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Modified Adult Measles in Outbreaks in Japan, 2007–2008

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Different genotypes of C1, D3, D5, and H1 were isolated in outbreaks of 1984, 1987–1988, 1991–1993, and 2001, respectively, when the previous circulating genotype was replaced successively by a new genotype, through molecular studies of measles since 1984 in Japan. In March 2007, several patients with measles were observed in outpatient clinics, who were all young adolescents in high school and university students. The outbreak expanded subsequently throughout Japanese districts in May and is still ongoing in 2008. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) was used to detect the measles genome from 18 clinical samples obtained from patients suspected of modified measles infection with a very mild febrile illness. The measles genome was detected in nine patients by reverse transcription polymerase chain reaction (RT-PCR) and in 12 patients by RT-LAMP. Six measles strains were isolated in the 2007–2008 outbreak and identified as the D5 genotype (MVi/Bangkok.THA/93 type) different from the D5 sub-cluster (MVi/Palau.BLA/93 type) isolated in 1990–2005. Similar Bangkok type D5 strains were isolated in Phnom Penh in 2002 and in Taiwan in 2003, suggesting that the D5 strains might have been introduced via South East Asia, rather than resulting from the accumulation of mutations in the D5 strains of 1990–2005. One D9 strain was isolated from a sporadic case in Aichi in 2006. There was no difference in the antigenicity of the D9 and D5 strains in comparison with the vaccine strain. Infrastructure of systematic laboratory-based surveillance system should be established in order to confirm measles virus infection in Japan. ***J. Med. Virol. 81:1094–1101, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: measles virus; measles vaccine; modified measles; genotype; neutralization test antibody

INTRODUCTION

Measles is still a major killer among infants in developing countries, and the World Health Assembly endorsed a resolution to achieve the goal of reduction of measles deaths that occurred in 1999 by half by the end of 2005 [WHO, 2002]. WHO/UNICEF estimates indicated that global routine measles vaccination coverage increased from 72% in 2000 to 80% for the first dose in 2006 and that the number of measles-related deaths decreased from 873,000 in 1999 to 345,000 in 2005 and to 242,000 in 2006, and so the tentative goal for 2005 was achieved on schedule [WHO, 2002; CDC, 2007]. Indigenous outbreaks of measles were eliminated in the USA by the implementation of a two-dose measles–mumps–rubella trivalent vaccine (MMR) program and the sporadic cases reported in the USA were caused by importation from areas where measles is not yet controlled, such as Africa and Asian countries, including Japan [Rota et al., 1998, 2002; Strebel et al., 2004].

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When measles was introduced to the USA, extensive transmission was controlled within two generations of secondary transmission by maintaining high-level vaccine coverage, effective surveillance, and a prompt outbreak response [CDC, 2008a,b]. The two-dose strategy has eliminated measles without indigenous measles transmission chain [Janaszek et al., 2003; Meissner et al., 2004]. Thus, the proportion of countries offering children a second dose of measles vaccine is increasing, and 168 (88%) countries implement the two-dose strategy [WHO, 2006]. The new goal is a 90% reduction of measles mortality by 2010 compared with the mortality in 1999 [WHO, 2006].

As for the reporting system for measles surveillance in Japan, through 3,000 sentinel clinics or hospitals for pediatric infectious diseases and 450 clinics for adult measles surveillance, patients with clinically suspected measles are reported to the Regional Health Care Center mostly without any systematic virological studies. The last measles outbreak was observed in 2001 in Japan. Among 33,812 reported cases, most patients were under 5 years of age and had not been vaccinated. Through a vaccination campaign to increase immunization coverage at 1 year of age, the number of reported cases decreased to 545 in 2005. The Japanese Government implemented a two-dose, combined measles and rubella vaccine (MR) for children at 1 and 6 years of age in 2006 [National Institute of Infectious Diseases, 2007]. Therefore, elimination of measles would be expected. However, patients with measles were reported increasingly in March 2007, and this outbreak expanded subsequently throughout the Japanese districts, peaking in the middle of May. Further, several reports indicated measles transmission by Japanese travelers or participants in an international sporting event [Delaportel et al., 2007; CDC, 2008a].

This outbreak showed different characteristics, demonstrating that most patients were young adults or adolescents attending high school and university students, with a much lower proportion of young infants, at the early stage of the outbreak. Finally, cases of measles were reported in all age groups, and a total of 3,105 pediatric cases and 959 adult patients were reported in 2007 and the outbreak is still ongoing. The actual number of cases of measles was estimated to be 10 times higher than the number of reported cases for pediatric measles, and 50–60 times higher for measles in adults. Thus, the number of patients with measles was suspected to be approximately 31,000 for pediatric and 50,000 for measles in adults. The age distribution was quite different from the previous outbreak in 2001. The number of patients with measles was the highest at 1–4 years of age, accounting for 40–50% in 2001, which decreased to 22% in the outbreak in 2007. A significant shift in the age distribution of cases of measles in 2007 was observed to be 10–14 years or higher, accounting for 44% in 2007 [National Institute of Infectious Diseases, 2007]. A relatively large proportion of adult patients with measles had a previous immunization history and, thus, typical measles symptoms were not observed, with patients only with mild fever and skin eruptions. In this report, virus isolation and detection of the measles virus genome by the reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) were examined in clinical samples. The genotyping and antigenicity of current circulating viruses were also investigated.

MATERIALS AND METHODS

Materials

Nasopharyngeal swabs were obtained from 22 patients suspected of measles on day 1 or 2 of the onset of the rash. Four patients had typical symptoms of measles with post-pigmentation, and 18 had atypical symptoms with fever for less than 3 days and mild eruptions, which did not satisfy the criteria of clinical measles by WHO, [2006].

Virus Isolation

B95a cells were cultured in RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS) and 0.1 ml of clinical samples was used to inoculate a monolayer of B95a cells in a 24-well plate. After two passages, samples without cytopathic effect (CPE) were considered negative. Seven strains were isolated in this study. MVi/Aichi.JPN/44.06 [D9] was isolated from a sporadic case in Nagoya City, a central district of Japan, in 2006. MVi/Tokyo.JPN/17.07 [D5] and MVi/Tokyo.JPN/18.07 [D5] were isolated in Tokyo and MVi/Mie.JPN/19.07 [D5] and MVi/Mie.JPN/23.07 [D5] in Mie Prefecture, a central district of Japan, in 2007. In addition, MVi/Mie.JPN/41.07 [D5] was obtained in the middle of the outbreak in 2007 and MVi/Mie.JPN/03.08 [D5] was isolated in 2008.

RT-PCR and Sequence Analysis

Total RNA was extracted from 200 μl of clinical samples with a magnetic bead RNA extraction kit (TOYOBO Co., Ltd., Osaka, Japan), and the RNA pellet was suspended in 30 μl of distilled water. The pellet was subjected to RT-nested PCR and RT-LAMP targeting the C-terminus of the N protein region, known as the most variable region [WHO, 2001]. The measles virus genome was first converted to cDNA with the N-430 (+) primer (5′-ATTAGTAGTGATCAATCCAGG) with AMV reverse transcriptase (Life Technologies Inc., Gaithesburg, MD). The first PCR was performed with a set of N-850 (+) (5′-TAGAAACTATGTATCCTGCT-3′) and MPX (−) (5′-AGGCCTGATTGAACCATGAT-3′) and the nested PCR was conducted with N1200 (+) (5′-GATCCAGCATATTTTAGATTAG-3′) and NP-P2 (−) (5′-AGGGTAGGCGGATGTTGTTCT-3′). PCR was performed using 1.25 U of *Taq* DNA polymerase (TaKaRa Bio-Medicals, Tokyo, Japan) with a TaKaRa thermal cycler (TaKaRa BioMedicals), with 30 rounds of thermal cycling conditions: denaturing at 93°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2.5 min. PCR products were confirmed by

electrophoresis through 1.5% agar stained with ethidium bromide, as previously reported [Nakayama et al., 1995; Yamaguchi, 1997; Zhou et al., 2003]. PCR products were excised from the gel and applied to sequence analysis by the dye terminator method using ABI 3130 (Applied Biosystems, Tokyo, Japan).

Measles Virus Loop-Mediated Isothermal Amplification (LAMP)

The LAMP method is characterized by auto-cycling strand displacement DNA synthesis with *Bst* DNA polymerase (New England Biolabs, Ipswich, MA) and a specially designed set of primers. Six LAMP primers were synthesized, recognizing eight different regions: F3 (5′-ACATTGGCATCTGAACTC), B3 (5′-TCCTCGACTCTGTTTGAC), FIP (5′-TGTCCTCAGTAGTATGCATTGCAGGTATCACTGCCGAGGATG), BIP (5′-AGCCCAAGTGTCATTTCTACACGGTGTCCTATCTTCCTTGCCCCCC), F Loop (5′-ATCTCTGAAACAAG), and B Loop (5′-CAAAGTGAGAATGAGCT). For the LAMP reaction, the mixture was made up to a total of 25 μl of reaction mixture, containing 40 pmol (each) of FIP and BIP, 5 pmol (each) of F3 and B3, 20 pmol (each) of Loop F and Loop B, 1.4 mM each of dNTPs, 0.8 M betaine, 20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween-20, 0.5 U AMV reverse transcriptase (New England Biolabs), 8 U *Bst* DNA polymerase (New England Biolabs), and 5 μl of sample RNA. The reaction mixture was subjected to real-time turbidimeter LA200 (TERAMECS, Kyoto, Japan) [Mori et al., 2004; Fujino et al., 2005] and the LAMP reaction was carried out at 63°C for 60 min. The turbidity measurement was closely related to the amplification of DNA and the turbidity >0.1 was considered LAMP-positive [Mori et al., 2004].

Neutralization Test (NT) Antibody

A total of 32 serum samples were used, which were obtained in April 2007, just before the peak of the outbreak, as part of a sero-epidemiological study on entering primary school at the age of 6 years, having received two doses of measles vaccine. The AIK-C vaccine strain [A], MVi/Tokyo.JPN/31.00-K [D5], MVi/Tokyo.JPN/17.07 [D5], and MVi/Aichi.JPN/44.06 [D9] were used as the challenge virus. Sera were treated at 56°C for 30 min to inactivate the complement activity and serially diluted by twofold, starting from 1:4 dilutions. Diluted sera were mixed with approximately 100 TCID50 of challenge virus at 37 C for 90 min and the mixture was placed on a monolayer of B95a cells. NT antibody titers were expressed as the reciprocal of the serum dilutions that neutralized the appearance of the CPE of measles virus [Okafuji et al., 2006].

RESULTS

Laboratory Examination of Patients With Non-Typical Measles Illness

From March 2007, several patients with measles were observed in outpatient clinics and 22 clinical samples were examined for virus isolation and genome detection and the results are shown in Table I. Four patients had typical measles with a marked fever for more than 3 days and typical measles eruptions with post-pigmentation. Three patients were adults and one had a history of vaccination. One patient was 8 years old and complicated with Gitelman syndrome during the measles illness. Two measles strains (MVi/Tokyo.JPN/17.07 and MVi/Tokyo.JPN/18.07) were isolated and the measles genome was detected in all patients by RT-PCR and RT-LAMP (Table Ia).

During the measles outbreak, 18 clinical samples were obtained from patients with non-typical measles with mild febrile illness and eruptions for less than 3 days. They were over 15 years of age and had a past history of single-dose measles immunization at 1–3 years of age. Measles virus was not isolated but the measles genome was detected in 9 (50%) by RT-PCR and in 12 (67%) by RT-LAMP (Table Ib). All RT-PCR positive samples were also positive on RT-LAMP.

MVi/Aichi.JPN/44.06, MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, and MVi/Mie.JPN/41.07 were isolated from patients with modified measles and MVi/Mie.JPN/03.08 was isolated from typical pediatric measles patient. Detection of the measles genome was not examined directly from the clinical samples in these five cases.

