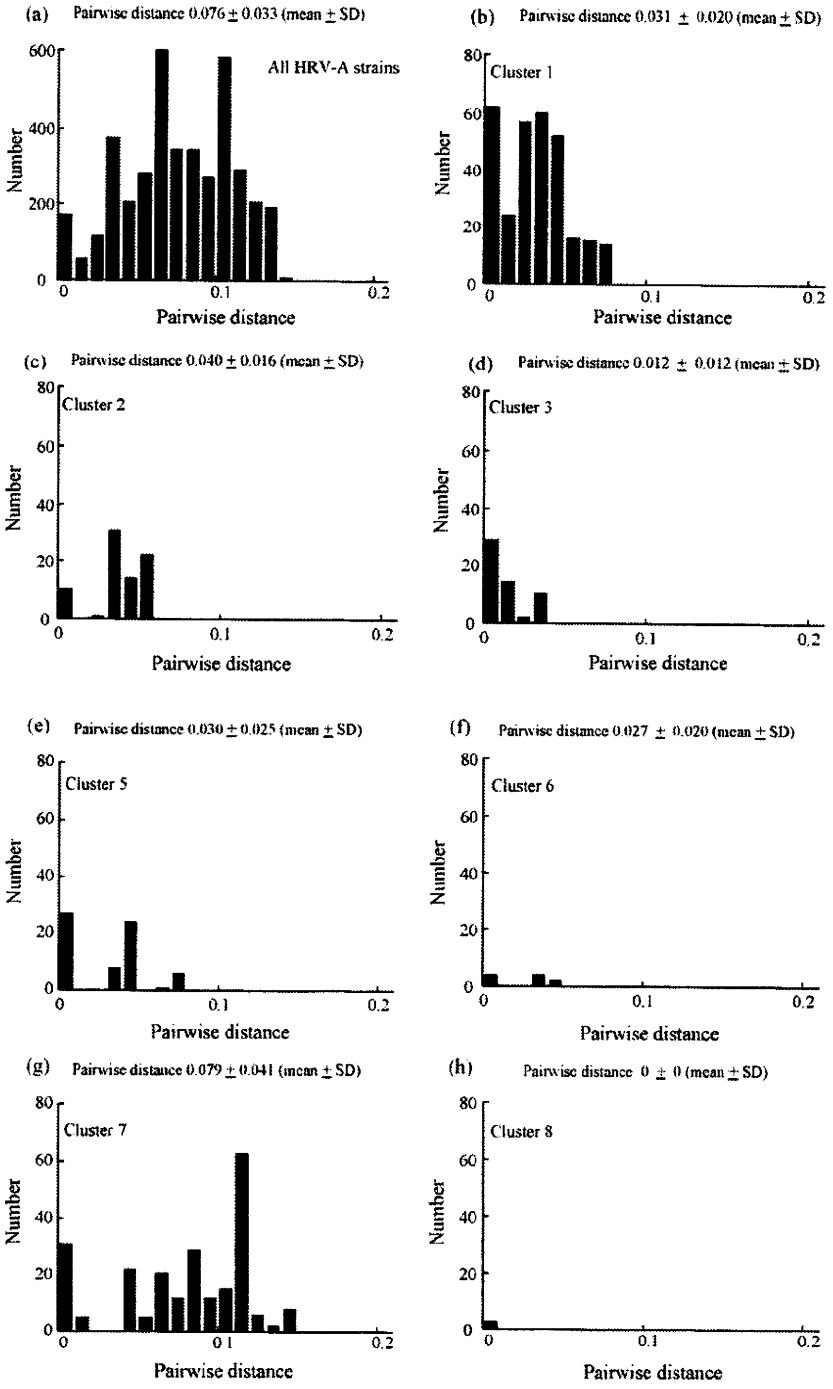

Fig. 3. Distributions of pairwise interspecies distances and pairwise distances for each intercluster for HRV-A based on deduced amino acid sequences of *VP4/VP2* coding region (130 aa). (a) Distribution of pairwise interspecies distances based on amino acid sequences of the *VP4/VP2* coding region. (b–h) Distributions of pairwise distances for each intercluster (Clusters 1–3 and 5–8).

domestic epidemiology of HRV more comprehensively large numbers of HRV strains derived from adults and large areas will be required.

In conclusion, various genetic types of HRV-A appear to be associated with ARI, including URI and wheezy bronchiolitis, in Japanese children. Further molecular epidemiologic investigations regarding the other HRV species (HRV-B and HRV-C) should be performed.

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NOTE

Endemicity of human metapneumovirus subgenogroups A2 and B2 in Yamagata, Japan, between 2004 and 2009

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ABSTRACT

To clarify a longitudinal epidemiology, we isolated 280 hMPV strains from patients with acute respiratory infections in Yamagata, Japan, between 2004 and 2009. We observed that the high season for hMPV was from winter to spring (between January and May) and the low season was in the fall (around September and October). A further molecular analysis revealed that subgenogroup A2 (A2) strains were the most commonly isolated (151/280; 53.9%), followed by B2 (108/280; 38.6%) and B1 (19/280; 6.8%). Our results suggested that A2 and B2 have been endemically in circulation as the major types almost every year, whereas other subgenogroups have appeared less frequently.

Key words acute respiratory infection, endemicity, human metapneumovirus, subgenogroup.

Human metapneumovirus (hMPV) was first described in 2001 following its isolation from infants and children with ARI (1). Based on genetic analyses, hMPV has been categorized as a member of the genus *Metapneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (2, 3). hMPV has been recognized as an important causative agent of respiratory tract disease worldwide, especially in the pediatric and elderly populations (2). hMPV is associated with the common cold and with lower respiratory tract illnesses, such as pneumonia, bronchiolitis, bronchitis, croup, and the exacerbation of reactive airway disease (2, 3). Serological studies have indicated that, by the age of 5 years, almost all children show evidence of hMPV infection (3–5). Based on genomic sequencing and phylogenetic analysis, hMPV appears to have a single serotype with two subgenogroups, A and B, which have extensive cross-reactivity and cross-protection (2, 3). Further, each genotype appears to have at least two distinct subgenogroups; A1 and A2, and B1 and B2 (3, 6). hMPV outbreaks occur in annual epidemics during late winter and early spring in temperate climates, although sporadic hMPV infection also occurs year round in temperate areas (2, 3, 7, 8). Although a longitudinal study is necessary to clarify the epidemiology of hMPV, including its seasonality, only a limited number of studies of more than 3 years in duration have been reported (9–11). Thus, we aimed to clarify the longitudinal epidemiology of hMPV based on virus isolation in a community such as Yamagata, Japan. Although we previously reported hMPV isolation using Vero E6 cell lines based on data obtained between 2004 and 2005 (12, 13), here we present the results for the entire study period, between 2004 and 2009, as a longitudinal study.

