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Clinical and Virological Characteristics of Rotavirus Gastroenteritis and Prevalence of Strains in Tochigi, Japan

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Abstract. *Aim: Rotavirus infection is a serious gastrointestinal infection that is usually prevalent during winter months and often seen in infants and young children. Studies on genotypes of prevalent rotavirus strains are quite important for preventing infection, developing vaccines, and its evaluation. A purpose of this study was to make an investigation of a rotavirus infection of Nasu Region of Tochigi, Japan and to compare to the other region. Materials and Methods: We examined the clinical findings in 147 patients who attended to the Department of Pediatrics at International University of Health and Welfare Hospital in Nasu-shiobara City, Tochigi Prefecture, Japan during the time of April 1, 2008 to March 31, 2010. Results: We analyzed the clinical findings of the 37 patients with a fecal sample positive for rotavirus antigen. Furthermore, viral genotypes were determined using rotavirus-positive samples from 27 of these 37 patients. The genotypes were determined as G1P [8] in 5 samples, G3P [8] in 5 samples, G9P [8] in 3 samples, and G6P [9] in 2 samples. We were able to analyze the phylogenetic trees of these genotypes. Conclusion: Of particular note, we detected G6P [9] which were extremely rare in human beings but common in cattle. Studies on changes in prevalent strains after vaccine introduction need to be conducted.*

Rotavirus (RV), is a dagerous viral pathogen causing gastroenteritis in humans. As most of infants have received the rotavirus vaccine rotavirus infections among infants and young children has since decreased significantly in developed countries (1). Each year, the vaccine prevents an estimated 40,000 to 50,000 hospitalizations among U.S. infants and young children. Rotavirus illness has also decreased among older children and adults that are not vaccinated; they are likely gaining indirect protection from rotavirus disease as vaccinated children are less likely to get the disease and spread it to others. Focusing on deaths of under 5 years in developing countries infectious gastroenteritis has critical roles. Nature of rotavirus in spans of many mammals and birds, infection of rotavirus infection in humans is virtually limited to humans.

Rotavirus, a member of the family Reoviridae, has 11 segments of double-stranded RNA as a genome, and the viral particle is composed of the outer capsid, inner capsid, and core (2). The outer capsid consists of two structural proteins, VP4 and VP7, which contain neutralization antigens. The inner capsid consists of structural protein VP6. Based on the antigenicity of the inner capsid protein VP6 and genomic characteristics, rotavirus is classified into seven groups (A-G), among which group A RV is the major etiologic agent in humans and animals. For epidemiological investigations of RV, a genetic classification system based on the outer capsid proteins VP7 (G type) and VP4 (P type) has been adopted (3). While at least 11 G genotypes have been isolated from humans, G1, G2, G3, G4, and emerging G9 are major genotypes of human rotaviruses. As human P genotypes, P [8] is the most common genotype worldwide, followed by P [4] and P [6]. Also, 6 non-structural proteins (NSP) are known.

While at least 11 G genotypes have been isolated from humans, G1, G2, G3, G4, and emerging G9 are major genotypes of human rotaviruses. As human P genotypes, P [8] is the most common genotype worldwide, followed by P [4] and P [6]. In human RVs, five common G and P genotype combinations (genogroups) have been identified: G1P[8], G2P [4], G3P [8], G4P [8], and G9P [8] on the Wa-like genome constellation (I1-R1-C1-M1-A1-N1-T1-E1-H1), and G2P[4] on the DS-1-like constellation (I2-R2-C2-M2-A2-N2-T2-E2-H2).

Rotavirus infects not only humans but non-human monkeys and bovine, pigs, horses, dogs, cats, rats and chicken. New

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Key Words: Rotavirus gastroenteritis, endemic strain, genotype, G6P [9], rotavirus vaccine.

introduced vaccines against rotavirus epidemic strains may change the cross-immunity of virus strains. Goto *et al* (4) discovered genotypes G1P [8] from the cerebrospinal fluid of children with rotavirus meningoencephalitis in the Tochigi Prefecture, Japan. Reports investigated the epidemic strain in local areas are very important to investigate the pandemic from the epidemic in the region (5).

