Also, hyperimmune rabbit antisera were raised against *E. coli*-expressed GI, GII, and GV N- and C-terminal VP1 in order to examine the cross-reactivities among SaV GI, GII, and GV rVP1 by Western blotting (Fig. 5 and Table 2). We found that the GI antisera cross-reacted with GV rVP1, but that GV antisera did not cross-react with GI rVP1, i.e., there was only a one-way cross-reactivity. Also, GII antisera cross-reacted weakly with GI rVP1, but GI antisera did not cross-react with GII rVP1, i.e., this too was a one-way cross-reactivity. Figure 2 shows that short continuous residues (8–9 amino acids) had 100% homology between VP1 sequences. These short residues may have represented particular target residues for the polyclonal antibodies that were raised against *E. coli*-expressed VP1. A similar result was observed by Yoda et al. [28] with *E. coli*-expressed NoV VP1. Yoda suggested specific conformational epitope(s) or limited continuous epitope(s) in the NoV VP1 residues that allowed for broad cross-reactivity between NoV GI and GII VP1, which generally have a low amino acid homology. The reason(s) for these SaV one-way cross-reactivities have not yet been determined, nor has the significance of the double (and triple) bands by Western blotting (Fig. 5). Comparable double bands are usually detected for NoV rVP1 expression and were found to include N-terminal cleavage products [2, 16]. However, these SaV extra bands have not yet been determined.

Interestingly, GI VLP rabbit antiserum cross-reacted weakly with GV rVP1 but did not cross-react with GII rVP1 by Western blotting (Fig. 5). Farkas et al. [6] showed that the SaV GV VP1 sequence was related more to GI than GII, based on phylogenetic distance analysis. Also, GI and GV strains possess a predicted ORF3 (VP3?), whereas the ORF3 is absent in GII strains. Further investigations are clearly needed to determine the significance of these novel findings.

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Characterizations of Adenovirus Type 41 Isolates from Children with Acute Gastroenteritis in Japan, Vietnam, and Korea

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Genetic and antigenic characterizations of 70 strains of adenovirus type 41 (Ad41), isolated between 1998 and 2001 from children in Japan, Vietnam, and Korea, were done by DNA restriction enzyme (RE) analysis, sequencing analysis, and monoclonal antibody (MAb)-based enzyme-linked immunosorbent assay (ELISA). Eight genome types were observed in the present study, among which D25, D26, D27, and D28 were novel genome types. These eight genome types were divided into two genome-type clusters (GTCs) based on phylogenetic analysis of the hypervariable regions (HVRs) of the hexon. GTC1 includes D1, D25, D26, D27, and D28, and the GTC2 contains D4, D12, and D22. The amino acid homologies among the members within a GTC were 97 to 100%, whereas between the members of different GTCs the homologies were 92 to 94%. The specificity of the GTC classification was confirmed by ELISA with MAb 1F, which was selected by the Ad41 prototype Tak strain. It was found that only the isolates of GTC1 but not of GTC2 reacted with MAb 1F. These results suggest that Ad41 isolates from the three countries should be classified into two subtypes. The accumulation of amino acid mutations located in HVRs of hexon are indicative for the classification of Ad41 subtype.

Adenoviruses are responsible for a wide range of disease symptoms. To date, there are 51 recognized serotypes of human adenovirus that are classified into six subgenera (A to F) based on several antigenic, morphological and molecular criteria. Among them, subgenus F, represented by the two serotypes adenovirus type 40 (Ad40) and Ad41, is associated with diarrhea in children; it is found in 1 to 20% of fecal specimens from children with acute gastroenteritis (3, 9, 12, 15, 22, 35). The two serotypes (Ad40 and Ad41) of subgenus F are termed enteric adenovirus (EAd) because of their tropism for the gastrointestinal tract (10, 32). The subgenus F adenoviruses grow poorly in most cell culture systems, in contrast to other cultivable "nonenteric" adenoviruses, and are therefore also termed "noncultivable" or "fastidious" adenovirus (7, 16).

Earlier surveys had shown that the occurrences of Ad40 and Ad41 are approximately equal (6, 11). However, several studies have recently reported a decrease in the rate of isolation of Ad40 and a concomitant increase in the rate of isolation of Ad41 (5, 8, 25, 34). After 1986, Ad41 infection became dominant over Ad40. Our previous studies confirmed that Ad41 was the prevailing serotype of adenovirus associated with acute diarrhea among children also in Japan, Vietnam, and Korea (19). The change in prevalence of Ad41 might have been caused by an antigenic drift, thus increasing the incidence of infection in susceptible individuals (3). Therefore, it is important to characterize these Ad41 strains. In the last decade, there were relatively few comprehensive epidemiological studies of subgenus F adenoviruses from children with diarrhea in Asian countries. The objectives of the present study were (i) to describe the genome types of Ad41 isolates by DNA restriction endonucleases (REs) analysis in Japan, Vietnam, and Korea and (ii) to describe the genetic and antigenic characterization of different genome types of Ad41 isolates in these three countries.

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MATERIALS AND METHODS

Fecal specimens. A total of 3,577 fecal specimens were collected from children between 1 month to 15 years of age with acute diarrhea from Japan, Vietnam, and Korea. These specimens included 1,991 specimens from Tokyo, Osaka, Saga, Maizuru, and Sapporo in Japan collected between July 1998 and June 2001; 1,355 specimens from Ho Chi Minh City in Vietnam collected between December 1999 and November 2000; and 231 specimens from Seoul in Korea collected between January 1998 and July 1999. By using polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (24) and the combination of PCR and restriction fragment length polymorphism methods (31), adenoviruses were detected and serotyped. A total of 158 adenovirus-positive specimens were identified. Of these specimens, 101 specimens were typed as Ad41, the most common serotype, followed by Ad40 (12 isolates), Ad8 (12 isolates), Ad2 (10 isolates), Ad3 (10 isolates), Ad5 (8 isolates), and Ad31 (1 isolate). Four specimens remained untypeable. These Ad41 specimens were used for analysis in the present study.

DNA RE analysis. ***(i) Virus isolation.*** Ad41 prototype strain Tak was obtained from the National Institute of Infectious Diseases of Japan. Ad41 specimens were isolated and propagated in Caco-2 cells (human colonic carcinoma cells). The inoculation was performed as previously described (26). The cultured fluids were harvested after infection at the time of maximal cytopathic effect (CPE).

(ii) Extraction of viral DNA. A total of 8 ml of the cultured fluids were centrifuged at 1,353 × *g* for 30 min, and the cells were pelleted. In order to avoid mixing of cellular nucleic acid in the subsequent extraction of viral DNA, ultracentrifugation of the cultured supernatant was performed (40,700 × *g* for 3 h).

TABLE 1. Oligonucleotide primers for PCR sequencing

Primer	Polarity	Sequence (5'-3')	Position[a]
HX5-3	+	cac atc gcc gga cag gat gct tcg gag ta	40–68
HX3-4	−	gtg ttg tga gcc atg ggg aag aag gtg gc	1819–1847
S-29	+	gcc agc acr twc ttt gac at	289–308
S-52	−	ccc atg ttg cca gtg ctg ttg tar tac a	986–1013
S-51	+	ccc aac aga ccc aay tac	937–956
S-53	−	aag ggg ttg acg ttg tcc at	1555–1574

[a] The sequence positions are based on the Ad3 hexon region.

After centrifugation, the supernatant was removed, and the viral pellet was obtained. Subsequent extraction was conducted by a modified Hirt method (44).

(iii) Digestion of DNA by RE. Approximately 1 μg of viral DNA was digested with 10 U of the REs BamHI, BglI, BstEII, EcoRI, HindIII, KpnI, PstI, SacI, SmaI, and XhoI (TaKaRa Biotechnology Co., Ltd.) according to the manufacturer's instructions. After digestion, reaction products were loaded onto 1.0% agarose gel containing ethidium bromide (0.5 μg/ml) and run at 50 V for 5 h in Tris-acetate (TAE) buffer. The gel was photographed under UV light. The genome typing system proposed by Adrian et al. (1) was used as the basis for our study. By comparing the resulting patterns with the published restriction profiles of prototype strains, genome type identifications could be achieved.

PCR sequencing analysis of HVRs. In order to understand the genetic relationships between different genome types of Ad41 strains isolated from the three countries in the present study, eight Ad41 representing strains were randomly selected from eight genome types, and their nucleotide sequences of hypervariable regions (HVRs) were determined.