Genotype Analysis

Seven measles strains were isolated and examined for sequence analysis of the C-terminal of the N protein region, as recommended by WHO, [2005a]. MVi/Aichi.JPN/44.06 was isolated from a sporadic case

TABLE I. Results of Laboratory Examinations

Case	Vac	V. Iso.	RT-PCR	LAMP
a. Four typical measles cases				
1 (23Y)	+	+	+	+
2 (19Y)	−	−	+	+
3 (18Y) Encephalitis	−	−	+	+
4 (8Y) Gitelman syndrome	−	+	+	+
	V. Iso. (+)	RT-PCR (+)	LAMP (+)	
b. 18 cases with mild fever and rash	0/18	9/18	12/18	

Vac: Past history of measles vaccination
V. Iso: Virus isolation

in Nagoya in 2006, a central district of Japan, before the nationwide outbreak, and this strain did not cause further transmission. MVi/Tokyo.JPN/17.07 and MVi/Tokyo.JPN/18.07 were isolated in Tokyo. MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, MVi/Mie.JPN/41.07, and MVi/Mie.JPN/03.08 were isolated in Mie Prefecture, a central area of Japan, during the outbreak 2007–2008. The results of phylogenetic analysis are shown in Figure 1. MVi/Aichi.JPN/44.06 was D9 and two stains isolated in Tokyo at the beginning of the outbreak in 2007 were identified as genotype D5. Four strains isolated in Mie Prefecture were also identified as genotype D5. MVi/Gunma.JPN/19.07 was isolated in Gunma Prefecture, located in the north direction of Tokyo, was also D5 [Morita et al., 2007]. Measles outbreaks last for more than a year and a relatively homologous strain has been circulating throughout Japan.

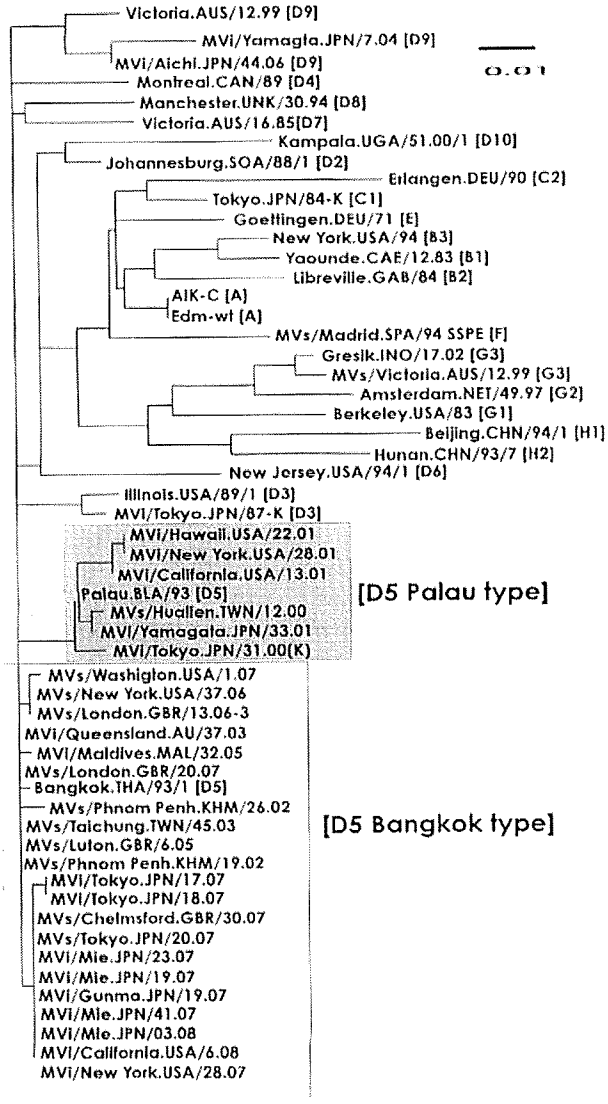

Fig. 1. Phylogenetic analysis of measles virus in the N gene. MVi/Aichi.JPN/44.06 was isolated in 2006 and MVi/Tokyo.JPN/17.07, MVi/Tokyo.JPN/18.07, MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, MVi/Mie.JPN/41.07, and MVi/Mie.JPN/03.08 were isolated during the outbreak 2007–2008 in this study. MVi/Gunma.JPN/19.07 was reported by Morita et al. [2007] and other strains were registered in GeneBank. The Palau type D5 strains are shown in gray square and the Bangkok type D5 strains in open square.

Two different reference strains, MVi/Bangkok.THA/93/1 and MVi/Palau.BLA/93, were identified as genotype D5 and the Palau type D5 was transmitted via a Japanese traveler in 1993 [Rota et al., 1998]. The Palau type D5 was a major circulating genotype from 1990 to 1997 and from 2002 and afterward [Nakayama et al., 2003; Zhou et al., 2003; Okafuji et al., 2006]. However, the D5 strains in 2007–2008 belonged to the Bangkok type D5. Among D5 sequences obtained in 2007–2008, there was one nucleotide difference in the target of the N gene. Accession numbers of the partial N gene sequence of the isolates in this study are AB426895–99.

In Switzerland, 11 cases of measles were reported between March and April 2007 and, thereafter, a large nationwide outbreak was reported with further expansion to other European countries and the USA [Delaporte et al., 2007; Richard et al., 2008; CDC, 2008b]. MVi/New York.USA/28.07 and MVi/California.USA/6.08 were registered as imported from Switzerland and classified into the Bangkok type D5. In 456 nucleotides of partial N gene, there was no difference among isolates in outbreak in Japan, 2007–2008 and those prevalent in Europe and the USA. The epidemiological linkage was not identified but would be suspected by molecular epidemiological data.

The entire length of the H gene was sequenced and analyzed together with reference strains and data from the previous reports. Four strains isolated in 2007 were classified as the Bangkok type D5, not Palau type (Fig. 2). Differences in the nucleotide and amino acid sequences of the H gene are shown in Table II. Sequence variations of the H gene consisted of 52 (2.8%) to 59 (3.2%) nucleotide differences among genotypes A (AIK-C vaccine strain) and D5 strains, and 66 (3.6%) between genotypes A and D9. There were 16–19 (2.6–3.1%) amino acid changes in D5 or D9 in comparison with genotype A. In the same D5 strains, MVi/Tokyo.JPN/17.07 [Bangkok type] showed 35–43 (1.9–2.3%) nucleotide differences in comparison with the Palau type D5 strains, MVi/Palau.BLA/93 or MVi/Tokyo/JPN/31.00-K, but 16 (0.9%) in comparison with MVi/Bangkok.THA/93/1. Accession numbers for the entire H gene sequence of the isolates in this study are AB426900–04.

Antigenic Differences

MVi/Tokyo.JPN/18.07 [Bangkok type D5], MVi/Tokyo.JPN/31.00-K [Palau type D5], MVi/Aichi.JPN/44.06 [D9], and the AIK-C vaccine strain [A] were used for the analysis of antigenicity. Five or six serum samples for each NT antibody titer were selected, for which the NT titers against the AIK-C strain had already been checked, and a total of 32 sera were used for the analysis of antigenicity. The challenge viruses were

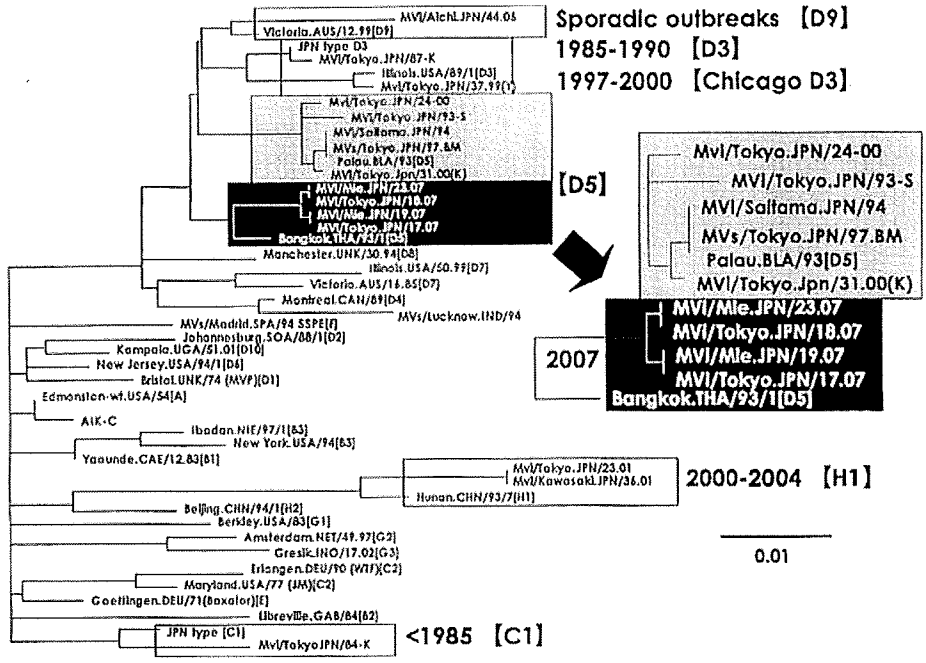

Fig. 2. Phylogenetic analysis of measles virus in the H gene. Strains in gray square are Palau type D5 and those in black square Bangkok type D5.

adjusted to 100 TCID50 and the results are shown in Figure 3. There was no significant difference in NT antibody titers within fourfold dilutions against the different genotypes of A, Palau type D5, Bangkok type D5, and D9.

Characteristics of Wild-Type Measles Virus

Seven wild-type measles virus strains were isolated and the current wild type did not induce cell fusion in Vero cells. D5 and D9 strains infected and replicated in Vero cells without demonstrating typical cell fusion. Infectious titers in Vero cell culture fluid detected 7 days after infection were similar or 1/2 or 1/3 lower than those in the B95a cell culture (data not shown).

Measles virus grew well at 35–37°C and virus growth at 39°C differs from strain to strain [Nakayama et al., 2003]. Virus growth of MVi/Aichi.JPN/44.06 at different temperatures of 33, 35, 37, and 39°C was investigated in B95a cells, and the results are shown in Figure 4. It grew well at 33, 35, and 37°C, but the infective titers at 39°C decreased to approximately 1/100 in comparison with those observed at 33 or 35°C. Three D5 strains isolated in 2007 and MVi/Tokyo.JPN/93-S [Palau type D5] were cultured in B95a cells at 35°C and 39°C and the infective titers on day 7 of culture are also shown in Figure 4. As previously reported, MVi/Tokyo.JPN/93-S grew well at 39°C as it did at 35°C, but the infectivity of the Bangkok type D5 was lower at 39°C than at 35°C.

DISCUSSION

The WHO global measles and rubella laboratory network (LabNet) has been established and the Western Pacific Region adopted the goal of measles elimination by increasing the use of laboratory testing as an integral component of its surveillance. They recommended that effective surveillance comprised laboratory confirmation by the detection of IgM antibody in single serum, together with virus isolation for genotyping. However, the sensitivity of virus isolation is low and depends on the timing of sample collection and transporting conditions. The detection of measles virus-specific IgM antibodies in serum is a standard serological diagnostic

TABLE II. Nucleotide and Amino Acid Differences in D5 and D9 Strains

	AIK-C	Palau	Bangkok	Tokyo, 2000	Tokyo, 2007	Aichi, 2006
AIK-C		52 (2.8%)	53 (2.9%)	59 (3.2%)	57 (3.1%)	66 (3.6%)
Palau	16 (2.6%)		25 (1.3%)	10 (0.5%)	35 (1.9%)	45 (2.4%)
Bangkok	18 (2.9%)	8 (1.3%)		33 (1.8%)	16 (0.9%)	39 (2.1%)
Tokyo.JPN/2000	17 (2.8%)	5 (0.8%)	11 (1.8%)		43 (2.3%)	54 (2.9%)
Tokyo.JPN/17.07	16 (2.6%)	8 (1.3%)	4 (0.7%)	11 (1.8%)		50 (2.7%)
Aichi.JPN/44.06	19 (3.1%)	11 (1.8%)	10 (1.6%)	14 (2.3%)	10 (1.6%)	

Values present diagonally below in the table body indicate amino acid differences and the values present diagonally above in the table body indicate nucleotide differences.

