TABLE I. Characteristics of 27 Enteroviruses Detected in Infants and Children With Diarrhea in Ho Chi Minh City, Vietnam During 2002 and 2003

						Laboratory diagnosis		Genetic sequence analysis		
No	Patient	Sex	Age	Dehydration grade	Isolate	RT-PCR	Cell culture	Highest identity (%)	Reference strain	Accession number
1	17	F	5 m	A	EV/17/VN/02	+	+	94	Coxsackievirus B2/10175	AY373053
2	35	M	4 m	A	EV/35/VN/02	+	−	95	Coxsackievirus A1/Tompkins	AF329684
3	85	M	15 m	A	EV/85/VN/02	+	−	95	Coxsackievirus A22/Chulman	AF329690
4	86	M	19 m	A	EV/86/VN/02	+	−	92	Poliovirus 1/NE/459/Spain/82	L76409
5	88	M	4 y	A	EV/88/VN/02	+	−	93	Coxsackievirus A18/G13/USA	AF499640
6	121	F	9 m	A	EV/121/VN/02	+	+	93	Coxsackievirus A11/Belgium/1	AF499636
7	143	F	13 m	A	EV/143/VN/02	+	−	95	Enterovirus 74/USA/CA75-10213[a]	AY556057
8	163	F	9 m	A	EV/163/VN/02	+	−	94	Echovirus 19/Djum/91/Finland	AF447480
9	178	F	13 m	B	EV/178/VN/02	+	−	97	Coxsackievirus A12/P-2200/Kanagawa/03	AB126210
10	179	F	6 m	A	EV/179/VN/02	+	−	97	Coxsackievirus A12/P-2200/Kanagawa/03	AB126210
11	195	M	13 m	A	EV/195/VN/02	+	−	93	Coxsackievirus A18/G13	AF499640
12	225	M	24 m	A	EV/225/VN/03	+	+	95	Echovirus 6/Kh7/92/Filand	AF447481
13	268	F	12 m	A	EV/268/VN/03	+	−	96	Enterovirus 74/USA/CA75-10213[a]	AY556057
14	405	F	NR	A	EV/405/VN/03	+	−	94	Coxsackievirus A1/Tompkins	AF329684
15	549	M	8 m	A	EV/549/VN/03	+	−	93	Coxsackievirus A18/G13/USA	AF499640
16	573	M	23 m	A	EV/573/VN/03	+	−	93	Poliovirus 3/1620/Netherland/58	L76406
17	617	M	3 y	A	EV/617/VN/03	+	−	95	Echovirus 19/Djum/91/Finland	AF447480
18	719	M	7 m	A	EV/719/VN/03	+	−	96	Echovirus 9/DM/Finland	AF524867
19	721	M	8 m	A	EV/721/VN/03	+	−	92	Coxsackievirus A1/Tompkins	AF329684
20	768	F	3 y	A	EV/768/VN/03	+	−	99	Coxsackievirus B3/P/2000/Germany	AY752946
21	866	M	8 m	A	EV/866/VN/03	+	−	97	Echovirus 2/HVN-1337/Hungary	AY167103
22	910	F	10 m	A	EV/910/VN/03	+	−	94	Coxsackievirus A4/P-2319/Kanagawa/03	AB162739
23	913	F	8 m	A	EV/913/VN/03	+	−	94	Echovirus 11/ROU-9191	AJ577594
24	935	F	18 m	A	EV/935/VN/03	+	−	94	Coxsackievirus A18/G13	AF499640
25	937	M	14 m	A	EV/937/VN/03	+	−	88	Echovirus 6/Lytic/Charles	U16283
26	994	F	6 m	B	EV/994/VN/03	+	−	91	Echovirus 31/Australia	AF412372
27	1002	F	14 m	A	EV/1002/VN/03	+	+	91	Enterovirus 71/R13223-IND-01	AY179601

No, number; M, male; F, female; y, year; m, month; NR, not recorded; A, no dehydration; B, mild dehydration.
[a]New strain.

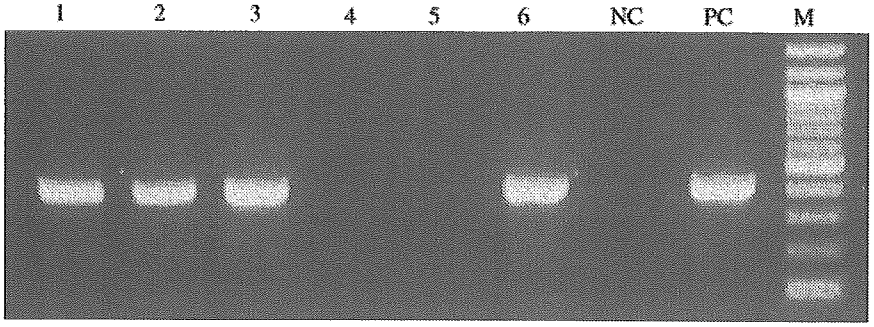

Fig. 1. First identification of enteroviruses in fecal specimens collected from infants and children admitted to hospital with acute gastroenteritis in Ho Chi Minh City, Vietnam during 1-year period (October 2002 and September 2003). **Lane**: **1**, **2**, **3**, and **6**; Vietnamese enteroviruses detected in feces by RT-PCR; **4** and **5**; No viral amplification in feces; NC, negative control; PC, positive control; M, 100 bp molecular marker.

available. The spectrum of clinical manifestations of enteroviral infection varies from asymptomatic infections to fatal cases. Interestingly, the identification of enteroviruses in feces from infants and children with diarrhea in Vietnam in the present study was high (9.8%). This result was supported by PCR with different primers, different protocols and by nested PCR as the most sensitive method (data not shown). The enterovirus-positive specimens were negative not only for common diarrheal enteropathogens (rotavirus, adenovirus, norovirus, sapovirus and astrovirus) but also for bacteria (campylobacter, clostridium, *escherichia coli* and salmonella). Taken together, the results support the assumption that enteroviruses were the causative agents of acute gastroenteritis in Vietnamese infants and children. It is also the first indication of the presence of enteroviruses in a pediatric population with diarrhea in Vietnam. These findings suggest that about 9.8% of acute gastroenteritis in infants and children in Ho Chi Minh City, Vietnam were due to enteroviruses and 90.2% caused by other etiologic agents. Interestingly,