Viral DNA diluted 1:100 was used as a template DNA for PCR. The primers were designed according to the method of Takeuchi et al. (37). In the first PCR, a pair of primers, HX5-3 and HX3-4, was used. The PCR product of 1,800 bp contained all seven HVRs. The second PCR was conducted by using the first PCR product as a template with a combination of the following two pairs of primers: S-29 and S-52 or S-51 and S-53. The primer pairs used are listed in Table 1. The cycling conditions of the PCR were modified in 35 cycles (94°C for 1 min, annealing at 45°C for 2 min, and primer extension at 72°C for 3 min, with a final product extension at 72°C for 7 min). The second PCR products were extracted from 1% agarose gel by using phenol and chloroform. The purified DNA was used to perform enzymatic extension reaction for DNA sequencing with a DNA sequencing kit (BigDye Terminate v3.0 cycle sequencing ready reaction; ABI Prism; Applied Biosystems, Warrington, Great Britain). After the reaction products were cleaned, samples were analyzed by autosequencer (ABI Prism 310 genetic analyzer). Evaluation of all sequence data and analysis of identity were conducted by using DNASIS software (version 1994; Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The sequence alignments of nucleotide and deduced amino acid were carried out by DDBJ (http://www.ddbj.nig.ac.jp) by using the CLUSTAL W program. The phylogenetic tree was constructed with MEGA version 2.1 (18) by using the neighbor-joining method and the bootstrap test (http://www.megasoftware.net/).

Nucleotide sequence accession numbers. Sequence data from the present study were entered in the GenBank/EMBL/DDBJ database under the following accession numbers: Ad41-D1-VN47 (AB103341), Ad41-D4-VN28 (AB103342), Ad41-D12-JP3171 (AB103343), Ad41-D22-Km079 (AB103344), Ad41-D26-JP3106 (AB103345), Ad41-D27-JP2149 (AB103346), Ad41-D25-Ks35 (AB103347), and Ad41-D28-VN1020 (AB103348).

ELISA based on MAb. ELISA based on the use of monoclonal antibodies (MAbs) was utilized to examine the antigen specificity of Ad41 strains. ELISA was performed as described by Nishio et al. (24). Anti-Ad40 rabbit serum was used as capture antibody. The MAbs 15D (adenovirus group-specific), 12D (Ad40 type specific), and 1F (Ad41 type specific) were used as detector antibodies. In each test, cell culture supernatants of Ad41 prototype strain were included as positive controls, and phosphate-buffered saline was used as a negative control. If the optical density value was >0.2, as well as 2-fold greater than the negative control well, the specimen was considered positive.

RESULTS

Genome types of Ad41 isolates from three countries. Seventy Ad41 fecal specimens were isolated successfully, and RE analyses were performed with 10 different REs: BamHI, BglI, BstEII, EcoRI, HindIII, KpnI, PstI, SacI, SmaI, and XhoI. To confirm the genome type, restriction patterns were compared to that of the Ad41 prototype Tak strain. The results of enzyme code and genome type determinations obtained in the present study are shown in Table 2. New restriction patterns were observed in cleavage with enzyme EcoRI, KpnI, PstI, SacI, and SmaI. According to the nomenclature system, the new patterns were named as follows: code 7 of EcoRI, code 4 of KpnI, code 4 of PstI, code 3 of SacI, and code 9 of SmaI (Fig. 1a to e).

Analysis of 70 Ad41 isolates from three countries revealed the existence of eight genome types in the present study. Of these genome types, four (D1, D4, D12, and D22) were previously reported by van der Avoort et al. (43). In addition, four novel genome types D25, D26, D27, and D28 are reported here for the first time. The photographs of representative strains of these novel genome types cleaved by the panel endonucleases are shown in Fig. 2. They accounted for 61% (43 of 70) of all Ad41 isolates. The novel genome type D26 had enzyme code 2212141341 and was closely related to another novel genome type D25, differing only in cleavage with BamHI.

In Japan, the novel genome type D26 predominates with 26 isolates, followed by D12 and D27 with 17 and 4 isolates, respectively. In 3 years, D26 and D12 were observed every

TABLE 2. Genome types of Ad41 isolates in Japan, Vietnam, and Korea

Country	Genome type[b]	Enzyme code[a]									Total no. of isolates
		BamHI	BglI	BstEII	EcoRI	HindIII	KpnI	PstI	SacI	SmaI	XhoI	
Japan	**D26**	2	2	1	2	1	**4**	1	**3**	4	2	26
	D12	2	1	2	1	5	1	1	1	2	1	17
	D27	2	2	1	7	1	1	1	1	4	2	4
Vietnam	**D28**	2	1	4	4	4	2	4	1	**9**	1	10
	D4	3	1	1	1	3	1	1	1	4	1	4
	D1	1	1	1	1	1	1	1	1	1	1	2
Korea	D22	2	1	2	1	5	1	3	1	2	1	4
	D25	1	2	1	2	1	**4**	1	**3**	4	2	1
	D26	2	2	1	2	1	**4**	1	**3**	4	2	2

[a] The boldface numbers indicate a novel pattern.
[b] Novel genome types are indicated in boldface.

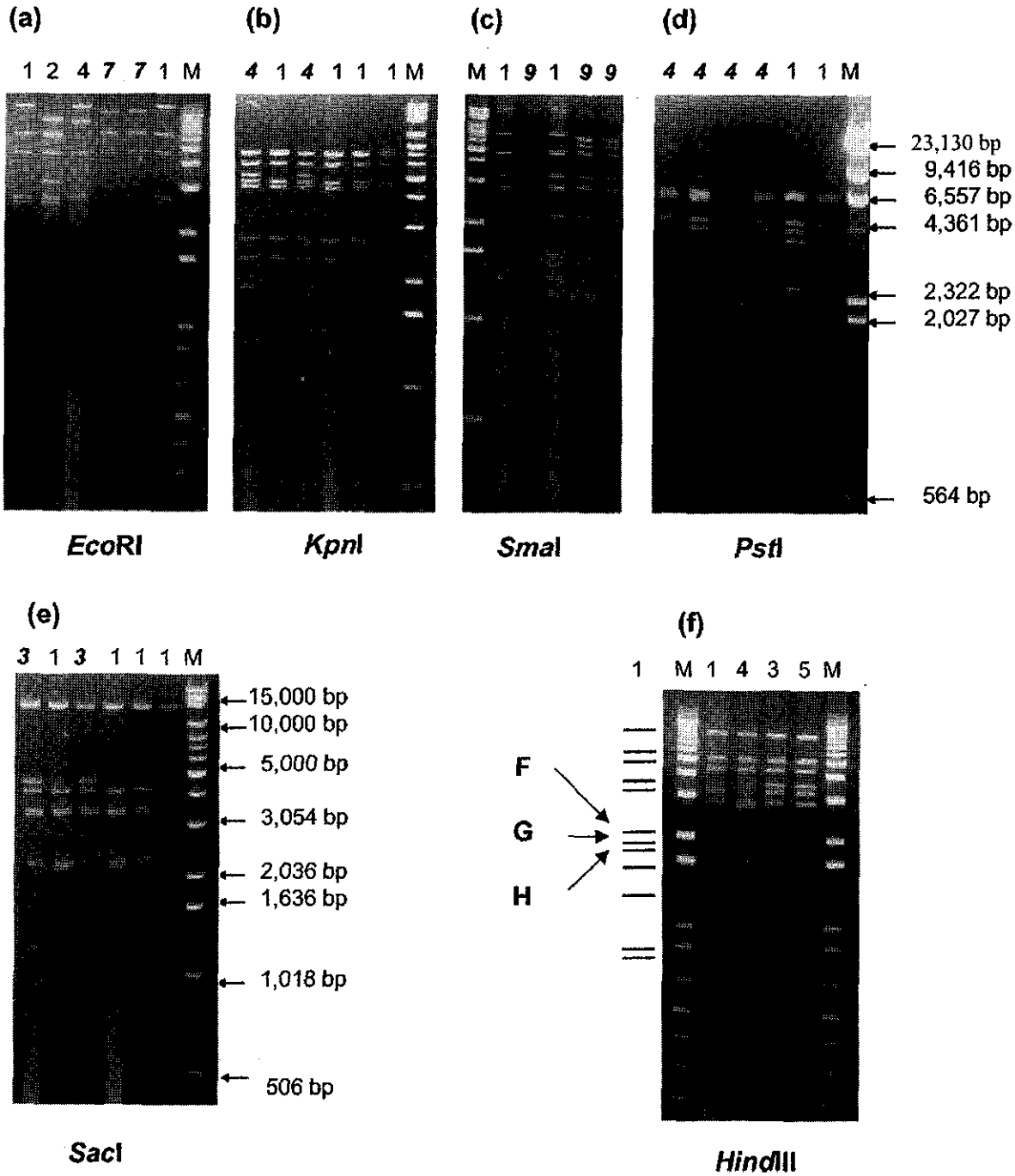

FIG. 1. Novel restriction patterns with EcoRI (a), KpnI (b), PstI (d), SacI (e), and SmaI (c). The numbers above the lanes are the enzyme code; lane M contains molecular weight standards (1-kb DNA extension ladder; Life Technologies), except for lane M of PstI. Lane M of PstI contains molecular weight standards (Marker II, λDNA HindIII digested; TaKaRa). Enzyme code "1" refers to the pattern of the prototype. The boldface italic numbers mark the novel patterns. (f) Restriction patterns with HindIII observed in the present study. The numbers above the lanes are the enzyme code; lane M contains molecular weight standards. The marker lines on the left give the model pattern of enzyme code 1. The arrowed letters F, G, and H refer to the names of the fragments according to the physical map given by Takiff et al. (40). The patterns of codes 3 and 5 lack fragment F.

year. From July 1999 to June 2000, the new genome type D27 appeared, but it was not found the next year. In Vietnam, D28, represented by 10 isolates, was the most common. D4 and D1 were observed in 4 and 2 isolates, respectively. In Korea, D22 predominated with four isolates. Interestingly, the novel genome type D26 was observed in Korea as well. It was present in two isolates, whereas only one isolate of novel genome type D25 was detected.