Between January 2004 and December 2009, 12 504 nasopharyngeal swab specimens were obtained from patients with ARI at pediatric clinics collaborating with the local

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List of Abbreviations: ARI, acute respiratory infection; CPE, cytopathic effect; hMPV, human metapneumovirus; RT-PCR, reverse-transcription PCR.

health authority of Yamagata Prefecture for the surveillance of viral diseases in Japan. Among them, 8112 (64.9%) were from patients <5 years old, 2918 (23.3%) were from patients between 5 and 9 years, 1064 (8.5%) were from patients between 10 and 14 years, 357 (2.9%) were from patients >14 years, and 53 (0.4%) were from patients of unknown age. The specimens were collected and placed immediately in tubes containing a transport medium, and then transported to the Department of Microbiology, Yamagata Prefectural Institute of Public Health for virus isolation (12).

Virus isolation was carried out using a modified microplate method (i.e. the HHVe6MRG plate) (12, 13). Briefly, human embryonic lung fibroblast (HEF), human laryngeal carcinoma (HEp-2), Vero E6, Madin-Darby canine kidney cells (MDCK), rhabdomyosarcoma (RD-18S) and green monkey kidney (GMK) cell lines were prepared on the wells of a 96-well microplate (Greiner Bio-One, Frickenhausen, Germany) (12, 13). After medium change, each specimen was centrifuged at 450 *g* for 20 min and 75 μL of the supernatant was inoculated onto two wells of each cell line. We observed the plates two to three times per week for CPE for approximately 1 month without passage or medium change. When a suspected hMPV CPE was observed, viral identification and genotyping were carried out by RT-PCR and sequence analysis (13). For RT-PCR and sequencing analysis, primers MPVF1f, MPVF1r, BF101, BF104 and HMPV-F1 were used (14–16). Sequence data for the isolates from Yamagata between 2004 and 2009 were registered under accession numbers AB251496-AB251574, AB518311-AB518476 and AB548217-AB548249 at GenBank. Sequence data were analyzed with CLUSTAL W version 1.83, and a phylogenetic tree was constructed by the neighbor-joining method (17) using the same software.

As a result, we succeeded in isolating a total of 280 hMPV strains, which included 79 strains reported in previous studies (12, 13), with a total isolation rate of 2.2% (280/12 504). Among 13 of these 280 samples, other respiratory viruses were co-isolated with hMPV by using the HHVe6MRG plate: adenovirus 1, echovirus 18, coxsackievirus A4, coxsackievirus B3 (three cases), coxsackievirus B4, parainfluenza 1, parainfluenza 2, parainfluenza 3, influenza B, and cytomegalovirus (two cases). The age distribution of the cases where hMPV was isolated was as follows: 208 (74.3%) were from patients <5 years old, 52 (18.6%) were from patients between 5 and 9 years, 14 (5.0%) were from patients between 10 and 14 years, and six (2.1%) were from patients >14 years. The results shown in Figure 1 are based on all 280 hMPV genotyped isolates, and those shown in Figure 2 are based on the four reference strains and 63 representative Yamagata isolates, most of which were analyzed in this study.

As shown in Figure 1, hMPV were only isolated over a 3-month period in 2004 and over a 6-month period in 2008. However, hMPV were isolated throughout most of the year between 2005 and 2007 and in 2009, although only a small number of hMPV were isolated in September and October.

As shown in Figures 1 and 2, phylogenetic analysis revealed that three hMPV subgenogroups (A2, B1 and B2) were isolated in each of the 6 years. Subgenogroup A2 (A2) strains were the most commonly isolated (151/280; 53.9%), followed by B2 (108/280; 38.6%) and B1 (19/280; 6.8%). A mixture of the three subgenogroups was observed only in 2005 and two subgenogroup strains co-circulated

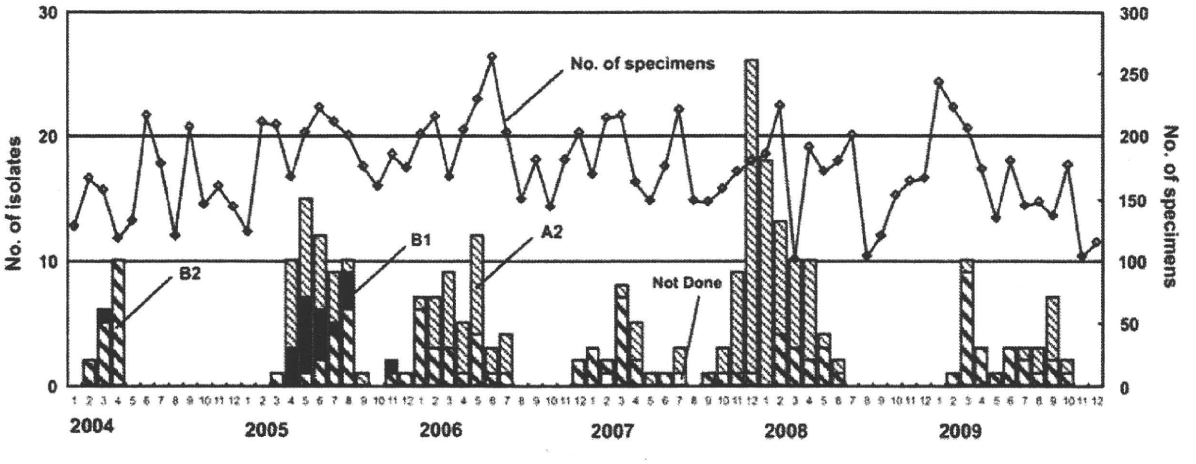

Fig. 1. Monthly distribution and subgenogroups of hMPV strains isolated in Yamagata, Japan, between 2004 and 2009. Subgenogroups, A2, B1, and B2, were grouped according to the phylogenetic analysis shown in Figure 2 and are based on reference 13.