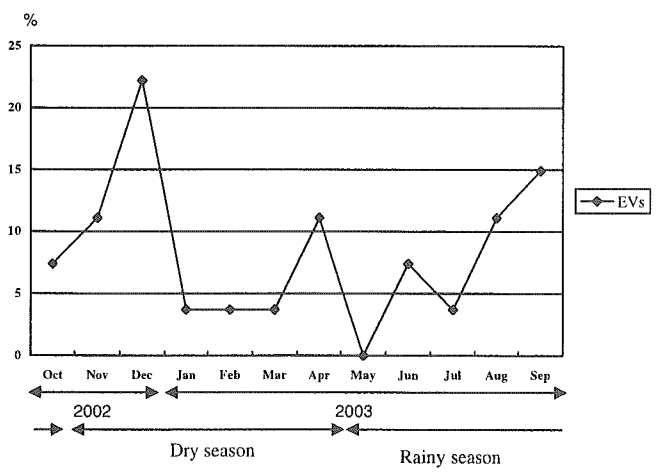

Fig. 2. Monthly distributions of enteroviral infection in infants and children admitted to hospital with acute gastroenteritis in Ho Chi Minh City, Vietnam. Enteroviruses were identified throughout the year except in May and the highest number was in December. The periods of dry season and rainy season were also indicated.

most infants and children suffering from acute gastroenteritis in association with enteroviral infection displayed no severe sign of dehydration. Furthermore, all these patients recovered and were discharged after the average 3–4 days in hospital (data not shown). Apparently, it seems that enteroviruses cause mild diarrhea in the pediatric population in Ho Chi Minh City. However, there was no clear difference in the clinical picture and laboratory results in certain infections. The climate in Ho Chi Minh City, Vietnam is distinctively seasonal. The dry season lasts from November to April, the hottest months are from February to May and temperatures may reach over 37°C. The rainy season characterized by sudden heavy rain begins in May and ends in October. The wettest months are from June to September. In this study, no specific seasonal pattern was presented even though enteroviruses were observed to be the most prevalent in December. These findings are different from other studies where enterovirus have been isolated during the rainy season in tropical countries such as Vietnam [Melnick, 1996; Mark and Raymond, 2001].

There are several different cell lines used for the cultivation of enteroviruses [Reigel, 1985; Chezzi, 1996; AbuBakar et al., 1999; Munemura et al., 2003; Moce-Llivina et al., 2004]. The use of the epidermal-like cell line CaCo2 and the efficient propagation of viruses from clinical material for diagnostic purposes have been reported [Reigel, 1985]. Fecal specimens were used for virus isolation using two cell lines CaCo2 and Vero. Of these only four enterovirus strains could be isolated by cell culture. Although virus isolation had been usually carried out to identify the causative viruses by different investigators, the detection rate for the presence of enteroviruses using this assay would have missed approximately 85.2% compared to RT-PCR. However, this method is complicated, labor intensive, time consuming, low sensitive, and requires the cell culture techniques, special cell lines from which certain viruses have been isolated [Ishiko et al., 2002]. Furthermore, attempts to isolate viruses are unsuccessful frequently because of the low viral titer in clinical specimens. In addition, some viruses grow poorly or are refractory to

