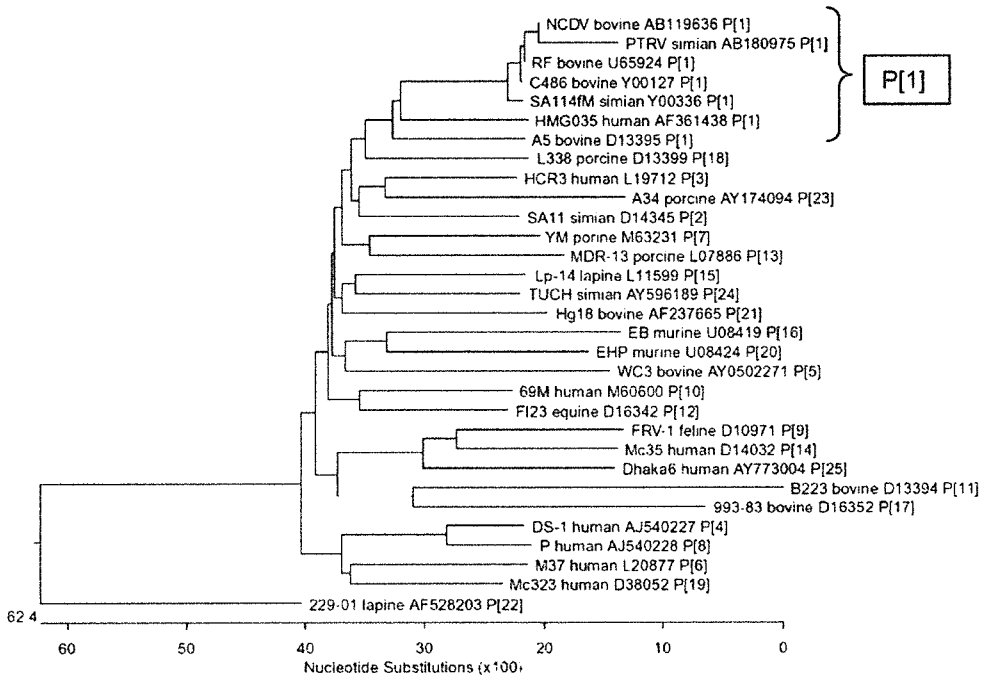

Fig. 4. Phylogenetic analysis of VP8* genes of the PTRV and other rotavirus strains representative of 25 P genotypes indicates that the PTRV VP8* gene belongs to P[1] type. Phylogenetic tree was constructed by employing the Clustal W algorithm of the MegAlign program in DNASTAR software package (Madison, WI). The length of each pair of branches represents the distance between sequence pairs.

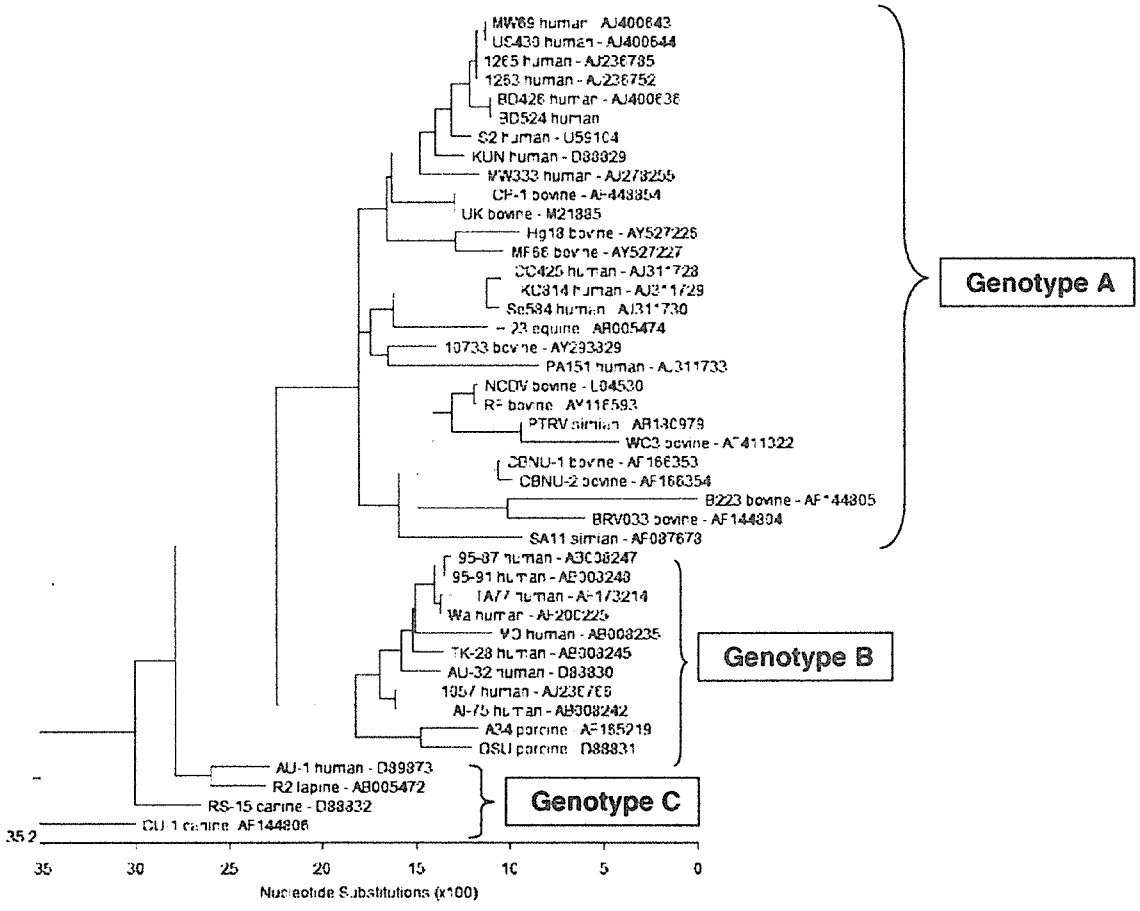

Fig. 5. Phylogenetic analysis of NSP4 genes of the PTRV and other rotavirus strains representative of 3 genotypes indicates that the PTRV NSP4 gene belongs to NSP4 genotype A. Phylogenetic tree was constructed by employing the Clustal W algorithm of the MegAlign program in DNASTAR software package (Madison, WI). The length of each pair of branches represents the distance between sequence pairs.