In the present study we examined the endemic strain of rotaviruses in the Nasu region, Tochigi Prefecture, Japan, for their regional characteristics. Comparison of the clinical features due to endemic strains in this region was performed with those due to global pandemic strains.

Materials and Methods

Patients. During the time of April 1, 2008 to Mar 31, 2010 children who suffered from fever , diarrhea, vomiting, abdominal pain, consulted the Department of Pediatrics , International University of Health and Welfare Hospital, Nasu-shiobara, Tochigi, Japan were investigated for clinical and laboratory findings, infectious disease epidemic situations and past medical histories. Considering the findings of 147 patients diagnosed with acute gastroenteritis, referring to the results from stool culture and simple rapid tests using immunochromatography (Sekisui Medical Co., Tokyo, Japan) of stool samples for rotavirus or adenovirus infections. Swabs of stool specimen and cerebrospinal fluid and blood specimens were collected and kept at –20˚C. None of the147 children had traveled abroad. Twenty-three patients out of 37 rapid tests using immunochromatography rotavirus antigens-positive admitted to the hospital.

Genetic analysis was performed with 24 fecal, 1 cerebrospinal and 2 blood specimens. Presence of rotavirus and adenovirus antigens in stool specimens was confirmed with immunochromatography. All specimens were stored at –80˚C until analyzed. G types [1-4, 8, 9, 12], P types ([4], [6], [8], [9]), VP6, and NSP4 genotypes were determined by the nested PCR with the primer sets reported previously (6-8).

Reverse transcribed-polymerase chain reaction (RT-PCR). Nucleotide sequences were determined directly with the cDNA products amplified by RT-PCR. As a template for RT-PCR, dsRNA was extracted from stool suspension with a commercially available kit (RNAID kit, BIO101, Inc., La Jolla, CA, USA) according to the manufacturer's instructions. RT–PCR was performed with reverse transcriptase (AMV) (Seikagaku Co., Tokyo, Japan) and thermostable DNA polymerase (Expanded High Fidelity PCR System, Roche, Mannheim, Germany) with the primers for individual gene segments prepared based on the human rotavirus strains reported previously (9). For all the gene segments, full-length sequences except for primer binding regions at 50- and 30-end were amplified and sequenced. PCR products were purified by WizardR SV GEL and PCR Clean-Up System (Promega, Inc., Madison, WI). Sequencing reaction was performed with fluorescent dideoxy chain termination chemistry using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA).

Sequence was determined by ABI Prism 3100 genetic analyzer (Applied Biosystems). GENETYX-Win version 5.1 (Software Development, Tokyo, Japan) was used to calculate the identity of gene segments. Phylogenetic analysis was performed with MEGA software version4.1 based on the neighbor-joining method and the Kimura two-parameter model. Phylogenetic trees were supported statistically by bootstrapping with 1,000 replicates. The nucleotide sequences of 11 gene segments of the KF17 strain determined in this study were deposited in the GenBank database under accession numbers JF421975-JF421985 (10).

Specimen collection was performed by the authors' responsibilities under the agreement based on ethical guidelines on clinical research (issued by the Ministry of Education and Science and the Ministry of Health, Welfare and Labor of Japan) and the Helsinki Declaration (World Medical Association). The study plan was approved by the Research Ethics Committee of International University of Health and Welfare.

Results

Age distribution of patients was from 7 months to 2 years. Adenovirus was detected in 10 children and not during the summer months (Figure 1). Rotavirus infections were mainly distributed to the age of 7 months to 2 years and adenovirus infections in 7 to 11 months. Monthly incidence and age distribution of patients with infectious gastroenteritis and with rotavirus infections showed similar trends with previous reports (1, 3).