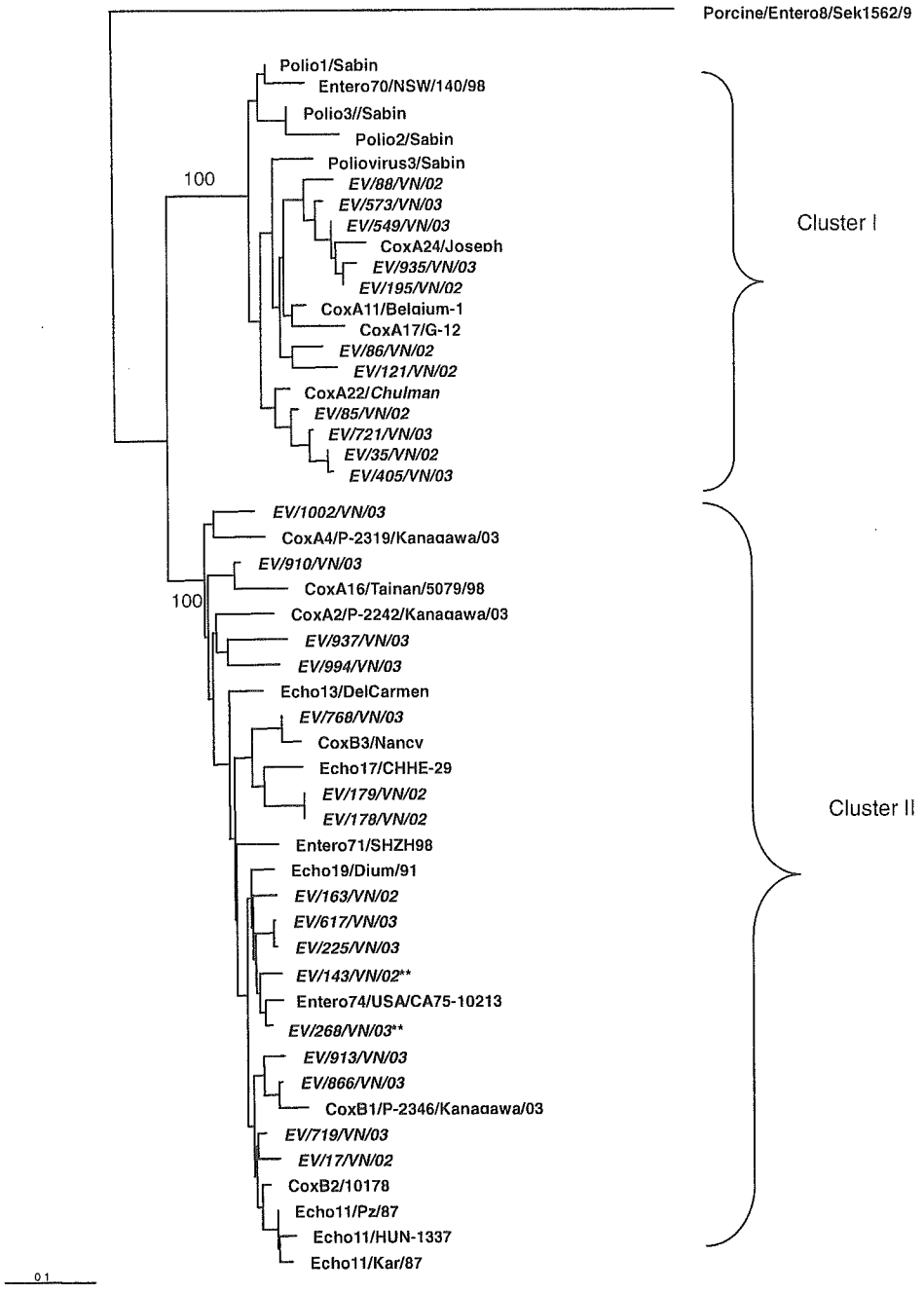

Fig. 3. Phylogentic tree of nucletotide sequences of 27 enterovirus isolates based on the enterovirus 5′ noncoding region (5′ NCR) classification scheme of Siafakas et al. [2002]. The numbers in the branches indicate the bootstrap values. Enteroviruses detected in the present study were highlighted in italic. Porcine/Entero8/Sek1562/98 was used as an out-group strain for phylogenetic analysis. Note: **Vietnamese enterovirus isolate had the closest march with new enterovirus 74 (known as Entero74/USA/CA75-10213).

growth in cell culture [Chonmaitree et al., 1982; Wilfert et al., 1983]. There are therefore many disadvantages, which limit the usefulness of cell culture [Guney et al., 2003]. RT-PCR is used successfully to detect most enteroviruses, including those, which cannot be propagated readily in cell culture [Rotbart, 1990]. The results are in agreement with other published reports indicating that RT-PCR is the most sensitive and reliable method, compared to virus isolation in cell culture for the detection of enteroviral infection in clinical fecal specimens collected from infants and children with acute gastroenteritis and can be a useful tool for diagnosis purposes and molecular epidemiological study of the diversity of viruses causing diarrhea. At present,

amplification of the genome by RT-PCR in clinical specimens has been introduced as a more convenient and powerful alternative to molecular diagnosis [Rotbart, 1990; Oberste et al., 1998]. In addition, genome amplification allows further characterization of enteroviruses by sequencing analysis [Casas et al., 1997; Arola et al., 1999].

The results of this study showed that all Vietnamese enterovirus sequences belonged to two distinct genetic clusters I and II. The closest march at the nucleotide level between these enterovirus isolates detected by RT-PCR and reference strains registered previously in the DDBJ DNA/Genbank database dropped to 88%. It is also noteworthy that identity among Vietnamese enteroviruses was observed as low as only 58%. And the lowest homologies among those in the same cluster (I and II) also were 74% and 82%, respectively. Taken together, the findings clearly indicate that enterovirus strains co-circulating in infants and children with acute gastroenteritis in Ho Chi Minh City, Vietnam are diverse genetically. Furthermore, based on the genetic analysis of 5′ NCR sequences, the findings suggest the similarity of Vietnamese enteroviruses with coxsakieviruses and echoviruses. Another interesting feature of this study was the demonstration of two Vietnamese enterovirus isolates, EV/143/VN/02 and EV/268/VN/03 which had the closest marches at nucleotide level by BLAST with a new strain called enterovirus 74 (known as the Entero74/USA/CA75-10213).

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Changing distribution of group A rotavirus G-types and genetic analysis of G9 circulating in Japan

Brief Report

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Summary. A total of 1,797 fecal specimens from infants and children with acute gastroenteritis in Japan from July 2000 to June 2003 were tested for group A rotavirus by ELISA, RT-PCR, RNA-PAGE and latex agglutination methods. Of these, 439 were found to be positive for group A rotavirus and this presented 24.4%. In 2000–2001, G1 was the most prevalent (45.5%) followed by G2 (32.5%), G3 (12.3%), G9 (5.9%) and G4 (2.6%). However, G2 was found predominant with 40% in the following year (2001–2002). Interestingly, G9 had a rapid increase of infection up to 17.8%. In 2002–2003, G3 dominated over other G-types with 34%. Another interesting feature of the study was the demonstration of great genetic diversity among G9 strains in Japan. Worth of note was the first prevalence pattern of rotavirus G-types with an increase of G2, G3 as well as G9 and a decrease of G1 during the 20 year-survey of rotavirus infection in Japan.

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Viral gastroenteritis is a common disease with a high morbidity reported worldwide. Acute gastroenteritis consistently ranks as one of the top causes of all deaths. In the early 1980s, diarrheal disorders were the most severe child killers, responsible for an estimated 4.6 million deaths worldwide every year. Despite much progress in the understanding of pathogenesis and of management with the widespread use of oral rehydration therapies (ORT), diarrheal illness remains one of the most important causes of global childhood mortality and morbidity. The mortality among children due to acute gastroenteritis is greater in developing than

in developed countries [26]. Every year, 2.5 million children still die from diarrhea, almost all of them in developing countries. Among other enteropathogenic viruses, rotaviruses are recognized as the major etiologic agents of gastroenteritis in children and young animals [12, 13, 17, 18, 25]. Rotaviruses are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with group A, B and C rotaviruses [1, 21]. Of these, group A rotaviruses are the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [7]. In developing countries, the mortality rate following rotavirus infection is still high and there is so far no effective universal vaccine [12, 17].