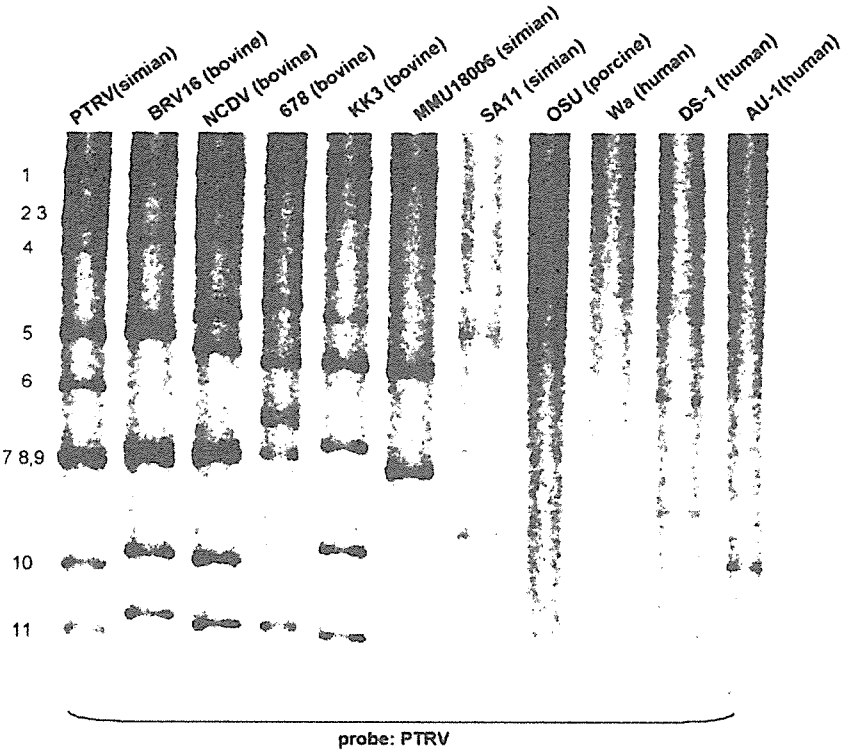

Fig. 6. Hybridization patterns between a ^{32}P-labeled PTRV RNA probe and RNAs of selected bovine, simian, porcine and human rotavirus strains (listed at the top).

the PTRV NSP4 gene clustered with that of genotype A bovine rotaviruses (Fig. 5).

Genetic analysis of the PTRV whole genome as determined by RNA–RNA hybridization in solution

The PTRV probe formed at least 9 hybrid bands with the genomic RNAs of US G6 bovine rotavirus NCDV strain and Japanese G8 bovine rotavirus BRV16 strain, 8 bands with those of Scottish G8 bovine rotavirus 678 strain and Japanese G10 bovine rotavirus KK3 strain (Fig. 6). Three to four hybrid bands were formed between the PTRV probe and the genomic RNAs of simian rotavirus RRV (MMU18006) or SA11, whereas one to four hybrid bands were formed between the PTRV probe and the genomic RNAs of human rotavirus Wa, DS-1 or AU-1 strain or porcine rotavirus OSU strain. These results indicated that the PTRV strain belonged to the bovine rotavirus genogroup rather than the simian rotavirus genogroup. Since, in general, a rotavirus genogroup native to one animal species is not detected in another animal species (Flores et al., 1986; Nakagomi and Nakagomi, 1993, 2002), this finding appears to suggest that the PTRV may be of bovine origin.

Rotaviruses bearing a P[1]:G8 specificity appeared to have been endemic in the monkey colony

As summarized in Table 4, all five serum samples derived from three pig-tailed macaques that were collected at Medical Lake colony between 1987 and 1994 had neutralizing antibodies to P[1]:G8 virus PTRV, suggesting that all 3 macaques in the colony were infected with a virus bearing a P[1] and/or G8 specificity. The finding that four of five serum samples had (i) antibodies to a reassortant RRV × 1290 (P[3]:G8) and (ii) no antibodies to RRV (P[3]:G3) indicated that the virus bore G8 specificity. Of note is the finding that two samples collected before 1990 as well as three samples collected after 1990 (when one macaque developed diarrhea from which the PTRV virus was isolated) had also neutralizing antibodies to the PTRV virus, suggesting a G8 virus such as

Table 4
Analysis by neutralization of serum samples collected from selected pig-tailed macaques in the colony

Rotavirus used for neutralization			60% PRN antibody titer of indicated serum (date blood collected)				
Strain/reassortant	G type	P type	Macaque #1		Macaque #2		Macaque #3
			(7/16/87)	(10/29/91)	(1/21/88)	(8/19/94)	(1/12/94)
PTRV	8	P[1]	320	320	1280	2560	640
RRV × 1290	8	P[3]	80	80	640	640	320
PTRV × DS-1	2	P[1]	80	160	1280	2560	320
MMU18006 (RRV)	3	P[3]	<40	<40	<40	80	<40
DS-1	2	P[4]	<40	<40	<40	40	<40

the PTRV strain was endemic among macaques in the colony during at least 1987 and 1994. It is interesting to note that (i) four of five serum samples did not have neutralizing antibodies (<1:40) to G3 virus RRV and (ii) only one sample had low level of antibodies (1:80) to the RRV virus which was probably induced by the G8 virus infection(s) due to the shared high level of amino acid identity in VP7 antigenic region C (amino acid residues 208–224) between the G3 and G8 viruses. This observation suggested that the Medical Lake monkey colony was free from G3 virus (the only G type detected thus far from non-human primates) for at least 8 years (from 1987 to 1994). Four of five serum samples had (i) antibodies to a reassortant PTRV × DS-1 (P[1]:G2) and (ii) no antibodies to DS-1 (P[4]:G2), indicating that the virus endemic in the colony bore a P[1] specificity. Thus, these data demonstrated that the virus that infected three monkeys tested bore a P[1]:G8 specificity like the PTRV strain. If the SA11 viruses bearing P[1] specificity (Pereira et al., 1984, 1986; Burns et al., 1989; Lópes and Arias, 1992) were derived from the original stool sample obtained from a healthy vervet monkey (Malherbe and Strickland-Chomley, 1967), then it would suggest that a virus bearing P[1] specificity can replicate in monkey small intestine. This assumption may render support to our finding that a PTRV-like virus bearing P[1] specificity was endemic among pig-tailed macaques for at least 8 years in the colony.

At the Medical Lake breeding facility, the macaques were kept indoors in breeding family groups of approximately 20. However, there was many transfer between groups with the possibility of moving pathogens throughout the facility.