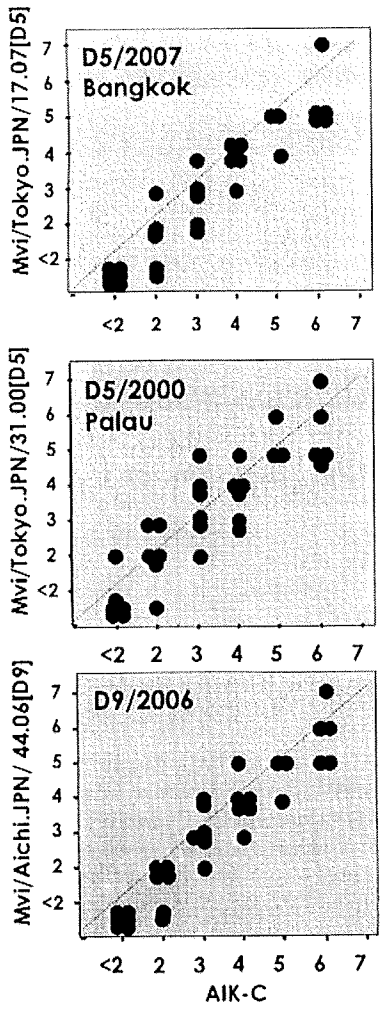

Fig. 3. Neutralization test antibodies against the AIK-C vaccine strain (genotype A), MVi/Tokyo.JPN/17.07 (genotype D5 Bangkok type), MVi/Tokyo.JPN/31.00 (genotype D5 Palau type), and MVi/Aichi. JPN/44.06 (genotype D9).

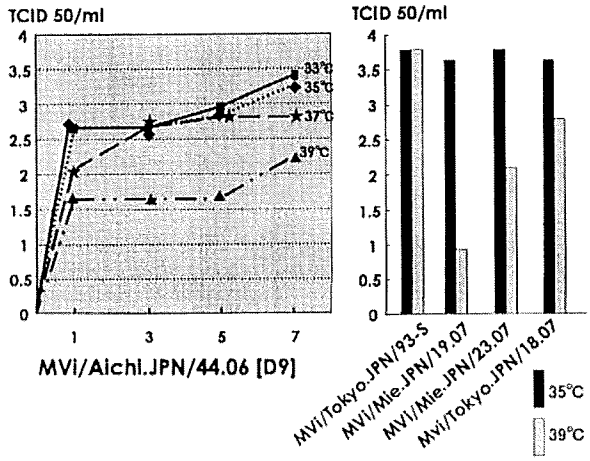

Fig. 4. Virus growth at different temperatures of 33, 35, 37, and 39°C.

method when serum samples are obtained on day 4 or 5 of rash onset and later [WHO, 2005b]. These methods were effective in the case of the primary infection causing typical measles illness. However, in the case of modified or subclinical measles, the detection rate of serum IgM antibodies was reported to be extremely low and the genomic detection by RT-PCR should be examined [Mosquera et al., 2005]. In this report, serum IgM antibody against measles virus was initially examined in several patients, but it was negative because all patients visited the clinics within day 1 or 2 after rash onset. In addition, the patients over 15 years of age having a single dose of measles immunization demonstrated very mild febrile illness with eruptions. Among 18 patients, 12 were diagnosed by RT-LAMP. The remaining six patients were not confirmed, because paired sera were obtained rarely from adults with mild symptoms. Detection of the measles genome is reliable and sensitive method and these six patients were supposed to have some other virus infections.

van den Hof et al. [2003] examined 791 serum samples from Dutch persons aged 2–49 years. The sero-prevalence and mean titers of measles EIA and NT were lower in the vaccinated populations than in older individuals infected naturally, and vaccine-acquired immunity weakened year by year. Glass and Grenfell [2004] developed a mathematical model to predict clinical and subclinical measles cases. Vaccination levels dropped from >90% in the 1990s to 84% in 2001/2002 in England, and they considered that clinical measles cases would remain fairly stable over time if vaccine coverage is maintained at 90%, but that there would be a pronounced increase in the numbers of clinical cases if vaccine coverage is around 84% or below. In any case, they predicted an increase in the number of subclinical measles. In the measles outbreak in Japan 2007–2008, a large proportion of cases of measles comprised adults or teenagers with very mild illness, and many cases went undiagnosed. These cases were confirmed by detection of the measles genome in this study and the diagnostic approach is now combining genome detection with virus isolation and the detection of IgM antibodies [CDC, 2008c].

Circulating wild-type measles virus genotypes have been investigated since 1984 and dynamic changes in the major circulating genotype have been reported [Nakayama et al., 1995; Yamaguchi, 1997; Zhou et al., 2003; Nakayama et al., 2004; Okafuji et al., 2006]. The dominant circulating genotypes changed drastically in large outbreaks in 1984, 1987–1988, 1991–1993, and 2001–2002 and each outbreak was caused by a different genotype in Japan, as summarized in Figure 5. Genotype C1 was an indigenous strain for a long period before 1985, D3 was involved in the 1987–1988 outbreak, and D5 in 1990–1993. The genotype of measles virus was studied in India from 1994 to 1997. The indigenous strain in India was D4, and a large outbreak was observed in 1997, caused by the Chicago type D3 strain [Nakayama et al., 2004]. In 1997, the Chicago type D3 was isolated in Japan, which was a different cluster from

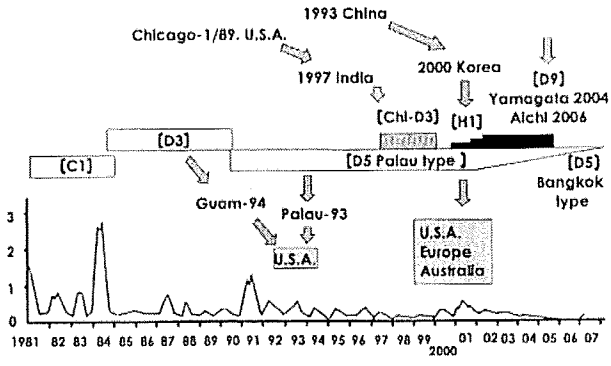

Fig. 5. Major circulating measles genotypes since 1984.

the D3 strains in 1987–1988. The Chicago-type D3 in 1997–1999 was supposed to have been imported from outside, and it was replaced by D5 in 2000, similar to the previous Palau type D5 before 1997. The H1 genotype was isolated from adult measles patients, and this genotype became a dominant strain after 2000 [Zhou et al., 2003]. In 2004, a sporadic outbreak was reported at a junior high school in Yamagata Prefecture and several measles strains were isolated. They were identified as genotype D9, which had not previously been reported in Japan [Mizuta et al., 2005]. In 2005, sporadic outbreaks were reported in Chiba and Ibaraki Prefectures, caused by the Palau type D5. In Asian countries, several different indigenous genotypes have been reported: H1 in China [Xu et al., 1998], G2 in Indonesia and Malaysia [Rota et al., 2000], D4 in India [Wairagkar et al., 2002], D3 in Papua New Guinea [Miki et al., 2002], and D7 in Australia [Chibo et al., 2003]. D8 in Nepal [Truong et al., 2001] and D9 in Australia [Chibo et al., 2003] were also reported. The sporadic outbreak caused by D9 in Yamagata in 2004 was suggested to have been imported from outside. In this study, D9 was also isolated in a sporadic outbreak in Aichi Prefecture in 2006, and seven nucleotide differences in the partial N gene (7/456: 1.5%) were observed between the two D9 strains of the MVi/Yamagata.JPN/7.04 and MVi/Aichi.JPN/44.06. There was no information on the isolation of D9 during 2004–2006 and no epidemiological linkage. Thus, they seemed to have been imported independently from other Asian counties, considering the narrow transmission chain in Japan.

The Palau type D5 was an indigenous strain since 1990 in Japan but was interrupted by outbreaks of the Chicago type D3 from 1997 to 2000 and of H1 from 2001 to 2004. Genotype D5 was detected in a sporadic outbreak in 2006 around Tokyo and transmitted to Okinawa, which was identified as the Bangkok type D5, which was different from the Palau type circulating during1990–2005 in Japan [National Institute of Infectious Diseases, 2007; Morita et al., 2007]. The Bangkok type D5 isolated in Japan in 2007 would have been imported from other Asian countries rather than being a result of the accumulation of mutations of the indigenous Palau type D5. Similar Bangkok type D5 strains (MVi/Queensland.AU/37.03, MVi/Maldives. MAL/32.05, MVs/Phnom Penh.KHM/19.02, MVs/Taichung. TWN/45.03) were reported in Asian countries and also in the UK (MVs/Chelmsford.GBR/30.07).

In 2007 and 2008, similar strains were reported in Europe and the USA and epidemiological linkage to the outbreak in Switzerland was identified [Delaportel et al., 2007; Richard et al., 2008; CDC, 2008b]. In this report they were thought to have been transmitted from Japan, demonstrating high-level sequence homology to Japanese isolates. Asia is the major reservoir of the wild measles virus and several Asian genotypes have been detected in the USA. and Europe. Global knowledge on the distribution of genotypes is imperative in identifying the geographical regions where more aggressive vaccination campaigns should be implemented in order to eliminate measles.

Minor antigenic changes in the Chicago type D3 and H1 strains were reported in comparison with the NT titers against the AIK-C vaccine strain [Zhou et al., 2003; Nakayama et al., 2004]. In this study, 1.3–1.8% amino acid differences were observed between the D5 Bangkok and Palau types. Therefore, the difference in the antigenicity was investigated and there were no significant differences in NT antibody titers against the Bangkok type D5, Palau type D5, and D9 strains in comparison with those against the AIK-C vaccine strain. No significant difference was demonstrated in terms of antigenicity. Some virus strains of the Chicago type D3 and Palau type D5 grew as well at 39°C as they did at 33, 35, and 37°C. However, the Bangkok type D5 and D9 showed poor growth at 39°C. The responsible genomic region(s) for virus growth at different temperatures is now under investigation.

Recommendations to prevent further transmission from the index case of importation noted that the outbreak investigation was conducted when the index case of a Japanese boy had a measles-like illness and only six additional cases were identified [CDC, 2008a]. Transmission was interrupted because of the high vaccination coverage rates of two-dose MMR among children and adolescents. The prompt response was sustained by strong and effective surveillance systems. The WHO has recommended that all children should be provided with a second opportunity for measles vaccination [WHO, 2006]. This second opportunity is scheduled just before entry to primary school in most countries, and a two-dose schedule of combined measles–rubella vaccine was launched in 2006 in Japan. The scheduled timing of the two doses is at the age of 12–24 months and 5–6 years. Supplemental immunization for the other populations was not considered, and most school children (over 8 years of age) did not have the benefit of the two-dose schedule. Okafuji et al. [2006] reported that NT antibodies decreased to undetectable levels in approximately 10% of vaccine recipients 6–7 years after vaccination when the measles outbreak was controlled. The outbreak in Japan 2007 highlighted the inadequacy of the immunization strategy, leading to a gap in immunization among teenagers without

supplemental immunization. Additionally, several had no immunization history because of a distrust of the vaccine due to the MMR scandal from 1989 to 1993 in Japan [Ueda et al., 1995]. All school children and young teenagers should have a second dose of measles vaccine to attain the goal of measles elimination. Thus, the Japanese government has decided to launch a catch-up campaign targeting young teenager at 13 and 18 years of age for the next 5 years, anticipating elimination of measles by 2012. The infrastructure for the surveillance system based on laboratory-based diagnosis is now in preparation.

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procedure, with no significant complications reported in the literature to date. The quality of the clinical sample obtained using EBUS-TBNA is higher than that for conventional TBNA and, unlike conventional TBNA, EBUS-TBNA has been shown to be useful for the diagnosis of sarcoidosis and lymphoma,[5] both of which are seen with increased frequency in HIV-infected patients. EBUS-TBNA has several advantages over traditional methods of tissue biopsy: diagnostic yield is frequently superior to mediastinoscopy[3]; it has an excellent safety profile[7]; use of the technique is not limited to adult populations[8]; and it can be performed as a day case, thereby reducing hospital costs.