Rotaviruses belong to the genus *Rotavirus* within the family *Reoviridae* and their genome consists of 11 double stranded RNA segments, which encode six structural and six non-structural proteins. Each segment codes for one protein, with the exception of segment 11, which codes for two proteins. The non-structural proteins are involved in genome replication, the assortment of genome sets and regulation of gene expression. The structural proteins form a triple-layered capsid around the dsRNA genome [5]. Among group A rotavirus, 14 G-types based on the VP7 gene have been described, with types G1 to G4 represented 95% of strains identified in humans globally [5, 15]. Vaccines under development are focused on these four G-types. The first rotavirus vaccine licensed for use was a live-attenuated tetravalent rhesus-human reassortant vaccine incorporating G1 to G4 specificity. This vaccine was, however, withdrawn from use after reports of association with intestinal intussusceptions. Moreover, study of the molecular epidemiology of the rotaviruses provides knowledge on the diversity of the specific VP7 types found in humans. For diarrheal disease control to be successful through vaccination, constant monitoring of the rotavirus types is needed [2].

Thus, the objectives of the present study were: to determine the incidence of group A rotavirus infections in infants and children with acute gastroenteritis in five different places of Japan during 2000 and 2003; to characterize the detected rotaviruses according to genotype and to describe the genetic diversity among them. Additionally, the age-related distribution and seasonal pattern of rotavirus infection were also described.

A total of 1797 fecal specimens were collected from infants and children with acute gastroenteritis in five places (Tokyo, Maizuru, Saga, Sapporo and Osaka) of Japan during July 2000 and June 2003. 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 and stored at −30 °C until use for the detection of group A rotavirus. The viral genomes were extracted from 140 μl of 10% fecal suspensions using a spin column technique according to the manufacturer's instructions (QIAGEN®, Germany).

Group A rotavirus was detected by enzyme linked immunosorbent assay (ELISA), Rotalex (a commercial latex agglutination), reverse transcription-polymerase chain reaction (RT-PCR), and RNA-polyacrylamide gel electrophoresis (RNA-PAGE) methods. For Rotalex test (Daiichi Kagaku Co., Ltd, Japan), fecal specimens were processed according to the manufacturer's instructions.

Electropherotyping of viral RNA was carried out in 10% polyacrylamide gel electrophoresis (PAGE), and silver staining was performed as described by Theil et al. [27]. ELISA with polyclonal antibodies specific for group A human rotavirus as previously reported was conducted [28, 29]. RT-PCR with specific primers (Beg9 and VP7-1′) was used to perform the identifications of rotavirus. The 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 [31].

G genotyping of group A rotavirus was conducted using the protocol from the method previously described by Das et al. [3]. The full-length of VP7 gene was reverse transcribed and then further amplified with 9con1 and End9 primers. The expected size of the PCR product generated from the nearly full-length VP7 gene was 1,025 bp in length. The second amplification was performed using the first PCR product as the template with G-genotype 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 bp, 224 bp, 466 bp, 403 bp and 110 bp for G1, G2, G3, G4 and G9, respectively [3]. PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr) for 20 min then visualized under ultraviolet light, and the results were recorded by photography.

To have a better understanding of the relationship among Japanese rotavirus G9 isolates in the present study and the previously detected strains worldwide, the nucleotide sequences of PCR products (DNA) positive for rotavirus G9 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 W (Version 1.6). Reference rotavirus strains and their accession numbers used in this study were as follows: 99-TK2091VP7/JP (AB091756), 99-TK2082VP7/JP (AB091755), 99-SP1904VP7/JP (AB091754), 99-SP1542VP7/JP (AB091753), 00-SG2864VP7/JP (AB091751), SD768/USA (AJ491191), EM41/USA (AJ491170), R160/Brazil (AF274971), R143/Brazil (AF274969), Arg1740 (AF323719), 632/97/UK (AJ401262), GOS51916/96/UK (AJ401244), 84/02-1/Italia (AY184813), AP13/India (AJ491154).

A total of 1,797 fecal specimens collected from Japanese infants and children with acute gastroenteritis from 2000 to 2003 were examined for the presence of group A rotavirus. Out of 1,797 diarrheal fecal specimens, 439 were found to be positive for group A rotavirus and this presented 24.4%. Among group A rotavirus detected in 2000–2001, G1 was the most prevalent (45.5%) followed by G2 (32.5%), G3 (12.3%) and G4 (2.6%). Only 9 cases were determined as G9 by RT-PCR and it accounted for 5.9%. However, G2 was found predominant with 40% in the following year (2001–2002). G1 was the next with 20%. Interestingly, G9 had a rapid increase of infection up to 17.8%. Table 1 shows that in 2002–2003, G3 and G4 dominated over other G-types with 34% and 28%, respectively. It was surprising that the incidence of G1 and G2 known as the leading types in previous years dropped rapidly and were responsible for only 6% and 5.2%, respectively

Table 1. Distribution of group A rotavirus G-types circulating in infants and children with acute gastroenteritis in Japan during a 3-year period (2000–2003)

Year	No. of specimen tested	No. of viral positive (%)	Mono-infection (%)					Mixed infection (%)						
			G1	G2	G3	G4	G9	G1/3	G2/4	G2/9	G3/4	G3/9	G4/9	G3/4/9
2000–2001	645	154 (23.8)	70 (45.5)	50 (32.5)	19 (12.3)	4 (2.6)	9 (5.9)	–	1 (0.6)	1 (0.6)	–	–	–	–
2001–2002	595	135 (22.6)	27 (20)	54 (40)	16 (11.9)	13 (9.6)	24 (17.8)	–	–	1 (0.7)	–	–	–	–
2002–2003	557	150 (26.9)	9 (6)	8 (5.2)	51 (34)	42 (28)	28 (18.7)	1 (0.7)	1 (0.7)	–	7 (4.6)	1 (0.7)	1 (0.7)	1 (0.7)
Total	1,797	439 (24.4)	106 (24.2)	112 (25.5)	86 (19.6)	59 (13.4)	61 (13.9)	1 (0.2)	2 (0.5)	2 (0.5)	7 (1.6)	1 (0.2)	1 (0.2)	1 (0.2)