A rotavirus strain bearing a G8 specificity

G8 rotaviruses were first detected in Indonesia from children with diarrhea during 1978–81 (Matsuno et al., 1985; Albert et al., 1987). Prototype strains (69M and B37) of such Indonesian G8 viruses with a characteristic "super short" electropherotype (Fig. 1) were reported later to bear a P4[10] specificity. Since then, the G8 viruses have been isolated sporadically from patients with diarrhea in various parts of the world in association with various P types including P[4], P[6] and P[14] (Adah et al., 1997; Bishop et al., 2001; Cooney et al., 2001; Gerna et al., 1990; Holmes et al., 1999; Jagannath et al., 2000; Kang et al., 2002; Kelkar and Ayachit, 2000; Palombo et al., 2000). Recently, however, the G8 viruses in conjunction with P[4] or P[6] specificity have been detected in diarrheal patients at higher frequency especially in certain countries in Africa (Adah et al., 2001; Armah et al., 2001; Cunliffe et al., 2000, 2001, 2002; Fischer et al., 2003; Nakata et al., 1999; Santos and Hoshino, 2005; Steele et al., 2002). For example, (i) in Blantyre, Malawi, during the 1997–1999 season, the G8 viruses were the most prevalent G type (34.8%) followed by G1 (30%) and (ii), in Maiduguri, Nigeria, during the 1999–2000 season, the G8 was the second most prevalent (27.7%) after G1 (39.3%). Thus, a rhesus- or bovine (UK)-based single VP7 (G8) gene substitution reassortant vaccine candidate has been constructed (Hoshino et al., 2003). Such a G8 vaccine component could be added to the existing tetravalent vaccine to formulate a pentavalent (G1, G2, G3, G4 and G8) or hexavalent (G1, G2, G3, G4, G8 and G9) vaccine (Kapikian et al., 2005).

The G8 serotype is perhaps the third most common G type found in cattle after G6 and G10 (Chang et al., 1996; Falcone et al., 1999; Fukai et al., 1999, 2002; Okada and Matsumoto, 2002; Parwani et al., 1993; Snodgrass et al., 1990; Suzuki et al., 1993). The bovine G8 viruses have been detected in conjunction with P[1], P[5], P[11] or P[14] (Adah et al., 2003; Fukai et al., 1999, 2004; Lu et al., 1995; Sato et al., 1997; Snodgrass et al., 1990; Taniguchi et al., 1991). In 1996, 1 of 121 rotavirus-positive field samples collected from foals with or without diarrhea was reported to bear a P[1]:G8 specificity (Isa et al., 1996). Recently, the P[1]:G8 viruses have been detected, albeit limited in number, in humans in parts of India (Jagannath et al., 2000) and Nigeria (Adah et al., 2001). Since a close association of people with cattle in such regions is reported to be common, these bovine-like P[1]:G8 human rotavirus strains are suspected to be examples of the direct transmission of such viruses from cattle to humans. More recently, the P[1]:G8 virus was detected in newborn guanacos (*Lama guanicoe*) with acute diarrhea in Argentina (Parreño et al., 2004). In the present study, we reported the isolation of a P6[1]:G8 virus (designated as PTRV strain) from a pig-tailed macaque with diarrhea. The PTRV strain is the first simian rotavirus shown to carry a non-G3 specificity in conjunction with a P6[1] specificity. RNA–RNA hybridization assay suggests that the simian PTRV strain may be of bovine origin. However, this P[1]:G8 virus, as shown in the present serology study, was endemic among pig-tailed macaques in the colony.

Recently, there is renewed interest in establishing a non-human primate model (Chege et al., 2005; McNeal et al., 2005; Westerman et al., 2005a, 2005b) (i) to better understand the mechanisms involved in protective rotavirus immunity and (ii) to evaluate safety and efficacy of rotavirus vaccine candidates prior to clinical trials in humans. One of the obstacles in establishing such a homologous simian model is that there is only one G type (G3) existing in simian rotaviruses. Recently, Chege et al. reported that five of five infant vervet monkeys and one of two infant baboons that were given by gastric intubation 2 ml of 10–20% stool suspension containing a P[6]:G8 virus obtained from a child with diarrhea did not develop any clinical signs but shed virus for 2–8 days and seroconverted (Chege et al., 2005). These findings suggest that a virus bearing G8 specificity can replicate in monkey small intestine and thus can be used in challenge studies. The PTRV strain with P6[1]:G8 specificity, which was demonstrated in this study to be able to infect pig-tailed macaques endemically, may prove to be a valuable virus in challenge studies in a monkey model.

Materials and methods

Rotavirus strains, cell cultures, culture medium, virus neutralization assay, subgroup determination, hyperimmune antiserum and polyacrylamide gel electrophoresis

Table 2 summarizes the reference rotavirus strains used in this study. Primary or secondary cultures of African green monkey kidney (AGMK) cells (Diagnostic Hybrids, Athens, OH) were used for genetic reassortment. An established monkey kidney MA104 cell line was used for virus amplification, plaque purification, virus titration and plaque reduction neutralization (PRN) assay. Eagle's minimum essential medium supplemented with 0.5 μg/ml trypsin (Sigma γ-irradiated trypsin, Sigma Chemical, St. Louis, MO) and antibiotics was used as maintenance medium, and Leibovitz L-15 medium (Quality Biological. Gaithersburg, MD) supplemented with antibiotics was employed when preparing virus or serum dilutions. PRN assay was performed in a six-well plate (Costar, Corning Inc., Corning, NY) using 50–60 plaque-forming units per 250 μl of the virus as described previously (Hoshino et al., 1998). Agarose (SeaKem ME, BMA, Rockland, ME) was used as a solidifying reagent in overlay medium. Hyperimmune antiserum to each rotavirus strain or reassortant was raised in specific pathogen-free guinea pigs (Charles River, Wilmington, MA) which were free of rotavirus neutralizing antibodies (titer < 1:20 versus PTRV) as determined by PRN assay. At least two guinea pigs were used for antiserum production against each virus or reassortant in an attempt to obtain a more accurate neutralization profile. Sera were inactivated before use by heating at 56 °C for 30 min. Rotavirus immunogens were prepared as previously described (Hoshino et al., 2005; Wyatt et al., 1982). Rotavirus genomic double-stranded (ds) RNAs were extracted with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1 v/v, GIBCO Invitrogen Corp, Carlsbad, CA), precipitated with ethanol and analyzed in a 10% polyacrylamide gel as reported previously (Jones et al., 2003). Subgroup specificity was determined by subgroup-specific monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) as described previously (Greenberg et al., 1983).