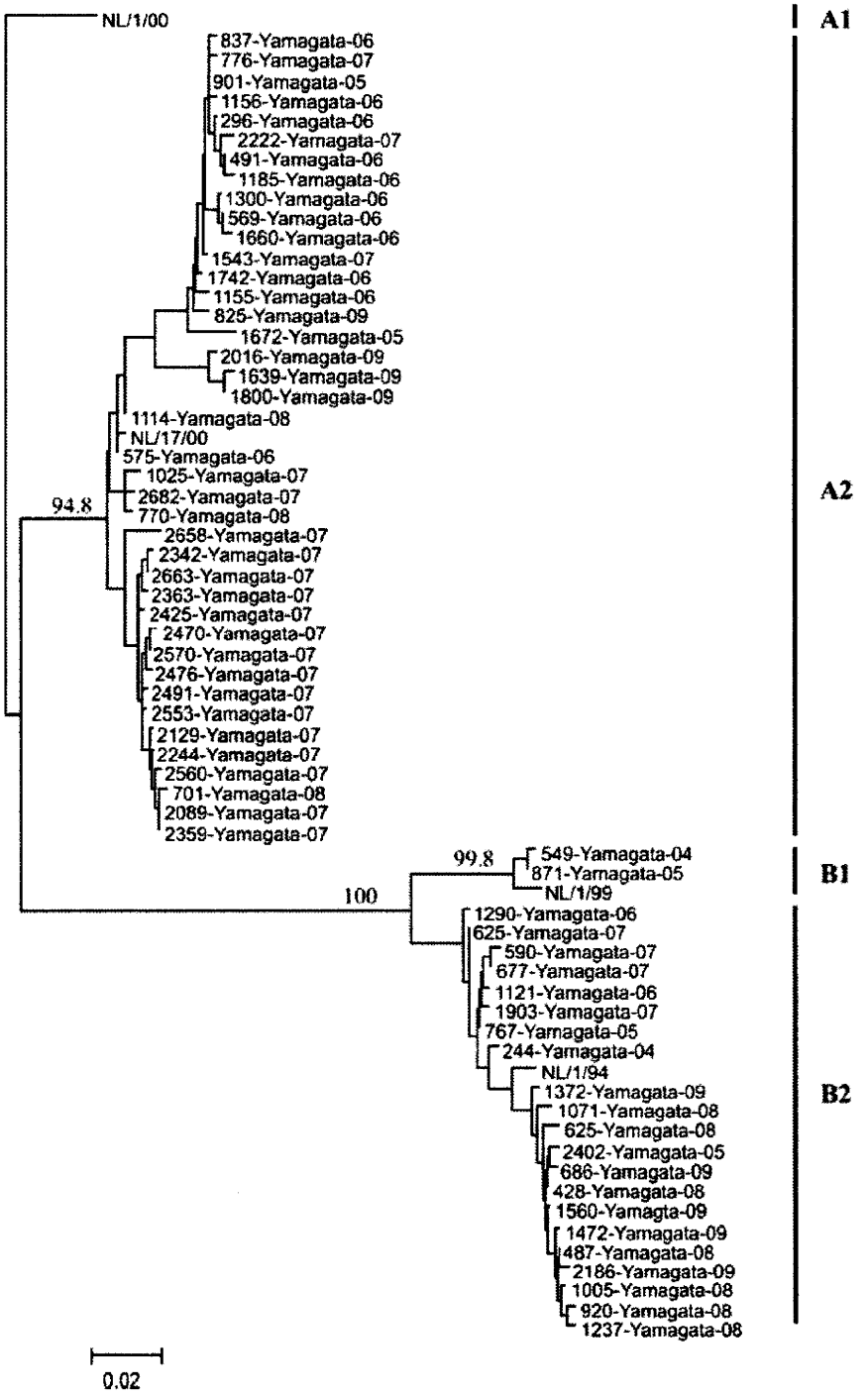

Fig. 2. Phylogenetic tree for the partial (441 bp) sequence of the fusion region of representative hMPV strains isolated in Yamagata, Japan between 2004 and 2009 as well as for reference strains. Details for the 2004–2005 Yamagata isolates are given in reference 13 and reference strains (NL/1/00, NL/17/00, NL/1/99 and NL/1/94) were based on reference 15. Branch lengths are proportional to the number of nucleotide differences. Numbers are the bootstrap probabilities (%). The marker denotes the measurement of relative phylogenetic distance.

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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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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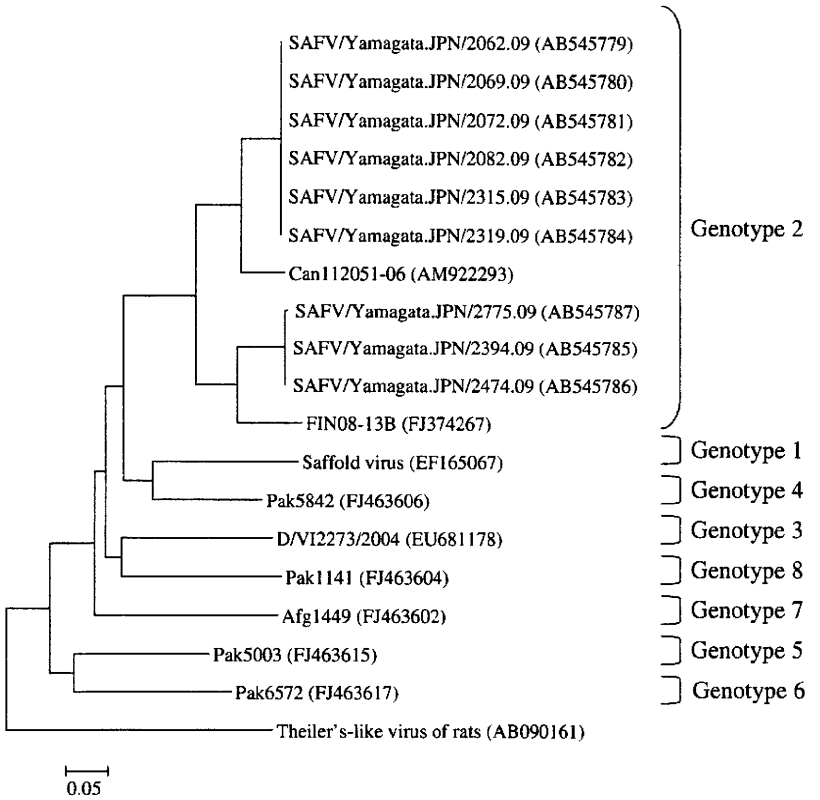

Figure 1. A phylogenetic tree of Saffold cardiovirus (SAFV) based on *VP1* gene (327 nt), constructed using *VP1* gene (GenBank accession numbers are given in parentheses).

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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Original Article

Genotyping and Phylogenetic Analysis of the Major Genes in Respiratory Syncytial Virus Isolated from Infants with Bronchiolitis

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SUMMARY: We performed the genotyping and phylogenetic analysis of respiratory syncytial virus (RSV) isolated from 17 infants with bronchiolitis in Kanagawa Prefecture, Japan in 2005 and 2006. The major genes in these samples (attachment [*G*] glycoprotein gene, fusion [*F*] protein gene, and nucleoprotein [*N*] gene) were sequenced and analyzed genetically. Phylogenetic analysis of these genes revealed that 7 and 10 strains could be classified into subgroups A and B, respectively. Phylogenetic analysis of the *G* gene revealed that the subgroup A and B strains were unique genotypes GA2 and BA, respectively. Moreover, the amino acid sequences for these genotypes suggested a relatively high frequency of amino acid substitutions in the G and F proteins in these strains, whereas the N protein was highly homologous. These results suggest that RSV genotypes GA2 and BA may be associated with bronchiolitis in the cases studied here.