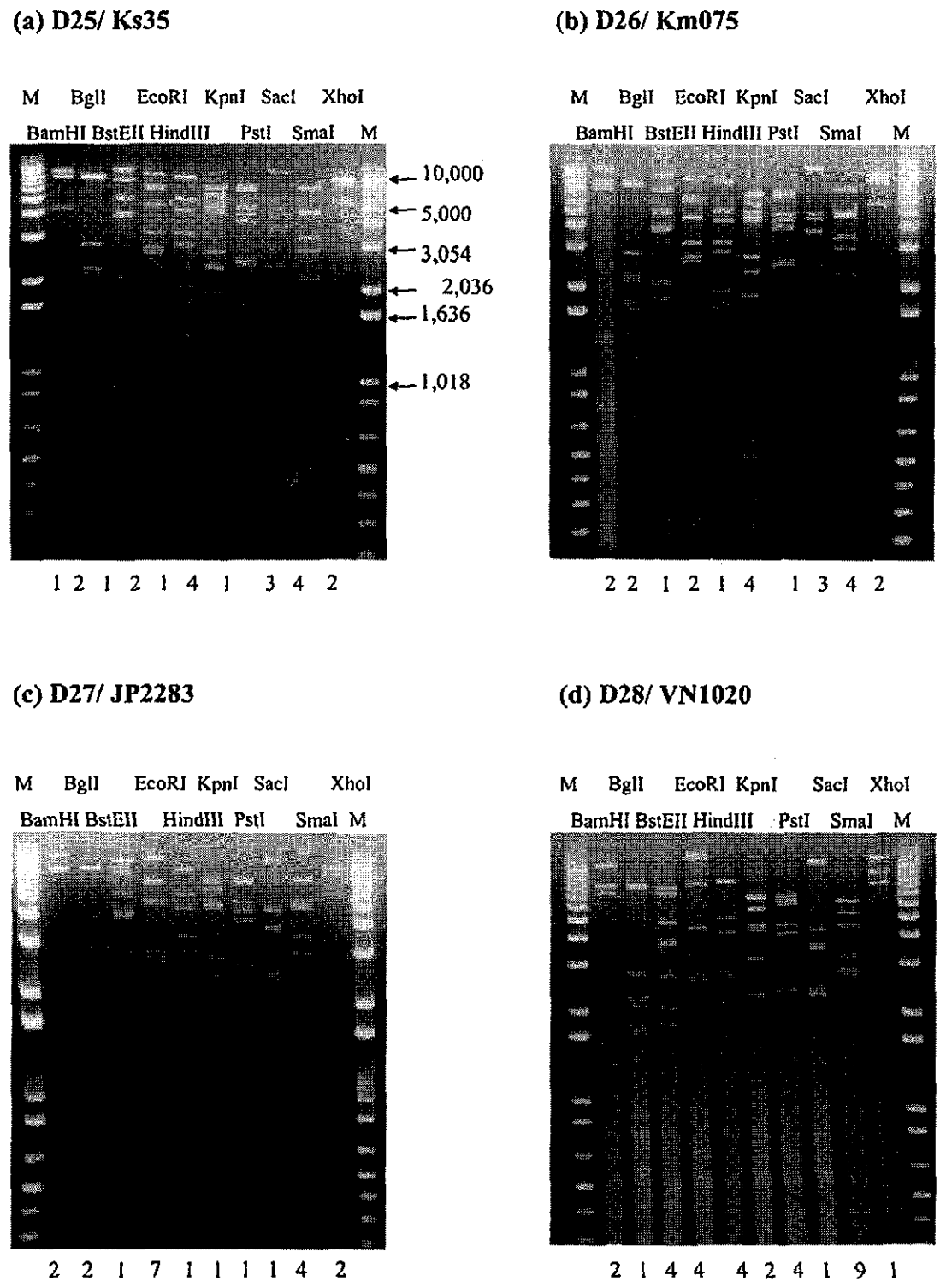

FIG. 2. (a to d) Restriction profiles of novel Ad41 genome type strains isolated from Japan, Vietnam, and Korea, obtained in the present study. (a) D25/Ks35; (b) D26/Km075; (c) D27/JP2283; (d) D28/VN1020. The numbers under the lanes are the enzyme code; lane M contains molecular weight standards.

Genetic analyses of HVRs for eight genome types of Ad41. The HVRs of eight representative strains from eight genome types obtained in the present study were sequenced. The DNA sequences, containing seven hexon HVRs, with a total of 1,232 to 1,235 nucleotides, from the eight strains were determined. Alignments of the predicted amino acid sequences were performed. A phylogenetic tree based on the deduced amino acid sequences of eight representing genome types was subse-

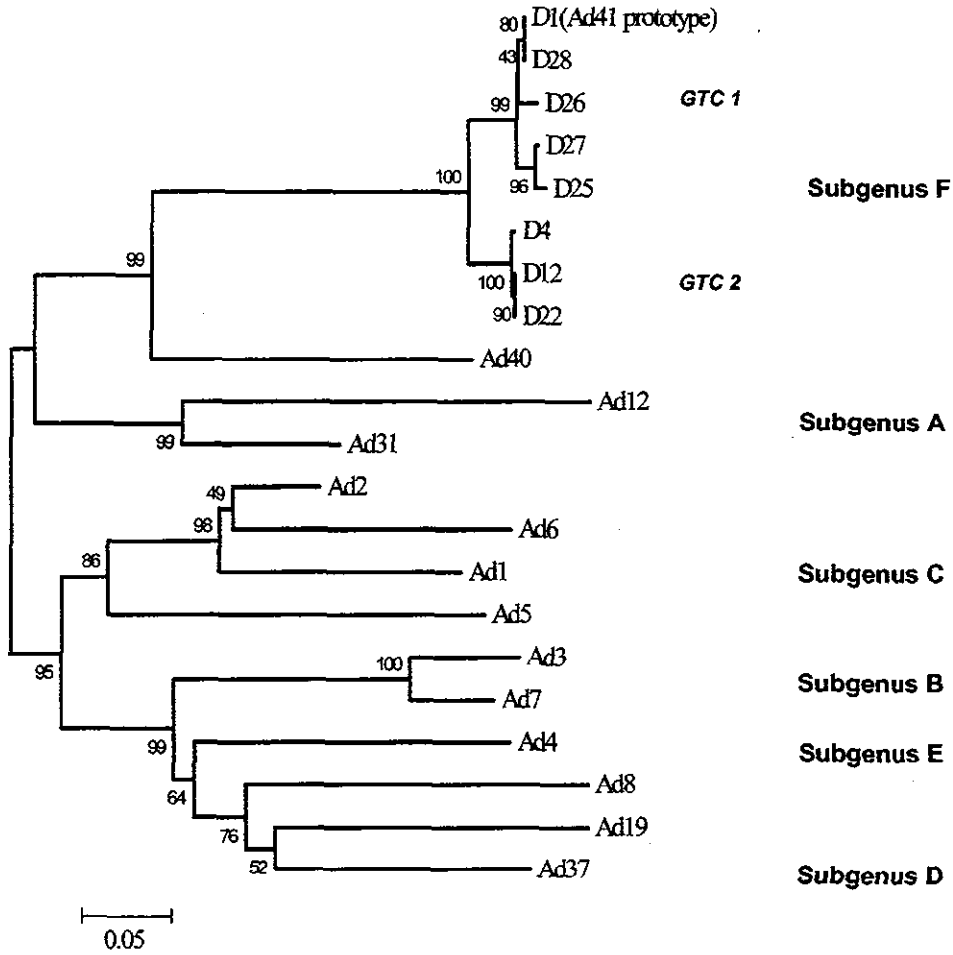

FIG. 3. Phylogenetic tree based on the deduced amino acid sequences of HVRs of Ad reference strains and Ad41 strains isolated from Japan, Vietnam, and Korea. Two GTCs, GTC1 and GTC2, are shown. The sequences of reference strains previously reported are listed in GenBank under the indicated accession numbers: Ad12 (X73487) (36), Ad31 (74661) (29), Ad1 (X67709) (28), Ad2 (J01917) (2), Ad5 (M73260) (17), Ad6 (X67710) (28), Ad3 (X76549) (30), Ad7 (Z48571) (21), Ad4 (X84646) (30), Ad8 (X74663) (29), Ad19 (X98359) (43), Ad37 (X98360) (43), Ad40 (X51782) (42), and Ad41 (X51783) (41).

quently constructed (Fig. 3). It revealed that in the eight genome types representing strains, two genome type clusters (GTCs) are present. GTC1 includes D1 (Ad41 prototype Tak strain), D28, D26, D27, and D25, and GTC2 consists of D4, D12, and D22. The amino acid homology rates in all seven HVRs among the eight representative strains are shown in Table 3. The highest amino acid homologies were observed within a GTC (97 to 100%). Between the members of different GTCs, the homologies were 92 to 94%. The alignments of HVRs predicted amino acid sequences are shown in Fig. 4. The same substitutions in amino acid (aa) 54 (HVR1), aa 74 (HVR2), aa 100 to 101 (HVR3), aa 133 (HVR4), aa 155 to 156, aa 160 (HVR5), aa 190 (HVR6), aa 314 to 316, and aa 323 (HVR7) were presented between GTC1 and GTC2 strains. The members of GTC2 have a common deletion in aa 73 and an insertion in aa 320.