Construction, identification and characterization of single VP7 gene substitution PTRV × DS-1 reassortant

Roller tube cultures of primary AGMK cells were coinfected at a multiplicity of infection of approximately one with the human rotavirus DS-1 strain and the pig-tailed macaque rotavirus PTRV strain. When approximately 75% of the infected cells exhibited cytopathic effects, the cultures were frozen and thawed once, and the lysate was plated onto MA104 cells in a six-well plate in the presence of hyperimmune antiserum to the 69M strain (P4[10]:G8) for selection of the desired PTRV × DS-1 (P6[1]:G2) reassortant. The desired single VP7 gene substitution reassortant was selected and identified and then plaque-purified three times. The origin of genes of the reassortant was identified by polyacrylamide gel electrophoresis (PAGE) of its genomic dsRNAs. The origin of certain genes which was not able to be determined with certainty by PAGE was studied further by constant denaturant gel electrophoresis as previously described (Jones et al., 2003). Hyperimmune antiserum to each reference rotavirus strain or reassortant was analyzed for VP4- or VP7-specific antibodies to selected human and animal rotavirus strains or reassortants by 60% PRN assay.

Sequence and phylogenetic analyses of the gene encoding VP7, VP8 or NSP4 of the PTRV strain*

Full-length cDNA of the VP7 or NSP4 gene or 876 basepairs (bp) of the VP4 gene of the PTRV strain were amplified using primer pair Beg9 and End9 (Gouvea et al., 1990) (for VP7 gene), Wa10F and Wa10R (for NSP4 gene) or con2 and con3 (Gentsch et al., 1992) (for 876-bp fragment of VP4) as previously described (Hoshino et al., 2005). The PCR products were gel-purified using a microcentrifuge method with a Wizard SV Gel and PCR Clean-up System protocol (Promega, Madison WI). The nucleotide sequence of the entire open reading frame of the VP7 gene and NSP4 gene as well as the VP8* gene were sequenced at least twice using the BigDye terminator cycle sequencing kit (Applied Biosystems) with ABI PRISM 310 automated DNA sequencer. Phylogenetic tree of VP7, VP8* or NSP4 gene of the PTRV strain and other selected rotavirus strains was constructed by employing the Clustal W algorithm of the MegAlign program in DNASTAR software package (Madison, WI). The nucleotide sequences of the PTRV genes are retrievable from GenBank under the accession numbers AB180973 (VP7), AB180975 (VP8*) and AB180979 (NSP4).

RT-PCR genotyping

The genomic dsRNA extracted from approximately 20% original diarrhea stool suspension was analyzed by RT-PCR assay for determination of G and P genotypes using primers reported previously (Isegawa et al., 1993; Gouvea et al., 1994).

RNA–RNA hybridization in solution

Analysis of the genetic composition of the PTRV whole genome by RNA–RNA hybridization in solution was carried out as described previously (Flores et al., 1982; Nakagomi et al., 1989). Briefly, the ^{32}P-labeled single-stranded probe of the PTRV virus was hybridized to the denatured genomic RNAs of selected simian, bovine, porcine and human rotaviruses for 16 h at 65 °C, and the resulting hybrids were separated by PAGE. Autoradiography of each hybrid pattern was compared with the homologous pattern for analysis of relative reactions of the whole genome (Nakagomi and Nakagomi, 1993).

Analysis by neutralization of serum samples of selected pig-tailed macaques in the colony

The pig-tailed macaque from which the PTRV strain was isolated developed diarrhea in October 1990. Unfortunately,

serum samples derived from this specific macaque were no longer available for analysis. However, serum samples collected from other pig-tailed macaques in the same colony were available, and thus we analyzed a total of five serum sampled derived from three macaques (#1, #2 and #3) by neutralization. A pair of samples collected before and after 1990 from macaques #1 and #2 as well as a single sample collected in 1994 from macaque #3 were included (Table 4).

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A high incidence of intussusception in Japan as studied in a sentinel hospital over a 25-year period (1978–2002)

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SUMMARY

The development of second-generation rotavirus vaccines requires knowledge of baseline incidence rates for intussusception in infants prior to vaccine introduction. To obtain such estimates we reviewed clinical records in a hospital that served as the major provider of paediatric beds in a local community in the northern part of Japan. During the 25-year period (1978–2002), there were 91 hospitalizations due to radiologically confirmed intussusception in children <5 years of age, of which 45% were <1 year of age. Assuming that all children with intussusception in the area had been admitted to this hospital, there were an average of 185 and 78 hospitalizations per 100 000 person-years for children <1 year old and 5 years old respectively. There was period-to-period variability with no long-term secular trend in the incidence of intussusception. The incidence rate in Japan was among the highest thus far reported, providing further evidence of geographic variability.

INTRODUCTION

Rotavirus diarrhoea causes substantial morbidity and mortality worldwide. To reduce such disease burden, the first rhesus-human reassortant rotavirus tetravalent (RRV-TV) vaccine was licensed in the United States in 1998. However, in 1999 after less than 10 months of use the recommendations by paediatric and public health authorities were withdrawn. The products were recalled from the market because it was suspected of increasing the risk of intussusception at a rate of $\sim 1/11\,000$ during the week following immunization [1]. An accurate knowledge of the incidence of intussusception is critically important because it serves as a baseline for intussusception cases among those who will receive the second-generation rotavirus vaccines.

Intussusception is a pathological condition in which one portion of the intestine invaginates into an adjacent segment of the intestine, leading to a strangulating obstruction. It is a paediatric emergency in infants and children with its peak incidence at 3–9 months, and is characterized by palpable abdominal mass, colicky abdominal pain, vomiting, and the passage of bloody stool [2]. Intussusception is a rather rare disease and its incidence varies depending on geographic location and study periods. A recent and extensive review by the World Health Organization on intussusception concluded that in developed countries the baseline incidence of intussusception is between 0·5 and 4·3 cases/1000 live births or 0·66–1·2

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Table. *Numbers and incidence rates (per 100 000 person-years) of intussusception-associated hospitalizations among two age categories of infants and children in Akita, Japan, over the 25-year period (1978–2002)*

	0–11 months			0–59 months		
Period	No. of cases	Incidence rate	95% CI	No. of cases	Incidence rate	95% CI
1978–1982	15	250·0	140·0–412·3	28	89·8	59·7–129·8
1983–1987	5	97·3	31·5–227·1	11	47·9	23·9–85·8
1988–1992	5	124·9	40·5–291·6	12	67·2	34·7–117·4
1993–1997	9	244·3	111·8–463·6	26	165·2	107·9–242·0
1998–2002	7	210·6	84·5–433·8	14	96·8	52·9–162·4
Total	41	185·1	132·7–251·0	91	77·8	62·6–95·5

cases/1000 children <1 year of age [3]. However, there are no data in Japan and only one study from Asia is available in which 0·77 intussusception cases/1000 live births were reported on the basis of retrospective data from five hospitals in Taiwan during the period 1955–1964 [4].