INTRODUCTION

Respiratory syncytial virus (RSV; genus *Pneumovirus*, family *Paramyxoviridae*) causes acute respiratory infection (ARI), which is the most common disease in children around the world (1,2). RSV can also cause severe obstructive pulmonary disease such as bronchiolitis and bronchopneumonia (3–5). However, despite the fact that RSV is a common cause of bronchiolitis or pneumonia, particularly in infants (aged under 1 year), the underlying mechanisms leading to such conditions remain unknown (6). RSV infections can be life-threatening in these cases (2,7), and recent studies have suggested that RSV infections might trigger or exacerbate airway hyperresponsiveness, including asthma (8,9). Indeed, it is generally recognized by pediatricians that virus-induced asthma caused by RSV or other viruses can result in refractory airway disease (10).

The RSV genome encodes around 10 proteins, the major antigens of which are attachment (G) glycoprotein and fusion (F) glycoprotein (2). As nucleocapsid (N) protein is also an essential structural protein of antigenomic replicative intermediate RNA (2), these major proteins might mediate infection of the host cell and RNA replication.

Molecular epidemiological studies focusing on the analysis of *G* gene have suggested that RSV is classified into subgroups A and B (11). On the basis of this classification, it has been suggested that a specific genotype, GA3, might be associated with a significantly greater severity of illness (12), although other reports have suggested that the severity of illness is linked to the amount of RSV in nasopharyngeal aspirate rather than the viral subgroup (13,14). Thus, the association between a specific virus type and the severity of RSV infections, including bronchiolitis, has not been accurately addressed. In light of this, we performed genetic analysis of the major genes (*G*, *F*, and *N* genes) of RSV isolated from infants (aged under 1 year) with RSV-related bronchiolitis.

MATERIALS AND METHODS

Patients: Each of the 17 patients (mean age ± standard deviation [SD], 4.7 ± 3.4 months) examined for this study exhibited fever and wheeze on initial examination. Chest X-rays of all patients showed the typical hyperinflation and reticulogranular pattern indicative of bronchiolitis. None of the patients had a history of contact with each other. All patients were admitted to the Fujisawa City Hospital or the National Hospital Organization Yokohama Medical Center and treated

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Table 1. Summary of patient data, subgroups, and genotypes of RSV isolates

Patient no.	Age (mo)	Onset date	Strain	Subgroup	Genotype	Genbank accession no.		
						G gene	*F* gene	*N* gene
1	2	Sep 7, 2005	RSVi/Kanagawa.JPN/Sep.05/Ka-1	B	BA	AB500659	AB500676	AB370030
2	6	Sep 7, 2005	RSVi/Kanagawa.JPN/Sep.05/Ka-2	B	BA	AB500660	AB500677	AB370031
3	3	Sep 9, 2005	RSVi/Kanagawa.JPN/Sep.05/Ka-3	A	GA2	AB500655	AB500672	AB370032
4	2	Oct 1, 2005	RSVi/Kanagawa.JPN/Oct.05/Ka-4	B	BA	AB500661	AB500678	AB370033
5	11	Oct 2, 2005	RSVi/Kanagawa.JPN/Oct.05/Ka-5	B	BA	AB500664	AB500681	AB370034
6	3	Oct 11, 2005	RSVi/Kanagawa.JPN/Oct.05/Ka-6	B	BA	AB500662	AB500679	AB370035
7	5	Oct 11, 2005	RSVi/Kanagawa.JPN/Oct.05/Ka-7	B	BA	AB500665	AB500682	AB370036
8	9	Oct 20, 2005	RSVi/Kanagawa.JPN/Oct.05/Ka-8	B	BA	AB500663	AB500680	AB370037
9	8	Nov 4, 2005	RSVi/Kanagawa.JPN/Nov.05/Ka-9	A	GA2	AB500649	AB500666	AB370038
10	6	Dec 2, 2005	RSVi/Kanagawa.JPN/Dec.05/Ka-10	A	GA2	AB500650	AB500667	AB370039
11	11	Dec 6, 2005	RSVi/Kanagawa.JPN/Dec.05/Ka-11	A	GA2	AB500651	AB500668	AB370040
12	1	Jan 1, 2006	RSVi/Kanagawa.JPN/Jan.06/Ka-12	A	GA2	AB500652	AB500669	AB370041
13	4	Jan 2, 2006	RSVi/Kanagawa.JPN/Jan.06/Ka-13	B	BA	AB500656	AB500673	AB370042
14	1	Jan 2, 2006	RSVi/Kanagawa.JPN/Jan.06/Ka-14	A	GA2	AB500653	AB500670	AB370043
15	1	Jan 10, 2006	RSVi/Kanagawa.JPN/Jan.06/Ka-15	B	BA	AB500657	AB500674	AB370044
16	2	Feb 3, 2006	RSVi/Kanagawa.JPN/Feb.06/Ka-16	B	BA	AB500658	AB500675	AB370045
17	4	Feb 10, 2006	RSVi/Kanagawa.JPN/Feb.06/Ka-17	A	GA2	AB500654	AB500671	AB370046

with infusion, oxygen, and beta2-agonist or epinephrine nebulization. Patient data are summarized in Table 1.

Virus isolation: HEp-2 (ATCC CCL-23), HEL (WI-38 strain, ATCC CCL-75), RD (ATCC CCL-136), and MDCK cells (ATCC CCL-34) were obtained from the American Tissue Culture Collection (Rockville, Md., USA) and grown in Dulbecco's modified Eagle minimal medium (Invitrogen, Carlsbad, Calif., USA) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 for cell culture and viral isolation (15). Throat swabs from patients were inoculated into these cell lines grown in 24-well microplates (Corning, Corning, N.Y., USA) and incubated at 33°C (for isolation of influenza virus) or 36°C in a humidified atmosphere of 5% CO_2 (15). Viruses were isolated as described previously (15). No significant presence of any pathogen other than RSV was detected in any of the patients.

Reverse transcription-polymerase chain reaction (RT-PCR): Viral suspensions (isolates) were centrifuged at 3,000 × *g* at 4°C for 30 min for RNA extraction, RT-PCR, and sequence analysis. The supernatants (culture supernatants) were used for RT-PCR and sequence analysis, as described previously (16–19). Briefly, RSV RNA was extracted from 140 μL of the culture supernatants using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA). The resulting RNA solution was treated with DNase I (Takara, Tokyo, Japan), and the reverse transcription reaction mixture was incubated with random hexamer primers at 42°C for 75 min, followed by incubation at 99°C for 5 min, and then amplified by thermal cycling.

