Table 2
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP7 of strain CMP034 with those of 15 known G rotavirus genotypes

Strain	Species	G genotype	Identity (%)	
			Nucleotide	Amino acid
KU	Human	G1	72.4	75.1
DS-1	Human	G2	81.3	88.0
S2	Human	G2	82.6	87.7
TA20	Human	G2	82.9	88.0
KUN	Human	G2	82.0	88.0
906SB/98	Human	G2	83.0	88.0
92C	Human	G2	83.5	88.0
34461-4	Porcine	G2	90.9	94.7
CMH222	Human	G3	73.6	76.6
Hochi	Human	G4	72.5	72.3
OSU	Porcine	G5	74.1	76.0
NCDV	Bovine	G6	72.3	75.7
Ch2	Avian	G7	61.0	56.9
B37	Human	G8	73.5	57.1
116E	Human	G9	77.0	77.3
61A	Bovine	G10	71.7	74.2
YM	Porcine	G11	72.9	75.1
L26	Human	G12	74.8	75.1
L338	Equine	G13	72.0	72.6
CH3	Equine	G14	73.5	73.9
Hg18	Bovine	G15	72.1	72.3

The GenBank accession numbers of the following strains are given in parentheses: KU (D16343), DS-1 (AB118023), S2 (M11164), TA20 (AF106281), KUN (D50124), 906SB/98 (AY261347), 92C (U73949), 34461-4 (AY766085), CMH222 (AY707792), Hochi (AB012078), OSU (X04613), NCDV (M12394), Ch2 (X56784), B37 (J04334), 116E (L14072), 61A (X53403), YM (M23194), L26 (M58290), L338 (D13549), CH3 (D25229), Hg18 (AF237666).

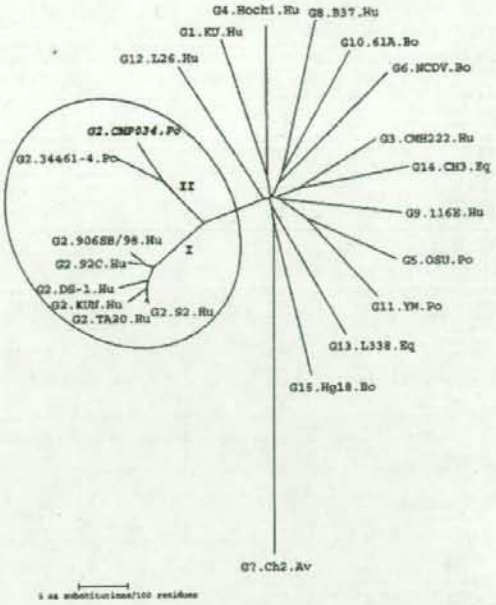

Fig. 4. Phylogenetic tree based on the VP7 nucleotide sequences. The tree displays the relationships among strain CMP034 and of the 15 known G genotypes. GenBank accession numbers of the VP7 sequences of all known 15 G genotypes are given in the legend to Table 2. The tree was generated on the basis of the neighbor-joining method using program MEGA 3.1.

season to verify whether P[19] was really circulating in the pig population in Chiang Mai city. Therefore, we conducted a rotavirus surveillance in both humans and pigs simultaneously in the same epidemic season in Chiang Mai city during the year 2000–2001, and we found P[19] strains circulating in pig populations in Chiang Mai city (Maneekam et al., 2006). These PoRV strains were G3P[19], and shared a level of VP4 P[19] sequence identity with those of P[19] Mc323 and Mc345 as high as 95.4–97.6% nucleotide sequence identity. This finding indicates that gene VP4 of Mc323 and Mc345 might have been derived through reassortment from VP4 P[19] of PoRV strains that circulated in this region. However, P[19] rotavirus was not detected in humans in the study that was carried out in the same epidemic season (Khamrin et al.,

	A		B		C		F	
	87	101	142	152	208	221	235	242
G2.CMP034	IYYPTEAKNEISDTE		NVVLMRYDTTS		GIGCKITEVNTFET		NGVNHKIS	
G2.34461-4	.....		.....K.....		.....		.....	
G2.92C	L.....D.		.....N..		.....D.S...I		.....N	
G2.906SB/98	L...A.....D.		.....GN..		.....D.S....		.....N	
G2.S2	L...A.....D.		.....N..		.....D.D...I		.....N	
G2.TA20	L...A.....D.		.....N..		.....D.D...I		.....N	
G2.KUN	L.....D.		.....N..		.....D.....I		.....N	
G2.DS-1	L...A.....D.		.....N..		.....D.....I		.....N	
G1.KU	L.....STQ.N.GD		.L...K..QSL		....Q..N.DS..M		D.I....N	
G3.CMH222	L.....AT..N.NS		.....K..A.L		....L..DTT...E		D.....LD	
G4.Hochi	L...S..PTQ.....		....I.PASGE		....Q..NTA....		DS....LD	
G5.OSU	L...N..AT..A..K		.I...K..GNL		....S..DI.S...		D.....LD	
G6.NCDV	L...V..S..MA...		.L...K..S.Q		....LI.NPD....		D.....LN	
G7.Ch2	....KA.DT..A.P.		.I..AH.TNDV		....Q..NTD...I		D.....VD	
G8.B37	L...V..ET..A.SS		.I...K.NAN.		R...L..DTT...E		D...Y..N	
G9.116E	L...I..STQ.G...		....VK.NS.L		....T..NTA...E		D.....LD	
G10.61A	L.....RT..N.N.		.I.....NSSL		....Q..NTR...E		D.....LD	
G11.YM	L...H..ATQ.A.DK		.I...K..GN.		....L..DPT...E		D.....LD	
G12.L26	L...NSVTT..T.PD		.I..VQ.QNSL		....T..D.A...E		D.....N	
G13.L338	L...N.VVS.LN.DS		.I.VVK.S.EL		....L..DTE...E		D.....N	
G14.CH3	L.S....ATQ.D.SS		.....K..EAL		....L..N.E...E		D..D...N	
G15.Hg18	.....I.S.DLA.PD		.I...K.ESDL		....L..DTSS...		D..S..LD	

Fig. 3. Comparison of the amino acid sequences of antigenic regions A, B, C, and F of porcine CMP034 with those of 15 known G genotypes. Dots indicate amino acid residues identical with those in the sequence of strain CMP034.

Table 3
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP6 of strain CMP034 with those of known subgroups

Strain	Species	Subgroup	Identity (%)	
			Nucleotide	Amino acid
BRV033	Bovine	I	76.8	90.1
NCDV	Bovine	I	79.1	90.9
UK	Bovine	I	80.0	91.4
SA11	Simian	I	78.7	91.1
I321	Human	I	80.7	90.9
US1205	Human	I	78.6	90.1
1076	Human	I	79.7	91.6
RMC321	Human	I	90.8	98.9
RMC/G60	Human	I	90.7	98.4
RMC/G7	Human	I	90.9	98.4
4F	Porcine	I	91.0	98.9
4S	Porcine	I	90.9	98.9
JL94	Porcine	I	92.6	98.4
A253	Porcine	I	86.0	99.2
A131	Porcine	I	87.5	99.2
OSU	Porcine	I	86.9	98.9
H-1	Equine	I	87.7	97.9
Wa	Human	II	82.1	93.7
116E	Human	II	83.4	93.9
TK159	Human	II	82.9	91.9
Gottfried	Porcine	II	83.6	93.7
FI-14	Equine	I+II	78.9	91.1
L338	Equine	I+II	80.5	92.1
H-2	Equine	Noni/nonII	78.9	90.2
FI-23	Equine	Noni/nonII	78.5	90.2

The GenBank accession numbers of the following strains are given in parentheses: BRV033 (AF317126), NCDV (AF317127), UK (X53667), SA11 (AY187029), I321 (X94618), US1205 (AF079357), 1076 (D00325), RMC321 (AF531913), RMC/G60 (AY601552), RMC/G7 (AY601551), 4F (L29184), 4S (L29186), JL94 (AY538664), A253 (AF317122), A131 (AF317124), OSU (AF317123), H-1 (AF242394), Wa (K02086), 116E (U85998), TK159 (AY661888), Gottfried (D00326), FI-14 (D00323), L338 (D82974), H-2 (D00324), FI-23 (D82971).

2006a). In this epidemiological study of the distribution of PoRV G and P genotypes in Chiang Mai province, we detected an unusual rotavirus strain, which appears to carry a novel P[27] genotype. As revealed by the analysis of the amino acid sequence, gene VP4 of PoRV strain CMP034 shares less than 77% amino acid sequence identity with the 26 known P genotypes.

It is well established that the VP4 sequence of animal rotaviruses contains 776 amino acid residues, while most human rotavirus strains contain 775 residues, with the loss of one residue between positions 134 and 136 of the VP8* fragment of gene VP4 (Gorziglia et al., 1988; Kantharidis et al., 1987). Analysis of the amino acid sequence of VP4 revealed that CMP034 has lost one amino acid residue at position 135, similar to human rotaviruses. Nevertheless, the deletion of three other amino acid residues in the hypervariable region of the VP5* portion of gene VP4, like those found in bovine rotavirus strain B223, implies an animal origin of CMP034. This unusual feature of amino acid deletion in the VP4 gene of CMP034, therefore, makes the origin of gene VP4 unclear. Nevertheless, it is interesting to note that sequence analyses of VP6 and NSP5/6 genes of CMP034 reveal a porcine-like specificity, whereas the NSP4 gene has some characteristics consistent with a human origin.