In a study of 23 admitted patients with rotavirus infections age distribution observed in the one month to 4 year of age, most frequent 7 cases in 1 year of age (Figure 2). The male-female ratio was 14 boys and 9 girls. Initial symptom was diarrhea in 8 cases, vomiting, diarrhea and fever at the same time in another 8. Poor feeding was initial symptom in a patient of one month of age. There was no high serum Na but 3 low serum Na by laboratory examinations. High BUN value was seen in 3 and more than half of the patients showed high value of serum uremic acid. Liver dysfunction was observed in 4, encephalitis in one, convulsions due to gastroenteritis in 2 and secondary lactose intolerance in 2 cases. Duration of hospitalization was 4 to 11 days and the average was 6.8 days. There was no death case and all patients discharged without sequelae.

RV was detected in 27 specimens examined by RT-PCR, although three NSP4 genotypes were positive (21 B, 1 C, 1 E3) (Table I). Subsequent sequence analysis revealed that this genotype E3 (KF17) strain had the G6-VP7 gene which is rarely found in human rotavirus, and the full-length VP7 gene sequence and partial VP4 gene sequence of the KF17 were preformed. Therefore, nearly full-length sequences of all 11 RNA segments were determined for the KF17 strain. Strains KF17 were obtained from 3 year-old female outpatient (Case 24) in February 2010. Two G6-positive patients (Cases 24 and 25) were from different families, and lived in the same Tochigi prefecture town, one which has plenty of cattle farms. However, there was no evidence that they were in contact with each other, or with cattle or other animals. Trisomy 13 had been diagnosed for the Case 24.

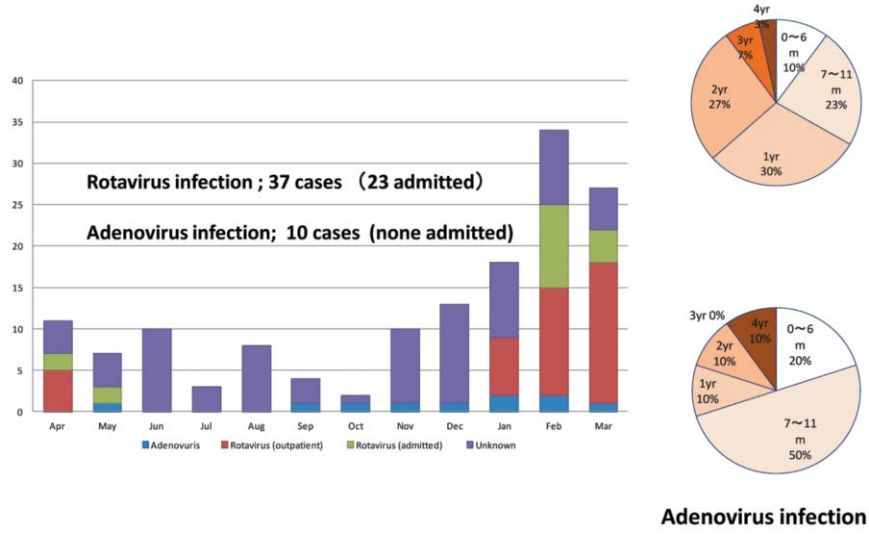

Figure 1. *Age distribution of 147 patients with acute gastroenteritis*.