The *G* gene of RSV was the target for the external and seminested PCRs. External PCR was carried out with primers ABG490 and F164. The forward primer, ABG490 (5′-ATGATTWYCAYTTTGAAGTGTTC-3′), corresponded to bases 497–519 of the G protein gene of strain A2, and bases 491–513 of the G protein gene of strain 18537. The reverse primer, F164 (5′-GTTATGACACTGGTATACCAACC-3′), corresponded to bases 164–186 of the F protein gene of strain 18537 (with one mismatch with the G protein gene of strain A2), and has previously been used to amplify the G protein genes of both groups (16). Amplification was carried out at 94°C for 1 min, followed by 35 cycles of 94°C for 40 s, 50°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. One microliter of diluted external PCR product was used for the seminested PCR. Subgroup A-specific primer AG655 (5′-GATC YCAAACCTCAAACCAC-3′), corresponding to bases 655–674 of the G protein gene of strain A2 for subgroup A, and subgroup B-specific primer BG517 (5′-TTYGTT CCCTGTAGTATATGTG-3′), corresponding to bases 517–538 of the G protein gene of strain 18537 for subgroup B, were used as forward primers, and F164 was used as the reverse primer for the seminested PCR (17). Amplification was carried out at 94°C for 1 min, followed by 40 cycles of 94°C for 40 s, 54°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. The nested amplicons were 450/585 bp and 645 bp for group A/B and BA viruses, respectively. The primers for *F* gene were designed as follows: the anterior half of the *F* gene (nucleotides 3–1,068 of the N terminal region) was amplified at 94°C for 1 min, followed by 35 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 60 s, with a final extension at 72°C for 10 min, using the RSV-U primer (5′-GGCAAATAACAATGGAGTTG-3′) and the RSV-4R primer (5′-AAGAAAGATACT GATCCTG-3′) (18). The primers for *N* gene were as follows: RSVN3 (5′-GCG GCA GCT ACT ACA AGC AG-3′), corresponding to bases 426–451 of the *N* gene of strain A2, and RSVN5 (5′-TCG GGC GGG ACC TGG ACC TC-3′), corresponding to bases 748–773 of the *N* gene of strain A2 (19). Amplification was carried out at 94°C for 3 min, followed by 35 cycles at 94°C for 40 s, 68°C for 30 s, and 72°C for 45 s, ending with extension for an additional 10 min at 72°C. Purification of the DNA fragments and determination of the nucleotide sequences was performed as described previously (20).

Phylogenetic and deduced amino acid sequence analysis: Phylogenetic analysis based on the nucleotide sequences of the *G*, *F*, and *N* genes was performed as described previously (11,16). The nucleotide sequence of *G* gene spanned bases 673–912 (240 bp) of prototype strain A2 (GenBank accession no. M11486) (11). For group B viruses, the sequence corresponded to bases 670–963 (294 bp) of a genotype BA strain from Argentina (strain BA4128/99B; GenBank accession no. AY333364) (11). The *F* gene is located within the F2 subunit and the 550 nucleotides of *F* gene (nucleotides 23–572 of the N-terminal region) were found to be distinct (21,22). The nucleotide sequences of the partial *N* gene of RSV (positions 452–747: 296 bp) were analyzed as described previously (19) using the CLUSTAL W program on the DNA Data Bank of Japan (DDBJ) homepage (http://www.ddbj.nig.ac.jp/Welcome-j.html) and TreeExplorer (version 2.12) (http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbor-joining (NJ) method (20,23). The reliability of the tree was estimated using 1,000 bootstrap replications and intercluster frequency distributions were calculated using pairwise genetic distances for all strains, as described previously (24).

Prototype strains were used to compare the deduced amino acid sequences of these genes encoding the proteins, as described previously (11,16). Thus, strains A2 (for subgroup A, GenBank accession no. M11486) and BA4128/99B (for subgroup B, GenBank accession no. AY333364) (11) were used to compare G protein sequences, whereas strains A2 (for subgroup A, GenBank accession no. M11486) and 18537 (for subgroup B, GenBank accession no. D00334) (16) were used to compare F protein. Finally, strains A2 (for subgroup A, GenBank accession no. M11486) and 18537 (for subgroup B, GenBank accession no. D00736) were used to compare N protein sequences.

RESULTS

Phylogenetic and amino acid analysis of *G* gene/*G* protein: The phylogenetic tree based on the nucleotide sequences of the *G* gene is shown in Fig. 1. Seven and 10 of the 17 RSV strains were classified to subgroups A and

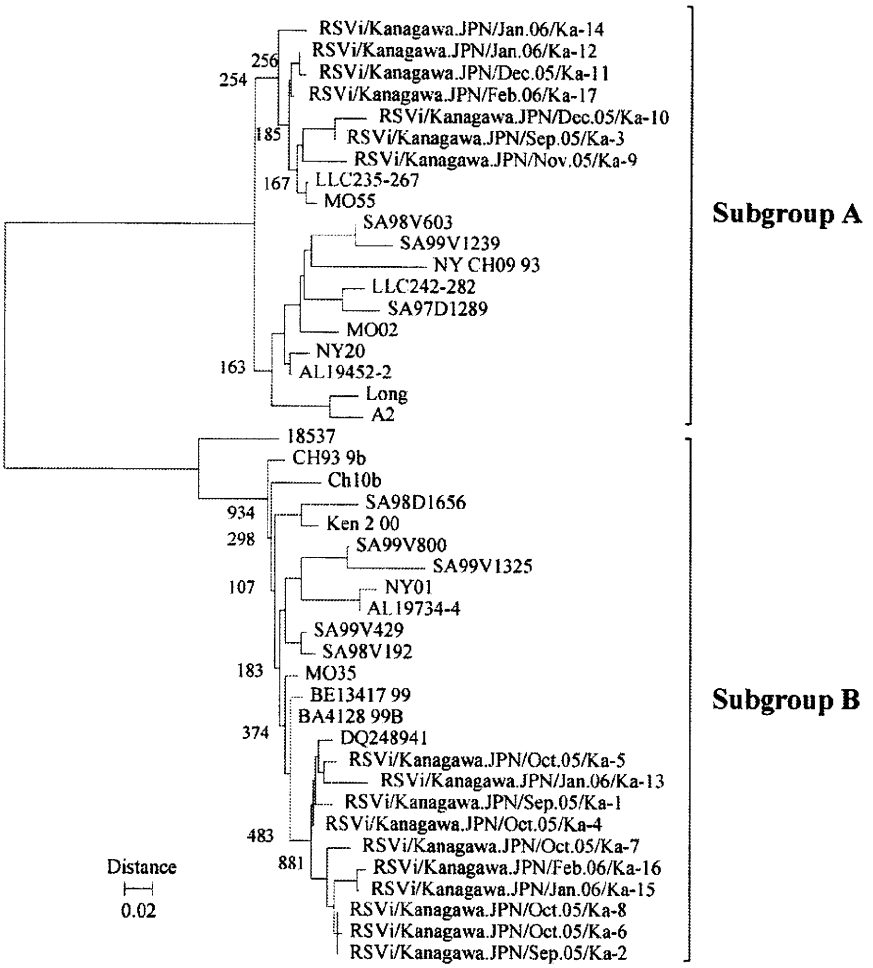

Fig. 1. Phylogenetic tree based on *G* gene sequences of various RSV strains. Evolutionary distance was calculated using Kimura's two-parameter method, and the tree was plotted using the NJ method. Numbers at main branches indicate the bootstrap values of the clusters supported by that branch. GenBank accession numbers of the strains are AB500649 to AB500665.