To our knowledge this is the first report to demonstrate the feasibility and high diagnostic yield of EBUS-TBNA for the investigation of mediastinal intrathoracic lymphadenopathy in HIV-infected patients. EBUS-TBNA, where available, should be considered for the investigation of mediastinal intrathoracic lymphadenopathy in patients that do not have lesions more amenable to diagnostic sampling.

Conflict of interest

None declared.

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Phylogenetic analysis of human bocavirus (HBoV) detected from children with acute respiratory infection in Japan

Dear Editor

Human bocavirus (HBoV) belongs to family *Parvoviridae* and causes acute respiratory infection (ARI) in humans.[1] HBoV is an emerging virus discovered by Allander et al. in 2005 from a Swedish patient with ARI.[2] Although the clinical course of HBoV infection in most patients is good, it is suggested that HBoV is associated with lower respiratory infection including bronchiolitis and pneumoniae[3–5]; however, its epidemiology remains poorly understood at present.[3] In this study, in an attempt to clarify the epidemiology, we performed phylogenetic analysis of HBoV detected from Japanese children with ARI in recent years.

We obtained 7 amplicons of HBoV from patients (age range, 6 months–2 years) with ARI. Their inhabitant areas were Yamagata prefecture (3 patients), Shiga prefecture (2 patients), Fukushima prefecture (1 patient), and Tokyo metropolitan (1 patient). We initially extracted DNA from throat swabs and amplified the full-length HBoV genome as previously described.[6] The obtained amplicons were then sequenced and aligned.[6] We next performed phylogenetic analysis, after which evolutionary distances were estimated using Kimura's two-parameter method and phylogenic trees were constructed using the neighbor-joining (N-J) method.[6]

As shown in Fig. 1, 6 strains (3 from upper respiratory infection, 1 from bronchiolitis, and 2 from pneumonia)

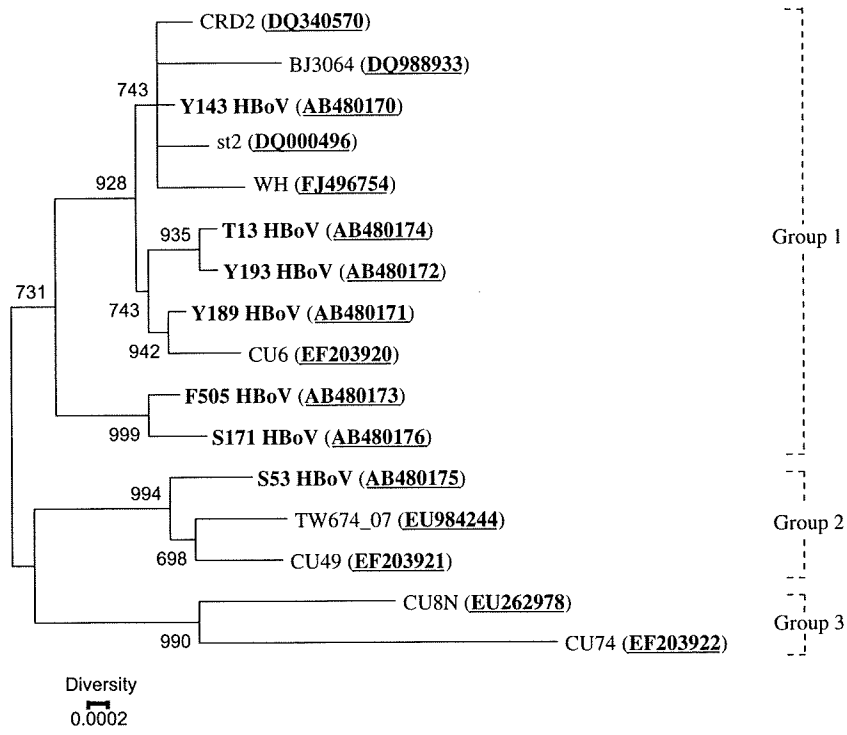

Figure 1 Phylogenetic tree of human bocavirus (HBoV) based on HBoV full-length genome. The tree was constructed using the full-length genome of HBoV (nucleotide positions: 1–5299). Numbers in parentheses indicate the GenBank accession number. The reliability of the tree was estimated using 1000 bootstrap replications. The numbers at each branch indicate the bootstrap value for the clusters supported by that branch.

and 1 strain (from upper respiratory infection) of HBoV were classified into group 1 or 2. In the present tree, strains belonging to groups 1 and 2 were subdivided into some clusters. In both groups 1 and 2, the present strains were classified into the same clusters of the strains detected in USA or Thailand. In addition, among the present strains (7 strains), homology was 99.5–99.96%. No other viruses were detected using the cell culture method (Vero, RD18, HEL, MDCK, and HEp-2 cells). The results suggest that in Japan as well as in some other countries HBoV may be partially responsible for the development in some patients of ARI, including upper respiratory infection, bronchiolitis, and pneumonia.[3–5] The clinical significance of HBoV as an etiologic agent of ARI is not yet completely clarified and thus additional epidemiologic studies are needed.

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Predominance of methicillin-resistant *Staphylococcus aureus* among HIV positive subjects with pyrexia of unknown origin in Chennai, Southern India

Dear Editor,

HIV infection reportedly leads to a plethora of ill-effects on the cellular and humoral arms of immunity and predisposes the infected individuals to a myriad opportunistic infections (OIs), especially involving bacteria.[1] As well as vulnerable to a broad array of OIs, the HIV infected patients also risk to development of incidental bacterial infections, which intriguingly have rarely been reported as comprehensively as the former. Blood stream infection (BSI) owing to bacteria is a frequent complication in HIV infected patients, which often is associated with a poor prognosis, especially under certain conditions, namely intravenous drug abuse, and a low CD4 T-cell count.[2]

Invasive pneumococcal disease caused by *Streptococcus pneumoniae* and the Non-typhoidal Salmonellae (NTS) is reportedly the common cause of bacteraemia in United States of America, tropical Africa and other South East Asian countires.[3–5] The specific association between HIV and NTS bacteraemia was first described by Bottone et al., in New York, and further reports from the US linked NTS bacteraemia to AIDS with all of them reporting bacteraemic relapse in 25–66% of cases. Subsequently recurrent NTS bacteraemia was added to the CDC case definition for AIDS in 1987.[4]

According to a recent study by Burkey et al., methicillin-resistant *Staphylococcus aureus* (MRSA) represents a large and growing proportion of *S. aureus* bacteraemia among the urban cohort of HIV patients in the US. A significant increase in the incidence of MRSA was observed over the study period from 2000 to 2004.[6]

Hence, we investigated the aetiology and features of BSI in 180 HIV infected subjects that presented with symptoms of bacteraemia at the YRG Centre for AIDS Research and Education (YRG CARE), Chennai, India between January 2003 and July 2008. The microbiology laboratory of YRG CARE practices Good Clinical Laboratory Practices and is certified by the College of American Pathologists, Illinois, USA since the year 2002. The HIV status of the patients was determined by the algorithm recommended by World Health Organization South East Asia Office (WHO-SEARO), New Delhi and National AIDS Control Organization (NACO), New Delhi. Blood samples were collected aseptically from patients with suspected BSI. A 5 mL blood sample from each patient was directly inoculated into the brain heart infusion (BHI) biphasic blood culture bottles at the bed-side and transported immediately to the laboratory where, they were incubated at 37 °C. On suspicion of growth observed, microscopic examination after gram staining and subculture on routine laboratory media were performed. Further identification was done by cultural morphology and biochemical methods.[7] Antibiotic susceptibility testing of the isolates was performed following Kirby–Bauer disk diffusion method, adhering to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

The age range of the patients with symptoms of bacteraemia was 1–72 years. The male:female ratio was 3.7:1 with a median age of 35 years. Of the total 180 study subjects analysed, 142 were males (mean age 36 ± 9.9 years) and 38 were females (mean age 29.5 ± 10.8 years). Bacteraemia was observed in 18 cases (10%) and 7.9% of females and 10.6% of males presented with BSI. The patients with blood stream infections generally presented with low CD4 T-cell counts and the median CD4 T-cell count of those patients was 60 cells/μL (Range 16–241 cells/μL).

S. aureus and coagulase negative staphylococci (CONS) predominated as the common aetiological agents of blood stream infections followed by *Escherichia coli*, *Acinetobacter* spp. and alpha-hemolytic streptococci. The aetiological agents of BSIs observed in HIV patients are listed in Table 1. Five of the 7 (71%) strains of *S. aureus* were resistant to oxacillin (methicillin-resistant *S. aureus*) and generally highly susceptible to netilmicin, ofloxacin, ciprofloxacin and gentamicin and chloramphenicol. *Staphylococcus epidermidis* strains exhibited relatively less resistant profile and were highly susceptible to gentamicin, oxacillin, ciprofloxacin and ofloxacin.

Table 1 The aetiological agents of blood stream infections (BSIs) observed in HIV patients.

Aetiological agent	Number of isolates (%)
Staphylococcus aureus	7 (3.9)
Coagulase negative *Staphylococcus*	7 (3.9)
Escherichia coli	2 (1.1)
Acinetobacter spp.	1 (0.6)
Alpha-hemolytic streptococci	1 (0.6)

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Stability of the seven hexon hypervariable region sequences of adenovirus types 1–6 isolated in Yamagata, Japan between 1988 and 2007

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ABSTRACT

Seven hexon hypervariable regions (HVRs) of adenoviruses (Ads) were identified by comparing the regions among different serotypes; however, no one has compared HVR sequences among the identical serotypes, except for adenovirus type 3 (Ad3).

To examine a variability between the HVRs for each serotype, we compared the sequences of Ad1–6 isolates, respectively, isolated between 1988 and 2007 in Yamagata, Japan. We selected 23–43 isolates randomly and sequenced 894–987 bp regions.

Except for strains with insertions and deletions, the sequence identities among Ad1–6 were 99–100%, excluding that between the two Ad5 groups (approx. 94%). Even the insertions and deletions were likely to be established, as these changes were repeatedly observed. The obtained phylogenetic tree indicated that Ad isolates and reference strains branched depending on serotype. The Yamagata isolates had similar sequences or amino acid arrangements to the reference strains as well as to other strains isolated in different areas. HVRs have been stably conserved as serotype-specific regions for a long period with only minor genomic variations. Therefore, we herein recommend that these regions be hereafter referred to as "serotype-specific regions", which might be a more appropriate title with which to characterize the epidemiological nature of these sites than the current "HVRs".

1. Introduction

There are 51 human adenovirus (hAd) serotypes that are subdivided into six subgroups A–F on the basis of certain biochemical and serological criteria (De Jong et al., 1999; White and Fenner, 1994). Certain serotypes and/or subgroups were found to be associated with respiratory, ocular, enteric and genitourinary infections (White and Fenner, 1994). Adenovirus types 1–7 (Ad1–7) were commonly isolated from patients with Ad infections (Schmitz et al., 1983). However, Ad7 has rarely been isolated since the 1980s in Japan (Mizuta et al., 2006). Furthermore, between 2000 and 2007 in Japan, a total of 11 025 Ad1–6 strains were isolated from throat swab specimens, whereas only 194 other serotype strains were detected (Infectious Disease Surveillance Center, 2008). Thus, Ad1–6 are currently the most commonly isolated Ads related to acute respiratory infections (ARIs), especially in Japan (Infectious Disease Surveillance Center, 2008).

The hexon, which is the major capsid protein of the Ad particle, carries the group-specific (α) and the type-specific (ε) antigenic

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determinants (Norrby, 1969). Analyses using monoclonal antibodies revealed that there are several group-specific and type-specific antigens on the surface of the hexon (Ádám et al., 1986, 1995).