The distribution of rotavirus G and P genotypes in pigs has been reported from throughout the world (Estes, 2001; Liprandi et al., 1991; Pongsuwanna et al., 1996; Winiarczyk et al., 2002). On the basis of the accumulated evidence of transmission of rotaviruses between pigs and other animal species, including human, pigs are regarded as the potential reservoir for the emergence of unusual or novel strains of rotaviruses (Palombo, 2002; Martella et al., 2005, 2006b). Several novel strains of P genotypes have been identified recently in pigs, such as P[23] (Liprandi et al., 2003) and, most recently, P[26] (Martella et al., 2006a). In the present study, a novel P genotype has been isolated from a diarrheic piglet in Thailand.

Although G2 strains of rotavirus are commonly found in humans, it has not been identified from other animal sources (Estes, 2001), except for one recent report from Spain of a

Table 4
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein NSP4 of strain CMP034 with those of known NSP4 genetic groups

Strain	Species	NSP4 genetic group	Identity (%)	
			Nucleotide	Amino acid
KUN	Human	A	78.6	78.8
UK	Bovine	A	79.0	80.0
NCDV	Bovine	A	78.1	80.0
SA-11	Simian	A	78.9	78.2
Wa	Human	B	85.1	84.0
RV4	Human	B	84.5	88.2
ST3	Human	B	83.1	81.1
M37	Human	B	83.9	81.7
116E	Human	B	85.9	83.4
97'SZ37	Human	B	85.4	83.4
GR856/86	Human	B	86.8	86.2
GR1106/86	Human	B	86.6	85.1
A131	Porcine	B	79.8	85.4
A253	Porcine	B	81.8	85.7
A411	Porcine	B	81.2	82.8
H-1	Equine	B	81.9	83.4
OSU	Porcine	B	85.7	85.1
A34	Porcine	B	86.0	85.1
FRV-1	Feline	C	77.8	77.1
AU1	Human	C	77.9	76.5
GRV	Caprine	C	61.0	78.2
CMH222	Human	C	78.0	77.1
CU-1	Canine	C	73.3	77.7
EW	Murine	D	66.2	60.0
EHP	Murine	D	66.1	59.4
EC	Murine	D	66.0	58.2
Ty-1	Avian	E	49.8	30.2
Ty-3	Avian	E	46.8	29.7
Ch-1	Avian	E	51.3	32.5

The GenBank accession numbers of the following strains are given in parentheses: KUN (D88829), UK (K03384), NCDV (X06806), SA11 (K01138), Wa (AF093199), RV4 (U59108), ST3 (U59110), M37 (U59109), 116E (U78558), 97'SZ37 (AF284778), GR856/86 (AF170832), GR1106/86 (AF170833), A131 (AF144798), A253 (AF144797), A411 (AF144799), H-1 (AF144800), OSU (D88831), A34 (AF165219), FRV-1 (D89874), AU-1 (D89873), GRV (AB055968), CMH222 (DQ288660), CU-1 (AF144806), EW (U96335), EHP (U96336), EC (U96337), Ty-1 (AB065285), Ty-3 (AB065286), Ch-1 (AB065287).

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PoRV strain bearing a G2-like genotype (strain 34461-4) in a piglet (Martella et al., 2005). The VP7 sequence analysis of our CMP034 strain revealed a genetically close relationship to the G2-like PoRV strain 34461-4. A close genetic relationship between VP7 from strains CMP034 and 34461-4 was demonstrated by analysis of the VP7 antigenic regions A, B, C, and F. The only difference observed between the two strains was a Lys to Arg change at position 147 in the antigenic region B. The relatedness between the sequence of VP7 in strains CMP034 and 34461-4 was confirmed repeatedly by the phylogenetic analysis of the VP7 sequence, showing that CMP034 and 34416-4 are clustered closely together in a branch separate from that of other human G2 reference strains. These findings suggest that VP7 of PoRV strain CMP034 may have originated from the same ancestor as those of strain 34461-4. The isolation of two strains of rotaviruses with a close genetic relatedness of gene VP7 from two countries, Thailand and Spain, which are geographically far apart, may indicate that the gene VP7 of G2 specificity may have already been introduced into the porcine rotaviruses worldwide. To verify this notion, the epidemiological rotavirus surveillance in pigs may need to be performed extensively in several other regions of the world.

Materials and methods

Rotavirus antigen detection

Porcine rotavirus strain CMP034 was isolated during the surveillance of porcine rotavirus infection in Chiang Mai province, Thailand from June 2000 to July 2001. A total of 175 fecal specimens were collected from diarrheic piglets from six different farms and the age of the piglets ranged from 7 days to 49 days old. Group A rotavirus antigen was detected by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody against group A rotavirus as described previously (Hasegawa et al., 1987). Out of 175, 39 (22.3%) were positive for group A rotaviruses (Maneekarn et al., 2006). PoRV strain CMP034 was identified as group A rotavirus in stool sample from a diarrheic piglet of 49 days old at a farm in Mae Rim district, Chiang Mai province. Despite numerous attempts, using RNA PAGE of phenol/chloroform or acid phenol/guanidinium thiocyanate/chloroform RNA extraction methods, we were unable to visualize the dsRNA electrophoretic pattern.

RNA extraction, RT-PCR, and multiplex-PCR for G and P genotyping

The G and P genotypes of CMP034 were determined by RT-PCR and multiplex-PCR. Viral dsRNA was extracted from 10% fecal supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). Viral dsRNA was denatured in 50% (v/v) dimethyl sulfoxide at 95 °C for 5 min. The RT-PCR was carried out with a OneStep RT-PCR Kit (Qiagen, Hilden, Germany). For PCR amplification of the VP7 gene, a 1062 bp fragment was generated using Beg9 (forward) and End9 (reverse) primers (Gouvea et al., 1990). For PCR amplification of the VP4 gene, an 876 bp fragment was generated using Con3 as a forward primer and Con2 as a reverse primer (Gentsch et al., 1992). The G genotyping was performed using different pools of primers specific for G genotypes of human and animal rotaviruses (G1–G6 and G8–G11) as described previously (Das et al., 1994; Gouvea et al., 1990, 1994a; Winiarczyk et al., 2002). The VP4 characterization was performed using different pools of P genotype-specific primers for P[1], P[4]-P[11], and P[14] (Gentsch et al., 1992; Gouvea et al., 1994b; Mphahlele et al., 1999; Winiarczyk et al., 2002).

Amplification and sequence analysis of VP4, VP7, VP6, NSP4, and NSP5/6 genes

We could not initially identify the P genotype of PoRV CMP034 strain by multiplex PCR using several sets of genotype-specific primers, so the reverse primer 170 (Martella et al., 2006a), was used in combination with Con3 (forward primer) for amplification of gene VP4 (2341 bp). The full-length VP7 gene (1062 bp) was reverse transcribed and amplified using the primer pair of Beg9 and End9 (Gouvea et al., 1990). The G genotype of CMP034 strain was not

Table 5
Oligonucleotide primers used for the amplification and sequencing of genes VP4, VP6, VP7, NSP4, and NSP5/6

Primer	Gene	Sequence 5′ to 3′	Sense	Position	Reference
Con3	VP4	TGGCTTCGCTCATTTATAGACA	+	11–32	Gentsch et al. (1992)
170	VP4	GGTCACAWCCTCTAGMMRYTRCTTA	−	2362–2383	Martella et al. (2006a)
Con2	VP4	ATTTCGGACCATTTATAACC	−	868–887	Gentsch et al. (1992)
Con2R[a]	VP4	GGTTATAAATGGTCCGAAAT	+	868–887	Gentsch et al. (1992)
VP4-3R	VP4	CAATTCTRTTHCGAATTATTGGRTT	−	2287–2311	Khamrin et al. (2006a)
P34F665	VP4	GATTGCCACCAATACAGAAC	+	665–684	This study
P34F2089	VP4	GAGTAGACACGTTTGAGGAGG	+	2069–2089	This study
Beg9	VP7	GGCTTTAAAAGAGAGAATTTCCGTCTGG	+	1–28	Gouvea et al. (1990)
End9	VP7	GGTCACATCATACAATTCTAATCTAAG	−	1036–1062	Gouvea et al. (1990)
VP6-5F	VP6	GGCTTTTAAACGAAGTCTTC	+	1–20	Shen et al. (1994)
VP6-3R	VP6	GGTCACATCCTCTCACTA	−	1339–1356	Shen et al. (1994)
NSP4 1a	NSP4	GGCTTTTAAAAGTTCTGTTCCG	+	1–22	Kudo et al. (2001)
NSP4 2b	NSP4	GGTCACATTAAGACCGTTCC	−	731–750	Kudo et al. (2001)
GEN-NSP5F	NSP5/6	GGCTTTTAAAGCGCTACAG	+	1–24	Matthijnssens et al. (2006)
GEN-NSP5R	NSP5/6	GGTCACAAAACGGGAGTG	−	650–667	Matthijnssens et al. (2006)

[a] Con2R primer was modified from the original Con2 primer described by Gentsch et al. (1992).