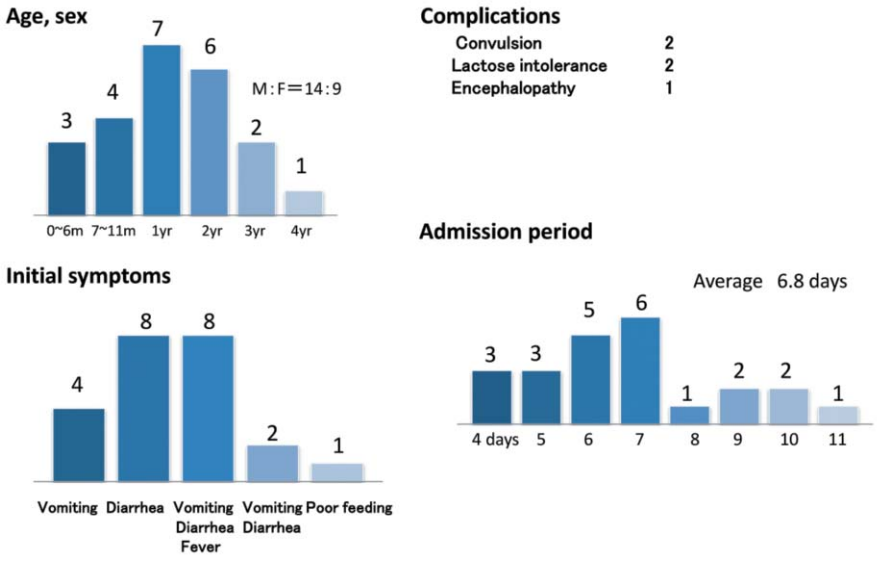

Figure 2. *Summary of 23 admitted patients with rotavirus infections*.

Out of the 15 RV strains which were determined for their G or P types; two strains were identified as P [9] and 13 as P [8]. Strains were determined as G1 (five specimens), G3 (five specimen) and G9 (three specimens). Immune electrophoresis results showed G genes (VP7) in Cases 2, 3. 21, 22, 16 were G1 (618 bp), Cases 4, 5, 9 were G9 (179bp) and Cases 7, 16, 17, 18, 23 were G3 (682 bp) (Figure 3). The P gene (VP4) was determined as [8] (345 bp) in all cases of 2-5, 7, 9, 16-18, 21-26. VP6 genotype was determined as II (485bp) in all cases of 2-5, 7, 9-20, 22-24, 26, 27. NSP4 genes in Cases 2-4,7,9-20,22, 23, 26,27 were B (618 bp), Cases 24 was E3 and Case 25 was C. The genotypes of the current classification are now in progress.

Possible genotyping by direct sequencing showed 5 G1P [8], 5 G3P[8] and 3 G9P [8] . By the phylogenetic analysis 2 samples were determined as G6P [9]. Especially G6P [9] per 11 gene phylogenetic analysis available was performed. In the phylogenetic tree VP7 gene of Case 3 G1P [8] was located close to the epidemic strain in Japan and China (Figure 4). This G1 was closer to the virus that was

Table I. *Summary of genetic analysis of rotavirus strains*.

Case	Age	Gender	Specimen	G type (VP7)	P type (VP4)	Sub group (VP6)	NSP4
1	3	F	CSF				
2	1	F	feces	G1	P[8]	I2	B
3	3	M	feces	G1	P[8]	I2	B
4	2	M	feces	G9	P[8]	I2	B
5	1	F	feces	G9	P[8]	I2	B
6	1	F	blood				
7	0	F	feces	G3	P[8]	I2	B
8	0	F	blood				
9	1	F	feces	G9	P[8]	I2	B
10	2	M	feces			I2	B
11	2	F	feces			I2	B
12	2	M	feces			I2	B
13	1	F	feces			I2	B
14	2	M	feces			I2	B
15	1	F	feces			I2	B
16	2	F	feces	G3	P[8]	I2	B
17	1	M	feces	G3	P[8]	I2	B
18	5	F	feces	G3	P[8]	I2	B
19	7	F	feces			I2	B
20	3	M	feces			I2	B
21	2	F	feces	G1	P[8]		
22	1	M	feces	G1	P[8]	I2	B
23	1	M	feces	G3	P[8]	I2	B
24	3	F	feces	G6	P[9]	I2	E3
25	0	F	feces	G6	P[9]		C
26	1	F	feces	G1	P[8]	I2	B
27	0	F	feces			I2	B

Table II. *Genomic constellations of KF17 from Case 24, G6 P[9] rotaviruses, and prototype strains*.