Sequence analysis of Ad2 and Ad5 indicated that the Ad hexon contains both variable and conserved regions (Kinloch et al., 1984; Pring-Åkerblom and Adrian, 1993). Comparisons of the hexon sequence among different serotypes and subgroups revealed that there are seven hypervariable regions (HVRs) at loops 1 and 2, which exist on the exterior surface of the hexon (Crawford-Miksza and Schnurr, 1996a; Gall et al., 1998; Pring-Åkerblom et al., 1993, 1995a,b; Toogood et al., 1989). These regions vary not only in sequence but also in length among subgroups (Crawford-Miksza and Schnurr, 1996a). The presence of unique serotype-specific sequences in the seven HVRs, together with the results of serological study, suggests that these regions contain the type-specific neutralization epitopes (Crawford-Miksza and Schnurr, 1996a; Pring-Åkerblom et al., 1995b; Toogood et al., 1989, 1992). Furthermore, Crawford-Miksza et al. reported that the seven HVRs are hot spots for illegitimate recombination events and single-base mutations (Crawford-Miksza and Schnurr, 1996b) and Rux et al. proposed that HVR7 should be defined more precisely as HVR 7–9 (Rux et al., 2003).

We focused our interest on the variability of the Ad HVRs of a single serotype, and sought to clarify whether these HVRs function as a serotype-specific region as described above. Except for Ad3, to date no one has compared HVR sequences among a single serotype chronologically within a community (Choi et al., 2006; Fujimoto et al., 2008). In this study, we compared the hexon sequence that included HVRs 1–7 from Ad1–6 strains, isolated from children with ARIs in Yamagata, Japan between 1988 and 2007.

2. Materials and methods

2.1. Clinical specimens

Between 1988 and 2007, 62 778 nasopharyngeal swab specimens were collected from patients with ARI at pediatric clinics collaborating with the local health authority of Yamagata Prefecture as part of the national surveillance of viral diseases in Japan. Most of the patients were under 15 years old and were clinically diagnosed as having ARI with fever and/or cough and/or rhinorrhea. Specimens were placed immediately in tubes containing 3 ml of transport medium, including minimum essential medium (Nissui, Pharmaceutical Co., Ltd., Tokyo, Japan), penicillin G (50 U/ml, Meiji Seika, Ltd., Tokyo, Japan) and streptomycin (0.4 mg/ml, Meiji Seika, Ltd., Tokyo, Japan) (Mizuta et al., 2003). The specimens were collected and transported to the Virus Research Center, Sendai Medical Center, Sendai, Japan between 1988 and 1998 and to the Department of Microbiology, Yamagata Prefectural Institute of Public Health, Yamagata, Japan between 1999 and 2007 for virus isolation.

2.2. Virus isolation and identification

Virus isolation was carried out using a microplate method. Briefly, human embryonic lung fibroblast (HEF), human laryngeal carcinoma (HEp-2), Vero, and Madin-Darby canine kidney (MDCK) cell lines were prepared on the wells of a 96-well microplate (Greiner Bio-One, Frickenhausen, Germany) (Numazaki et al., 1987). In 2001, we added the rhabdomyosarcoma (RD-18S) and green monkey kidney (GMK) cell lines to the above four original cell lines (Mizuta et al., 2003). Furthermore, we substituted the Vero E6 cell line for the regular Vero cell line in 2004 to isolate human metapneumoviruses (hMPVs) (Abiko et al., 2007). The composition of the growth and maintenance media was described previously (Mizuta et al., 2008). When the monolayer of each cell line was ready for specimen inoculation, the plates were washed with phosphate-buffered saline without calcium or magnesium, and 100 μL of maintenance medium was added to each well of the plate. After centrifugation of the specimens at 3000 rpm for 15 min, 75 μL of supernatant was inoculated directly onto two wells of each cell lines. The inoculated plates were centrifuged for 20 min at 2000 rpm, incubated at 33 °C in a 5% CO_2 incubator, and assessed for cytopathic effect (CPE) for 14 days, except for the wells containing the Vero E6 cell line, which were observed for one month to isolate hMPVs (Abiko et al., 2007). In this system, Ads were mainly isolated on the HEF, HEp-2 and Vero/Vero E6 cell lines. When a typical CPE was observed, viral identification was carried out by neutralization test. Antisera against Ad1–6 were prepared at the Virus Research Center, Sendai Medical Center. The isolates were stocked at −80 °C after the cells were passaged two to three times.

2.3. Preparation of viral DNA

Twenty-three to 43 Ad isolates obtained in Yamagata were selected randomly for further sequence analysis and these strains are shown in Table 1. Ad1–6 prototypes, which were provided by Dr. T. Inada, National Institute of Infectious Diseases, Tokyo, Japan and Ad3a and Ad3c, which were provided by Drs. Q. Li and G. Wadell, Umeå University, Sweden, were also sequenced as reference laboratory strains (Li and Wadell, 1988).

Viral DNA extraction was carried out based on the method described previously (Mizuta et al., 1994). After the addition of proteinase K (Wako Pure Chemical Industries, Osaka, Japan) at a final concentration of 120 μg/ml, 300 μL of the viral culture fluid were incubated at 37 °C for 3 h, mixed with 75 μL of 5 M NaCl and then left at 4 °C for 30 min. After centrifugation at 15 000 rpm for 5 min, the supernatant fluid was extracted with an equal volume of Tris–EDTA saturated phenol and phenol–chloroform–isoamyl alcohol (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions, and the viral DNA was precipitated with 99% ethanol and recovered by centrifugation at 15 000 rpm for 30 min at 4 °C. The precipitate was washed with 70% ethanol and dried at room temperature. The dried precipitants were redissolved in 20 μL of distilled water for a further PCR step.

2.4. PCR and sequence analysis

The target sequence encompassed the seven HVRs, which locate from amino acid (A.A.) 137 to A.A. 465 based on the Ad2 sequence reported previously (Crawford-Miksza and Schnurr, 1996a). To amplify this region, we prepared the primer pairs, Ad3HexF5 (TgTAggCgACAACAgAgTgC) and Ad3HexR4 (TgTTggTgTTAgTgggCAAA), for Ad3, and Ad2HexF1 (CAACCgTgTgCTTgATATgg) and Ad2HexR1 (AgTCgTAggTgTTggggTTg), for Ad1, Ad2, Ad4, Ad5 and Ad6. AdCH4 was also used for Ad1 (Okada et al., 2007). A total of 60 μL of fluid, containing 6 μL of 10× buffer, 3 μL of 2 mM dNTP, 3 μL of 10 μM forward and reverse primer, 0.3 μL of Taq polymerase (5 U/μL) and water, was reacted at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min (40 cycles), followed by a final extension at 72 °C for 10 min (1 cycle).

The PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and then sequenced using a Big Dye Terminator V1.1 cycle sequencing kit on an ABI Prism 310 (Applied Biosystems, Foster City, CA) automatic sequencer. The same primers were used for both sequencing and PCR. Sequence data for the isolates from Yamagata were registered with GenBank under the following accession numbers: Ad1 (AB433286–AB433297 and AB433299–AB433327), Ad2 (AB433690–AB433729), Ad3 (AB067664–AB067672 and AB366402–AB366431), Ad4 (AB433734–AB433756), Ad5 (AB434210–AB434252) and

Table 1
Results of sequence analysis (base and amino acid) at seven hexon hypervariable regions of Ad1–6 reference strains and isolates obtained between 1998 and 2007 in Yamagata, Japan.

Serotype	Strain	894–987 bp analysis	298–329 A.A. analysis
Ad1	Y88[a]–282,Y88–6178,Y89–5283,Y91–49,Y91–1231,Y97–174,9–Yam agata–00[a],676–Yam agata–00,656–Yam agata–01,549–Yam agata–02,50–Yam agata–05	identical to Y88–282	identical to Y88–282
	Y89–1015,Y90–1932,Y96–1181,Y98–603,Y98–986,968–Yam agata–99,789–Yam agata–00,1903–Yam agata–01,2015–Yam agata–04,2323–Yam agata–04,1847–Yam agata–05,143–Yam agata–06,660–Yam agata–07,226–Yam agata–07,Ad1P	9 bp substitutions from Y88–282	
	Y90–3227,Y92–1417,Y93–1560,Y97–832,31–Yam agata–02,46–Yam agata–03	1 bp substitution from Y88–282	
	Y94–176,Y94–291,3209–Yam agata–02,3217–Yam agata–02,36–Yam agata–03,1632–Yam agata–03,Y96–1829	1 bp substitution from Y89–1015	
	Y92–246,Y93–560	9–15 bp (3–5 A.A.) deletions compared with Y89–1015	
	720–Yam agata–06	15 bp (5 A.A.) deletions compared with Y88–282	
Ad2	Y88–1257,Y93–380,Y93–680,Y97–249,Y97–1728,647–Yam agata–00,112–Yam agata–02,1999–Yam agata–02,522–Yam agata–07	identical to Y88–1257	
	Y88–1668,Y89–5154,Y90–2538,Y91–1154,Y92–747,Y94–714,Y94–976,Y95–633,Y95–983,Y96–1108,Y96–1869,Y96–1872,Y98–331,Y98–1589,508–Yam agata–01,1110–Yam agata–01,556–Yam agata–03,201–Yam agata–04,224–Yam agata–04,2209–Yam agata–04,17–Yam agata–05,16–Yam agata–06,Ad2P,Y89–186,Y90–1356,Y91–100,Y92–950,2643–Yam agata–05,340–Yam agata–00,235–Yam agata–06,54–Yam agata–07	1–3 bp (0–2 A.A.) substitution from Y88–1257	
	1525–Yam agata–03	3 bp (1 A.A.) insertions and 17 bp (4 A.A.) substitutions compared with Y88–1257	
Ad3	Y88–507,Y88–608,Y88–5309,Y89–762,Y89–2632,Y89–4067,Y90–3080,Y90–4578,Y91–2489,Y91–3246,Y92–189,Y92–884,Y93–58,Y93–930,Y94–2330,Y94–2591,Y95–171,Y95–2087,Y96–224,Y97–1022,989–Yam agata–99,Ad3a,Ad3c	identical to Y88–507	
	Y97–1721,Y98–681,1370–Yam agata–01,1874–Yam agata–03,3098–Yam agata–03,2070–Yam agata–04,2133–Yam agata–04,246–Yam agata–05,571–Yam agata–05,1382–Yam agata–06,2435–Yam agata–06	4 bp (4 A.A.) substitutions from Y88–507	
	1091–Yam agata–00,1115–Yam agata–00,98–Yam agata–01,57–Yam agata–07,285–Yam agata–07,Y98–9,2191–Yam agata–02	1 bp (1 A.A.) substitution from Y88–507	
	Ad3P	11 bp (8 A.A.) substitutions from Y88–507	
Ad4	Y91–164,Y91–2667,Y91–2101,Y92–123,Y92–706,Y92–1169,Y95–3486,Y97–817,Y97–1256,Y97–1428,K98–262,K98–465,1512–Yam agata–01,1632–Yam agata–01,1684–Yam agata–01,939–Yam agata–02,2011–Yam agata–02,2172–Yam agata–04,547–Yam agata–07,696–Yam agata–07	identical to Y91–164	
	617–Yam agata–02	1 (1 A.A.) bp substitution from Y91–164	
	1597–Yam agata–03,1699–Yam agata–06	12 bp (4 A.A.) deletions and 18 bp (9 A.A.) substitutions from Y91–164	
	Ad4P	1 bp (1 A.A.) substitution from 1597–Yam agata–03 without deletion	
Ad5	Y88–158,Y92–1128,Y93–1452,Y96–1374,Y97–104,33–Yam agata–01,2681–Yam agata–02,2863–Yam agata–02,2197–Yam agata–04,1676–Yam agata–05,489–Yam agata–06	identical to Y88–158	
	Y88–4866,Y89–5682,Y89–6660,Y90–1350,Y91–456,Y92–668,Y93–464,Y93–1629,Y94–1162,Y96–1939,Y97–1657,Y98–821,Y98–1499,2103–Yam agata–01,350–Yam agata–03,2116–Yam agata–04,2319–Yam agata–04,1572–Yam agata–06,Ad5P,3101–Yam agata–03,53–Yam agata–06,Y91–1628,163–Yam agata–00	0–2 bp (0–1 A.A.) substitutions from Y88–158	
	Y88–5197,Y94–225,1174–Yam agata–01,19–Yam agata–02,1986–Yam agata–03,474–Yam agata–06,1073–Yam agata–07	57 bp (11 A.A.) substitutions from Y88–158	identical toY88–5197
	Y90–790	1 bp substitution from Y88–5197	
	489–Yam agata–05,443–Yam agata–07	1–5 bp (1–2 A.A.) substitutions from Y88–5197	
Ad6	Y88–71,Y88–4586,Y89–2224,Y89–2275,Y89–3121,Y90–2710,Y90–2751,Y91–1807,Y92–491,Y93–866,Y93–1754,Y94–1090,Y94–2526,Y94–2595,Y94–2598,Y96–1757,Y96–2200,Y97–185,Y97–265,Y97–737,Y98–534,Y98–882,1052–Yam agata–01,1931–Yam agata–02,106–Yam agata–04,1444–Yam agata–06,1480–Yam agata–06	identical to Y88–71	identical to Y88–71
	Y90–2162,Y91–1815,Y98–1191,Ad6P	1–9 bp substitutions from Y88–71	
	Y88–3882,Y92–366,Y92–487,Y93–461,2114–Yam agata–02	two 3 bp (1 A.A.) and 6 bp (2 A.A.) deletions compared with Y88–71	

[a] Year of isolation

Ad6 (AB434175–AB434209). Sequence data for reference strains were also registered as AB067658, AB067659, AB366432, and AB436560–AB436564. Sequence data were analyzed with CLUSTAL W version 1.83, and a phylogenetic tree was constructed via the neighbor-joining method (Saitou and Nei, 1987) using the same software.