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determined, even by using several sets of genotype-specific primers. In order to determine the G genotype specificity, we analyzed the sequence of gene VP7. The full length of gene VP6 was amplified by primer pairs VP6-5F and VP6-3R, which were modified slightly from the original VP6-specific primers described by Shen et al. (1994). The NSP4 full-length gene was amplified by the NSP4-1a and NSP4-2b primer pair (Kudo et al., 2001). The full-length NSP5/6 gene was amplified with the pair of primers, GEN-NSP5F and GEN-NSP5R, described by Matthijnssens et al. (2006). The sequences of primers used for amplification and sequencing are shown in Table 5.

The PCR amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). The nucleotide and deduced amino acid sequences of genes VP4, VP7, VP6, NSP4, and NSP5/6 were compared with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using BLAST (Basic Local Alignment Search Tool) server (Altschul et al., 1990). Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 3.1 (Kumar et al., 2004).

Nucleotide sequence accession number

The nucleotide sequences of genes VP4, VP6, VP7, NSP4, and NSP5/6 of strain CMP034 have been deposited in GenBank with the accession numbers DQ534016, DQ534018, DQ534015, DQ534017, and DQ916134, respectively.

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An outbreak of *adenovirus serotype 41* infection in infants and children with acute gastroenteritis in Maizuru City, Japan

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Abstract

A total of 337 fecal specimens were collected from infants and children with acute gastroenteritis in Maizuru City, Japan from July 2004 to June 2005 and tested for the presence of *rotavirus*, *norovirus*, *sapovirus*, *astrovirus*, and *adenovirus* by RT-multiplex PCR. Among diarrheal viruses detected, *norovirus* was the most prevalent (13.6%, 46 of 337), followed by *adenovirus* (8%, 27 of 337), *group A rotavirus* (5%, 17 of 337), *astrovirus* (1.8%, 6 of 337), and *sapovirus* (1.8%, 6 of 337), respectively. *Adenovirus* was subjected to molecular genetic analysis by sequencing. *Adenovirus* detected in this study was classified into five serotypes, namely Ad1, Ad2, Ad3, Ad5, and Ad41. Of these, Ad41 was the most predominant serotype that accounted for 85.2% (23 of 27). It was noteworthy to point out that Ad41 infection was apparently confined only to the period of 4 months (October 2004 through January 2005). This pattern of infection implied the outbreak of Ad41 in these subjects, which was the first outbreak of acute gastroenteritis attributed to *adenovirus* in Maizuru City, Japan. Another interesting feature of the study was the existence of two Ad41 subtypes co-circulating in this outbreak. This report confirmed the presence of *adenovirus* as one of an important cause of acute gastroenteritis among Japanese infants and children.

Keywords: *Adenovirus*; Outbreak; Maizuru

1. Introduction

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths (Murray and Lopez, 1997). Among different kinds of diarrheal viruses, *rotavirus* is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide (Mulholland, 2004). *Adenovirus*, however, is also considered to be a significant enteropathogen in association with sporadic cases as well as outbreaks of gastroenteritis in such settings as kindergartens, schools, and hospitals (Chiba et al., 1983; Van et al., 1992; Akihara et al., 2005).

Human *adenovirus* belongs to the *Mastadenovirus* of the family *Adenoviridae*. *Adenovirus* causes a variety of diseases such as acute respiratory, gastrointestinal, and urinary tract infections. To date, 51 *adenovirus* serotypes have been recognized and classified into six subgenera from A to F. This classification scheme is generally consistent with subgroupings of *adenoviruses* on the basis of their physicochemical, biological and genetic properties (Hierholzer et al., 1988; Schnurr and Dondero, 1993; De Jong et al., 1999). Among six subgenera, subgenus F, represented by two *adenovirus* serotypes, *adenovirus* serotype 40 (Ad40) and Ad41, was the most important in association with acute gastroenteritis and accounting for 1–20% of cases. They had a global distribution and were of comparable prevalence both in outpatients and hospitalized children in both the developed and developing countries (Brandt et al., 1985; Shinozaki et al., 1991; Li et al., 2004).

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Over the past decades, neutralization test, ELISA, or virus isolation had been used for the detection and identification of *adenovirus* serotypes. However, these methods are relatively complicated, labor intensive, time consuming, of low sensitivity, and sometimes require the cell culture techniques. In addition, isolation of *adenovirus* is sometimes unsuccessful because of the low viral titer in clinical specimens (Van der Avoort et al., 1989; Takeuchi et al., 1999; Li et al., 2004). Those disadvantages lead to a limitation of their use. Amplification of the viral genome by RT-PCR has been introduced as a convenient and powerful alternative for molecular diagnosis. Highly sensitive and specific RT-PCR assay is currently available for the detection of *adenovirus*. Additionally, genome amplification allows further characterization of the *adenovirus* serotype by sequence analysis (Takeuchi et al., 1999; Phan et al., 2005b).

The objectives of this study were to determine the prevalence of diarrheal virus infection in infants and young children with acute gastroenteritis in Maizuru City, Japan, to identify the serotype and to characterize the genetic diversity among *adenoviruses* detected in this study. Additionally, the age-related distribution and seasonal pattern of *adenovirus* infection were also described.

2. Materials and methods

2.1. Fecal specimens

A total of 337 fecal specimens were collected from infants and children with acute gastroenteritis in a clinic in Maizuru City, Japan during the period of July 2004 to June 2005. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatants were collected for the detection of diarrheal viruses.

2.2. Extraction of viral genome

The viral genome was extracted from 140 μl of 10% fecal supernatant using a QIAamp spin-column technique according to the manufacturer's instructions (QIAGEN®, Hilden, Germany).

2.3. Reverse transcription (RT)

For reverse transcription (RT), 4 μl of extracted viral genome was added with a reagent mixture consisting of 5× First strand buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/μl) (Invitrogen, Carlsbad, CA, USA), random primer (1 μg/μl) (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase Inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and MilliQ water. The total volume of the reaction mixture was 8 μl. The RT step was carried out at 50 °C for 1 h, followed by 99 °C for 5 min and held at 4 °C (Phan et al., 2005b).

2.4. Detection of diarrheal viruses by polymerase chain reaction (PCR)

The first group of viruses, including *astrovirus*, *norovirus* (*GI*, *GII*), and *sapovirus* and the second group including *group A*, *B*, and *C rotaviruses*, and *adenovirus* were detected by RT-PCR with primers as previously reported by Phan et al. (2005b). The identification of the first group of viruses was performed with specific primers Beg9 and VP7-1, B5-2 and B3-3, G8NS1 and G8NA2, Ad1 and Ad2, for *group A*, *B*, and *C rotaviruses*, and *adenovirus* with four different amplicon sizes of 395 bp, 814 bp, 352 bp and 482 bp, respectively. For the detection of the second group of viruses, specific primers PreCAP1 and 82b, G1SKF and G1SKR, COG2F and G2SKR, SLV5317 and SLV5749 were utilized to specifically generate four different sizes of amplicons of 719 bp, 330 bp, 387 bp and 434 bp for *astrovirus*, *norovirus* (*GI*, *GII*), and *sapovirus*, respectively. PCR was carried out with 1 μl of cDNA in 10 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/μl), primers (33 μM/μl), Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA) and MilliQ water. PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

2.5. Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min, then visualized under ultraviolet (UV) light, and the results were recorded by photography.

2.6. Serotyping of adenovirus by PCR and sequence analysis

2.6.1. Amplification of hexon hypervariable regions (HVRs) by PCR

Seven hypervariable regions of the hexon gene of *adenovirus* were amplified by specific primers S29 (for sense 5′-GCCAGCACRTWCTTTGACAT-3′) and S53 (for antisense 5′-CCCATGTTGCCAGTGCTGTTGTARTACA-3′) to generate the amplicon size of 1286 bp (Takeuchi et al., 1999). PCR was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 7 min, and then held at 4 °C.

2.6.2. Nucleotide sequencing and phylogenetic analysis of HVRs

The nucleotide sequences of PCR products (DNA) positive for *adenovirus* were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.). Sequence analysis was performed using CLUSTAL X software (Version 1.6). A phylogenetic tree with 1000 bootstrap resamples of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTER X. The genetic distance was calculated using

Kimura's two-parameter method (PHYLIP). The nucleotide sequence data of *adenovirus* serotype 41 strains 5918/JP and 5950/JP had been submitted to GenBank and had been assigned accession number DQ336390 and DQ336391, respectively. Reference *adenovirus* strains and accession numbers used in this study were as follows: *adenovirus* serotype 31 (X74661), *adenovirus* serotype 1 (X67709), *adenovirus* serotype 2 (XJ01917), *adenovirus* serotype 5 (M73260), *adenovirus* serotype 6 (X67710), *adenovirus* serotype 3 (X76549), *adenovirus* serotype 4 (X84646), *adenovirus* serotype 8 (X74663), *adenovirus* serotype 19 (X98359), *adenovirus* serotype 37 (X98360), *adenovirus* serotype 41-subtype 1 (AB103349), *adenovirus* serotype 41-subtype 2 (AB103344), and *adenovirus* serotype 40 (X51782).