Fig. 2. Amino acid alignments of G protein gene from subgroup A (A) and subgroup B (B) strains. Alignments are shown relative to the sequences of prototype strain A2 and prototype strain BA4128/99B strain. Amino acids shown correspond to strain A2 G protein, positions 220 to 298 for the group A viruses, or to strain BA4128/99B G protein, positions 219 to 315 for the group B viruses. Identical residues are indicated by dashes.

B, respectively. Moreover, all strains belonging to subgroup A were located in genotype GA2, and all strains belonging to subgroup B were genotyped as BA. The homology between A2 (prototype strain) and the present GA2 type strains was 85.8–88.3% at the nucleotide level and 73.1–78.2% at the amino acid level, whereas the homology between the prototype of genotype BA (BA4128/99B strain) and the present genotype BA strains was 94.6–98.0% at the nucleotide level and 90.8–95.9% at the amino acid level. The mean (SD) intercluster distances in this tree were 0.049 ± 0.025 and 0.033 ± 0.018 between the present subgroup A and B strains, respectively. The amino acid sequences of subgroup A and B strains were compared to the prototype A2 and BA4128/99B strains, respectively (Fig. 2). Relatively frequent amino acid substitutions were seen in the present strains belonging to both subgroups. Moreover, when G protein amino acid sequences were compared for the present genotype GA2 strains and Long strain (a representative prototype subgroup A strain), the positively selected changes Pro226Leu, Ser269Thr, Pro289Ser, and Pro290Leu were seen in all the genotype GA2 strains (25).

Phylogenetic and amino acid analysis of *F* gene/F protein: The phylogenetic tree based on the nucleotide sequences of the *F* gene is shown in Fig. 3. Similarly to the *G* gene, 7 and 10 strains were classified into subgroups A and B, respectively. The homology between A2 strain and the present strains was 90.2–94.1% at the nucleotide level and 84.2–92.4% at the amino acid level, whereas the homology between the prototype of subgroup B (18537 strain) and the present strains was 94.7–97.3% at the nucleotide level and 88.0–98.4% at the amino acid level. The mean (SD) intercluster distances between the present subgroup A and B strains in this tree were 0.062 ± 0.026 and 0.031 ± 0.016, respectively. The amino acid sequences of subgroup A and B strains were compared to those of A2 and 18537 strains, respectively (Fig. 4), and clusters of amino acid substitutions of F protein were found in the present strains belonging to both subgroup A and B.

Phylogenetic and amino acid analysis of *N* gene/N protein: As was the case with the *G* and *F* genes, 7 and 10 strains were classified into subgroups A and B, respectively (Fig. 5). The homology of *N* gene between the A2 strain and the present strains was 96.6–97.0% at the nucleotide level and 98.0–99.0% at the amino acid level, whereas the homology between the prototype of subgroup B (18537 strain) and the present strains was 97.6–98.0% at the nucleotide level and 99.0–100% at the amino acid level. The mean (SD) intercluster distances in this tree were 0.010 ± 0.010 and 0.001 ± 0.001 between the present subgroup A and B strains, respectively. In addition, one amino acid substitution (at nt 1783, from histidine [His] to tyrosine [Tyr]; amino acid [aa] 216) was found in the present subgroup A strains with respect to the A2 strain (representative subgroup A strain); no substitution was found upon comparing our subgroup B strain with the 18537 strain (representative subgroup B strain). Thus, the present study appears to suggest that the high degree of homology for the *N* gene in RSV may be associated with bronchiolitis in infants.

DISCUSSION

Phylogenetic analysis of the *G*, *F*, and *N* genes from the 17 RSV isolates obtained from Japanese infants with bronchiolitis revealed that 7 and 10 strains could be classified into subgroups A and B, respectively. The phylogenetic tree based on the *G* gene sequences showed that all strains belonging to subgroups A and B could be classified into types GA2 and BA, respectively. There was greater nucleotide divergence between the *G* and *F* genes in the present subgroup A strains than in the subgroup B strains. In addition, the amino acid sequences of the *G* and *F* genes indicated that substitutions were relatively frequent with respect to the prototype strains. In contrast, the amino acid sequences of the *N* gene were highly conserved compared with the prototype

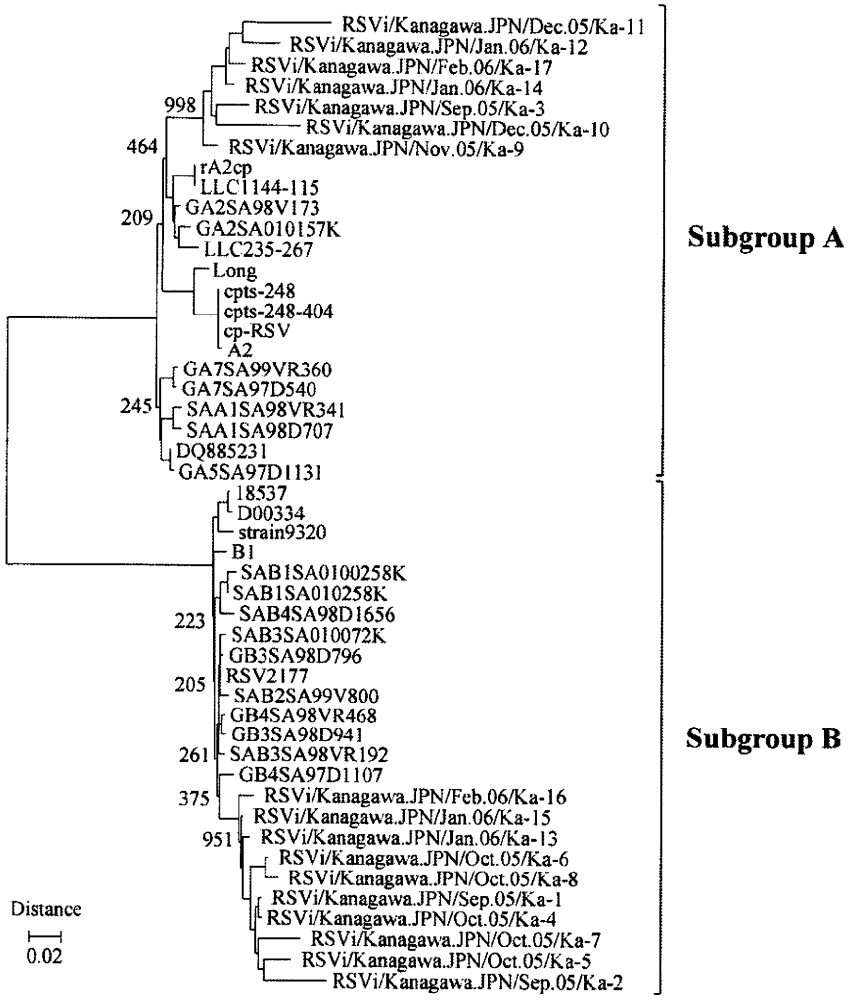

Fig. 3. Phylogenetic tree based on *F* gene sequences of various RSV strains. Evolutionary distance was calculated using Kimura's two-parameter method, and the tree was plotted using the NJ method. Numbers at main branches indicate the bootstrap values of the clusters supported by that branch. GenBank accession numbers of the strains are AB500666 to AB500682.

strains. In the present cases, RSV strains with a relatively wide genetic diversity as regards the *G* and *F* genes might be associated with bronchiolitis.