The aim of this study was to estimate the incidence rate of intussusception and to examine if there was any secular trend using retrospective analysis of clinical records in a hospital that served as the major provider of paediatric beds in a local community in the northern part of Japan.

METHODS

The Odate Municipal Hospital, where this retrospective study was conducted, possessed 497 beds of which 32 were for paediatric in-patients. The hospital is located in the centre of the north-eastern part of Akita prefecture, Japan, an area with a population of approximately 90 000. In this study, however, we conservatively defined the catchment area of this hospital as consisting of three administrative regions, i.e. Odate city and two adjacent towns (Tashiro-machi, and Hinai-machi). This restriction was made to fulfil the assumption that all children living in this area would have been admitted to this hospital if they had intussusception during the period of 1978–2002. Admission logbooks covering January 1978 to December 2002, together with excerpted case records, were reviewed and original medical charts were referred to in 65% (59/91) of entries to obtain detailed clinical information. One of the authors (Y.T.) was actually involved in the diagnosis and treatment of all patients with intussusception in and after 1986.

A case was defined as a child <5 years of age who was admitted to the Odate Municipal Hospital with the discharge diagnosis of acute intussusception that was confirmed by the radiological examination with liquid contrast enema. We included only those patients who lived in the three administrative regions defined above. We excluded those cases in which there occurred spontaneous reduction of intussusception suspected by signs and symptoms or only with diagnostic ultrasonography before the radiological diagnosis was established.

To calculate the incidence rate of intussusception-associated hospitalizations for each 5-year subset of the study period, the number of hospitalizations during each 5-year subset period was divided by the person-years. The person-years for each 5-year subset period were calculated as the sum of the numbers of the live births for that 5-year period without any adjustment. The Poisson model was used to calculate the 95% confidence interval (CI) of incidence rates. The number of live births was obtained from the vital statistics data of Akita prefecture. The birth cohort in the catchment of the hospital was in continuous decline, and the number of live births in Odate city and the two adjacent towns almost halved from 1279 in 1978 to 629 in 2002.

RESULTS

For a period of 25 years starting from January 1978 to December 2002, we identified 91 entries of radiologically confirmed intussusception-associated hospitalizations in children <5 years of age (Table). Of these, 41 entries accounting for 45% of the total intussusception-associated hospitalizations occurred in infants (<1 year of age) (Fig. 1). From these hospital records, we estimated that the incidence rate for intussusception-associated hospitalizations averaged 78 hospitalizations per 100 000 person-years (95% CI 62·6–95·5) for children <5 years of age, and 185 hospitalizations per 100 000 person-years (95% CI

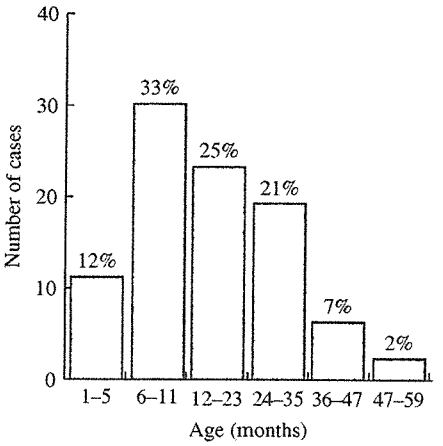

Fig. 1. Age distribution of intussusception-associated hospitalizations among infants and children in Akita, Japan, over the 25-year period (1978–2002).

132·7–251·0) in infants (<1 year of age) (Table). The average intussusception-associated hospitalization rates for every 5-year period varied from period to period with 48–165 per 100 000 person-years for the <5 years age group, and 97–250 per 100 000 person-years for the <1 year age group (Table). While incidence rates varied from period to period, there was no long-term secular trend for intussusception-associated hospitalizations over the 25-year period (Table).

Intussusception-associated hospitalizations showed a distinctive peak in the 6–11 months age group, accounting for 33% of the total hospitalizations (Fig. 1). While the hospitalizations in the <6 months age group accounted for 12%, only 3·2% occurred in the 3 months age group and none in the 0–2 months age group. Whereas the peak incidence occurred in the first year of life accounting for 45% of the total hospitalizations, there were similarly high percentages of hospitalizations (46%) occurring in the 12–35 months age group (Fig. 1).

Intussusception-associated hospitalizations showed no apparent seasonal pattern (Fig. 2). Of 91 entries for intussusception-associated hospitalizations, there were seven cases of readmissions and one case of three admissions. Thus, the total number of children who had intussusception was 82, of which 60% were male (male *vs.* female = 1·5:1). In every case hydrostatic reduction was undertaken, but 12 cases (13%) eventually received surgical treatment. Of 59 (65%) hospitalizations for which medical charts were available, 49 (83%) sought medical treatment within 12 h of the onset of disease, and 56 (95%) did so within 24 h of the onset of disease.

DISCUSSION

This retrospective study used highly specific methods to estimate a population-based incidence of intussusception among children who would be likely to receive second-generation rotavirus vaccines. Ensuring specificity may have led to underestimation of the true incidence in that only intussusception cases confirmed by radiological examination with liquid contrast enema were included whereas suspect cases (in which spontaneous reduction had occurred before radiological examination) were excluded. All 91 cases of intussusception-associated hospitalizations identified in this study occurred in children living in the administrative regions that we defined as the catchment area of the hospital. This study may also underestimate the true incidence rate of acute intussusception, because the possibility was not completely ruled out that some cases of intussusception among infants and children living within this defined region were admitted to other remote hospitals outside this region.

Despite the likelihood of underestimation, the incidence rate of intussusception-associated hospitalizations obtained in this study (185 hospitalizations per 100 000 person-years <1 year of age) ranks the highest among such incidence rates from various countries, and definitely exceeds the rates from the United States which ranged from 18 to 56 per 100 000 person-years <1 year of age [5–9]. However, it should be noted that intussusception rates in the United States varied by race, the highest being among infants of other races (112–217 per 100 000 person-years) than in black infants (32–50 per 100 000 person-years) and white infants (27–35 per 100 000 person-years) [6]. Thus, the incidence rate for intussusception-associated hospitalizations among Japanese infants is closer to that of infants of races other than whites and blacks. In this regard, it will be interesting to examine whether the incidence rate of intussusception in Korean infants is equally high because the incidence rate is predicted to be high in Korea based on a previous publication stating that on average as many as 64 patients presented annually to a single hospital in Korea [10]. Such variability in the incidence of intussusception by ethnic group, if proven, may reflect genetic or ethnic predisposition to the disease and should be addressed further when untoward effects of upcoming rotavirus vaccines are evaluated.