3. Results

3.1. Molecular epidemiology of diarrheal viruses

A total of 337 fecal specimens were collected from infants and children with acute gastroenteritis in Maizuru City, Japan, during the period of July 2004 to June 2005. For the pediatric population, the lowest age was 3 months, the highest was 14 years, and the average age was 1.3 years (15 months). Among all children with acute gastroenteritis, 92% were aged less than 36 months. Moreover, the number of males accounted for 53.4%. RT-multiplex PCR was performed to test all fecal specimens for the presence of *rotavirus*, *norovirus*, *sapovirus*, *astrovirus*, and *adenovirus*. The results shown in Table 1 revealed that diarrheal viruses were detected in 102 out of 337 (30.3%) specimens tested. Among the diarrheal viruses detected, *norovirus* was the most prevalent (13.6%), followed by *adenovirus* (8%), *group A rotavirus* (5%), *astrovirus*, and *sapovirus* (1.8% each), respectively. No *group B and C rotaviruses* were found in these subjects. Since *adenovirus* was detected with a high prevalence, it was interesting to further characterize its serotypes and genetic relationships.

3.2. Nucleotide sequencing and phylogenetic analysis of adenovirus

The PCR products of *adenovirus* were sequenced in order to further characterize the genetic relationship among the *adenovirus* isolates detected in infants and children with acute gastroenteritis in Maizuru City, Japan. Their nucleotide sequences containing seven hypervariable regions of the hexon gene were compared to each other as well as to those of reference *adenovirus* strains available in GenBank by BLAST. A total of 27 *adenovirus* sequences were analyzed by phylogenetic analysis using the recent seven-hypervariable regions of the hexon gene-based classification scheme of Li et al. (2004). *Adenoviruses* detected in the present study were classified into five serotypes, Ad1, Ad2, Ad3, Ad5 and Ad41. Of these, Ad41 predominated over other serotypes and represented 85.2% (23 of 27) while one of each was Ad1, Ad2, Ad3 and Ad5, respectively (Fig. 1). Using CLUSTAL X, it was also noticed that these *adenoviruses* had a high identity on the nucleotide level as well as on the amino acid level with corresponding *adenovirus* reference strains previously registered in GenBank ranging from 94% to 100%.

Table 1
Distribution of diarrheal viruses detected in infants and children with acute gastroenteritis in Maizuru City, Japan during 2004 and 2005 (number of fecal specimens tested: 337)

Target virus				
Norovirus	*Adenovirus*	*Group A rotavirus*	*Sapovirus*	*Astrovirus*
46	27	17	6	6
13.6%	8%	5%	1.8%	1.8%

3.3. Outbreak of adenovirus serotype 41

The results shown in Fig. 2 revealed that although the fecal specimens were collected over a period of 12 months (July 2004

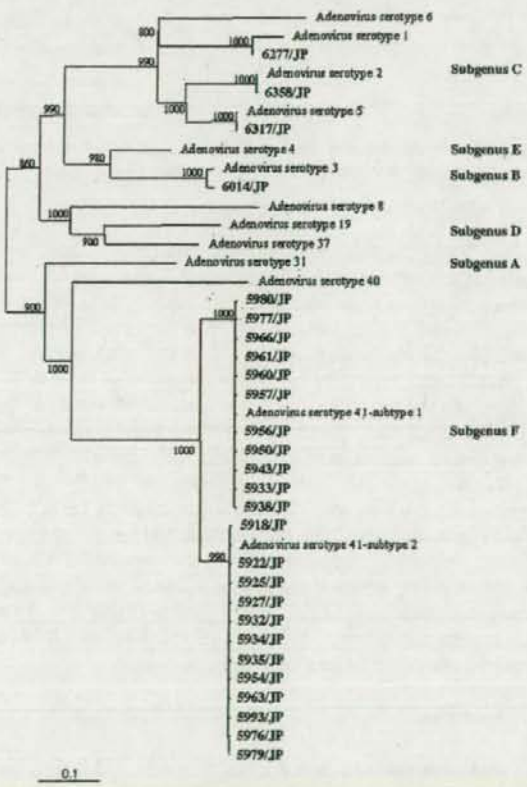

Fig. 1. Phylogenetic tree of nucleotide sequences of *adenoviruses* detected in acute gastroenteritis infants and children in Maizuru City, Japan, in 2004–2005. The tree was constructed from nucleotide sequences of seven hypervariable regions of the hexon gene of *adenovirus* isolates detected in Maizuru City, Japan. Reference strains of human *adenovirus* were selected from GenBank under the accession number indicated in the text. *Adenoviruses* detected in this study were highlighted in bold. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values.

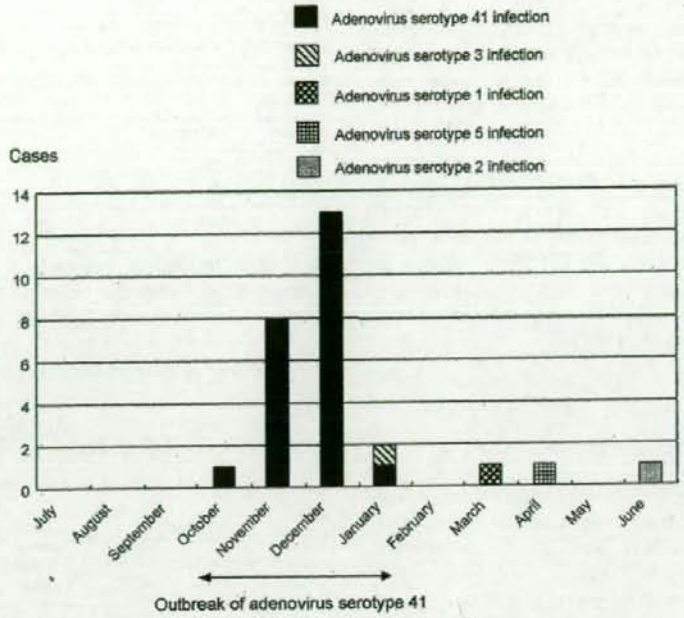

Fig. 2. Monthly distribution of *adenovirus* infection in infants and children with acute gastroenteritis in Maizuru City, Japan during the period of July 2004 to June 2005. The duration of outbreak of Ad41 infection was shown.

to June 2005), the Ad41 infection was apparently confined to a period of 4 months (October 2004 through January 2005). This pattern of infection indicated an outbreak of Ad41 in these subjects, and this would be the first outbreak of acute gastroenteritis attributed to *adenovirus* in Maizuru City, Japan. A phylogenetic tree of the nucleotide sequences of these Ad41 isolates and the reference strains was constructed and all of 23 Ad41 isolates formed two distinct subtypes 1 and 2. It was also found that the nucleotide as well the as amino acid sequences of HVRs among Ad41 isolates in each subtype were of significantly high identity (99–100%). Altogether, the results clearly indicated that two subtypes of Ad41 had been co-circulating in this outbreak. In addition, the majority (91.3%, 21 of 23) of Ad41 infected cases were confined to infants and young children aged less than 3 years (Table 2). This observation demonstrated that Ad41 infection in this outbreak occurred mainly in infants and young children.

4. Discussion

Viral gastroenteritis is still a health burden and one of the most frequently encountered problems in developed and developing countries (Murray and Lopez, 1997). In this study, diarrheal viruses were detected in 30.3% of fecal specimens tested. These findings suggested that about 30% acute gastroenteritis in infants and children in Maizuru City might be due to the diarrheal viruses and 69.7% caused by other etiologic agents. Interestingly, *norovirus* dominated over *group A rotavirus* and became a leading cause of viral gastroenteritis among infants and children in the present study. According to the epidemiological survey (1996–2003) of diarrheal viruses conducted in Maizuru City, the incidence of *group A rotavirus* was always higher than that of *norovirus*, ranging from 10.2% to 23.5% (Zhou et al., 2003; Phan et al., 2005a; Yoshinaga et al., 2006). Taken together, there was a changing predominance of viruses causing diarrheal illness among infants and children in Maizuru City. It was possible that infants and children in Japan might have enough antibody protection against *rotavirus*, which was triggered by the previous group A rotavirus infection. However, it might be due to the co-existence of multiple factors such as changes of climate, water, and others. Further research should be conducted in order to investigate this phenomenon.

In Maizuru City, the detection rate of *adenovirus* infection ranged from 3.8% to 4.8% (Zhou et al., 2003; Li et al., 2004). In this study, it was interesting that *adenovirus* infection was identified with a high incidence (8%) and was recognized as the second common agent of acute gastroenteritis in Maizuru City during 2004–2005. Sequence analysis showed that *adenovirus* detected in this study belonged to three distinct subgenera (B, C and F) with five serotypes (Ad1, Ad2, Ad3, Ad5 and Ad41). Of note, high prevalence (85.2%) of Ad41 with a sudden appearance and disappearance pattern was confined to a short period of 4 months (October 2004 through January 2005) suggesting an outbreak of Ad41 in Maizuru City. By contrast, only one *adenovirus* was found from October to January of previous years (2002–2004) in diarrheal fecal specimens of children collected in Maizuru City, Japan (data not shown).