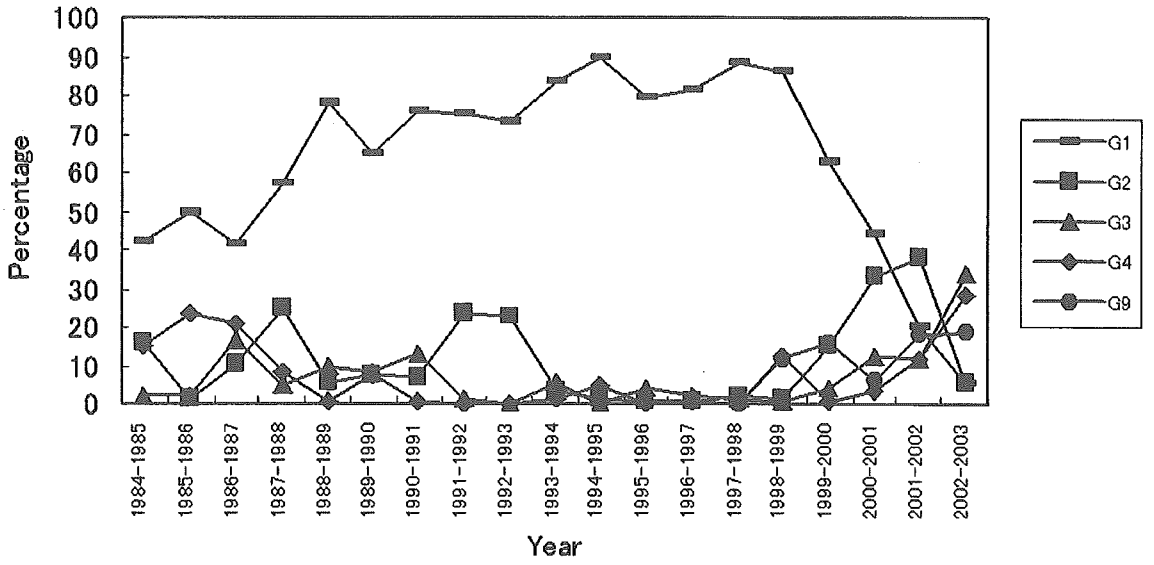

Fig. 1. Prevalence pattern of group A rotavirus G-types with an increase of G2, G3 as well as G9 and a decrease of G1 among infants and children with acute gastroenteritis in five places (Tokyo, Maizuru, Saga, Sapporo and Osaka) of Japan since 1984

(Fig. 1). Of interest, the relatively high rate (3.4%) of viral mixed infections, including one triple infection (G3, 4 and 9) was demonstrated in this study. In addition, it was revealed that the majority of mixed infection (66.7%, 10 of 15) was the combination between rotavirus G3 and other serotypes.

In the present study, group A rotavirus was identified every month of year except in September. The high prevalence of rotavirus infection lasted from February (12%, 215 of 1797) to June (6%, 107 of 1797) and was found highest in April (32%, 575 of 1797). For the pediatric population, the lowest age was 2 months, the highest was 14 years, and the average age was 2.9 years (35 months). It was found that the highest incidence of rotavirus was in the 12–23 month old group (39.9%), and the lowest fell into the infants aged less than 6 months (5.2%). Infants and children aged less than 3 years had a rather high rate of rotavirus infection (65.9%). Moreover, the number of male child infected with rotavirus accounted for 48.1% (211 of 439).

A total of 66 rotavirus G9 isolates (including mono-infection and mixed infection) sequences were analyzed by phylogenetics and grouped using the rotavirus G9 VP7 region classification scheme [22]. In this study, all of the G9 sequences were classified into only one distinct lineage III. Our results clearly indicated that G9 lineage III was a dominant group in Japan. All Japanese G9 isolates detected in 2000 and 2003 had the high homology at the nucleotide as well as the amino acid ranging from 99% to 100% each other and to those in previous year (1999–2000). However, it was found that these G9 isolates had the closest match of only 93%–94% and 86%–88% at the nucleotide level to those

detected during 1998 and 1999 and to G9 prototype strains (F45, AU32, WI61 and 116E), respectively. Moreover, the high identity (97%–100%) between G9 isolates in 2000–2003 and recent G9 strains worldwide was also noted. Compared with Japanese G9s in 1998–2000, they further demonstrated the substitutions at amino acid 9 (V to I), 11 (I to T), 44 (V to A), 46 (P to S), 100 (N to D), 143 (T to K), 144 (H to Y), 208 (T to I), 212 (A to T) and 267 (E to D). Interestingly, the change at 100 was revealed to be located in antigenic region A, 143 and 144 in antigenic region B, 208 and 212 in antigenic region C. However, only one amino acid (46, S to P) was identified different from global G9 strains previously registered in the DDBJ DNA database (Table 2).

Rotavirus is one of the global causes of viral gastroenteritis and also is associated with sporadic cases and outbreaks of gastroenteritis worldwide. Rotavirus infection causes acute gastroenteritis in all age group, through it occurs predominantly in infants and young children [12, 17]. Out of 1,797 fecal specimens in this study, 439 were determined to be positive for rotavirus and the majority of infants and children with rotavirus infection (65.9%) were aged less than 36 months. This result was consistent with previously published reports on rotavirus epidemiology worldwide in which its prevalence was shown to be ranging from 13.7 to 65.6%

Table 2. Comparison of amino acid sequence of VP7 gene among Japanese and global group A rotavirus G9 strains