Antigen specificity of Ad41 isolates. MAbs were used to examine the antigen specificity in all Ad41 isolates belonging to eight different genome types. All of these isolates reacted with MAb 15D; none of them reacted with MAb 12D. On the other hand, two antigenic subtypes among eight genome types were distinguished on the basis of the specificity of the MAb 1F selected by Ad41 prototype Tak strain. The isolates that belonged to genome types D1, D28, D26, D27, and D25, were recognized by MAb 1F, but the mem-

TABLE 3. Homology comparison of HVRs between Ad41 isolates and prototype strains Ad40 and Ad41

HVR	% Homology[a] with HVR:								
	Ad40	D1	D26	D27	D28	D25	D4	D12	D22
Ad40 (prototype)		72	72	72	72	72	72	72	72
D1 (Ad41 prototype)	68		98	98	100	98	94	95	95
D26 (JP3106)	68	98		98	98	98	94	94	94
D27 (JP2149)	67	98	99		98	98	94	94	94
D28 (VN1020)	68	100	98	98		98	94	95	95
D25 (Ks35)	67	98	97	99	98		94	94	94
D4 (VN28)	66	94	93	93	94	92		99	99
D12 (JP3171)	66	94	93	93	94	93	99		99
D22 (Km079)	66	94	93	93	94	93	99	100	

[a] The upper right data are the percent homologies of the nucleotide sequences; the lower left data are the percent homologies of the amino acid sequences.

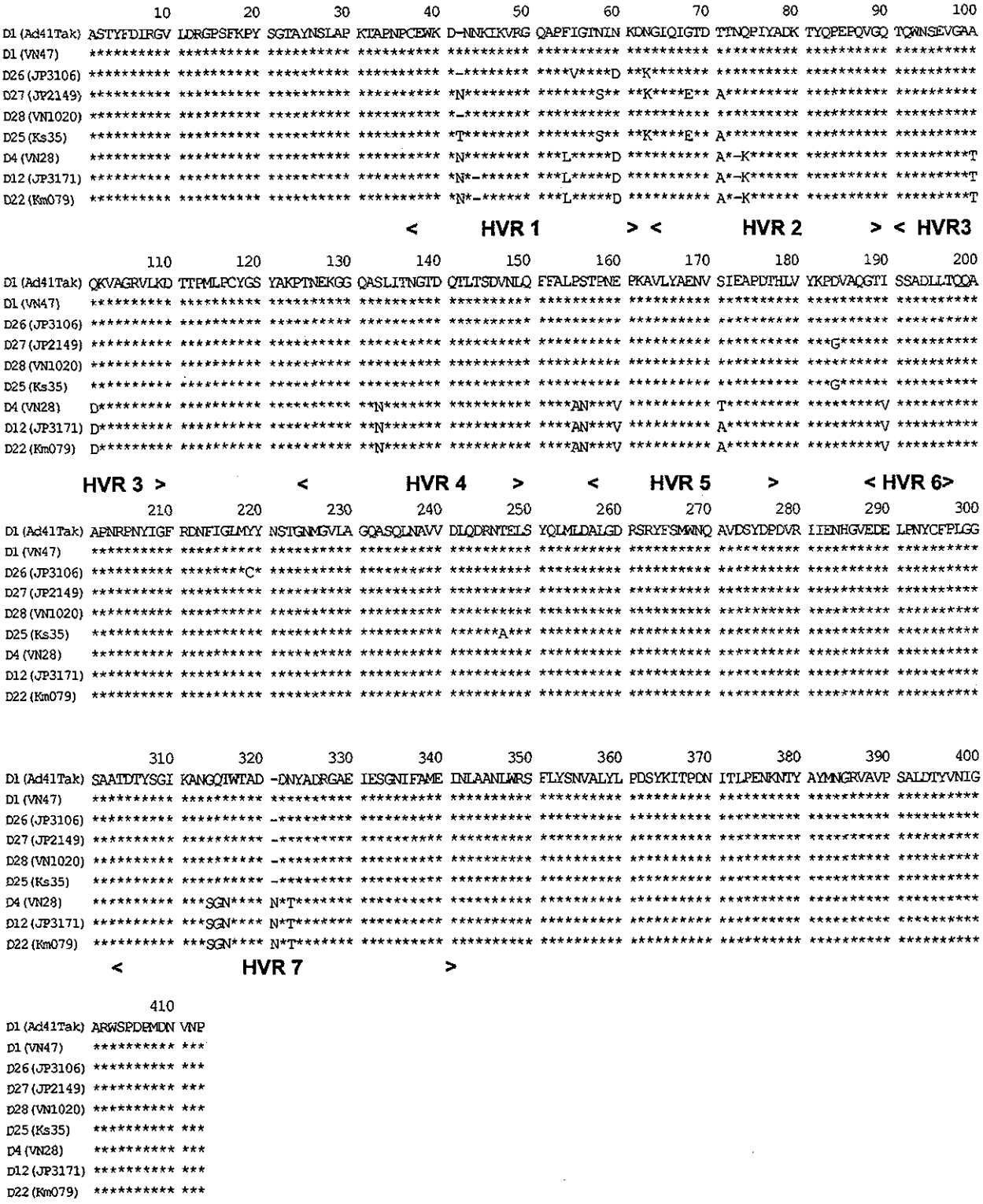

FIG. 4. Alignments of the deduced amino acid sequences of hexon, including seven HVRs from eight Ad41 genome type strains. The regions of seven HVRs are given. The borders of the seven HVRs are in accordance with the literature (37). Asterisks indicate the identical residues to the sequence of D1.

bers of D4, D12, and D22 could not be distinguished with the same MAb 1F.

DISCUSSION

In this study, the genome types of Ad41 strains isolated from fecal specimens in Japan, Vietnam, and Korea were determined. In addition, the genetic and antigenic characterizations are given.

EAd are known as fastidious adenoviruses since they do not grow well in conventional cell lines. There are varied reports in the literature concerning the ability of EAd to replicate in different cell lines, such as Chang conjunctival cells, Hep-2 cells, 293 cells, and PLC/PRF/5 cells. The 293 cells, an Ad5-transformed HEK cell line that contains and expresses the Ad5 early region E1A and E1B, were considered useful for the growth of many Ad41 strains (40), but infection yields are still low. Recently, Pinto et al. (27) have reported the use of Caco-2 cell line in isolating laboratory strains of human enteric viruses, including the group A rotavirus type 3, astrovirus serotype 1, and Ad5, Ad40, and Ad41. In their study, for EAd, apparent CPE was observed with laboratory strains, whereas wild-type Ad40 and Ad41 strains failed to induce CPE in Caco-2 cells, though the amplification of viral nucleic acid was confirmed by dot blot hybridization. In our study, Caco-2 cells were used to propagate EAd from fecal specimens. Seventy Ad41 and ten Ad40 (unpublished data) strains from clinical specimens were successfully isolated. The present study confirmed that Caco-2 cells are a useful cell line for research on diarrheal viruses.

Among these genome types observed in Japan, Vietnam, and Korea, four novel genome types (D25, D26, D27, and D28) were observed for the first time, and the other four genome types (D1, D4, D12 and D22) were reported in earlier studies (13, 14, 43). Genome type D12 was present in 17 isolates in Japan over a period of 3 years. This genome type was first isolated in 1982 from The Netherlands and was highly prevalent in the 1980s (43). In Sweden, an identical D12 was also reported, specimens of which were mainly obtained from an outbreak of gastroenteritis in a long-term pediatric ward at a hospital of Stockholm in 1988; it was then found sporadically for several years afterward (13). Another genome type D22 observed in Korea was first isolated during 1984 in The Netherlands, too (43). In the present study, genome type D4 identified in Ho Chi Minh City, Vietnam, was also first found in Malaysia in 1979 (14). These phenomena may indicate that genome types of Ad41 have a scattered global distribution. It is quite possible that the novel genome types observed in the present study are also present in Europe and other continents. Another possibility is that, because of limited research work these novel genome types were not detected.