Table 2
Characteristics of *adenovirus* type 41 outbreak in infants and children with acute gastroenteritis in Maizuru City, Japan

No.	Patient	Age	Sex	Date of stool collection	Laboratory findings			
					Adenovirus	*Adenovirus* type	Subtype	Other virus
1	5918	2 y	M	25 October 2004	+	41	2	–
2	5922	1 y 11 m	M	2 November 2004	+	41	2	–
3	5925	7 m	M	8 November 2004	+	41	2	–
4	5927	1 y 11 m	F	16 November 2004	+	41	2	–
5	5932	10 m	F	24 November 2004	+	41	2	–
6	5933	1 y 5 m	M	24 November 2004	+	41	1	–
7	5934	1 y 1 m	F	24 November 2004	+	41	2	–
8	5935	2 y 9 m	F	24 November 2004	+	41	2	–
9	5938	2 y 2 m	M	25 November 2004	+	41	1	–
10	5943	1 y 7 m	F	2 December 2004	+	41	1	–
11	5950	2 y 4 m	M	11 December 2004	+	41	1	–
12	5954	10 m	F	13 December 2004	+	41	2	–
13	5956	2 y 5 m	F	13 December 2004	+	41	1	–
14	5957	2 y 3 m	M	13 December 2004	+	41	1	–
15	5960	6 y 8 m	M	15 December 2004	+	41	1	–
16	5961	1 y 2 m	F	15 December 2004	+	41	1	–
17	5963	1 y 3 m	F	16 December 2004	+	41	2	*Norovirus*
18	5966	9 y 3 m	M	18 December 2004	+	41	1	–
19	5976	9 m	F	22 December 2004	+	41	2	–
20	5977	3 y 1 m	F	22 December 2004	+	41	1	–
21	5979	3 m	M	24 December 2004	+	41	2	–
22	5980	1 y 2 m	F	24 December 2004	+	41	1	–
23	5993	3 y 6 m	F	6 January 2005	+	41	1	–

Note. M, male; F, female; y, year; m, month; +, positive; –, negative.

This is the first report of an outbreak attributed to Ad41 infection among infants and young children in Maizuru City, Japan. Another interesting feature of the study clearly demonstrated that two distinct Ad41 subtypes, subtypes 1 and 2, were co-circulating in this outbreak.

Although it has been reported that the prevalences of Ad40 and Ad41 were approximately equal (Shinozaki et al., 1991; Phan et al., 2004), none of Ad40 was detected in the present study. However, this finding was in line with recent studies that reported a decrease in the detection rate of Ad40 and a concomitant increase of Ad41 to become the predominant serotype. This phenomenon might reveal the occurrence of an antigenic drift of Ad41. Such changes of antigenicity might have allowed the Ad41 to escape from acquired immunity and cause an increase of Ad41 infection for the susceptible individuals within the community (Van der Avoort et al., 1989; Li et al., 2004).

In this outbreak, the majority of infants and children with Ad41 infection (91.3%) were aged less than 36 months. This observation was consistent with the studies on *adenovirus* epidemiology worldwide in which *adenovirus* infection associated with acute gastroenteritis occurs predominantly in infants and young children (Chiba et al., 1983; Jarecki-Khan et al., 1993; Akihara et al., 2005). Our findings also confirmed *adenovirus* as one of the enteropathogens responsible for viral gastroenteritis among infants and children in Japan. According to some studies conducted in Japan, *adenovirus* infection has been found mainly in summer (Li et al., 2004; Akihara et al., 2005). In contrast, the present study demonstrated the outbreak of *adenovirus* in the cold season, spanning from October 2004 to January 2005. This observation clearly indicates that *adenovirus* infection can occur not only in the hot season but also in the cold season.

In conclusion, this report provided further evidence of the existence of the multiple co-circulating viruses in causing diarrheal illness in Maizuru City, Japan. It is also the first, to our best knowledge, demonstrating an outbreak associated with the *adenovirus* 41 infection in infants and children with acute gastroenteritis in Maizuru City and warns of the threat it poses.

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ARTICLE

Emergence of rare sapovirus genotype among infants and children with acute gastroenteritis in Japan

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Abstract A total of 1,154 fecal specimens from infants and children with acute gastroenteritis in five cities in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka), collected from July 2003 to June 2005, were tested for the presence of diarrheal viruses by reverse transcriptase multiplex PCR. Overall, 469 of 1,154 (40.6%) were positive for diarrheal viruses, of which 49 (10.4%) were positive for sapovirus. The peak of sapovirus infection shifted from April–June in 2003–2004 to October–December in 2004–2005. The observations show that maximum sapovirus prevalence can occur during warmer seasons. Sapovirus was subjected to molecular genetic analysis by sequencing. The results indicated that sapovirus genogroup I was a dominant group (100%). Sapovirus strains detected in this study were further classified into four genotypes (GI/1, GI/4, GI/6, and GI/8). Of these, sapovirus GI/1 was the most predominant, followed by sapovirus GI/6; these accounted for 93% (13 of 14) and 7% (1 of 14), respectively, in 2003–2004. However, it was noteworthy that sapovirus GI/6 suddenly emerged to become the leading genotype, accounting for 77% (27 of 35) of isolates in 2004–2005. This is believed to be the first report of the changing distribution of sapovirus genotypes and of the emergence of the rare sapovirus GI/6.

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Introduction

Acute gastroenteritis is a common illness in humans worldwide and has a great impact on public health [1]. It is recognized as one of the leading causes of death by infectious disease [1, 2]. The mortality of infants and children due to acute gastroenteritis is greater in developing than in developed countries [3]. While rotavirus is recognized as the most important cause of severe gastroenteritis in infants and young children worldwide, sapovirus (SaV) is also considered a significant global enteropathogen of acute gastroenteritis [4–7].

SaV, previously referred to as Sapporo-like virus, is a distinct genus within the family *Caliciviridae*. The prototype strain of SaV is the Sapporo virus (Hu/SaV/Sapporo virus/1977/JP), which was derived from an outbreak in a home for infants in Sapporo, Japan, in 1977 [8]. SaV has a positive-sense single-strand RNA genome surrounded by an icosahedral capsid. The SaV genome is 7.3–7.5 kb long and contains two main open reading frames. The open reading frame 1 encodes a polyprotein that undergoes protease processing to produce several nonstructural proteins, including an RNA-dependent RNA polymerase and a capsid protein. The open reading frame 2 encodes a small basic protein with unknown function [8]. On the basis of the sequence analysis of the capsid gene, SaV is divided into five genogroups, among which only genogroups I, II, IV, and V are known to infect humans [9–12]. Recently, the diversity of SaV was described by Akihara et al. [13], who found that SaV genogroup I and genogroup II could be classified into eight and five genotypes, respectively. Immunological and seroepidemiologic studies have indicated a worldwide distribution of SaV. The age-related prevalence of antibody against this virus also has shown that infections commonly occur in children under 5 years of age. Furthermore, it was

found that serum antibody level to SaV was lowest in the first year of life but increased after 2 years of age [14–16].

The objectives of this study were to determine the incidence of diarrheal virus infections in infants and children with acute gastroenteritis in five different cities in Japan during 2003–2005; to characterize the SaV detected according to genogroup and genotype; and to describe the genetic diversity among them. Additionally, the age-related distribution and the seasonal pattern of SaV infection were determined.

Materials and methods

Fecal specimens

A total of 1,154 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics from five different cities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan during the period of July 2003 to June 2005. The ages of the subjects ranged from 2 months to 15 years, the median age being 28 months. This age distribution in the periods 2003–2004 and 2004–2005 was similar. The fecal specimens were suspended in distilled water to prepare 10% suspensions, which were clarified by centrifugation at 10,000×*g* for 10 min; the supernatants were stored at −30°C.

Extraction of viral genome

The genomes of both RNA and DNA viruses were extracted from 140 μl of 10% fecal suspensions using a single QIAamp spin-column kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Reverse transcription

In reverse transcription (RT), 4 μl of extracted viral genome was added to a reagent mixture consisting of 5× first strand buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/μl) (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (1 μg/μl) (Takara, Shiga, Japan), RNase inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and MilliQ water. The total volume of reaction mixture was 8 μl. The RT step was carried out at 50°C for 1 h, then 99°C for 5 min, after which it was stored at 4°C [17].

Viral detection and polymerase chain reaction

Two multiplex polymerase chain reaction (PCR) procedures using mixtures of primers previously reported were used to identify two groups of diarrheal viruses. The first group includes astrovirus, norovirus (GI, GII), and SaV, and the second group includes group A, B, and C rotaviruses and adenovirus [17]. In the first multiplex PCR, the primer set included equimolar mixes of PreCAP1 and 82 b for astrovirus, G1SKF and G1SKR for norovirus genogroup I, COG2F and G2SKR for norovirus genogroup II, and SLV5317 and SLV5749 for SaV. The respective products of these reactions consisted of amplicons of 719, 330, 387, and 434 bp for astrovirus, norovirus (GI, GII), and SaV, respectively. In the second multiplex PCR, the primer set included equimolar mixes of Beg9 and VP7-1, B5-2 and B3-3, and G8NS1 and G8NA2 for group A, B, and C rotaviruses, respectively, and Ad1 and Ad2 for adenovirus. The respective products of these reactions consisted of amplicons of 395, 814, 352, and 482 bp for group A, B, and C rotaviruses and adenovirus, respectively. The PCR was performed at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 7 min, after which the products were stored at 4°C.