Strain	Host	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
KF17	Human	G6	P[9]	I2	R2	C2	M2	A3	N2	T3	E3	H3
B1711	Human	G6	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
Se584	Human	G6	P[9]	I2	R2	C2	M2	A3	N2	T1	E2	H3
Hun5	Human	G6	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
PA169	Human	G6	P[14]	I2	R2	C2	M2	A3	N2	T6	E2	H3
NCDV-Lincoln	Bovine	G6	P[1]	I2	R2	C2	M2		N2	T6	E2	
WC3	Bovine	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
Au-1	Human	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
T152	Human	G12	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6
DS-1	Human	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
Wa	Human	G1	P[8}	I1	R1	C1	M1	A1	N1	T1	E1	H1

circulating in Japan and China. VP7 gene of Case 7 G3P [8] was located close to the endemic strain spreading to China. This G3 is very close to the lineage expands distribution in China and Southeast Asia in recent years. VP7 gene of Case 5 G9P [8] was located in single lineage extends to the world. VP7 gene of Case 24 G6P [9] (KF 17 strain) was partially located closer to the cattle stock (Figure 5). Phylogenetic analysis of KF 17 was performed on VP4, VP6, VP1, VP2, VP3 ,NSP1, NSP2, NSP3, NSP4 and NSP5 genes and determined as G6P[8], I2-R2-C2-M2-A3- N2-T3-E3-H3. KF 17 strain of Case 24 showed G6 type of bovine strains and P [9] was admitted to reconcile with human strains (Table II). Per match ratio in each gene of VP6, VP1, VP2, VP3, and NSP4 showed a high match rate (84.1-86.4%) with DS-1

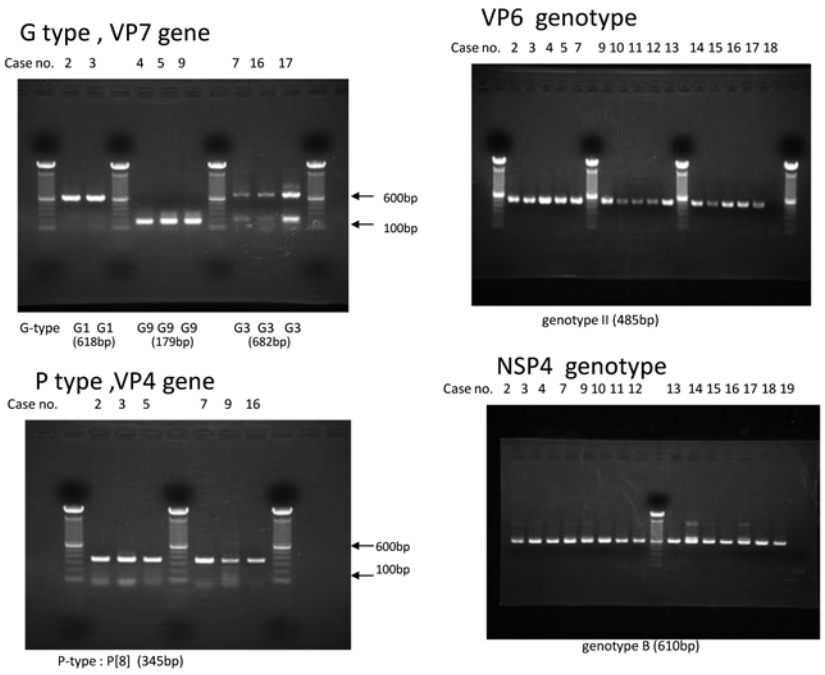

Figure 3. *Genotyping of clinical specimens*.