Strain	Place	Time	Amino acid identity									
							A (87–101)	B (143–152)		C (201–221)#		
			9	11	44	46	100	143	144	208	212	267
99-TK2082V	Japan	1998–1999	V	I	V	P	N	T	H	T	A	E
99-TK1904V	Japan	1998–1999	V	I	V	P	N	T	H	T	A	E
00-OS2986V	Japan	1999–2000	I	T	A	S	D	K	Y	I	T	D
00-SP2737V	Japan	1999–2000	*	*	*	*	*	*	*	*	*	*
JP44251	Japan	2000–2003	*	*	*	*	*	*	*	*	*	*
JP4243	Japan	2000–2003	*	*	*	*	*	*	*	*	*	*
SD768	USA	1998–1999	*	*	*	P	*	*	*	*	*	*
EM41	USA	1999–2000	*	*	*	P	*	*	*	*	*	*
AT694	USA	2000–2001	*	*	*	P	*	*	*	*	*	*
R136	Brazil	1998	*	*	*	P	*	*	*	*	*	*
R160	Brazil	1999	*	*	*	P	*	*	*	*	*	*
Arg1742	Argentina	1998–1999	*	*	*	P	*	*	*	*	*	*
GOS51916	UK	1996	*	*	*	P	*	*	*	*	*	*
480	UK	1997	*	*	*	P	*	*	*	*	*	*
0.7	UK	1998	*	*	*	P	*	*	*	*	*	*
84	Italy	2001–2002	*	*	*	P	*	*	*	*	*	*
BD524	Bangladesh	1996	*	*	*	P	*	*	*	*	*	*
6342LPSA	South Africa	1999	*	*	*	P	*	*	*	*	*	*

Note: #, A, B and C antigenic regions of rotavirus VP7 gene; *amino acids identical with the G9 strain 00-OS2986V/Japan/1999–2000

and usually much higher than norovirus [4, 16, 19, 20]. Our findings also confirmed rotavirus as one of the enteropathogens responsible for viral gastroenteritis among infants and children in Japan. According to some reports, rotavirus was prevalent in cold season, and several studies did not find a seasonal correlation [4, 14, 16]. In Japan, the highest prevalence time of a rotavirus is about three months late from two in recent years [24]. In this study, rotavirus was identified throughout the year except in September and was highest in April as spring. It was found that the highest incidence of rotavirus infection fell into the 12–23 month old group, and the rate of incidence decreased with increasing age over 24 months. Quite possibly, children aged from 12 to 23 months might lack antibody protection to rotavirus; whereas by the time children have reached 24 months they have begun to acquire viral immunity.

To date, the mixed infections with different rotavirus serotypes have been reported by several groups of investigators [4, 16, 30]. Interestingly, the relatively high incidence (3.4%) of viral mixed infections with different G-types was detected. Of these, one triple infection (G3, 4 and 9) was identified in a male patient with 1 year old. These results were in good agreement with other previously published reports in which the mixed infection among different rotavirus G-types is not rare [4, 16, 30].

During the period from 1984 through 2000, changed prevalence pattern of rotavirus G-types was found with an increase of G2 and a decrease of G1, although G1 remained the prevailing serotype. In the meanwhile, G2 still increased and became the leading in 2001–2002 but being taken a sharp decline in 2002–2003. In contrast, G3 had a dramatic increase to be the predominant serotype in 2002–2003. It was worthy to point out the first prevalence pattern of rotavirus G-types with an increase of G2 as well as G3 and a decrease of G1 during the 20 year-survey of rotavirus infection in Japan. G2 in 2001–2002 and G3 in 2002–2003 were further analyzed by sequencing. It was found that Japanese G2 and G3 in this study were genetically closer to the current isolates in China than those in Japan several years ago (data not shown). However, it might be due to co-existence of multiple factors such as changes of climate, water and others. The further research should be conducted in order to investigate this phenomenon.

Rotavirus G9 has been recognized as the most widespread of the emerging serotypes since 1996 as the frequent cause of severe acute gastroenteritis in infants and children from many countries covering all continents of the world [8, 10, 11, 23]. In Japan, only one G9 was firstly detected in Tokyo, Japan in 1996–1997. And then, serotype G9 was unexpectedly determined to be the prevailing serotype in 1998–1999 and 1999–2000 with 11.6% and 15.6%, respectively as presented by Zhou et al. [30]. In our study, it was interestingly noticed that the incidence of G9 infection continued to increase up 18.7% during 2002 and 2003. Our findings clearly indicated that G9 was one of important rotavirus serotypes along with G1–G4 causing acute gastroenteritis in Japanese children. To understand the genetic evolution of Japanese G9, the characterization of VP7 gene was subjected to sequencing analysis. Japanese G9 isolates detected in 2000–2003 were highly identical to each other and to those in 1999–2000 as well as recent G9 strains

worldwide. However, these G9 isolates had the low identity in comparison with those in 1998–1999 and G9 prototype strains (F45, AU32, WI61 and 116E). It was interesting that 10 amino acid substitutions of G9 VP7 genes were identified among Japanese strains. However, residues 9, 11, 44 and 46 were not involved in the virion after a cleavage between amino acids 50 and 51. Therefore, amino acid changes at other positions in our study were considered to be responsible for changes in antigenic specificity. According to the study of neutralization sites on VP7 of G9 strain (F45), the motif NTA (positions 211–213) in the antigenic C region was the critical region of neutralizing monoclonal antibody (Mab) F45:1 [9]. Therefore, the change at position 212 of G9 VP7 found in the present study influenced the neutralization of this Mab. Because the antigenic regions A and C are close in the three-dimensional structure of VP7 protein, they can interact with each other. A change in region C (for example: residue 213) of the F45 strain can lead to resistance to neutralization by Mab F45:9 which is targeted to region A [9, 25]. In addition, using fragment of VP7 expressed in phage display libraries to map rotavirus VP7 epitopes, the region between amino acid 233–283, especially amino acids 263–270, was implicated as immunodominant [6]. Thus it was possible that that region could induce a protective immune response. Taken together, the results clearly indicated that the amino acid changes of G9 VP7, especially in regions A and C played important roles in altering antigenicity that might cause the emergence of G9 strains in Japan due to immune escape.

In conclusion, group A rotavirus is still an important agent causing diarrhea in Japan. It is the first, to the best our knowledge, changing epidemiology of rotavirus serotype since 1984 in Japan. These changes indicate the diversity of rotavirus strains, which could be effective potential vaccine candidates. Our results underscore that vaccines should be constructed against a broad range of rotavirus strains and confirm the presence as well as the importance of G9 and warn of the threat they pose in future.