Genotype of group A rotavirus

Genotyping of group A rotavirus was conducted using the G-type specific primers from the method described previously by Das et al. in 1994 [18]. The full-length of *VP7* gene was reversely transcribed and then further amplified with Beg9 and End9 primers. The expected size of the PCR product generated from the full-length *VP7* gene was 1,062 bp in length. The second amplification was performed using the first PCR product as the template with G-type specific mixed primers (9T1-1, 9T1-2, 9T-3P, 9T-4, and 9T-B) for downstream priming and 9con1 for upstream priming in an amplification of *VP7* genes of G1–G4 and G9, respectively. These primers specifically generated five different sizes of amplicons of 158, 224, 466, 403, and 110 bp for G1, G2, G3, G4, and G9, respectively.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, after which they were stained with ethidium bromide for 20 min and then examined under ultraviolet light. The results were photographed.

Latex agglutination test

The Diarlex test (Orion Diagnostica, Espo, Finland), a commercial latex agglutination test, was used for the detection of group A rotavirus infection as a confirmatory test in the fecal specimens that were found by RT-PCR to harbor coinfection with SaV and group A rotavirus. A drop of the fecal supernatant was mixed with a drop of test latex on a slide, and the reaction was observed after 2 min. Development

of distinct agglutination in the Diarlex reagent was considered a positive result. If agglutination was seen in the negative control latex, the result was considered uninterpretable.

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of RT-PCR products (DNA) positive for SaV and norovirus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Clustal X software, version 1.6. A phylogenetic tree with 1,000 bootstrap resamples of the nucleotide alignment datasets was generated using the neighbor-joining method with Clustal X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). The sequences of SaV strains detected in the present study—5786/Osaka/JP, 5800/Osaka/JP, 5836/Osaka/JP, 5862/Osaka/JP, 5862/Osaka/JP, and 5821/Osaka/JP—had been submitted to GenBank and had been assigned accession numbers AB242322, AB242323, AB242324, DQ401095, AB242325, and AB242326, respectively. Reference SaV strains and accession numbers used in this study were as follows: PEC (AF182760), Lyon/598/97/F (AJ271056), London/92 (U95645), Mex340/90 (AF435812), Cruiseship/00 (AY289804), Hou7-1181/90 (AF435814), Arg39 (AY289803), Parkville/94 (U73124), Sapporo/82 (U65427), Manchester/93 (X86560), Karachi/730/1992 (AB126249), Karachi/874/1992 (AB181129), Karachi/928/1994 (AB181128), Karachi/1017/1990 (AB181227), Karachi/876/1993 (AB181132), Houston/90 (U95644), Stockholm/97 (AF194182), 12/DCC/Tokyo/Japan/44 (AB236380), Karachi/872/1991 (AB181231), 4408/Maizuru/JP (AB180209), 4724/Osaka/JP (AB180212), and Mex14917/2000 (AF435813).

Results

Molecular epidemiology of viral infections

RT-PCR analysis revealed the presence of viruses in 469 fecal specimens (Table 1). The ages of the infants and children with viral gastroenteritis ranged from 2 months to 11 years. The highest rate of viral infection was in the age group 12–23 months (42%) and the lowest rate was in the age group <6 months (2%). There was no difference in age distribution between the periods 2003–2004 and 2004–2005. In 2003–2004, group A rotavirus was the most prevalent (43.3%, 78 of 180), followed by norovirus (GI and GII) (29.5%, 53 of 180). Interestingly, norovirus (GI and GII) dominated over group A rotavirus and became the leading cause of acute gastroenteritis in 2004–2005, accounting for 45.3% (131 of 289) of cases. Moreover, a

Table 1 Distribution of viral infection in infants and children with acute gastroenteritis in five cities in Japan

Year	No. of specimens tested	No. (%[a]) positive for viruses	Monoinfection (%[b])								Mixed infection (%[b])							
			RV			NoV		SaV	Ade	AsV	Ade+ AsV	SaV + RA	Ade+ NoV GII	RA + NoV GII	Ade + RA	SaV + NoV GII	AsV + RA
			A	B	C	I	II										
2003–2004	402	180 (44.8)	78 (43.3)	0 (0)	1 (0.6)	1 (0.6)	52 (28.9)	12 (6.7)	22 (12)	5 (2.8)	1 (0.6)	2 (1.1)	3 (1.7)	2 (1.1)	1 (0.6)	0 (0)	0 (0)
2004–2005	752	289 (38.4)	77 (26.6)	0 (0)	0 (0)	2 (0.7)	129 (44.6)	30 (10.4)	32 (11.2)	8 (2.8)	0 (0)	0 (0)	1 (0.3)	2 (0.7)	1 (0.3)	5 (1.7)	2 (0.7)
Total	1,154	469 (40.6)	155 (33)	0 (0)	1 (0.2)	3 (0.6)	181 (38.6)	42 (9)	54 (11.5)	13 (2.8)	1 (0.2)	2 (0.4)	4 (0.9)	4 (0.9)	2 (0.4)	5 (1.1)	2 (0.4)

RV rotavirus, *NoV* norovirus, *SaV* sapovirus, *Ade* adenovirus, *AsV* astrovirus, *NoV GI* norovirus genogroup I, *NoV GII* norovirus genogroup II, *RA* group A rotavirus

[a] Refers to total number of specimens tested

[b] Refers to number of viral-positive specimens tested

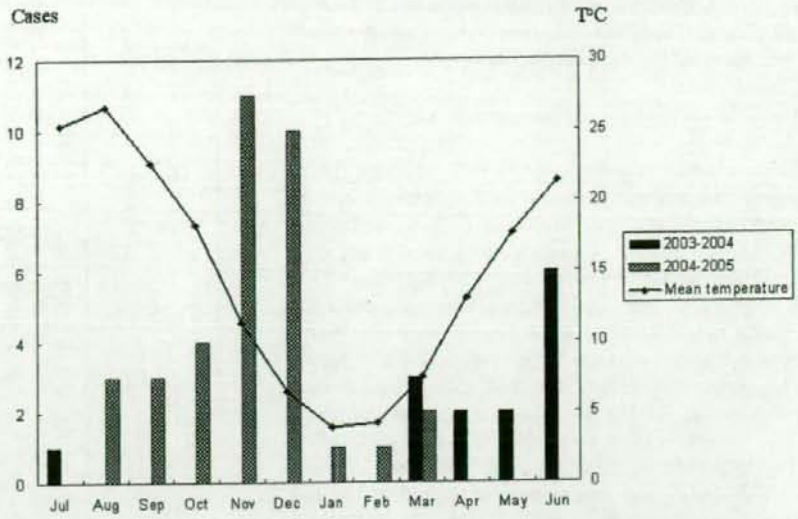

Fig. 1 Monthly distribution of SaV infection in infants and children with acute gastroenteritis in five different cities of Japan, July 2003 to June 2005. The mean temperature in Japan during the molecular epidemiology study of SaV infection is shown

rather high rate (4.3%, 20 of 469) of viral coinfections was shown in this study during 2003–2005. It was found that the number of SaV infections increased sharply from 14 cases in 2003–2004 to 35 cases in 2004–2005. The incidence of SaV was highest in the 12–23-month-old group (45%, 22 of 49) and lowest in infants aged <6 months (2%, 1 of 49). Of note, most of the SaV infections (82%, 40 of 49) occurred in infants and children <3 years of age.

Seasonal variation of sapovirus infection

A comparison of rate of detection of SaV between the periods 2003–2004 and 2004–2005 is shown in Fig. 1. The monthly mean temperature in the five cities in Japan is also shown. The summer lasts from June to September, and the hottest temperature was recorded in August. The coldest month is January, when the temperature might dip as low as 4°C. The incidence of SaV was highest in April–June (ten cases) and was second highest in January–March (three cases). The lowest rate of detection was recorded in October–December (zero cases) in 2003–2004 ($p<0.001$). In contrast, SaV infection was identified continuously from August to February in 2004–2005, and the highest number of cases was recorded in October–December (25 cases), followed by July–September (six cases) ($p<0.001$). The peak of SaV infection shifted from April–June in 2003–2004 to October–December in 2004–2005.