Few papers have examined secular trends of intussusception in a defined population. In the United States, the data from the Indian Health Service

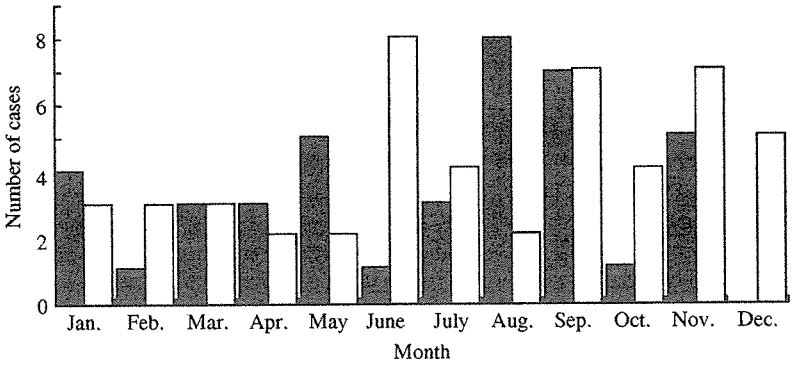

Fig. 2. Monthly incidence of intussusception-associated hospitalizations among two age categories of infants and children in Akita, Japan, over the 25-year period (1978–2002). ■, 0–11 months; □, 12–59 months.

showed a declining tendency of the incidence rate over the 17-year period (1980–1997), whereas the data from California, with a much larger dataset, did not indicate such decline at least for the 7-year period for which data were available [6]. We did not observe any declining trend or any recognizable pattern over the 25-year period (Table). Thus, caution must be exercised when the data only from two separate time points were used to address the question of whether the incidence rate of intussusception-associated hospitalizations was declining or not.

As to age distribution of intussusception-associated hospitalizations, the peak incidence occurred in the first year of life, particularly in the 6–11 months age group. However, it should be noted that there were similarly high percentages of hospitalizations (46%) occurring in the 12–35 months age group. This large proportion of intussusception-associated hospitalizations should be given attention, when the overall attributable risk of rotavirus vaccines for inducing intussusception is addressed. Under her assumption that some strains of human rotavirus may trigger intussusception, Nakagomi repeatedly raised the possibility that the RRV-TV vaccine might prevent subsequent severe rotavirus diarrhoea, thereby preventing potential cases of intussusception that might otherwise have been incurred by infection with intussusception-inducing strains of human rotavirus [2, 11]. Addressing such questions will require follow-up surveys of the cohort at least up to 35 months of age, and not up to 12 months of age, in order to see whether the overall reduction of intussusception cases occurs or not.

The proportion of cases that were successfully treated with hydrostatic enema under radiological monitoring was 87%, which may seem high, however, this did not result from the inclusion of unconfirmed suspect cases with spontaneous reduction but probably resulted from the early presentation of the patient for medical treatment. It is well known that late presentation of the patient often requires surgical intervention, sometimes leading to resection of the diseased intestine [2].

In conclusion, this study provides for the first time an estimate for the incidence rate of intussusception-associated hospitalizations in Japan. Our estimates rank the highest among the incidence rates for intussusception-associated hospitalizations that have been reported thus far. While there was period-to-period variation, there was no long-term secular trend of intussusception-associated hospitalizations over the 25-year study period, suggesting considerable geographic, ethnic and racial, and short-term temporal variability in the occurrence of intussusception. Thus, trying to generalize from any particular study or group of studies may result in misleading conclusions. Our study also implies that future vaccine trials or post-licensure vaccine efficacy studies need to be based on knowledge of current incidence of intussusception in the population being studied.

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Molecular Characterization of Rotavirus Gastroenteritis Strains, Iraqi Kurdistan

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Of 260 children with acute diarrhea in Erbil, Iraqi Kurdistan, 96 (37%) were infected with rotavirus. Reverse transcription–polymerase chain reaction identified G1, G4, G2, G9, P[8], P[6], and P[4] as the most common genotypes. Eight G/P combinations were found, but P[8]G1 and P[4]G2 accounted for >50% of the strains.

Rotavirus is the single most important cause of severe gastroenteritis in young children throughout the world. Globally, an estimated 702,000 children die each year due to rotavirus diarrhea (*1*). This large impact of rotavirus disease has speeded the development of rotavirus vaccines, and 2 live, attenuated rotavirus vaccines are expected to be available for global use within the next few years (*1*). Therefore, determining the prevalence and types of rotaviruses within regions is essential to prepare for introducing a vaccine.

Rotavirus, a member of the family *Reoviridae*, has a triple-layered capsid that contains 11 segments of double-stranded genomic RNA. While protective immunity against rotavirus infection is not completely understood, serotype-specific immunity is believed to play a major role (*1*). Rotavirus serotypes are defined by genome segment 4 for the P (protease-sensitive protein) type and by genome segment 9 (or 7 or 8, depending on the strain) for the G (glycoprotein) type. Fourteen G types exist, of which G1–G4 are commonly found in children with diarrhea, but a recent increase in the detection of serotype G8 and G9 strains has captured considerable attention (*2–4*). While >24 P types have been reported in the literature, only P[4], P[6], and P[8] are commonly found among human rotaviruses (*1–3*).

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In Iraq, the death rate in children <5 years of age was reported to be 130/1,000 for boys and 120/1,000 for girls in 2003 (*5*). Diarrhea is a major cause of illness and death in Iraqi children; however, little information exists about the origin of childhood diarrhea. Only a single study showed that rotavirus accounted for 24% of acute diarrhea in hospitalized children in Basrah (*6*).

The Study

This descriptive, cross-sectional study of 6 weeks' duration was undertaken at Erbil Paediatric Hospital in Iraqi Kurdistan between March and May 2005. The study recruited 260 children from 1 month to 5 years of age who were admitted with acute diarrhea (defined as the passage of watery or loose stools ≥3 times per day for <2 weeks' duration). Basic demographic, epidemiologic, and clinical information were collected prospectively, according to a pro forma. Ethical approval for the research was obtained from the review boards of the Liverpool School of Tropical Medicine and Erbil Paediatric Hospital. The hospital serves a population of ≈1.5 million, and ≈3,116 births per month occur in this population.