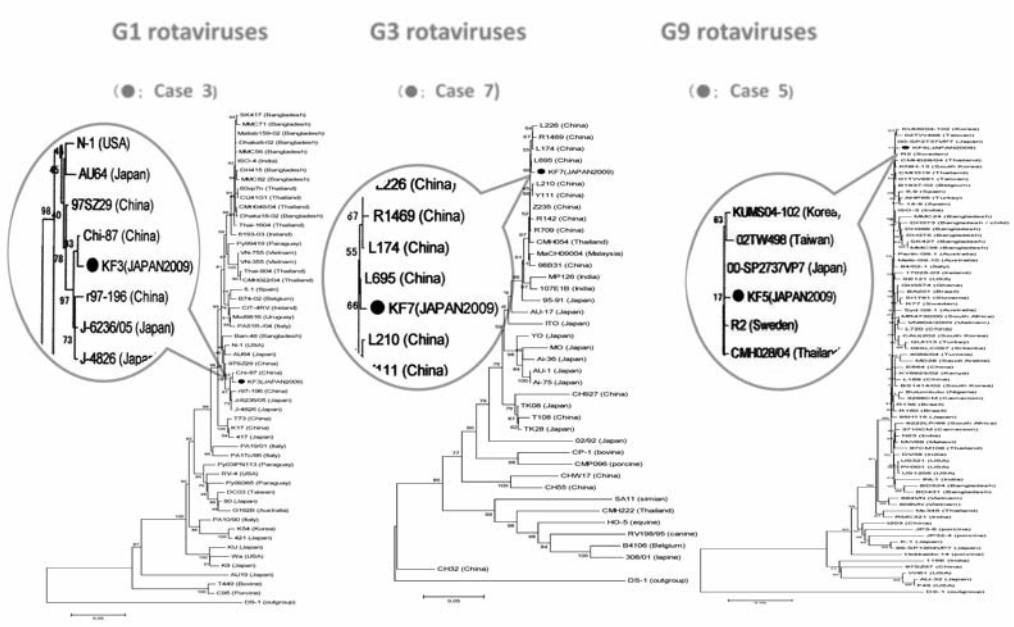

Figure 4. *Phylogenetic analysis of VP7 genes*.

type. Per match ratio in each gene of VP4, NSP1, NSP3, NSP4 and NSP5 also showed a high match rate (94.2-96.9%) with AU-1 type. Bovine type strain gene segments become reassortant rotaviruses major two genes in these stocks.

Discussion

Rotavirus gastroenteritis is a serious disease in children from 6 months to 2 years-old occurring mainly during the winter

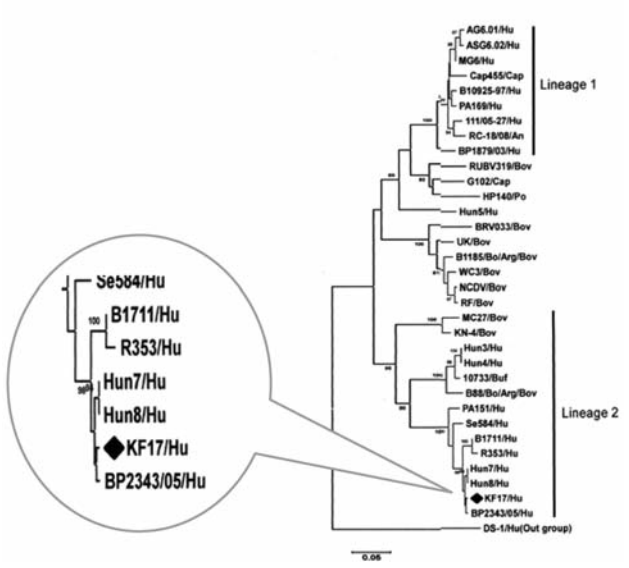

Figure 5. *Phylogenetic trees of the VP7 gene segments of the KF17 strain from Case 24.*