In studying the profiles of Ad41 isolates obtained with 10 restriction enzymes in three countries, it was found that almost all restriction patterns that appeared in Korean strains were also present in Japanese strains and, in contrast, Vietnamese isolates always exhibited different patterns. For example, Japanese and Korean Ad41 isolates have the patterns 1 and 5 upon cleavage with enzyme HindIII, but Vietnamese strains showed patterns 3 and 4, along with pattern 1. In addition, novel genome type D26 was observed in both Japan and Korea. These data might represent a gradient of antigenic drift reflecting increased travel activities between the two countries.

The occurrences of novel genome types reflect the proceeding mutation of viral DNA. Hexons are important capsid proteins of adenovirus. It was reported that seven HVRs located in hexons take part in the neutralization reaction and contain the epitopes of serotype determination (4). It is interesting to estimate what role these regions might have in identical adenovirus serotypes. Previous studies revealed that hexon contains HVRs that are conserved in different genome types of Ad3 and Ad7 belonging to the subgenus B (20, 38). In the present study, the sequence analyses of HVRs of different genome types of Ad41 isolates from three countries also demonstrated a high level of conservation. The homologies ranged from 92 to 100% and the eight genome types of Ad41 were divided into two clusters according to the predicted amino acid sequences of HVRs. The classification of GTCs had a curious coincidence with the result of ELISA with MAb 1F. It has been confirmed that the MAb 1F may react with hexon protein of Ad41 (24). Therefore, the results presented here suggest that HVRs of hexons may contain epitopes that are associated with the Ad41 subtype.

In comparing the ten enzyme codes, one difference can be found in cleavage with HindIII between GTC1 and GTC2. The members of GTC1 have the HindIII code 1 and 4; the members of GTC2 have codes 3 and 5. Physical map of Ad41 was published by Takiff et al. (39). Tak strain HindIII fragments F and H were determined to lie between map units 51 and 60, which is within the confines of the hexon gene. van der Avoort et al. (43) revealed that D4, D12, and D22 could not react with MAbs 1-23, 3-10, 3-18, and 7-14 and lacked fragment F in common (Fig. 1f). However, in the present study, the nucleotide sequence analysis of hexon-containing HVRs confirmed that the guanine-for-adenine substitution in nucleotide 696, which leads to failure of HindIII-cut viral DNA of GTC2 isolates, was a conservative substitution.

Recently, ELISA with MAbs specifically reacting with Ad40 and Ad41 is widely used for the diagnosis of EAd in fecal specimens. It is a rapid, readily available means for identification of Ad40 and Ad41. MAb 1F was used successfully for screening Ad41 in clinical specimens (24). In the present study, the Ad41 isolates belonging to genome types D4, D12, and D22 did not react with MAb 1F. The application limitation of MAbs was reported in earlier studies (5, 23, 33, 43). Due to virus strain mutation, the MAb may be safely used for screening purposes only temporarily. The genetic variation within one type of adenovirus can evidently affect the diagnosis capability of highly specific MAbs. It has been reported that a commercial MAb-based ELISA could not demonstrate the presence of a highly prevalent genome type of Ad41 in Canada (33). In South Africa, the locally circulating adenoviruses were not detected with a commercial kit utilizing MAbs directed against type-specific epitopes on the adenovirus hexon (23). For Japanese specimens, MAb 1F distinguished all of the Ad41 strains isolated from 1982 to 1988 (24) but failed to react with some Ad41 isolates in 1998 to 2001. These results also confirmed that antigenic drift on the surface of adenovirus occurred frequently. Hexon and fiber compose most of the external capsid of adenovirus. It may be difficult to find other, better targets for reagent antibodies except the hexon and

fiber. The use of pooled MAbs to hexon may provide a practicable way to avoid omissions in detection such as the ones described above when a single MAb was used.

In conclusion, eight genome types of Ad41 were found in Japan, Vietnam, and Korea. Four novel genome types were investigated for the first time in the present study. The existence of two GTCs and the antigenic difference between them may reflect the accumulation of amino acid mutations located in HVRs of the hexon. These amino acid mutations may affect classification of the Ad41 subtype. These data contribute to our understanding of the molecular epidemiological characterization of adenoviruses in these countries. Further studies are needed to obtain additional established strains of the Ad41 genome types in order to clarify the genetic and antigenic relationships between them.

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Virus Diversity and An Outbreak of Group C Rotavirus Among Infants and Children With Diarrhea in Maizuru City, Japan During 2002–2003

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A total of 236 fecal specimens collected from infants and children with gastroenteritis in Maizuru city, Japan from July 2002 to June 2003, were tested for the presence of rotaviruses, noroviruses, sapoviruses, astroviruses, and adenoviruses by RT-PCR, PAGE, RPHA, and latex agglutination methods. Among diarrheal viruses detected, group A rotavirus was the most prevalent (32.2%; 76 of 236) followed by norovirus GII (21.2%; 50 of 236), group C rotavirus (10.2%; 24 of 236), adenovirus (3.8%; 9 of 236), sapovirus (2.5%; 6 of 236), astrovirus (1.3%; 3 of 236), and norovirus GI (0.8%; 2 of 236), respectively. It is noteworthy that group C rotavirus infection was apparently confined only within the period of 5 months (December 2002 through April 2003). This pattern of infection implied that the outbreak of group C rotavirus in these patients, which was the first outbreak of gastroenteritis attributed to group C rotavirus in Maizuru city. Moreover, about half (12 of 24) of group C rotavirus infected cases were confined to infants and young children less than 3 years old. Another interesting feature of the study was the demonstration of the mixed infections with group C rotavirus and group A rotavirus, as well as group C rotavirus and norovirus GII in 20.8% (5 of 24) and 8.3% (2 of 24), respectively. This is the first report of gastroenteritis associated with the mixed infections with group C rotavirus and other viral enteropathogens such as norovirus. The results indicate that group C rotavirus could infect not only older children and adults but also infants and young children under 3 years old. ***J. Med. Virol. 74:173–179, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: multiplex PCR; genotype; enteropathogen

INTRODUCTION

Acute gastroenteritis is a major cause of morbidity and mortality among infants and young children in both developed and developing countries. Among other enteropathogenic viruses, rotaviruses are recognized as the major etiologic agents of gastroenteritis in children and young animals. Rotaviruses are classified into seven groups (A–G) on the basis of distinct antigenic and genetic properties [Bridger, 1994; Saif and Jiang, 1994]. Human infection has been reported with group A, B, and C rotaviruses. Of these, group A rotaviruses are the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [Kapikian et al., 2001]. Group B rotaviruses have been found primarily in China, where they have caused major outbreaks of severe gastroenteritis predominantly involving adults but also with mild diarrhea in children and neonates [Hung et al., 1984; Wang et al., 1985].

Group C rotavirus was first identified in the feces of young pigs with diarrhea in 1980 [Saif et al., 1980] and later was confirmed as a human pathogen by Bridger et al. [1986]. In fact, group C rotavirus infection in humans has been reported both in developed and developing countries [Rodger et al., 1982; Espejo et al., 1984; Brown et al., 1988; Chen et al., 1988; Jiang et al., 1995].

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Acute gastroenteritis associated with group C rotavirus was initially found in Sweden and then emerged in several parts of the world such as England and Malaysia [Bridger et al., 1986; Rasool et al., 1994; James et al., 1997]. In addition, the group C rotavirus has been reported in South Africa as sporadic diarrheal cases and a family diarrheal outbreak [Steele and James, 1999].

Group C rotavirus infection in Japan was first detected in Matsuyama city by Oseto et al. [1986] and thereafter Ushijima et al. [1989] reported the detection of this virus in Tokyo. Since then, group C rotavirus was found in other regions of Japan such as Chiba, Okayama, and Saga [Kuzuya et al., 1998]. The positive rates of group C rotavirus infection in Japan in sporadic cases and outbreaks of acute gastroenteritis ranged from 2.7 to 13.3%, which were considerably lower than those of group A rotavirus infection [Kuzuya et al., 1998].

The objective of this study was to determine the incidence of group C rotavirus infection in infants and young children with acute gastroenteritis in Maizuru city, Japan. Additionally, the age-related, and geographical distribution, as well as seasonal pattern of group C rotavirus infections are also described.

MATERIALS AND METHODS

Fecal Specimens

Two hundreds thirty six fecal specimens were collected from infants and children with acute gastroenteritis in Maizuru city, Japan, during the period of July 2002 to 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 diarrheal viruses.

Extraction of Viral Genomes

The viral genomes were extracted from 10% fecal suspensions using a spin column technique according to the manufacturer's instructions (Qiagen®, Hilden, Germany)

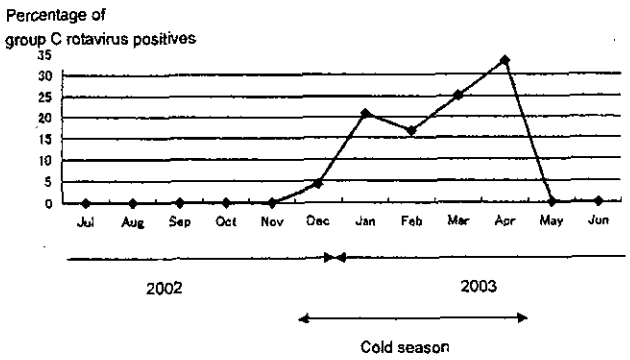

Fig. 1. Monthly distributions of group C rotavirus infection in infants and young children in Maizuru city, Japan. The group C rotavirus infected cases were apparently confined to a period of 5 months (December 2002–April 2003). The period of cold season was also indicated.