A commercial enzyme-linked immunosorbent assay (ELISA) was used to detect rotavirus antigen (Rotaclone, Meridian Diagnostics, Cincinnati, OH, USA). Stool samples were then stored frozen in the laboratory of the study hospital until they were transported to Liverpool for rotavirus genotyping and electropherotyping. All samples (66) with an absorbance equal to or greater than the positive control for the ELISA were subjected to genotyping. Rotavirus genomic RNA was extracted with guanidine isothiocyanate, followed by adsorption to and elution from silica particles according to the method described by Gentsch et al. (*7*). The purified RNA was then used to determine the P type and G type of rotavirus present in the stool specimens by reverse transcription-polymerase chain reaction as described by Gentsch et al. (*7*) and by Gouvea et al. (*8*). Rotavirus electropherotypes were determined by polyacrylamide gel electrophoresis according to the method described by Koshimura et al. (*9*), with some modifications.

Of 260 stool specimens tested by ELISA, 96 (37%) were positive for rotavirus. Rotavirus-positive patients had a mean age (SD) of 9.3 (8.5) months compared to 11.1 (10.1) months in the rotavirus-negative patients. These results suggest that rotavirus positive cases were slightly younger, although the difference was not statistically significant ($p = 0.14$). Rotavirus-positive patients were similar to rotavirus-negative patients in most of the epidemiologic and clinical characteristics (data not shown). However, rotavirus-positive patients were more likely to exhibit vomiting and have a shorter duration of diarrhea ($p<0.01$ for both analyses).

Of the 66 rotavirus strains that underwent molecular characterization, 25 (38%) were G1, 11 (17%) were G2, 13 (20%) were G4, and 7 (11%) were G9. Four (6%) were mixed infections (3 G1/G2, 1 G2/G4), and 6 (9%) were G nontypeable. A total of 7 (11%) were P[4], 10 (15%) were P[6], and 45 (68%) were P[8]. One showed mixed P[4] and P[8] genotypes (mixed with G1/G2), and 3 (5%) were P nontypeable. None of the rotaviruses was both G and P nontypeable.

A total of 8 different P and G genotype combinations were detected (Table). The most common combinations were P[8]G1 (19, 33%), P[8]G4 (12, 21%), P[4]G2 (6, 11%), P[6]G1 (6, 11%), and P[8]G9 (6, 11%). The unusual combination of P[6]G9 was detected in 1 of the patients.

An electropherotype was obtained for 50 of the 66 genotyped strains. Of these, 11 (22%) had a short electropherotype, and 39 (78%) had a long electropherotype (Table). Most of the short electropherotypes were the expected G2 strains; however, 1 strain (P[8]G9) also had a short electropherotype.

Table. Rotavirus genotypes and electropherotypes*

Genotype	No.(%) fully typeable strains	Electropherotype†
P[4]G2	8 (15)	Short (7/8)
P[6]G1	6 (11)	Long (5/6)
P[6]G4	1 (2)	ND
P[6]G9	1 (2)	Long
P[8]G1	19 (33)	Long (13/19)
P[8]G4	12 (21)	Long (12/12)
P[8]G9	6 (11)	Long (4/6); short (1/6)
P[6]GNT	2	Long (2/2)
P[8]GNT	4	Long (2/4)
P[NT]G2	3	Short (3/3)

*Four rotavirus infections were mixed: P[8]G1/G2 (2), P[4]G2/G4 and P[4]/[8]G1/G2. †Indicates number of strains electropherotypeable in the genotype combination; ND, not determined.

Conclusions

The only other study of viral gastroenteritis from Iraq (Basrah in the south) demonstrated that 24% of children with acute gastroenteritis were infected with rotavirus (*6*). This figure is somewhat lower than the 37% detection rate in our study. Moreover, the prevalence we found is similar to those reported from neighboring countries such as Iran (35%) (*10*), Jordan (33%) (*11*), Kuwait (40%) (*12*), and Turkey (37%) (*13*). However, our study was undertaken over a 6-week period from the end of March to the beginning of May 2005. No information is available on the seasonal prevalence of rotavirus infection in Iraq, and a longer study is warranted to determine the true prevalence of rotavirus infection and its seasonality in northern Iraq. However, the peaks of rotavirus infection in Iran, Kuwait, and Turkey were February–March, March–May, and December, respectively (*10,12,13*). More than 75% of our cases of rotavirus diarrhea occurred in children <1 year of age, with an overall mean age of slightly more than 9 months. This pattern is similar to that in many developing countries. In Jordan the mean age of children with rotavirus diarrhea was 7.2 months (*10*). However, in other countries in the region the distribution was different; 30% of the infants with rotavirus in Iran were <1 year of age (*10,12*), 50% in Kuwait were <1 year of age, and 63% in Turkey were <2 years of age (*13*).

Although this study period was brief, we detected a variety of rotavirus strains. Four of the major global human rotavirus genotypes (G1, G2, G4, G9) were detected, as were each of the major P genotypes (P[4], P[6], P[8]). In Iran, in a study undertaken in 2001 and 2002, only G1 and G2 rotaviruses were detected, and the only P types were P[4] and P[8] (*10*), and in Turkey over a 2-year period (2000–2002), G types G1–G4 and G9, as well as each of the 3 major human P types were found (*14*). In Iraq, the combinations P[8]G1 and P[8]G4 accounted for >50% of the strains of rotavirus. In Iran, P[8]G1 accounted for 95% of the strains, but P[8]G4 was not detected (*10*). In Turkey, P[8]G4 (42%) and P[8]G1 (27%) accounted for more than two thirds of the strains (*14*). G3 rotaviruses were not detected in Iraq or Iran, and in Turkey only 1 of the 65 strains was of genotype G3. Genotype G9 was detected in 13% of the Iraqi strains, a similar finding to results in Turkey (*14*). We also detected mixed rotavirus infections in 6% of our patients, again similar to the findings in Turkey (*14*). The presence of mixed rotavirus infections indicates that new rotavirus strains may evolve by reassortment (*1–3*).

Finally, among the G9 strains, one P[6]G9 had a long electropherotype, and one P[8]G9 had a short electropherotype. The P[6]G9 and P[8]G9 strains were both cultured and subgrouped by ELISA with monoclonal antibodies and found to be of subgroup II. Partial sequences (831 bp) were obtained for their VP7 genes (AB247941 and AB247943; available from the DNA Data Bank of Japan: www.ddbj.nig.ac.jp). They showed 99.4% similarity to each other and >99% similarity to strains from Australia (AY307087), Belgium (AY487858, AY487856), and India (RG9491165). A strain similar to our P[6]G9, called variant 3, was first detected in India, and strains similar to our P[8]G9, called variant 2, have been described in Bangladesh and in the United States (*15*).

Although the major global genotypes (except for G3 strains) were detected, clearly, rotavirus strains are continuing to diversify in Iraq and other parts of the region. This circumstance may pose challenges to the efficacy of rotavirus vaccines.