3. Results

3.1. Number of Ad isolates

A total of 2587 Ad1–6 strains were isolated between 1988 and 2007 in Yamagata. Ad3 was the most commonly isolated Ad serotype (1000 isolates), followed by Ad2 (670), Ad1 (532), Ad5 (246), Ad6 (72), and Ad4 (67).

3.2. Sequence analysis of Ad1–6 isolates

3.2.1. Ad1

A total of 975 bp were analyzed for Ad1. Since we found 9 identical base substitutions at identical positions, 2 groups (Y88-282 group and Y89-1015 group) were identified among the 41 analyzed strains (Table 1). Strains, belonging to the same group, showed only 0–2 base differences from each other, except for three strains having deletions. Two strains (Y92-246 and Y93-560), having sequences similar to that of Y89-1015, had 15- and 9-bp (5 and 3 A.A.) deletions, respectively, and 720-Yamagata-06, having a sequence similar to that of Y88-282, had a 15-bp (5 A.A.) deletion (Table 1). All three of these deletion sites were located at an identical position in HVR1 (Fig. 1). Apart from those with deletions, sequence identities among the analyzed Ad1 strains were 99.0–100%. Interestingly, all base substitutions for Ad1 strains occurred at the third position of the triplet code and all but the three strains with deletions had an identical A.A. arrangement (100% identity). Ad1P was identical to Y89-1015.

3.2.2. Ad2

A total of 987 bp were analyzed for Ad2. Among the 40 analyzed strains from Yamagata, all except for 1525-Yamagata-03 had 0–3 bp (0–2 A.A.) substitutions compared with Y88-1257 (Table 1). One unique strain 1525-Yamagata-03 had 3-bp insertions as well as 17-bp substitutions compared with Y88-1257. Apart from the 1525-Yamagata-03 strain, the sequence identities among the Ad2 strains analyzed were 99.6–100%. These Ad2 isolates had 0–2 A.A. substitutions at HVR1, HVR2, and between HVR3 and HVR4 compared with Y88-1257 (99.4–100% identity). 1525-Yamagata-03 had one A.A. insertion at HVR1 and four A.A. substitutions compared with Y88-1257: one A.A. substitution occurred between HVR3 and HVR4, another between HVR5 and HVR6, and two substitutions were located at HVR7 (Fig. 1). Ad2P was identical to Y88-1668.

3.2.3. Ad3

A total of 918 bp were analyzed for Ad3. Among the 39 analyzed strains from Yamagata, all isolates obtained between 1988 and 1996, Y97-1022, and 989-Yamagata-99 were identical to Y88-507 (Y88-507 group) (Table 1). Y98-681, 1370-Yamagata-01 and all isolates obtained between 2003 and 2006 were identical to Y97-1721 (Y97-1721 group), which had four base substitutions compared with Y88-507. 1115-Yamagata-00, 98-Yamagata-01, 57-Yamagata-07, and 285-Yamagata-07 strains were identical to 1091-Yamagata-00 (1091-Yamagata-00 group), which had one base substitution compared with Y88-507. Y98-9 and 2191-Yamagata-02 had one base substitution compared with Y88-507, though at a different position. Among the analyzed Ad3 strains, the sequence identities were 99.6–100%. Y97-1721 group had four A.A. substitutions (98.7% identity) and others had one A.A. substitution (99.7% identity) compared with strains in the Y88-507 group. These A.A. substitutions were located at HVR2-4 and HVR7 (Fig. 1). Y88-507 was identical to Ad3a and Ad3c, and had 11-bp (8 A.A.) substitutions compared with Ad3P.

3.2.4. Ad4

A total of 894 bp were analyzed for Ad4. All except 3 of the 23 strains analyzed had completely identical sequences to Y91-164 (Table 1). One strain (617-Yamagata-02) had one base substitution compared with Y91-164. Two strains (1597-Yamagata-03 and 1699-Yamagata-06) had identical sequences and showed 12-bp deletions as well as 18-bp substitutions compared with Y91-164. Except for 1597-Yamagata-03 and 1699-Yamagata-06, the sequence identities among the analyzed Ad4 strains were 99.9–100%. 617-Yamagata-02 had one A.A. substitution at HVR1 (99.7% identity), and 1597-Yamagata-03 and 1699-Yamagata-06 showed four A.A. deletions at the beginning of HVR3 and nine A.A. substitutions at HVR1, HVR3, between HVR3 and HVR4, and at HVR4, HVR5, and HVR6, compared with Y91-164 (Fig. 1). Ad4P showed one base and one A.A. substitution, but not the deletions, compared with 1597-Yamagata-03.

3.2.5. Ad5

A total of 939 bp were analyzed for Ad5. As we found 54 identical base substitutions at identical positions, we classified the 43 analyzed strains into 2 groups (the Y88-158 group and Y88-5197 group) (Table 1). The 34 strains, belonging to the Y88-158 group differed from each other by 0–3 base (0–2 A.A.) substitutions (99.7–100% sequence identity), whereas the 10 Y88-5197 group strains differed from each other by 0–6 base (0–3 A.A.) substitutions (99.4–100% sequence identity). Between the representative strains (Y88-158 and Y88-5197) of the two groups, there were 57-bp and 11 A.A. differences (93.9% sequence identity) (Fig. 1). Ad5P was identical toY88-4866.

3.2.6. Ad6

A total of 972 bps were analyzed for Ad6. Among the analyzed 35 strains, 27 isolates had completely identical sequences to Y88-71 (Y88-71 group) (Table 1). Three strains (Y90-2162, Y91-1815 and Y98-1191) had 1- or 9-bp substitutions compared with Y88-71, respectively (99.0–100% identity). On the other hand, Y88-3882, Y92-366, Y92-487, Y93-461, and 2114-Yamagata-02, which had identical sequences to each other, had unique sequences compared with Y88-71 (Y88-3882 group). These strains had 3-bp (1 A.A.) deletions at HVR1, 3-bp (1 A.A.) deletions at HVR2, and 6-bp (2 A.A.) deletions at the HVR7 compared with Y88-71. With regards to A.A. arrangement, Y90-2162, Y91-1815 and Y98-1191 were identical to Y88-71 (100% identity), whereas there were 63 differences apart from deletion sites between Y88-71 and Y88-3882 (Fig. 1). Ad6P had an identical A.A. arrangement to Y88-71, although it had 2-bp substitutions.

3.3. Phylogenetic analysis of Ad1–6 isolates

A phylogenetic tree was drawn using sequence data that included the seven HVRs (Fig. 2). Representative strains and reference strains were found to branch according to serotype.

4. Discussion

The present study demonstrated that the so-called seven HVRs among Ad1–6 isolates, which are perpetuated in communities such

as Yamagata, have been stably conserved over two decades as a single serotype. Primarily, the fact that apart from differences between two Ad5 groups and between strains having insertions and deletions, the sequence identities of Ad1–6 were 99.0–100% among identical serotype strains, respectively, strongly indicated this stability. Second, the sequence identity and similarity between the Ad1–6 isolates obtained in Yamagata between 1988 and 2007 and the reference strains, which were originally isolated in the mid 20th century, also supports the stability of these regions for each serotype (Enders et al., 1956; Li and Wadell, 1988). Third, the availability of Ad strains obtained from various places yet still having similar sequences or A.A. arrangements to isolates from Yamagata,

		<HVR1 >	<HVR2 >	<HVR3 >
Ad1	Y88-282	EEPTQE-MAEELEDEEEAEEEEAEEEAEAPQADQKVKKTHVYAQAPLAGEKITAN	GLQIV S--DTQTEG-NPV	FADPTYQPEPQVGESQW NEAEATA--S
	Y92-246	EEPTQE-MAEELEDEEEAEEE-----AEAPQADQKVKKTHVYAQAPLA*EK*TAN	*LQ*V S--DTQTEG-NPV	F**PTY*****V*ESQW NEAEATA--S
	Y93-560	EEPTQE-MAEELEDEEEAEEEE---EAEAPQADQKVKKTHVYAQAPLA*EK*TAN	*LQ*V S--DTQTEG-NPV	F**PTY*****V*ESQW NEAEATA--S
	720-Yamagata-06	EEPTQE-MAEELEDEEEAEEE-----AEAPQADQKVKKTHVYAQAPLA*EK*TAN	*LQ*V S--DTQTEG-NPV	F**PTY*****V*ESQW NEAEATA--S
Ad2	Y88-1257	TEDSGRAVAEDEEEE-DEDEEEEEEE--QNARDQATKKTHVYAQAPLS*ET*TKS	*LQ*G S-DNAETQA-KPV	Y**PSY*****I*ESQW NEADANA--A
	Y88-1668	TEDSGRAVAEDEEEE-DEDEEEEEEE--QNARDQATKKTHVYAQAPLS*ET*TKS	*LQ*G S-DNAETQA-KPV	Y**PSY*****I*ESQW NEADANA--A
	Y89-186	TEDSGRAVAEDEEEE-DEDEEEEEEE--QNARDQATKKTHVYAQAPLS*ET*TKS	*LQ*G S-DNAETQA-KP**I**	Y**PSY*****I*ESQW NEADANA--A
	Y91-100	TEDSGRAVAEDEEEE-D**K**DEEEEEEE--QNARDQATKKTHVYAQAPLS*ET*TKS	*LQ*G S-DNAETQA-KPV	Y**PSY*****I*ESQW NEADANA--A
	1525-Yamagata-03	TEDSGRAVAEDEEEE**E**DEDEEEEEEE--QNARDQATKKTHVYAQAPLS*ET*TKS	*LQ*G S-DNAETQA-KPV	Y**PSY*****I*ESQW NEADANA--A
Ad5	Y88-158	AATALEINLEEEDDDNED----------EVDEQAEQQKTHVFGQAPYS*IN*TKE	*IQ*G V--EGQT----PK	Y**KTF*****I*ESQW YETEINH--A
	Y88-5197	AATALEINLEEEDDDNED----------EVDEQAEQQKTHVFGQAPYS*IN*TKE	*IQ*G **N**--**DAGT**----PK	Y**KTF*****I*ESQW **NE**TEINH--A
	Y91-1628	AATALE**M**NLEEEDDDNED----------EVDEQAEQQKTHVFGQAPYS*IN*TKE	*IQ*G V--EGQT----PK	Y**KTF*****I*ESQW YETEINH--A
	163-Yamagata-00	AATALEINLEEEDDDNED----------EVDEQAEQQKTHVFGQAPYS*IN*TKE	*IQ*G V--EGQT----PK	Y**KTF*****I*ESQW YETEINH--A
	489-Yamagata-05	AATALEINLEEEDDDNED----------EVDEQAEQQKTHVFGQAPYS*IN*TKE	*IQ*G **N**--**DAGT**----PK	Y**KTF*****I*ESQW **NE**TEINH--A
	443-Yamagata-07	AATALE**M**NLEEEDDDNED----------EVDEQAEQQKTHVFGQAPYS*IN*TKE	*IQ*G **N**--**DAGT**----PK	Y**KTF*****I*ESQW **NE**TEINH--A
Ad6	Y88-71	NETAQVDAQELDEEENEANEA-------QAREQEQAKKTHVYAQAPLS*IK*TKE	*LQ*G TADATVAGAGKEI	F**KTF*****V*ESQW NEADATA--A
	Y88-3882	**DD**T-QV**QVAAEDDQDDDEEEE**-------**QLPQQRNG**KKTHVYAQAP**FA*EA*NKN**	*LQ*G **TNGAATEGN**-KEI	**Y**KTF*****I*ESQW NEA**ESSV**--A
Ad3	Y88-507	TTNRDNAVTTTTNTFGIASMK----------*DN*TKE	*LQ*G KDITTTEGEEKPI	Y**KTY*****V*EESW TDTDVTNEKF
	Y97-1721	TTNRDNAVTTTTNTFGIASMK----------*DN*TKE	*LQ*G KDITTTEGEEKPI	Y**KTY*****V*EESW TDTD**G**TNEKF
	Y98-9	TTNRDNAVTTTTNTFGIASMK----------*DN*TKE	*LQ*G KDITTTEGEEKPI	Y**KTY*****V*EESW TDTD**G**TNEKF
	1091-Yamagata-00	TTNRDNAVTTTTNTFGIASMK----------*DN*TKE	*LQ*G KDITTTEGEEKPI	Y**KTY*****V*EESW TDTDVTNEKF
	2191-Yamagata-02	TTNRDNAVTTTTNTFGIASMK----------*DN*TKE	*LQ*G KDI**N**TTEGEEKPI	Y**KTY*****V*EESW TDTDVTNEKF
	Ad3P	TT**N**GDNAVTTTTNTFGIASMK----------*DN*TKE	*LQ*G KDITTTEGEEKPI	Y**KTY*****V*EESW TDTD**G**TNEKF
Ad4	Y91-164	DANSKMHTFGVAAMP-GVT----------*KK*EAD	*LP*R IDSTS--GTDTVI	Y**KTF*****V*NDSW VDTNDAEEKY
	617-Yamagata-02	DANSKMHTFGVAAM**S**-GVT----------*KK*EAD	*LP*R IDSTS--GTDTVI	Y**KTF*****V*NDSW VDTNDAEEKY
	1597-Yamagata-03	D**SD**SKMHTFG**A**AAMP-GVT----------*KK*EAD	*LP*R IDSTS--GTDTVI	Y**KTF*****V*ND-- --TNGAEEKY
	Ad4P	D**SD**SKMHTFG**A**AAMP-GVT----------*KK*EAD	*LP*R IDSTS--GTDTVI	Y**KTF*****V*NDSW VDTN**G**AEEKY