Coinfection with sapovirus and other viral enteropathogens

In total, 49 fecal specimens were determined to be positive for SaV by RT-PCR. Seven (14%) of these specimens harbored coinfection with other viral enteropathogens: norovirus in five cases and group A rotavirus in two. The

Table 2 Characteristics of seven cases of mixed infection with SaV and other enteropathogens among the Japanese pediatric population

Case no.	Patient no.	Sex	Age (months)	City	Month	Year	Sapovirus		Other diarrheal viruses detected			
							Genogroup	Genotype	RT-PCR	Diarlex[a]	Sequence[b]	Genotype[c]
1	5,060	F	14	Maizuru	Apr	2004	I	1	Group A rotavirus	Positive	ND	G3
2	5,299	F	17	Maizuru	Apr	2004	I	1	Group A rotavirus	Positive	ND	G3
3	5,720	F	46	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
4	5,721	F	31	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
5	5,735	M	49	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
6	5,797	F	10	Osaka	Oct	2004	I	6	Norovirus	ND	GII/3	ND
7	5,806	M	11	Osaka	Nov	2004	I	6	Norovirus	ND	GII/4	ND

ND not done
[a] Used only to detect group A rotavirus
[b] Used only to determine genotype of norovirus
[c] Used only to genotype group A rotavirus

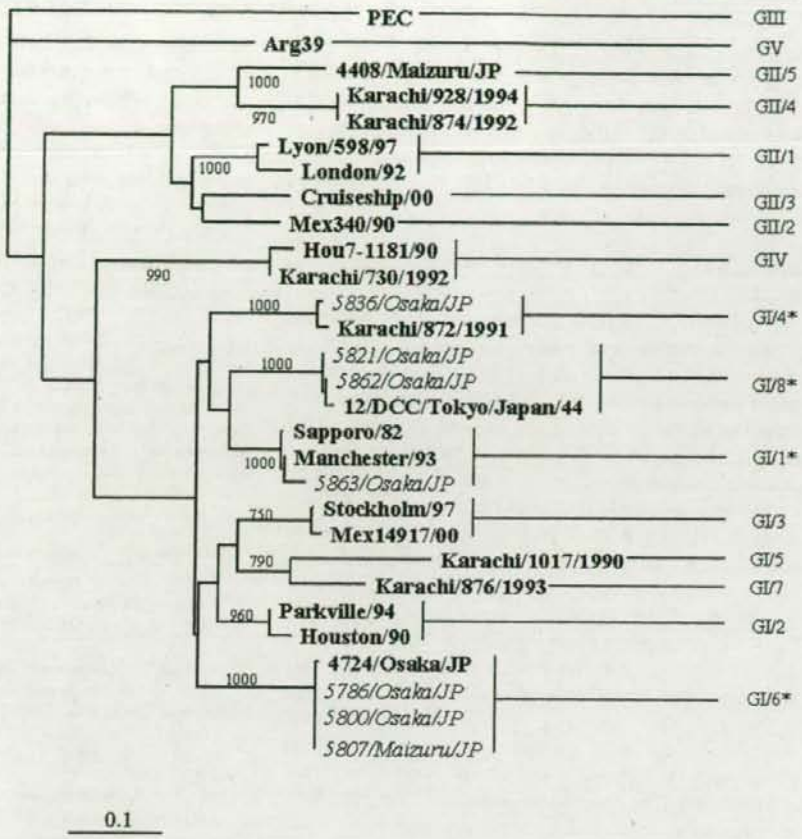

Fig. 2 Phylogenetic tree of nucleotide sequences of SaV strains detected in infants and children with acute gastroenteritis in five different cities in Japan. All of the SaV sequences were classified into only one distinct genogroup (I), and the SaV genogroup I sequences were clustered into four genotypes (GI/1, GI/4, GI/6, and GI/8). The tree was constructed from partial amino acid sequences of the capsid region of SaV strains. Reference SaV strains were selected from GenBank under the accession numbers indicated in the text. Japanese SaV is highlighted in *italic*. The PEC strain was used as an outgroup strain for phylogenetic analysis. The scale indicates amino acid substitutions per position. The numbers in the branches indicate the bootstrap values. *Genotype contains Japanese SaV detected in the study

five coinfections with norovirus were detected in Osaka and the two with group A rotavirus in Maizuru. All diarrheal viruses detected as coinfections with SaV (norovirus and group A rotovirus) were confirmed and further characterized by G typing, latex agglutination test, or sequencing analysis. The five noroviruses belonged to genogroup I, genotype 4 in four cases and genogroup I, genotype 3 in one case, while both group A rotaviruses were identified as G3 genotype 3 (Table 2).

Nucleotide sequencing and phylogenetic analysis of sapovirus

The PCR products of SaV were sequenced in order to further characterize the genetic relationship among the SaV strains detected in infants and children with acute gastroenteritis in Japan. The sequence of the 5′ end of the *SaV* capsid gene was targeted for genotyping [10, 13]. Their partial amino acid sequences were compared to each other as well as to those of reference SaV strains available in GenBank by BLAST. A total of 49 SaV sequences were analyzed by phylogenetic analysis and were grouped using the recent SaV capsid region classification scheme of Akihara et al. [13]. All of SaV sequences clustered into a single genogroup, genogroup I (GI). Most of the SaV GI sequences (93%, 13 of 14) in 2003–2004 belonged to genotype 1 (SaV GI/1) (typified by the Manchester virus cluster). Only one SaV GI sequence (7%, 1 of 14) clustered

Table 3 Distribution of SaV genotypes based on the sequencing genetic analysis among infants and children with acute gastroenteritis in five different cities in Japan

Date of fecal specimen collection	No. of specimens positive for sapovirus	No. (%) identified as sapovirus genogroup I			
		Genotype 1	Genotype 4	Genotype 6	Genotype 8
July 2003–June 2004	14	13 (93)	0 (0)	1 (7)	0 (0)
July 2004–June 2005	35	4 (11)	2 (6)	27 (77)	2 (6)

into the 4724/Osaka/JP virus cluster (known as SaV GI/6) (Fig. 2). In 2004–2005, SaV strains were classified into four distinct genotypes (GI/1, GI/4, GI/6, and GI/8). Interestingly, SaV GI/6 emerged as the predominant genotype in 2004–2005, representing 77% (27 of 35) of the strains, followed by SaV GI/1 (11%, 4 of 35) (Table 3).

Discussion

SaV infection causes acute gastroenteritis in all age groups, through it occurs predominantly in infants and young children [5, 6, 19, 20]. Overall, 49 of 1,154 fecal specimens tested were positive for SaV, and positive specimens were found in all age groups of the subjects included in the study. However, most (82%) of the SaV infections occurred in infants and children <3 years of age. These results were in line with previously published reports on SaV epidemiology worldwide, in which SaV prevalence was shown to range from 0.3 to 9.3%, far below the prevalence of either rotavirus or norovirus [19–22]. Our findings also confirmed SaV as one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. According to surveillance on pediatric cases of viral gastroenteritis in Japan, the main peak of SaV infection was in winter [6, 9]. Interestingly, the highest number of SaV infections in our study was identified in the April–June period in 2003–3004 and in the October–December period in 2004–2005. The observations show that maximum SaV prevalence can occur during warmer seasons.

To date, coinfections with various enteric viruses have been widely reported [12, 20]. Interestingly, we found a rather high rate (14%, 7 of 49) of coinfections with SaV and other viral pathogens. Coinfection of SaV with rotavirus and with norovirus was confirmed by the Diarlex test and G typing. These results underscore that coinfection with SaV and other enteropathogens is not rare.

All of the Japanese SaV sequences belonged to only one SaV genogroup I with four distinct genotypes (GI/1, GI/4, GI/6, and GI/8). The findings clearly indicated that SaV GI was the dominant group to cause acute gastroenteritis among the pediatric population in Japan. In strong agreement with previous reports [7, 10, 13], SaV strains most frequently detected in sporadic gastroenteritis in infants and children in 2003–2004 belonged to the Manchester cluster (GI/1). Of note, SaV GI/6 strains were the most predominant (77%) in 2004–2005 and closely homologous to each other, suggesting that they came from the same source of infection. Moreover, only two SaV GI/6 strains were found in 2002–2003 during a 7-year (1996–2004) survey of SaV infection in diarrheal fecal specimens from Japanese infants and children [20]. In the present study, only one SaV GI/6 was detected in 2003–2004. Taken together, there was an emergence of rare SaV GI/6, and this was the first evidence of the changing distribution of the SaV genotype in association with acute gastroenteritis in Japan. This sudden emergence of SaV GI/6 indicates that the pediatric population in Japan might lack antibody protection to these strains and that these rare strains could be more virulent than those usually associated with pediatric gastroenteritis. Continuous surveillance of SaV infection in Japan is recommended in order to determine whether these rare strains remain dominant in the coming years.

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Genomic analysis of diverse rubella virus genotypes

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Based on the sequence of the E1 glycoprotein gene, two clades and ten genotypes of *Rubella virus* have been distinguished; however, genomic sequences have been determined for viruses in only two of these genotypes. In this report, genomic sequences for viruses in an additional six genotypes were determined. The genome was found to be well conserved. The viruses in all eight of these genotypes had the same number of nucleotides in each of the two open reading frames (ORFs) and the untranslated regions (UTRs) at the 5′ and 3′ ends of the genome. Only the UTR between the ORFs (the junction region) exhibited differences in length. Of the nucleotides in the genome, 78 % were invariant. The greatest observed distance between viruses in different genotypes was 8.74 % and the maximum calculated genetic distance was 14.78 substitutions in 100 sites. This degree of variability was similar among regions of the genome with two exceptions, both within the P150 non-structural protein gene: the N-terminal region that encodes the methyl/guanylyltransferase domain was less variable, whereas the hypervariable domain in the middle of the gene was more divergent. Comparative phylogenetic analysis of different regions of the genome was done, using sequences from 43 viruses of the non-structural protease (near the 5′ end of the genome), the junction region (the middle) and the E1 gene (the 3′ end). Phylogenetic segregation of sequences from these three genomic regions was similar with the exception of genotype 1B viruses, among which a recombinational event near the junction region was identified.