time. 39.2~48.5% of patients with acute gastroenteritis less than 5-year-old admitted to hospitals in the Mie Prefecture, Japan was due to rotavirus infection (11). The age distribution was <6 months: 4.2%, <1-year-old: 24.8%, <2 years: 63.5%, of patients. Rotavirus-positive cases in sporadic gastroenteritis patients of 683 adult cases in 2001 were 97 and the equivalent of 14% of the overall and age distribution was 13~95 (mean=45 y ± 17 SD) year of age (12). Group receiving inpatient treatment (50 years ± 21months) was of significantly higher age (p=0.002) compared to those receiving outpatient treatment group (39 years ± 15 months).

Neurological symptoms associated with rotavirus infections less than 2 years old were estimated to be approximately 2% in the following (13). Although rotavirus was detected from all CSF specimens in cases of convulsion by RT-PCR assay (14), but was not detected in case 1. In 147 cases of this study monthly occurrence, trends in different age groups and clinical symptoms were not significantly different to previous reports. The was no sequelae of any cases of encephalopathy, seizures, secondary lactose intolerance complications.

Current genotypes of rotavirus are reported that most worldwide G1P [8] as 52%, followed by G2P [4] as 11% and G4P [8] as 8% (2). G1 type had been most prevalent since 1984 to 2007 in Japan, G3 was most prevalent only in 2004. In the same year there was difference in G1, G2 or G3 type endemic according to regions. G3 type had been predominant during the winter time of 1987 to 1988 in the Gifu Prefecture of Japan, but G4 type was predominant in 1987 to 1988. Serotype distribution of 196 samples during 10 years in Kurume was G1 68.4% (136 samples), G3 10.2% (20 specimens), G4 5.6% (11 samples), G2 3.6% (7 samples), and unclassified 12.2% (24 samples) (15).

Rotavirus G9 type was found in the United States in 1983 and from 1985 during the 1989 succession confirmed in Bangladesh, Japan, India and Thailand. This type caused the epidemic in Thailand and Japan from 2000 to 2003 (16-19). Other emerging human G12 type was endemic in the 200 2004 season in Nepal (5, 20-24). New viruses such as G8, G10 and G5 type emerged in Brazil and China (25, 26). The bovine type G6 geno type detected in this study was very rare. G6P [14] in Egypt (27) and G6P [9] in Burkina Faso (28) in either was reported as a rare virus. Case 24 without previous contact with obvious bovine did not identify the source of infection.

In the phylogenetic analysis the KF17 VP7 gene was located in a human lineage including other human G6 strains. Similarly, all other KF17 genes except for the NSP3 gene were relatively closely related to at least one of the human G6 RVs reported in Europe and the U.S. These findings suggest that human G6 RVs which had occurred by reassortment between human and bovine RVs are distributed worldwide, despite low prevalence. Since genotypes of a few gene segments are different among those human G6 strains, this suggests that G6 rotaviruses may occur independently in different locales/countries through reassortment among local strains.

Vaccine introduction caused changes in virus serotypes. In Brazil rotavirus vaccine was introduced in 2006, G2P [4] increased from 7% before vaccine introduction to 95% after introduction in 2009 (29). With the introduction of rotavirus vaccines now expected decreases in patients, but could change the epidemic fashion of infections (3, 30). In Japan should be investigation of changes in epidemiologic fashion, after the introduction of the rotavirus vaccine.

Conclusion

Clinical and virological characteristics of rotavirus infection in the Nasu Region, Tochigi Prefecture, Japan were investigated. Cases were most frequently common in February and March and in 7 months to 2 years of age. There were no sequelae of any cases of encephalopathy, seizures, and secondary lactose intolerance complications. Three of four rotavirus genotypes found in the present study were worldwide-circulating. A very rare G6P [9] type related to bovine rotaviruses was also detected. Further epidemiological studies are necessary concerning this strain. Investigation of the epidemic strain of rotavirus after introduction of a vaccine is also considered necessary.

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