		<HVR4 >	<HVR5 >	<HVR6
Ad1	Y88-282	GGRVLKKTTPMKPCYGSYARPTNKNGGQ GIL------VANNQGALESK	VEMQFF APSGTAMNER-NAVQPS	IVLYSEDVNMETPDTHISYKP SKTDENS
	Y92-246	G**V**KT*P*******Y*R***KN*** GIL------VANNQGALESK	VEMQ** APSGTAMNER-NAVQ*S	I*L*S*D*NME***T*IS*K* SKTDENS
	Y93-560	G**V**KT*P*******Y*R***KN*** GIL------VANNQGALESK	VEMQ** APSGTAMNER-NAVQ*S	I*L*S*D*NME***T*IS*K* SKTDENS
	720-Yamagata-06	G**V**KT*P*******Y*R***KN*** GIL------VANNQGALESK	VEMQ** APSGTAMNER-NAVQ*S	I*L*S*D*NME***T*IS*K* SKTDENS
Ad2	Y88-1257	G**V**KS*P*******Y*R***PF*** SVL------VPDEKGVPLPK	VDLQ** SNTTSLNDRQGNATK*K	V*L*S*D*NME***T*LS*K* GKGDENS
	Y88-1668	G**V**K**T***P*******Y*R***PF*** SVL------VPDEKGVPLPK	VDLQ** SNTTSLNDRQGNATK*K	V*L*S*D*NME***T*LS*K* GKGDENS
	Y89-186	G**V**K**T***P*******Y*R***PF*** SVL------VPDEKGVPLPK	VDLQ** SNTTSLNDRQGNATK*K	V*L*S*D*NME***T*LS*K* GKGDENS
	Y91-100	G**V**K**T***P*******Y*R***PF*** SVL------VPDEKGVPLPK	VDLQ** SNTTSLNDRQGNATK*K	V*L*S*D*NME***T*LS*K* GKGDENS
	1525-Yamagata-03	G**V**K**T***P*******Y*R***PF*** SVL------VPDEKGVPLPK	VDLQ** SNTTSLNDRQGNATK*K	V*L*S*D*N**L**E***T*LS*K* GKGDENS
Ad5	Y88-158	A**V**KT*P*******Y*K***EN*** GIL------VKQQNGKLESQ	VEMQ** STTEAAAGNG-DNLT*K	V*L*S*D*DIE***T*IS*M* TIKEGNS
	Y88-5197	A**V**KT*P*******Y*K***EN*** GIL------VKQQNGKLESQ	VEMQ** STTEAAA**V**NG-DNLT*K	V*L*S*D*DIE***T*IS*M* TIKEGNS
	Y91-1628	A**V**KT*P*******Y*K***EN*** GIL------VKQQNGKLESQ	VEMQ** STTEAAAGNG-DNLT*K	V*L*S*D*DIE***T*IS*M* TIKEGNS
	163-Yamagata-00	A**V**KT*P*******Y*K***EN*** GIL------VKQQNGKLESQ	VEMQ** STTEAAAGNG-DNLT*K	V*L*S*D*DIE***T*IS*M* TIKEGNS
	489-Yamagata-05	A**V**KT*P*******Y*K***EN*** GIL------VKQQNGKLESQ	VEMQ** STTEAAA**V**NG-DNLT*K	V*L*S*D*DIE***T*IS*M* TIKEGNS
	443-Yamagata-07	A**V**KT*P*******Y*K***EN*** GIL------VKQQNGKLESQ	VEMQ** STTEA**V**A**V**NG-DNLT*K	V*L*S*D*DIE***T*IS*M* TIKEGNS
Ad6	Y88-71	G**V**KT*P*******Y*R***SN*** GVM------V-EQNGKLESQ	VEMQ** STSTNATNEV-NNIQ*T	V*L*S*D*NME***T*LS*K* KMGDKNA
	Y88-3882	G**V**KT*P*******Y*R***SN*** GVM------V-EQNGKLESQ	VEMQ** STS**V**NA**M**NE**A**-NA**I**Q*K **L***L*S*D*NME***T*LS*K* **GKSDDNS**
Ad3	Y88-507	G**A**PA*N*******F*R***IK*** AKNRKVKPTTEGGVETEEPD	IDME** DGRDAVAG----ALA*E	I*L*T*N*NLE***S*VV*K* GTSD-NS
	Y97-1721	G**A**PA*N*******F*R***IK*** AKNRKVKPTTEGGVETEEPD	IDME** DGRDAVAG----ALA*E	I*L*T*N*NLE***S*VV*K* GTSD-NS
	Y98-9	G**A**PA*N*******F*R***IK*** AKNRKVKPTTEGGVETEEPD	IDME** DGRDAVAG----ALA*E	I*L*T*N*NLE***S*VV*K* GTSD-NS
	1091-Yamagata-00	G**A**PA*N*******F*R***IK*** AKNRKVKPTTEGGV**E**IEEPD	IDME** DGRDAVAG----ALA*E	I*L*T*N*NLE***S*VV*K* GTSD-NS
	2191-Yamagata-02	G**A**PA*N*******F*R***IK*** AKNRKVKPTTEGGVETEEPD	IDME** DGRDAVAG----ALA*E	I*L*T*N*NLE***S*VV*K* GTSD-NS
	Ad3P	G**A**PA*N*******F*R***IK*** AKNRKVKPTTEGGVETEEPD	IDME** DGRDAVAG----ALA*E	I*L*T*N*NLE***S*VV*K* **ETSN**-NS
Ad4	Y91-164	G**A**DT*N*******F*K***KE*** ANL----KDSETAATTPNYD	IDLA** DGKN**I**VAN-----YD*D	I*M*T*N*DLQ***T*IV*K* GKEDTSS
	617-Yamagata-02	G**A**DT*N*******F*K***KE*** ANL----KDSETAATTPNYD	IDLA** DGKN**I**VAN-----YD*D	I*M*T*N*DLQ***T*IV*K* GKEDTSS
	1597-Yamagata-03	G**A**DT*K*******F*K***KE*** ANL----KDSE**P**AATTPNYD	IDLA** D**S**K**T**IVAN-----YD*D	I*M*T*N*DLQ***T*IV*K* G**T**EDTSS
	Ad4P	G**A**DT*K*******F*K***KE*** ANL----KDSE**P**AATTPNYD	IDLA** D**S**K**T**IVAN-----YD*D	I*M*T*N*DLQ***T*IV*K* G**T**EDTSS

Fig. 1. Amino acid (A.A.) alignment of the seven hexon hypervariable regions (HVRs) for Ad1–6 representative isolates from Yamagata, Japan, obtained between 1988 and 2007 and reference strains based on Table 1. A.A. locations were modified based on references (Crawford-Miksza and Schnurr, 1996a; Pring-Åkerblom et al., 1995b; Rux et al., 2003) Asterisks indicate conservations with Ad1 sequence. Sites different from those of the top strain for each serotype are indicated by bold characters.

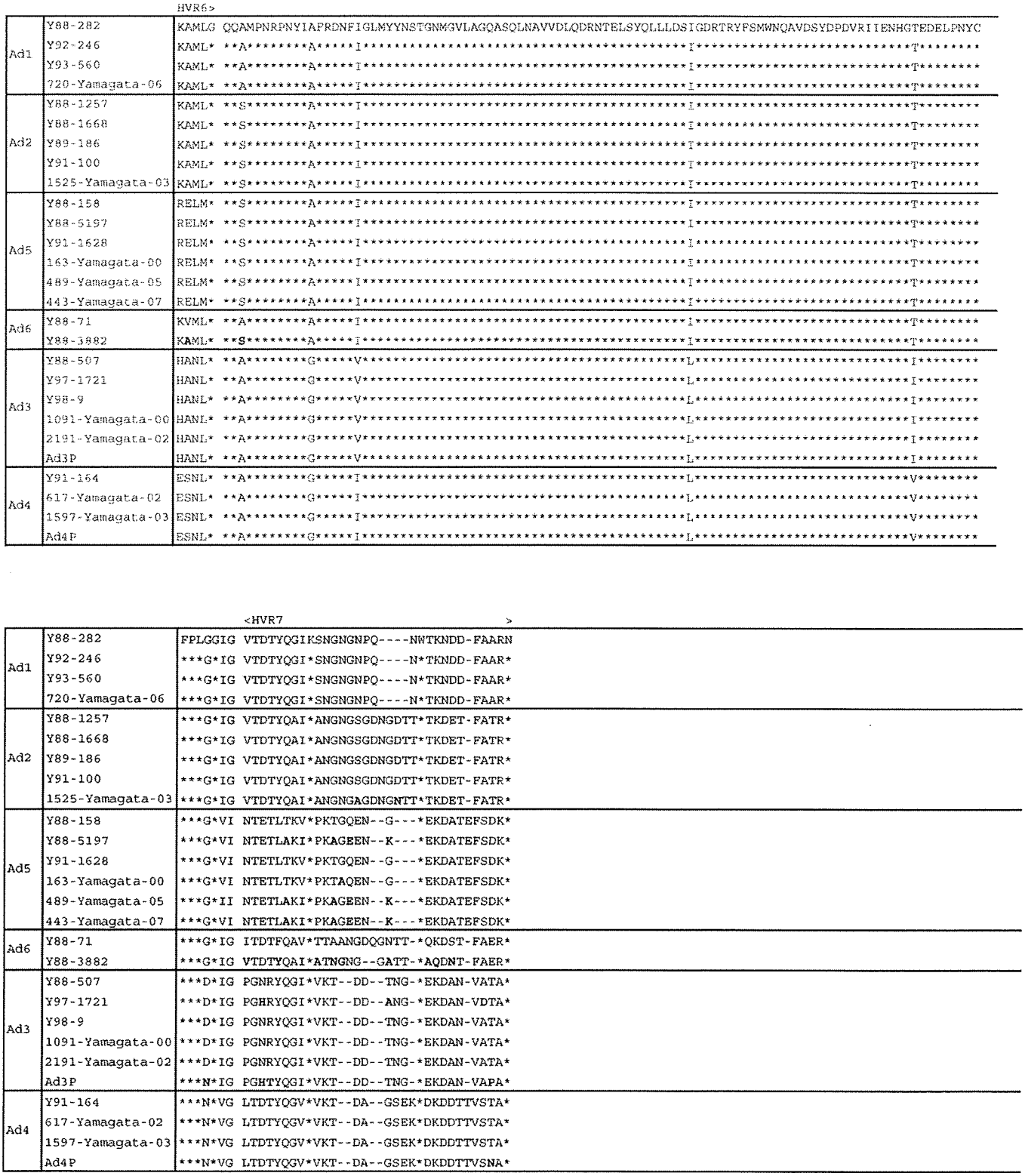

Fig. 1. (*Continued*).