Multiplex RT-PCR

The first group of viruses including human astrovirus, norovirus (GI, GII), and sapovirus and the second group including Group A, B, and C rotaviruses and adenovirus were detected by RT-PCR. The identification of the first group of viruses was performed by using multiplex RT-PCR with specific primers as reported previously [Yan et al., 2003]. 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 [Gouvea et al., 1990; Ushijima et al., 1992; Xu et al., 2000; Kobayashi et al., 2001; Kuzuya et al., 2003]. These primers were specifically generated four different sizes 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 of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C.

Reverse Passive Hemagglutination Test

All fecal specimens positive for group C rotavirus by multiplex PCR were screened further for group C rotavirus by another method of different principle, a reverse passive hemagglutination (RPHA) test. The techniques were carried out according to the manufacturer's instructions (Denka Seiken Co., Ltd., Japan). Fixed sensitized red blood cell with purified anti-group C rotavirus antibody can be agglutinated with group C rotavirus in fecal supernatants. Based on this principle, fecal specimens were reacted with sensitized red blood cell in microplates. Hemagglutination titers were observed after 2 hr.

Latex Agglutination Test

The Rotalex test, a commercial latex agglutination test, was used as a confirmation test for the detection of group A rotaviruses. Fecal samples were processed according to the instruction provided in the Rotalex kit insert (Daiich Kagaku Co., Ltd., Japan). A drop of the fecal supernatant was mixed with a drop of test latex on a slide, and reaction was observed after 2 min. Development of distinct agglutination in the Rotalex reagent was treated as positive. If agglutination was seen in the negative control latex, the test was considered uninterpretable.

Polyacrylamide Gel Electrophoresis

Electropherotyping of viral RNA was carried out in 10% polyacrylamide gels (PAGE), and silver staining was performed as previously described [Theil et al., 1981].

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for group C rotavirus and norovirus GII 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). The sequence data of group C rotavirus and norovirus strains detected in this study are freely available from the corresponding author. Reference strains and accession numbers used in this study were as follows: OT-99 (AB086969), KW290 (AB086967), KW408 (AB086968), S-1 (U20995), E93 (U20992), Santiago (U20996), Fuan (U20987), Uppsala/1004 (AF225560), Belem (X77256), A93M (AY392447), Preston (X77258), Bristol (X77257), Cowden (M61101), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Girllington (AJ277606), Hillington (AJ277607), Toronto (U02030), Leeds (AJ277608), Lordsdale (X86557), Seacroft (AJ277620).

RESULTS

Detection of Diarrheal Viruses

A total of 236 fecal specimens were collected from infants and children with acute gastroenteritis in Maizuru city, Japan, during the period of July 2002 to June 2003. The youngest age was 2 months, the highest was 14 years, and the average age was 2.7 years (32 months). Among all children with acute gastroenteritis, 72% were aged less than 36 months. Moreover, the number of male (57%) was higher than that of female (43%). All fecal specimens were tested for the presence of rotaviruses, noroviruses, sapoviruses, astroviruses, and adenoviruses by multiplex PCR, PAGE, RPHA, and latex agglutination methods. The results shown in Table I revealed that diarrheal viruses were detected in 152 out of 236 (64.4%) specimens tested. Among diarrheal viruses detected in this study, group A rotavirus was the most prevalent (32.2%) followed by 21.2% of norovirus genogroup II (NV GII), 10.2% of group C rotavirus, 3.8% of adenovirus, 2.5% of sapovirus, 1.3% of astrovirus, and 0.8% of norovirus genogroup I (NV GI), respectively. No group B rotavirus was found in these patients.

Outbreak of Group C Rotavirus

The cases positive for group C rotavirus by RT-PCR (24 of 236; 10.2%) were tested further to confirm the presence of group C rotavirus by RPHA and PAGE, and to confirm the presence of group A rotavirus by a commercial latex agglutination test (Rotalex Kit). The results shown in Table II revealed that although the fecal specimens were collected over a period of 12 months (July 2002 to June 2003) the group C rotavirus infection was apparently confined within a period of five months (December 2002 through April 2003) (Fig. 1). This pattern of infection indicated an outbreak of group C rotavirus in these patients and this would be the first outbreak of acute gastroenteritis attributed to group C rotavirus in Maizuru city. In addition, about half (12 of 24) of group C rotavirus infected cases were confined to infants and young children under 3 years old. This observation demonstrated that group C rotavirus infection occurred not only in adults and elder children but also in infants and young children under 3 years old.

Another interesting feature of this study was the demonstration of mixed infections with group C rotavirus and group A rotavirus, and group C rotavirus and norovirus GII. There were five cases of mixed infection with group C rotavirus and group A rotavirus (patient no. 6, 9, 14, 21, and 22) as determined by multiplex PCR.

The mixed infections have been confirmed by showing typical electrophoretic patterns of group A (known as 4-2-3-2 profile) and group C rotaviruses (known as 4-3-2-2 profile) simultaneously in the same samples of three patients (patient no. 6, 21, and 22). However, the other two patients (patient no. 9 and 14) only typical electrophoretic pattern of group A rotavirus but not group C rotavirus were demonstrated. The group A rotavirus co-infection in these patients (no. 6, 9, 14, 21, and 22) was confirmed by Rotalex test. Furthermore, there were two cases of mixed infection between group C rotavirus and norovirus GII (patient no. 1 and 5), which had also been detected by multiplex PCR.

Nucleotide Sequence and Phylogenetic Analyses of Group C Rotavirus Isolates

The PCR products of their *VP7* gene were sequenced in order to characterize further the genetic relationship among the 24 isolates of group C rotavirus detected in infants and children in Maizuru city. Their partial nucleotide sequences were compared to each other as well as to those of reference strains available in the GenBank database by BLAST. It was found that the nucleotide sequences among 24 isolates were of high identity ranging from 98 to 100% (data not shown). A phylogenetic tree of the nucleotide sequences of these isolates and the reference strains was constructed (Fig. 2) and all of 24 group C rotavirus isolates formed a cluster with the OT-99 reference strain isolated in an

TABLE I. Distribution of Diarrheal Virus Infection in Infants and Children With Acute Gastroenteritis in Maizuru City, Japan During July 2002 to June 2003

Number of specimen tested	Diarrheal viruses (%)							
	Rotavirus			Norovirus				
	A	B	C	I	II	Sapovirus	Astrovirus	Adenovirus
236	76 (32.2)	0 (0)	24 (10.2)	2 (0.8)	50 (21.2)	6 (2.5)	3 (1.3)	9 (3.8)

TABLE II. Characteristics of Group C Rotavirus Infection in Infants and Children With Acute Gastroenteritis in Maizuru City, Japan From July 2002 to June 2003

Number	Patient	Age (months)	Sex	Date of stool collection	Laboratory findings			
					RT-PCR	PAGE	RPHA	Rotalex
1	4,397	15	M	26/12/2002	Group C rotavirus NV GII	$+^{C}$	+	−
2	4,399	33	F	8/1/2003	Group C rotavirus	−	−	−
3	4,401	4	F	8/1/2003	Group C rotavirus	$+^{C}$	−	−
4	4,402	44	M	14/1/2003	Group C rotavirus	−	−	−
5	4,403	21	F	21/1/2003	Group C rotavirus NV GII	$+^{C}$	+	−
6	4,406	20	F	28/1/2003	Group C rotavirus Group A rotavirus	$+^{C,A}$	+	+
7	4,410	56	M	3/2/2003	Group C rotavirus	$+^{C}$	−	−
8	4,411	10	F	5/2/2003	Group C rotavirus	$+^{C}$	−	−
9	4,412	24	F	8/2/2003	Group C rotavirus Group A rotavirus	$+^{A}$	−	+
10	4,416	49	M	21/2/2003	Group C rotavirus	$+^{C}$	+	−
11	4,417	39	M	4/3/2003	Group C rotavirus	$+^{C}$	+	−
12	4,420	41	F	4/3/2003	Group C rotavirus	−	−	−
13	4,427	92	F	12/3/2003	Group C rotavirus	$+^{C}$	−	−
14	4,429	138	M	13/3/2003	Group C rotavirus Group A rotavirus	$+^{A}$	−	+
15	4,435	29	M	18/3/2003	Group C rotavirus	$+^{C}$	+	−
16	4,446	124	M	26/3/2003	Group C rotavirus	$+^{C}$	+	−
17	4,479	87	F	15/4/2003	Group C rotavirus	−	−	−
18	4,480	13	M	15/4/2003	Group C rotavirus	$+^{C}$	−	−
19	4,487	50	F	19/4/2003	Group C rotavirus	−	−	−
20	4,488	12	F	19/4/2003	Group C rotavirus	$+^{C}$	+	−
21	4,490	10	M	22/4/2003	Group C rotavirus Group A rotavirus	$+^{C,A}$	+	+
22	4,497	49	F	25/4/2003	Group C rotavirus Group A rotavirus	$+^{C,A}$	+	+
23	4,500	40	M	26/4/2003	Group C rotavirus	−	−	−
24	4,504	22	M	28/4/2003	Group C rotavirus	$+^{C}$	−	−