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Existence of multiple outbreaks of viral gastroenteritis among infants in a day care center in Japan

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Summary. A total of 921 fecal specimens collected from 44 infants in a day care center (DCC) in Tokyo, Japan during June 1999 to July 2000 were tested for the presence of rotavirus, norovirus, sapovirus, astrovirus and adenovirus by reverse-transcription-multiplex polymerase chain reaction (RT-multiplex PCR) and sequence analysis. Of 88 fecal specimens from infants with acute gastroenteritis, 51.1% (45) were found to be positive for diarrheal viruses. Astrovirus was the most prevalent (15.9%, 14 of 88), followed by norovirus GII (14.8%, 13 of 88), adenovirus (12.5%, 11 of 88), and sapovirus (2.3%, 2 of 88). Viral mixed infection accounted for 5.7% (5 of 88). Interestingly, 230 of 833 (27.6%) fecal specimens collected from asymptomatic infants were also infected with diarrheal viruses. Of these, astrovirus, norovirus GII, adenovirus and sapovirus were identified in 53, 46, 96 and 22 fecal specimens (23%, 20%, 41.7%, and 9.6%, respectively). Moreover, 13 of 833 (1.6%) normal specimens showed mixed viral infections. Surprisingly, no rotavirus (known as the most common causative agent of acute gastroenteritis in DCCs) was detected in those subjects. Another interesting feature was the demonstration of five separate outbreaks of acute gastroenteritis identified in a single DCC. Outbreak A was associated with both astrovirus serotype 1 and norovirus GII/3 (known as Toronto virus cluster); Outbreak B with adenovirus 12; Outbreak C with norovirus GII/4 (Lordsdale virus cluster); Outbreak D with sapovirus GIV and Outbreak E with astrovirus serotype 1. To our knowledge, this is the first proof of multiple outbreaks of viral gastroenteritis in Japanese infants in a single DCC. Our results confirm the presence as well as the importance of these viruses and warn of the threat they pose.

Introduction

Viral gastroenteritis is one of the most common illnesses in humans worldwide and it has a great impact on people [28, 29]. Acute gastroenteritis has been demonstrated as a major cause of morbidity and mortality among infants and young children in both developed and developing countries [22]. Acute gastroenteritis consistently ranks as one of the top causes of all deaths [12, 31]. Among other enteropathogenic viruses, rotaviruses are recognized as the major etiologic agents of gastroenteritis in children and young animals. Human infection has been reported with group A, B, and C rotaviruses. Group A rotavirus is the most important of these, being a major cause of severe gastroenteritis in infants and young children worldwide [3, 21]. Adenovirus, astrovirus, norovirus and sapovirus are also considered to be significant global enteropathogens [8–10, 18, 23, 24, 27, 30]. These viruses also are associated with sporadic cases and outbreaks of gastroenteritis in such settings as kindergartens, schools, and nursing homes for the elderly and among military recruits [13–16]. Transmission routes of these viruses are classified into foodborne, airborne, and person-to-person spread, and other unknown modes of transmission may exist [4, 5, 19].

A study in a day care center (DCC) showed that the incidence of diarrhea in infants and children attending DCCs might be twice as high as those staying at home [1]. Moreover, children not yet toilet-trained are at the greatest risk of transmitting diarrhea and other viral diseases, usually spread by the fecal-oral route or aerosol emissions [1]. It was found that infants in DCCs are particularly at risk of infection because of their immature immune systems. Other factors promoting spread of infections in this setting include lack of hygiene [6]. Diarrhea occurring in DCCs is usually mild and self-limiting. Good routines for washing hands and for changing nappies are considered to be the most important ways of controlling infection in DCCs [12]. Diarrhea caused by rotavirus is common in children in DCCs and often occurs in outbreaks, especially in the winter months [2, 26]. A child newly enrolled in a day care facility is at a particularly high risk for developing a diarrheal illness within the first month after enrollment, but the risk has been found to decrease as children remain in the same setting [20]. The occurrence of frequent outbreaks of infection in DCCs permits better studies of the immunologic factors protecting children against infection in this setting than can be achieved in outside group settings [15].

The objectives of this study were (i) to determine the presence of viral infection in infants with and without acute gastroenteritis in a DCC in Tokyo, Japan and (ii) to characterize the genome of the detected viruses. The age-related distribution, seasonal pattern of viral infections, and clinical manifestations are also described.

Materials and methods

Definition

The case definition of diarrhea used in this study was as at least three passings of unformed (loose and watery) stool a day in comparison with the usual bowel habits of the infants.

Caretakers, who understood the above definition of diarrhea, recorded the condition of every stool and also noted such symptoms as vomiting, fever, abdominal pain, blood or mucus in stool. An outbreak of acute gastroenteritis was defined as the occurrence of diarrhea and other symptoms in three or more infants within one week [17]. When acute gastroenteritis continued to the following week, the outbreak was also considered as one outbreak. A viral outbreak was defined as at least two infants with a specific virus detected by reverse-transcription-multiplex polymerase chain reaction (RT-multiplex PCR) [17].

Subjects and fecal collection

Fecal specimens were collected from infants enrolled in a DCC in Tokyo, Japan, from June 1999 to July 2000. In total, observations continued 61 weeks in the present study. One stool sample was collected from each infant once a week on Monday. If an infant did not have a bowel movement, no examination was performed for that infant on that particular day. When clinical manifestations of acute gastroenteritis were demonstrated, more than one specimen was collected in a week from that subject. In Japan, DCCs start a new semester in April, whereupon several infants will move to the senior class and new infants will enroll in the DCC. All infants who withdrew or enrolled during the research period were included in the present study. In total, there were 44 infants during the entire study. All infants were healthy when they enrolled in the DCC. Nine hundred and twenty-one fecal specimens were collected from infants with and without acute gastroenteritis during the period June 1999 to July 2000. Of these specimens, 88 were from diarrheal feces. 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 and stored at −30 °C until use for the detection of diarrheal viruses.