as described later, further indicates the stability of the HVRs. In this sense, it might be appropriate to use this region, which includes the seven HVRs, as the target for PCR to amplify the serotype-specific genome (Okada et al., 2007).

Although the seven HVRs were stably conserved as the serotype-specific region, there were several strains that showed deletions and insertions. For Ad1, three strains had 3–5 A.A. deletions at the identical portion of HVR1. 1525-Yamagata-03 (Ad2) had one A.A. insertion at HVR1, and Y88-3882 (Ad6) had one A.A. deletion at HVR1. Other deletions were observed at HVR2 (Y88-3882: Ad6), at HVR3 (1597-Yamagata-03: Ad4), and at HVR7 (Y88-3882: Ad6). As A.A. deletions and insertions were observed most frequently at

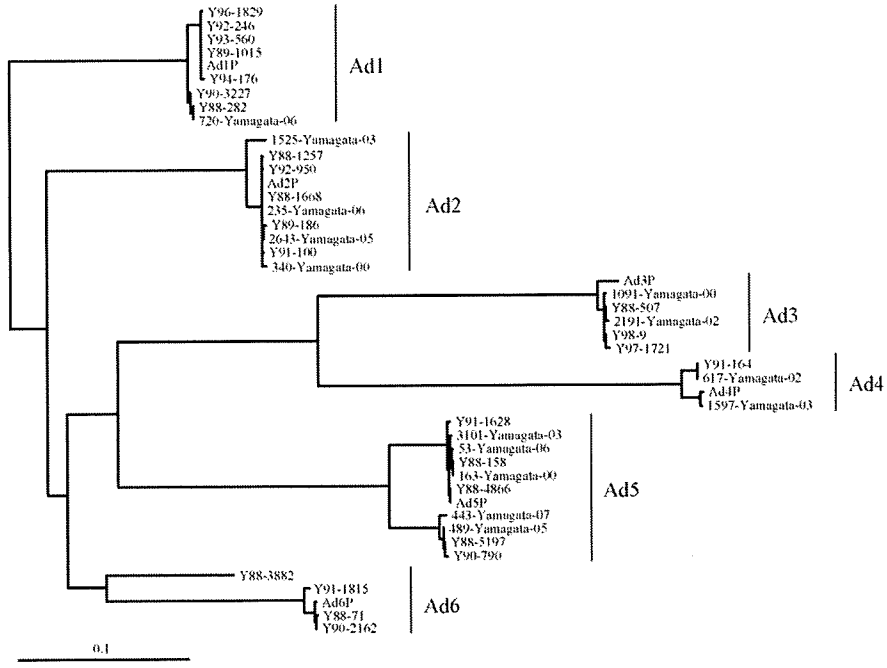

Fig. 2. Phylogenetic tree of representative Ad1–6 isolates from Yamagata, Japan obtained between 1998 and 2007 and reference strains based on Table 1. The marker denotes the measurement of relative phylogenetic distance.

HVR1, this region might be the most variable region. The variability in length of HVR1 and the marked differences between the two Ad6 groups also supports this point of view.

A number of interesting Y88-3882-group strains were identified in this study. When we attempted homology search using the BLAST method for this strain, we hit Ad6 strains, such as those with accession numbers Q3S8C4 and Q04966, which have similar A.A. arrangements to Y88-71. Between Y88-3882 and the Y88-71 group, there were not only 3 deletion sites but also 63 A.A. differences, and the phylogenetic tree also showed that Y88-3882 branched differently from other Ad6 group strains. Thus, the Y88-3882-group strains formed a unique cluster, although we identified them as Ad6 strains by neutralization test. Since five isolates (obtained in 1988, 1992, 1993 and 2002) had identical sequences to Y88-3882, it seems clear that this unique group has been perpetuated as a minor Ad6 group independently and concurrently with the major Y88-71 group in Yamagata. These findings suggest that even the strains with deletions and/or insertions are circulating as one established group of strains. As a unique strain, Adhikary et al. reported that Ad8I has only a 62% genomic identity to Ad8 in the HVRs, and it has major crossreactivity with Ad9 in neutralization test (Adhikary et al., 2004).

Fujimoto et al. analyzed the hexon sequence including the seven HVRs of Ad3 isolates obtained between 1994 and 2006 in Hyogo, Japan (Fujimoto et al., 2008) and found three genome types, Ad3Vx, Ad3Vy and Ad3Vz. Although their sequence data for the analyzed strains are not available, the reported sequence variations suggest that AdV3x is similar to Y97-1721, Ad3Vy is like 2191-Yamagata-02, and AdV3z is like Y88-507 or 1091-Yamagata-00. They reported that AdV3x first appeared in Hyogo in 2001 and suggested that this AdV3x strain was imported from Korea, where a large AdV3x outbreak occurred in 1998–1999 (Fujimoto et al., 2008). However, our results suggest that AdV3x-like strains already existed in Japan prior to the outbreak in Korea, since we had found Y97-1721-group strains, which are similar to AdV3x, in 1997. Either way, it appears Adv3x- and Y97-1721-like strains have been spreading across a wide area including Japan and Korea since around 1997–1998. We have not carried out antigenic analysis in order to compare the antigenicity between the Y88-507- and Y97-1721-group strains. Our observation that the major group changed from Y88-507 to Y97-1721 around 2003 coincides with their finding that AdV3x strains became dominant at that time. Such a substitution of the major group among Ad3 strains is quite unique when compared with in other serotypes, for which major and minor groups tend to co-circulate (Table 1). Further research is necessary to clarify the relationship between circulation pattern and epidemiological behavior (endemic and/or epidemic) according to serotype.

Crawford-Miksza et al. revealed that Ad7a, which belongs to subgroup B as well as Ad3, showed only 6 single-base differences in the hexon region among 18 strains over a 42-year period in California and other locations in the U.S.A. (Crawford-Miksza et al., 1999). Their data suggest that Ad7 had perpetuated with a stable hexon sequence over several decades in manner similar to that we observed for Ad1-6 in Yamagata. Analyzing Ad4 strains isolated between 1953 and 1997, they also reported that the evolution of Ad4 was more complex (Crawford-Miksza et al., 1999). Interestingly, our Y91-164 isolate had only one A.A. difference from their Ad4 variant (Z-G95-873), and 1597-Yamagata-03 had a similar A.A. arrangement to their isolate 88-16306, since the four A.A. deletions occurred at identical positions in HVR3. Thus, taking their finding together with ours, it can be suggested that Y91-164-like strains and 1597-Yamagata-03-like strains, which are also similar to Ad4P, have been co-circulating not only in Yamagata, but also around the world.

In conclusion, our findings suggest that even the so-called HVRs have been conserved as a single serotype for a long period of at least for two decades and that Ad1–6 have been perpetuated in a local area with only minor genomic variations or with established deletions and insertions. In other words, the sequence of the HVRs must have remained stable for Ad to exist as its serotype. On the other hand, we also confirmed that insertions, deletions and substitutions occurred mainly in the seven HVRs. These results suggest that Ads have evolved through the accumulation of insertions,

deletions and substitutions, which mainly occurred in the seven HVRs, over a reasonably long period of time. Unfortunately, the 20 years covered by this study is too short a time-frame in which to observe such evolution.

Finally, we propose that these regions should be hereafter referred to as "type-specific regions" rather than HVRs, since "type-specific regions" better represents the actual epidemiological characteristics of these sites in Ads.

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Genotypic and Phylogenetic Analysis of the G Gene of Respiratory Syncytial Virus Isolates in Okinawa, Japan, 2008

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Respiratory syncytial virus (RSV) is an important causative agent of acute respiratory infections (ARIs) in infants and young children (1). The prevalent season of RSV infection is from May to September in Okinawa Prefecture (Ryukyu Islands), while it is from November to February in other areas of Japan (2). This suggests that the subgroup of RSV prevalent in Okinawa may be different from those prevalent in the rest of Japan (1). The present study was performed to understand the molecular epidemiology of RSV in Okinawa.

Eight RSV isolates were obtained from children in Okinawa with ARIs in June and July 2008. The isolates were propagated in HEp-2, LLC-MK2, or Vero9013 cells. Virus RNA was extracted from the isolates 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 (PCR) was performed as described previously (3). Amplicons were purified using a QIAquick PCR Purification kit (Qiagen) and the nucleotide sequences were determined by direct sequencing (3). Partial nucleotide sequences (270 nt) of the G gene of RSV were analyzed phylogenetically using Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (4). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbor-joining (NJ) method (5). The reliability of the tree was estimated based on 1,000 bootstrap replications.

A phylogenetic tree was constructed including the Okinawa isolates (Fig. 1). Of the 8 new isolates, 7 were classified into subgroup A, and one into B. In subgroup A, the 7 isolates were classified into genotype GA2, and further into 2 subclusters: 5 with a Brazilian (JU1780/2007) isolate and 2 with Brazilian (SP1044/2006) and Belgian (BE/9600/05) isolates. In subgroup B, the Okinawa isolate was classified into genotype BA, and further in the subcluster with Indian (DEL/AFF/05), Belgian (BE/9382/05), and Brazilian (JU1324/2006) isolates. Two other isolates in Japan, S02-71 isolated in Sapporo in 2002 and NG-004-03 in Niigata in 2003, were located in a different subcluster within subgroup B. There is a 60-nt duplication in the second variable region of the G gene of the isolate (RSvi/OkinawaJPN/232.08). Strains with this unique duplication have recently spread in the world (6,7). Further, there was a novel stop codon which leads to a nucleotide substitution in the present isolate, corresponding to nucleotide position 881 in the reference strain (strain 18537, Genbank accession no. M17213); TTA to TAA; Leu to stop codon. The results suggest that RSV strains belonging to subgroup A were dominant in Okinawa in 2008, with those belonging to subgroup B as minor. These results were consistent with those of previous reports (7,8).

The prevalent season of RSV infection in Okinawa is summer, unlike that in other areas of Japan. In tropical regions of Asia, such as Hong Kong, the Philippines, and Thailand, epidemics of RSV infection occur in the rainy season (9). In northern tropical regions of South America, as observed in Cali, Columbia, RSV infection is epidemic year round (1). These reports suggest that the prevalent season of RSV infection differs depending on global region and climate (1,9) rather than to strain per se. Additional studies are needed in order to further understand the molecular epidemiology of RSV in Okinawa.

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