M, male; F, female; NV GII, norovirus genogroup II; RPHA, reverse passive hemagglutination test; RT-PCR, reverse transcription-polymerase chain reaction; +, positive; −, negative; $+^{C}$, typical profile of group C rotavirus; $+^{A}$, typical profile of group A rotavirus; $+^{C,A}$, mixed typical profile of group C rotavirus and group A rotavirus.

outbreak of acute gastroenteritis caused by group C rotavirus in a young education center in 2000 in Okayama Prefecture, Japan [Kuzuya et al., 2003]. The results indicated that group C rotavirus detected in infants and children in Maizuru city were closely homologous to the OT-99 reference strain and had a high identity to each other, suggesting that they probably came from the same source of infection. Moreover, the identities of 94–99% at the nucleotide sequence level among group C rotaviruses detected and other reference strains was also noted.

Nucleotide Sequence and Phylogenetic Analyses of Norovirus Genogroup II

Two norovirus genogroup II, which were associated with co-infections with group C rotavirus (patient no. 1 and 5), were characterized further for their genotypes and genetic relationship with the reference strains based on the recent norovirus capsid region classification scheme of Katayama et al. [2002]. The phylogenetic tree of nucleotide sequences of capsid region of the two norovirus GII isolates was constructed in comparison with the reference strains. The results shown in Figure 3 revealed that one isolate of noroviruses GII (4397/2002/Maizuru) formed a cluster with Lordsdale and Bristol reference strains. The other norovirus GII isolate (4403/2003/Maizuru) formed a cluster with Seacroft and Saitama U16 reference strains.

DISCUSSION

In this study, diarrheal viruses were detected in 64.4% of fecal specimens tested. These findings suggested that acute gastroenteritis in infants and children in Maizuru city about 64.4% might be due to the diarrheal viruses and 35.6% caused by other etiologic agents. Group A rotavirus was the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [Kapikian et al., 2001]. Among diarrheal viruses detected, group A rotavirus was the most prevalent and became a leading cause of viral gastroenteritis in infants and children in Maizuru city, Japan followed by norovirus (GI, GII), group C rotavirus, adenovirus, sapovirus, and astrovirus. These viruses also were considered to be significant global enteropathogens and were associated with sporadic outbreaks of gastroenteritis in such settings as kindergartens, schools, and nursing homes for the elderly and among military recruits [Rodger et al., 1982; Espejo et al., 1984; Carter and Willcocks, 1996; Bon et al., 1999; Koci et al., 2003].

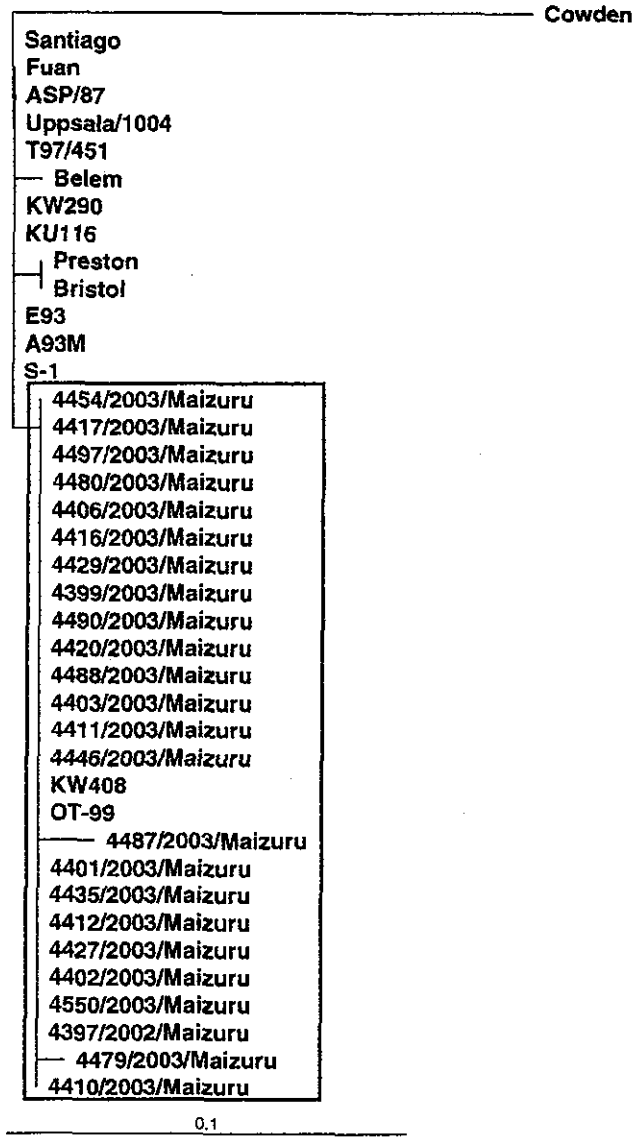

Fig. 2. Phylogenetic tree of nucleotide sequences of 24 isolates of group C rotavirus. The tree was constructed from partial nucleotide sequences of 24 isolates of group C rotavirus detected in Maizuru city, Japan. Reference strains of group C rotavirus were selected from DDBJ/GenBank under the accession number indicated in the text. Cowden strain was used as an out-group strain for phylogenetic analysis.

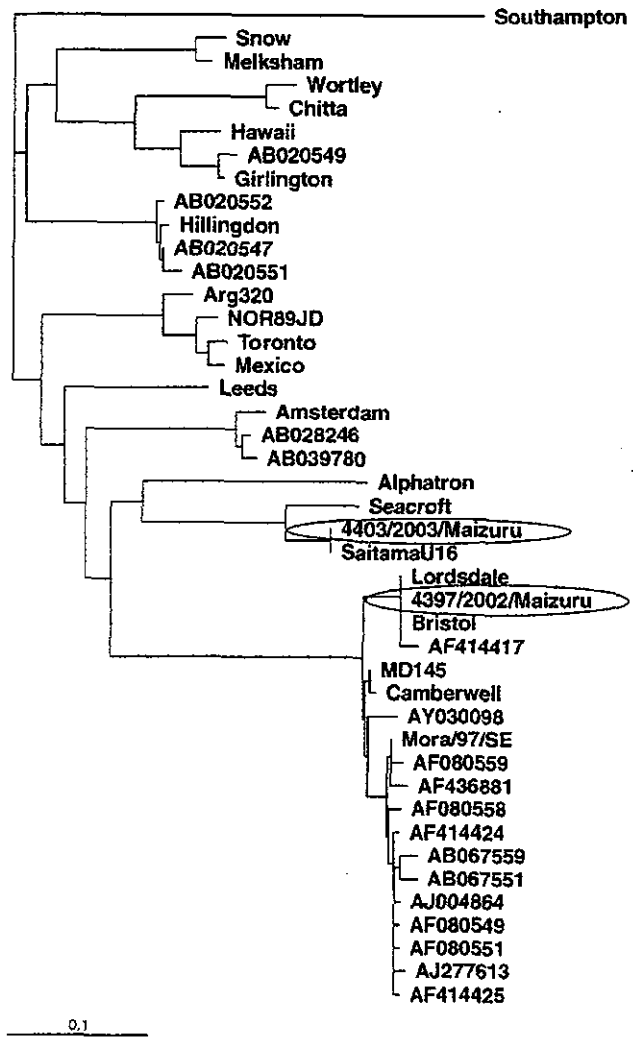

Fig. 3. Phylogentic tree of nucleotide sequences of two isolates of norovirus genogroup II (NV GII). The tree was constructed from partial nucleotide sequences of the capsid region of the two NVs GII, which were identified as mixed infections with group C rotavirus in Maizuru city, Japan. Reference strains of NV GII were selected from DDBJ/GenBank under the accession number indicated in the text. The NVs GII found in Maizuru city, Japan were indicated in the oval circle and belonged to different clusters known as Lordsdale and Seacroft reference strains, respectively. Southampton was used as an out-group strain for phylogenetic analysis.