Reverse transcription (RT)-multiplex PCR

The viral genomes were extracted from 140 μl of 10% fecal suspensions using a spin column technique according to the manufacturer's instructions (QIAGEN®, Germany). For RT, 7.5 μl of extracted RNA was added with a reaction mixture consisting of 2.05 μl of 5× First Strand Buffer (Invitrogen, USA), 0.75 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 μl of 10 mM DTT (Invitrogen), 0.75 μl (200 U/μl) of SuperScript Reverse Transcriptase II (Invitrogen), 0.375 μl (1 μg/μl) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 μl (33 U/μl) of RNase Inhibitor (Toyobo, Osaka, Japan), and 2.325 μl MilliQ water. The total reaction mixture was 15 μl. The RT step was carried out at 42 °C for 1 h, followed by 99 °C for 5 min and then held at 4 °C [33].

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 multiplex PCR. The identification of the first group of viruses was performed by using multiplex PCR with specific primers as previously reported [33]. In multiplex PCR, four pairs of specific primers published (PreCAP1 and 82b for amplifying astrovirus (AstV); G1SKF and G1SKR for norovirus genogroup I (NVGI); COG2F and G2SKR for norovirus genogroup II (NVGII), SLV5317 and SLV5749 for sapovirus (SV) were mixed. These primers specifically generated four different sizes of amplicons of 719 bp, 330 bp, 387 bp, and 434 bp for astrovirus, norovirus (GI, GII), and sapovirus, respectively [33].

In order to detect the second group of viruses, four pairs of published primers (Beg9 and VP7-1′, B5-2 and B3-3, G8NS1 and G8NA2 for amplifying VP7 gene of human group A, B, and C rotaviruses, respectively; Ad1 and Ad2 for hexon gene of all species from A to F adenoviruses) were used. These primers specifically generated four different sizes of amplicons of 395 bp, 814 bp, 352 bp 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 of 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C [34]. PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr) for 20 min then visualized under ultraviolet (UV) light, and the results were recorded by photography.

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of PCR products (DNA) positive for astrovirus, sapovirus, norovirus GII and 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 E-CLUSTAL W (Version 1.6). Viral strains and accession numbers used in this study were as follows: PEC (AF182760), Bristol/89 (AJ249939), Lyon/598/97/F (AJ271056), London/92 (U95645), Mex340/90 (AF435812), Cruise ship/00 (AY289804), Hou7-1181/90 (AF435814), Arg39 (AY289803), Mex14917/00 (AF435813), Houston/90 (U95644), Parkville/94 (U73124), Houston/86 (U95643), Sapporo/82 (U65427), Manchester/93 (X86560), Southampton (L07418), Karachi/877/1990 (AB181133), Karachi/730/1992 (AB126249), Karachi/879/1993 (AB181132), Karachi/874/1992 (AB126249), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Girlington (AJ277606), Hillington (AJ277607), Toronto (U02030), Leeds (AJ277608), Lordsdale (X86557), Seacroft (AJ277620).

Results

Detection of diarrheal viruses

A total of 921 fecal specimens were collected from infants with and without acute gastroenteritis. For the pediatric population, the lowest age was 1 month, the highest was 2 years, and the average age was 9.6 months at the time of DCC enrollment. In this study, 75% of all infants were less than 1 year old. The percentage of male infants was 54.5%. All fecal specimens were tested for the presence of rotavirus, norovirus, sapovirus, astrovirus, and adenovirus by RT-multiplex PCR method.

Of 88 fecal specimens from infants with acute gastroenteritis, 51.1% (45) were found positive for diarrheal viruses (Table 1). Among these viruses, astrovirus was most prevalent with 15.9% (14 of 88), followed by norovirus GII (14.8%, 13 of 88), adenovirus (12.5%, 11 of 88), and sapovirus (2.3%, 2 of 88). Five fecal specimens (5.7%) demonstrated mixed infection by both astrovirus and norovirus GII; no rotavirus or norovirus GI was detected in them. Interestingly, 230 of 833 fecal specimens (27.6%) collected from asymptomatic infants were infected with diarrheal viruses; from them, astrovirus, norovirus GII, adenovirus, and sapovirus were identified in 53, 46, 96, and 22 fecal specimens and represented 23%, 20%, 41.7% and 9.6%, respectively. Thirteen (5.7%) normal specimens had mixed viral infections. No rotavirus or norovirus GI was found in asymptomatic infants. It was found that of these 230 fecal specimens containing diarrheal virus, 75.2% (173) were collected from infants i) with no history of diarrhea before the date of fecal collection, or ii) with diarrhea of unknown etiology before the date of fecal collection or with virus different from that of the asymptomatic period, or

Table 1. Distribution of diarrheal virus infection in infants with and without acute gastroenteritis in a day care center (DCC) in Tokyo, Japan, during June 1999 to July 2000

Characteristics of fecal specimen	Number of fecal specimens	Viral positive specimens (%)	Diarrheal virus (%)								
			Mono-infection						Mixed infection		
			Rota	AstV	NV		SV	Ade	Ade/NVGII	Ade/AstV	AstV/NVGII
					GI	GII					
Diarrheal	88	45 (51.1)	0 (0)	14 (15.9)	0 (0)	13 (14.8)	2 (2.3)	11 (12.5)	0 (0)	0 (0)	5 (5.7)
Asymptomatic	833	230 (27.6)	0 (0)	53 (23.0)	0 (0)	46 (20.0)	22 (9.6)	96 (41.7)	4 (0.5)	6 (0.7)	3 (0.4)
Total	921	275 (29.9)	0 (0)	67 (7.3)	0 (0)	59 (6.4)	24 (2.6)	107 (11.6)	4 (0.4)	6 (0.6)	8 (0.9)

Note: Rota, Rotavirus; AstV, Astrovirus; NV, Norovirus, GI, Genogroup I; GII, Genogroup II; SV, Sapovirus; Ade, Adenovirus