Previous studies have suggested that G and F proteins are the major antigens of RSV and are linked to neutralization, antigenicity, and virulence (16,18,21). Thus, the phylogenetic analysis of RSV *G* gene by Martinello et al. showed that the GA3 genotype is associated with greater severity of illness in, for example, bronchiolitis and pneumonia (12). Similarly, correlations between certain strains and/or genotypes of RSV and slight differences in disease severity have been described by Hall et al. and Walsh et al. (26,27). In recent years, some genotypes, such as GA2, GA5, and BA, have been reported to be prevalent throughout the world (11,28–30). However, to gain a better understanding of the molecular epidemiology of RSV, it may be important to determine which RSV genotypes circulate in different areas. In the present study, two RSV genotypes (GA2 and BA) were found to be associated with bronchiolitis, in accordance with previous reports. In addition, the severity of illness caused by subgroup A isolates did not differ from that caused by subgroup B isolates in the present study. Indeed, previous reports have suggested that the severity of the illness is not linked to subgroups or genotypes but to the quantity of RSV in nasopharyngeal aspirate (13,31), and a very recent study suggested that the ectodomain of G protein was subject to strong positive selection, with 29 positively selected amino acid sites in RSV subgroup A (25). A comparison of the amino acid sequences of G protein between genotype GA2 and Long strain (a prototype subgroup A strain) found some positively selected sites (Pro226Leu, Ser269Thr, Pro289Ser, and Pro290Leu), whereas Pro226Leu defined genotype GA3 (25). In addition, some of these sites (226 and 290) are known epitopes in RSV subgroup A. Thus, variations of the amino acids at these sites may play a key role in severe respiratory infections such as bronchiolitis. The G protein is a major target (together with the F glycoprotein) of the RSV human immune response. Antigenic and genetic variations have been shown to occur more frequently in G protein than in F protein, thus suggesting a remarkable genetic flexibility in the G protein sequences

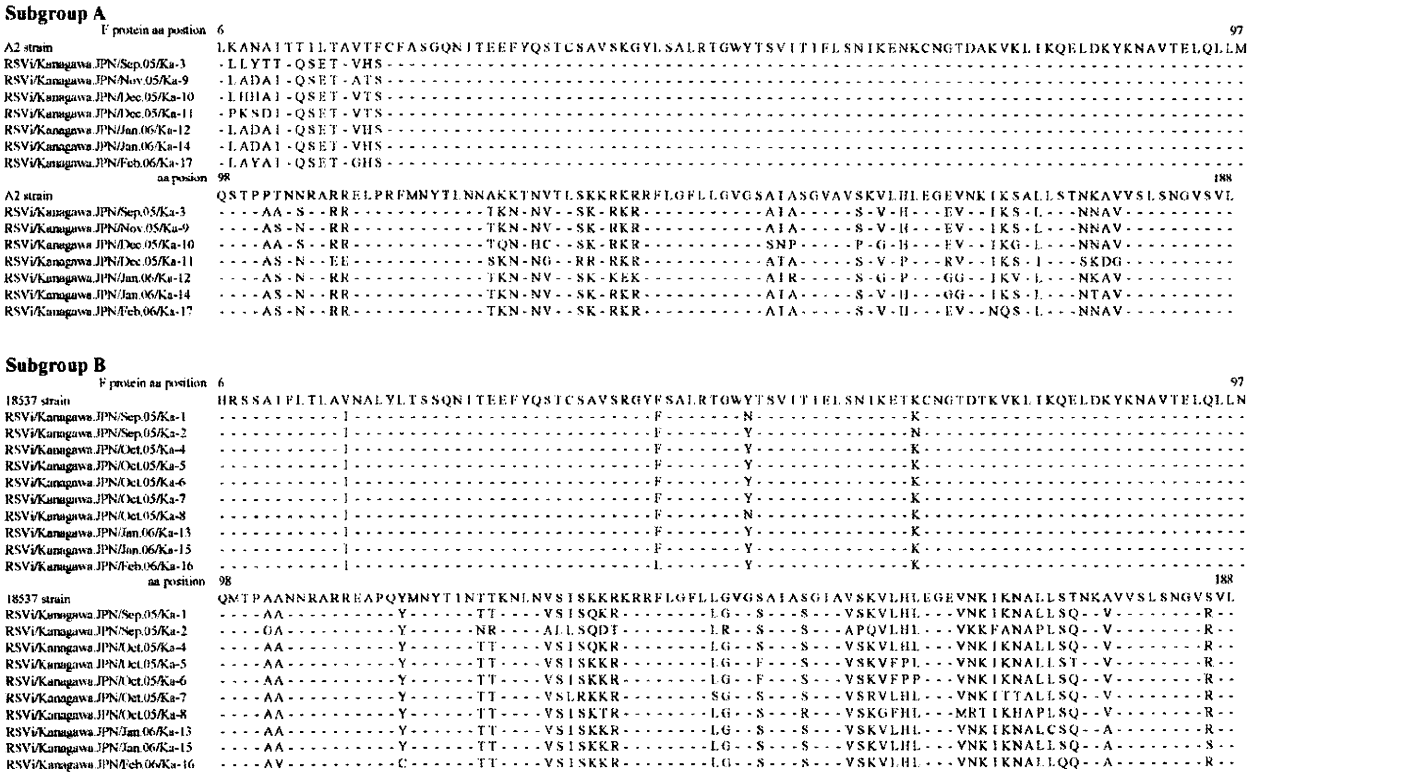

Fig. 4. Amino acid alignments of G protein gene from subgroup A (A) and subgroup B (B) strains. Alignments are shown relative to the sequences of prototype strain A2 and prototype strain 18537 strain. The amino acids shown correspond to strains A2 and 18537 F protein positions 6 to 188 for the group A and B viruses. Identical residues are indicated by dashes.

of RSV. Such a high level of genetic variation might be associated with the fact that G protein plays an important role in facilitating infection in RSV. Riccetto et al. have also demonstrated that the severity of illness of RSV infection in infants is associated with other factors such as body weight and prematurity (32). The association between RSV infection and severity of illness is not yet well understood, thus suggesting the need for additional studies.