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INTRODUCTION

Rubella virus is an important human pathogen that causes an acute, contagious disease known as rubella, 3-day measles or German measles, and severe birth defects (known as congenital rubella syndrome) when infection occurs during the first trimester of pregnancy (Chantler *et al.*, 2001). *Rubella virus* is the single member of the genus *Rubivirus* in the family *Togaviridae* and is an enveloped, single-stranded, positive-polarity RNA virus with a genome of approximately 10 kb. The genome contains two long open reading frames (ORFs): the 5′-proximal ORF (NSP-ORF) encodes two non-structural proteins, P150 and P90, that function in RNA replication, and the 3′-proximal ORF (SP-ORF) encodes three structural proteins: the capsid protein, C, and two envelope glycoproteins, E1 and E2. The SP-ORF is translated from a subgenomic RNA synthesized in infected cells (Frey, 1994). The genome also contains untranslated regions (UTRs) at its 5′ and 3′ ends and between the ORFs (known as the junction region).

Although rubella occurs worldwide, vaccination efforts with live-attenuated vaccines have been concentrated in developed countries. Currently, approximately 50 % of countries have national vaccination efforts against rubella (Robertson *et al.*, 2003). Isolation and genetic sequencing of rubella viruses has been most thorough in countries pursuing elimination (Bosma *et al.*, 1996; Frey *et al.*, 1998; Icenogle *et al.*, 2006; Katow, 2004; Katow *et al.*, 1997a, b; Reef *et al.*, 2002; Saitoh *et al.*, 2006); however, collections have recently been assembled from other regions of the world (Donadio *et al.*, 2003; Katow, 2004; Zheng *et al.*, 2003a, c). Recently, a standard taxonomy for rubella viruses was adopted based on sequences of a standard window within the E1 gene and supported by sequencing of the SP-ORF of selected viruses (WHO, 2005). The taxonomy consists of two clades [corresponding to the previous genotypes I and II (Frey *et al.*, 1998; Zheng *et al.*, 2003a)] containing a total of ten genotypes, seven in clade 1 (1a, 1B, 1C, 1D, 1E, 1F and 1g) and three in clade 2 (2A, 2B and 2c); the genotypes designated in lower case are provisional. Within the E1 gene, maximal variation among clade 1 viruses is 5.8 %, that among clade 2 viruses is 8.0 %, and it is 8.2 % between the two clades (Zheng *et al.*, 2003a). Geographically, clade 1 viruses circulate worldwide, whilst clade 2 viruses thus far have been restricted to Eurasia.

Thus far, ten complete genomic sequences of *Rubella virus* have been reported, which represent only two of the ten

Supplementary tables are available in JGV Online.

genotypes (eight sequences of genotype 1a viruses and two sequences of genotype 2A viruses). Among these sequences, genomic genetic variability is similar to that in the E1 gene, with the exception of a 'hypervariable region' (HVR) of greater variability in the middle of the P150 non-structural protein gene (Hofmann *et al.*, 2003; Zheng *et al.*, 2003b). Given the lack of representation of the majority of the genotypes in the current genomic database, the first goal of this study was to expand the number of genomic sequences, using viruses in our collection from six additional genotypes (1B, 1C, 1D, 1E, 2B and 2C). The second goal of this study was to extend phylogenetic analysis to 5′ regions of the genome, which had not previously been done. To this end, the sequence of the non-structural protease-encoding region within the P150 gene was determined and compared phylogenetically with the sequences of the junction region and the E1 gene from 43 viruses representing eight genotypes.

METHODS

Viruses, cells, RNA extraction, cDNA amplification and DNA sequencing. The viruses analysed in this study are listed in Table 1 (genomic sequences) and Supplementary Table S1, available in JGV Online (genomic regions). The Cba strain was provided by Dr Marta Zapata, University of Cordoba, Cordoba, Argentina. A monolayer of Vero cells (25 cm^2 T-flask or 60 mm plate) was infected with each virus. Three to five days post-infection, the culture medium was removed and total cellular RNA was extracted by using Tri-Reagent (Molecular Research Center) using the manufacturer's protocol. The RNA extracted from one 25 cm^2 T-flask or one 60 mm plate was resuspended in 50 µl double-distilled H_2O and stored at −80 °C until use. cDNA was synthesized in a 20 µl total reaction volume reaction containing 5 µl denatured (95 °C, 5 min) RNA template, 4 µl 5× Reverse Superscript buffer (Invitrogen), 4 µl 2.5 mM dNTPs, 1 µl 0.1 M dithiothreitol, 1 µl 4 µM 3′E1/808 reverse primer (5′-TTTTTTTTTCTATACAGCAAC-3′; T_9 followed by the complement of nt 9751–9762 of the rubella virus genome), 1 µl (40 units) RNasin (Promega) and 1 µl (200 units) Superscript reverse transcriptase III (Invitrogen). The reaction was incubated at 55 °C for 60 min and then stored at −20 °C prior to use in PCR. Each 50 µl PCR contained 25 µl 2× GC buffer I&II (TaKaRa), 8 µl 2.5 mM dNTPs, 3 µl cDNA template, 1 µl 40 µM appropriate forward and reverse primers and 0.5 µl (2.5 units) LA *Taq* polymerase (TaKaRa). Cycling parameters were determined according to the manufacturer's (TaKaRa) recommendations. For genomic sequencing, 10–11 overlapping fragments encompassing the entire genome were amplified by using appropriate primer pairs. The primers used to amplify the genomic regions are listed in Supplementary Table S2, available in JGV Online. Amplified fragments were purified following agarose-gel electrophoresis by using a QIAquick gel extraction kit (Qiagen). Sequencing reactions were performed bidirectionally by using appropriate primers and cycle-sequencing kits (ABI PRISM BigDye Terminator v. 3.1; PE Applied Biosystems) and resolved by using a 3100 Genetic Analyzer (Applied Biosystems). The 5′ and 3′ ends of the genome were determined by using a 5′/3′ FirstChoice RLM-RACE kit (Ambion Inc.).

Sequence analysis. For cataloguing and storage, sequences were input into free online sequence-alignment software (ALIGN Query, GENESTREAM SEARCH network server IGH, Montpellier, France; http://xylian.igh.cnrs.fr/bin/align-guess.cgi). The assembled nucleotide sequences were aligned by using the CLUSTAL_W multiple sequence-alignment program version 1.8 (Henikoff & Henikoff, 1994) and the PileUp program in the GCG software package (Genetics Computer Group, version 11.0; Accelrys Inc.). The TN93 substitution model (Tamura & Nei, 1993) with discrete gamma-distributed rate heterogeneity with eight gamma rate categories (Yang, 1994) (TN93+Γ model) was used as a substitution model for phylogenetic reconstruction, as it was found statistically to be the best fit for our datasets. Maximum-likelihood (ML) phylogenetic analysis

Table 1. Rubella virus genomic sequences used in this study

Virus	Isolation site and year	Genotype	GenBank accession no.	Reference
Fth_USA64	Connecticut, USA, 1964	1a	M15240	Dominguez *et al.* (1990)
RA27_USA64	Pennsylvania, USA, 1964	1a	L78917	Pugachev *et al.* (1997)
M33_USA61	New Jersey, USA, 1961	1a	X05259, X72393	Clarke *et al.* (1987)
CEN_BEL63	Belgium, 1963	1a	AF188704	Lund & Chantler (2000)
TO-W_JAP67	Toyama, Japan, 1967	1a	AB047330	Kakizawa *et al.* (2001)
TO-V_JAP67	Toyama, Japan, 1967	1a	AB047329	Kakizawa *et al.* (2001)
ULR_GER84	Leipzig, Germany, 1984	1a	AF435865	Hofmann *et al.* (2003)
SUR_SVK74	Bratislava, Slovakia, 1974	1a	AF435866	Hofmann *et al.* (2003)
Cba_ARG88	Cordoba, Argentina, 1988	1B	DQ085339	This report
GUZ_GER92	Stuttgart, Germany, 1992	1B	DQ388280	This report
Anim_MEX97	Baja California, Mexico, 1997	1C	DQ085341	This report
JC2_NZL91	Auckland, New Zealand, 1991	1D	DQ388281	This report
6423_ITA97	Pavia, Italy, 1997	1E	DQ085343	This report
BRDII_CN80	Beijing, China, 1980	2A	AY258323	Zheng *et al.* (2003b)
BRI_CN79	Beijing, China, 1979	2A	AY258322	Zheng *et al.* (2003b)
AN5_KOR96	Seoul, Korea, 1996	2B	DQ085342	This report
I-11_ISR68	Tel-Aviv, Israel, 1968	2B	DQ085338	This report
C74_RUS97	Moscow, Russia, 1997	2c	DQ085340	This report
C4_RUS67	Moscow, Russia, 1967	2c	DQ388279	This report