Group C rotavirus infection in Japan was reported initially by Oseto et al. [1986]. Thereafter, numerous reports of group C rotavirus infection in different regions of Japan have been published [Ushijima et al., 1989; Kuzuya et al., 1998]. In Japan, the group C rotavirus was detected mostly in winter and the incidence ranged from 2.7 to 13.3% [Kuzuya et al., 1998]. The results of this study are in line with the previous findings that group C rotavirus was detected in the cold season and accounted for 10.2% of acute gastroenteritis in infants and children. It is interesting to note that the group C rotavirus infection was confined only within a short period of five months (December 2002 through April 2003). Moreover, group C rotavirus was not found in a 5-year (1996–2001) survey of rotavirus infection in diarrheal fecal specimens of children collected from Maizuru city (data not shown). This is the first report of an outbreak attributed to group C rotavirus among infants and young children in Maizuru city.

Most studies reported that group C rotavirus infection has been demonstrated mainly in adults and children more than 3 years old [Mastumoto et al., 1989; Ishimaru et al., 1991; Steele and James, 1999; Nilsson et al., 2000]. In contrast, the present study has demonstrated that about 50% of acute gastroenteritis cases associated with group C rotavirus were confined to infants and young

children less than 3 years old. This observation clearly indicated that group C rotavirus infection could occur not only in adults and older children but also in infants and young children. The youngest patient in our study was 4 months old. Recently, an association with extrahepatic biliary atresia, a frequent form of a ductal cholestasis, observed in infants and young children and the detection of group C rotavirus in infants has been suggested [Schreiber and Kleinman, 1993; Riepenhoff et al., 1996].

All the fecal specimens that were positive for group C rotavirus by RT-PCR were tested further by RPHA and PAGE. It was found that only 41.7 and 66.7% of the samples were positive for group C rotavirus by RPHA and PAGE, respectively. Although the RPHA is a rapid and simple method used routinely for diagnosis in general clinical settings, a screening for the presence of group C rotavirus using this assay would have missed approximately 58.3% compared to RT-PCR. Using the percent of the PAGE method, even though it is relatively more sensitive than RPHA, the percent of false negatives was still as high as 33.3%. The failure of detection of group C rotavirus by RPHA and PAGE methods in the samples positive by RT-PCR might be due to a low concentration of group C rotavirus particles in those fecal specimens [Jiang et al., 1995]. Nevertheless, PAGE is a useful method that has been utilized for the detection of the rotavirus genome in fecal specimen and the RNA segment profiles (so-called genome patterns or electropherotypes) have been used widely to characterize the virus isolates from the outbreaks. Electropherotyping of group C rotavirus genome has allowed their genome profile to be classified into three distinct patterns, I, II, and III [Kuzuya et al., 1996, 1998]. The group C rotavirus isolates detected in our study showed the genome profile similar to pattern I. Taken together, the results clearly indicated that the RT-PCR was the most sensitive and reliable method, compared to PAGE and RPHA, for the detection and genotyping of group C rotavirus in clinical specimens and could be a useful tool for diagnosis purpose and the molecular epidemiological study of the diversity of diarrheal viruses.

Although mixed infections with different enteric viruses have been reported by several groups of investigators infection with group C rotavirus and other viral enteropathogens is rare [Kuzuya et al., 1998]. To date, only one mixed infection with group C rotavirus and group A rotavirus has been reported, among 1676 fecal specimens tested, in the United States by Jiang et al. [1995]. Interestingly, our study has demonstrated mixed infections with group C rotavirus and group A rotavirus, and group C rotavirus and norovirus GII in a relatively high percentage of 20.8 and 8.3%, respectively. This is the first report of acute gastroenteritis associated with the mixed infection of group C rotavirus and norovirus GII. The two noroviruses GII isolates belonged to different clusters known as Lordsdale and Seacroft reference strains, respectively. The norovirus GII that formed a cluster with Lordsdale was further confirmed by immunochromatography (IC) using a specific polyclonal antibody against norovirus GII genotype 1 (data not shown) [Okame et al., 2003]. Furthermore, all group A rotaviruses associated with mixed infections with group C rotaviruses were also further confirmed by the Rotalex test and RNA-PAGE. Our findings indicated that the mixed infections observed in our study were unlikely to be due to contamination by group A rotavirus and norovirus GII.

The partial nucleotide sequences of *VP7* gene of all 24 isolates of group C rotavirus detected in the present study had high nucleotide sequence homology not only with the OT-99 reference strain but also with other reference strains registered previously in the DDBJ DNA database. The results provide further evidence to support the hypothesis that the group C rotavirus strains worldwide might-constitute a single G genotype [Nilsson et al., 2000].

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第 52 回日本感染症学会東日本地方会総会学術講演会座長推薦論文

Development of RT-multiplex PCR Assay for Detection of Adenovirus and Group A and C Rotaviruses in Diarrheal Fecal Specimens from Children in China

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Abstract

Rotavirus, adenovirus, norovirus, sapovirus and astrovirus are considered to be significant global enteropathogens associated with sporadic cases and outbreaks of acute gastroenteritis. Therefore, a rapid and sensitive assay is preferred to screen for the presence of these viruses in diarrheal fecal specimens. In a previous study, we developed a reverse transcription single-round multiplex polymerase chain reaction (RT-smPCR) assay for the simultaneous detection of norovirus (genogroup I, genogroup II), sapovirus and astrovirus in fecal specimens (Yan et al., 2003). Recently, we developed another RT multiplex PCR for one-step amplification of all subgenera A to F adenoviruses, and group A and C rotaviruses. In this study, a total of 207 fecal specimens collected from children with acute gastroenteritis between December 2001 and April 2003 in Yunnan Province, China were examined for the presence of adenoviruses, and group A and C rotaviruses, by RT-multiplex PCR. The detection rate of these three viruses was 55.1% (114 out of 207 specimens), among which adenovirus and group A and C rotaviruses were identified in 11, 101 and 1 fecal specimen, respectively. Furthermore, one specimen was found to be positive for co-infection with adenovirus and group A rotavirus. An epidemic of acute gastroenteritis was also identified as peaking mainly in October and November.

Taken together, our results clearly indicate that this novel assay provides a potentially rapid and convenient tool for epidemiologic investigation of diarrhea caused by adenovirus and group A and C rotaviruses.

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平成16年 8 月20日

Introduction

Rotaviruses belong to the family *Reovidae*, which includes segmented, double-stranded RNA with 11 gene segments[8]. Rotaviruses are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties[3)19)]. Of these, group A rotavirus is recognized as the most important pathogen, causing an estimated 440,000 deaths per year worldwide, predominantly among children living in developing countries[16]. Group B rotavirus was reported to be responsible for severe diarrhea mainly in adults, but also in children[20)23)]. To date, prior to this study, group B rotavirus in association with human diarrhea has only been identified in China, India and Bangladesh[9)11)20)23)]. Group C rotavirus was first identified in the feces of young pigs with diarrhea in 1980[18] and was later confirmed as a human pathogen by Bridger et al[2]. in 1986. In fact, group C rotavirus infection mainly in older children and adults has been reported in both developed and developing countries[4)7)10)14)]. Adenoviruses (genus *Mastadenovirus*, family *Adenoviridae*) are double-stranded DNA viruses, with linear genomes enclosed within complex capsids composed of hexon, penton and penton fiber proteins[21]. Adenoviruses have been divided into six subgenera, A through F[21], of which subgenus A (serotypes 12, 18, 31) and subgenus F (serotypes 40, 41) have been implicated in infantile viral gastroenteritis, especially subgenus F which appears to be significantly associated with acute gastroenteritis[13)22)].

At present, RT-PCR is the most sensitive and reliable detection method available and is a useful tool for diagnostic purposes and molecular epidemiological studies of the diversity of diarrheal viruses[1]. To date, PCR assays with specific primers individually or combined for multiple human pathogens have proved to be comparable to or better than cell culture or immuno-diagnostic methods for detection of adenovirus and rotavirus. In the present study, we developed a RT-multiplex PCR assay for simultaneous amplification of all subgenera A to F adenoviruses and group A and C rotaviruses in fecal specimens collected from children with diarrhea in Yunnan Province, China. The results demonstrate this novel assay to have a higher sensitivity than enzyme immunosorbent assay (EIA). Our RT-multiplex PCR assay permits rapid, accurate and cost-effective detection of these three viral enteropathogens in fecal specimens.

Materials and Methods

Fecal specimens

A total of 207 stool samples were collected from pediatric outpatients (under 7 years old) with acute gestroenteritis in Yunnan Province, China, between December 2001 and April 2003. An approximately 10% stool suspension was made with sterilized MilliQ water (10,000g for 10 min at 4℃). The supernatant was kept frozen at −20℃ until testing.

Positive and negative controls

Positive controls in the present study were as follows. Two cultured adenovirus subgenus F type 40 (Ad40) and type 41 (Ad41), one cultured group A rotavirus serotype 1 (RVA-G1, Wa) and 9 diarrheal fecal specimens including 4 adenovirus-positives termed JP3018, JP3022, JP3026 and JP3135 ; 4 group A rotavirus-positives termed JP2998, JP3000, JP3011 and JP3209 and 1 group C rotavirus-positive (RVC, 83/175), all of which were collected from pediatric diarrheal patients in Japan. The viruses were previously detected by specific monoplex PCR, then used as positive controls in a multiplex PCR test and/or EIA