Recent studies have shown that RSV antigenic epitopes are insufficiently recognized by innate TLR4-expressing immune cells, which results in low antibody avidity for protective epitopes (33). Awomoyi et al., for example, have suggested that TLR4 polymorphism is linked to symptomatic RSV (34), therefore, both the antigenicity of RSV and the host immune conditions, such as innate immunity, may play important roles in the pathophysiology of severe respiratory infections such as bronchiolitis and pneumonia.

RSV is a major causative agent of severe respiratory infections such as bronchiolitis and pneumonia in infants. The results of the present and other studies suggest that bronchiolitis-associated RSV might show wide genetic diversity, although additional molecular epidemiologic studies are warranted to better understand such RSV infections.

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Conflict of interest None to declare.

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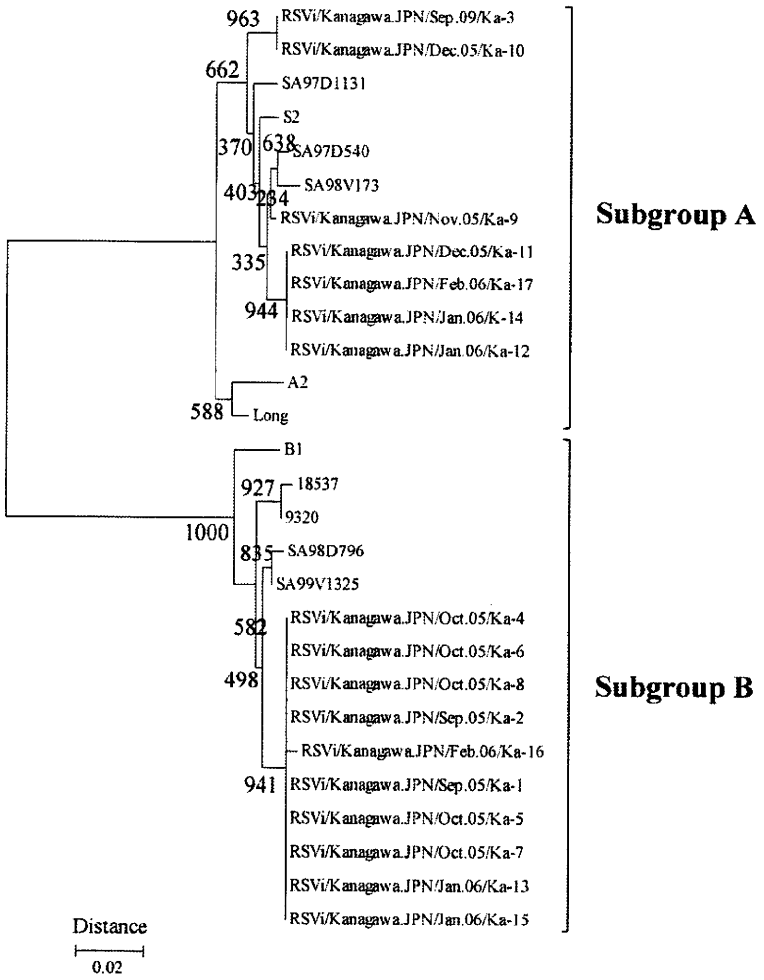

Fig. 5. Phylogenetic tree based on *N* gene sequences of various RSV strains. Evolutionary distance was calculated using Kimura's two-parameter method, and the tree was plotted using the NJ method. Numbers at main branches indicate the bootstrap values of the clusters supported by that branch. GenBank accession numbers of the strains are AB370030 to AB370046.

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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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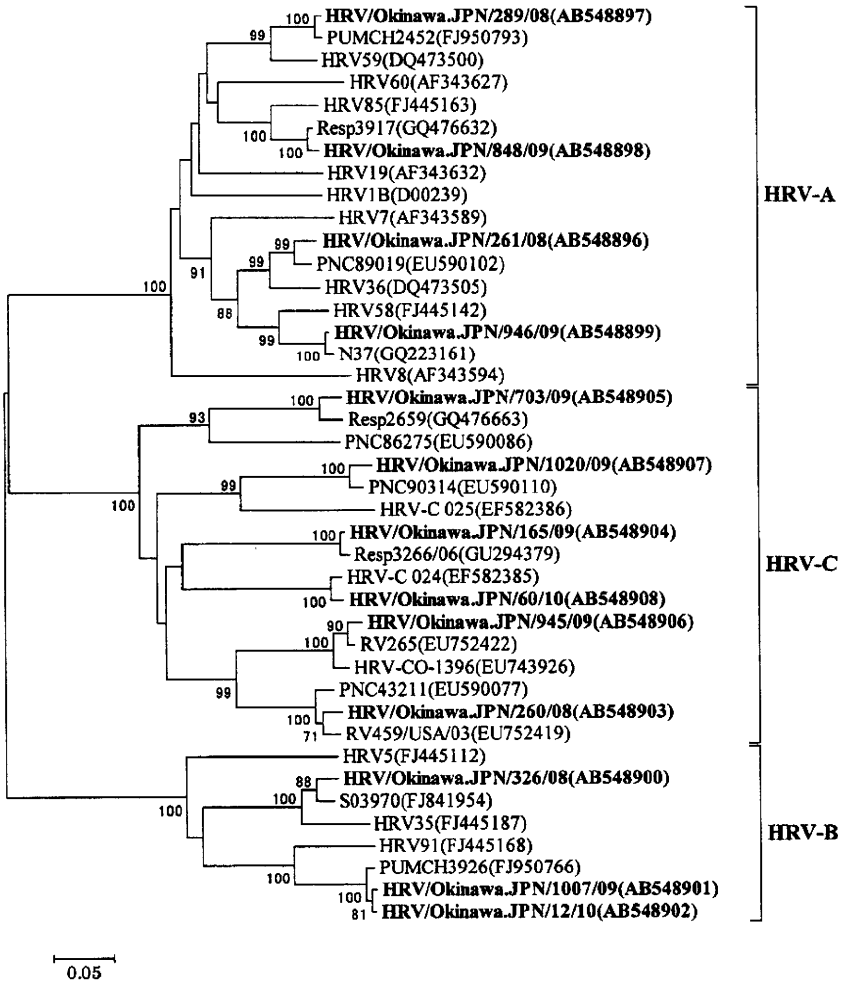

Fig. 1. Phylogenetic tree based on the *VP4/VP2* coding region sequences (393 nt) of the 41 human rhinoviruses (HRVs) including the present strains and reference strains. The present strains are shown as bold letters. Numbers in parentheses indicate the Genbank accession number. The numbers at each branch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