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Dr Ahmed is a pediatrician who conducted this research as part of the requirements for his master's degree in tropical pediatrics. His research interests are in viral gastroenteritis and respiratory tract infections.

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A lack of significant association between the electropherotype or G-serotype of the infecting strain and disease severity of rotavirus gastroenteritis

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Summary. Despite many previous studies, the question has not been settled as to whether some human rotavirus strains are more virulent than others. Since disease severity is most clearly reflected by the hospitalization status of the infected children, we examined whether there was any difference in the distribution of dominant strains between inpatient and outpatient groups. The study population comprised 763 children with acute diarrhea who were treated at a general hospital in Honjo City, Akita, Japan, during 1986–1997. Rotaviruses from stool specimens were classified into 77 electropherotypes using polyacrylamide gel electrophoresis. A single dominant strain or two co-dominant strains circulated simultaneously with some infrequent strains in most rotavirus seasons. Over the 11 rotavirus seasons, there was no significant difference in the relative frequencies of 15 rotavirus strains between the inpatient and the outpatient groups when strains of rotavirus were defined by their electropherotypes in polyacrylamide gel electrophoresis. However, infection with one G1 strain that co-dominated with a G4 strain carrying an identical electropherotype except the VP7 gene resulted in a statistically significantly reduced risk of hospitalization. There was no significant difference in the relative frequencies of four major G-serotypes or long/short RNA pattern. We conclude that the virulence or disease-causing potential of human rotavirus is not substantially different in the majority of strains.

Introduction

Group A rotavirus, species *Rotavirus A*, belonging to the genus *Rotavirus* within the family *Reoviridae*, has been established as the major etiological agent of

acute gastroenteritis in infants and young children worldwide [9]. Two outer capsid proteins of rotavirus, VP7 and VP4, can induce production of neutralizing antibodies, and define the G- and P-serotype, respectively. The inner capsid protein VP6, the most abundant viral protein, defines another antigenic specificity called subgroup (I or II), but antibodies directed against VP6 are not involved in virus neutralization. The genome of the rotavirus consists of 11 segments of double-stranded RNA that are readily resolved by polyacrylamide gel electrophoresis (PAGE). The resulting RNA migration pattern is termed electropherotype, and there are two major RNA migration patterns, "long" and "short", based on the relative migration rate of gene segments 10 and 11. The electropherotype is unique to an individual strain, and has long been used to track down the individual strains in epidemiologic studies of rotavirus infection [6]. Major observations made in such molecular epidemiologic studies include: (i) multiple strains co-circulate in the same epidemic season; (ii) only one or two strains predominate in each season; and (iii) different strains emerge in each epidemic season [8, 11, 15].

One lingering question that has been addressed is whether various characteristics of rotavirus strains such as G- and P-serotypes are associated with the severity of illness upon infection. There have been studies that aimed to determine whether the virulence of human rotavirus differs according to such virological parameters, but the results are inconclusive [1, 2, 12, 17, 19, 21]. Most of these preceding studies focused on serotypes or subgroups rather than individual strains. Furthermore, few studies have examined whether the virulence differed among multiple strains co-circulating in the same geographic region during the same epidemic season. In addressing this question, we made an assumption that, if there were virulent and less-virulent strains among human rotaviruses, there should be some detectable differences in the distribution between rotavirus strains isolated from hospitalized patients who had severer diarrhea and those isolated from outpatients who had less severe disease. Thus, the aim of this study was to determine whether there was any difference in the distribution among circulating strains, as identified by their electropherotypes, between rotaviruses isolated from hospitalized children with diarrhea and those isolated from children treated only at the outpatient department of the hospital.

Materials and methods

Stool specimens

Stool specimens were collected from patients with acute diarrhea (aged 15 years or younger), who had been treated at a general hospital in Honjo City, Akita Prefecture, Japan, between January 1987 and December 1996. In this study, children who required hospitalization were classified as the inpatient group, and those who did not require hospitalization and treated only at the outpatient department of the same hospital were classified as the outpatient group.

Because no rotavirus-positive specimens were found between August and September during the study period, each rotavirus season was defined as 12 months beginning in September and ending in August of the next year. Thus, the whole study period spanned 11 consecutive rotavirus seasons, although the first season (86–87) extended from January to August 1987 (8 months), and the last season (96–97) extended from September to December 1996 (four months).

Detection of rotaviruses, RNA extraction, and electrophoresis

The presence of rotaviruses in stool specimens was initially examined by the latex agglutination assay. From approximately 10% suspensions of rotavirus-positive fecal specimens, genomic RNA was extracted with phenol and chloroform, and precipitated in ethanol. For those specimens containing only a small amount of viral RNA, genomic RNA was extracted after ultracentrifugation using a Beckman TLA 100.4 rotor at 60,000 rpm for 1 hour. Genomic RNA was resolved by PAGE, with a 10% separating and a 4% stacking gel without sodium dodecyl sulfate in the Laemmli buffer system. After electrophoresis for 16 hours at a constant current of 8 mA per gel, gels were stained with ethidium bromide, followed by visualization of the RNA bands under UV illumination. Wa (G1, P1A [8]) and DS-1 (G2, P1B [4]) were used as reference strains.

Nomenclature of the electropherotypes was essentially the same as that described by Koshimura et al. [11]. However, a letter O was inserted after either LH (long RNA pattern) or SH (short RNA pattern) to denote strains only from the outpatient group. A strain was defined as the dominant strain when a single electropherotype was found in more than 50% of the inpatient or the outpatient groups in any one season. Two strains were defined as co-dominant if the electropherotype of each strain accounted for more than 25% but less than 50% of all strains in any one season.

Determination of G-serotype

The G-serotype of rotaviruses was determined by enzyme-linked immunosorbent assay (ELISA), using monoclonal antibodies for serotypes G1-4 (ROTA-MA, Serotec Co, Sapporo, Japan) [22] as capture antibodies. The procedure and the judging criteria were as described previously [11]. Reverse transcription (RT)-PCR was applied to specimens whose serotype could not be determined, using consensus and type-specific primers for G1-4 [4, 13].

Statistical analysis

The chi-square test was used to examine whether there was any difference in the distribution of rotavirus strains between the inpatient and outpatient groups. When the expected counts under the null hypothesis were less than five in 25% or more of the cells, Fisher's exact test was used. Statistical analysis of dominant strain distribution was performed for each rotavirus season, since previous studies showed that different strains emerge in each season [8, 11]. We combined minor strains into one category, since the data were statistically sparse, and infrequent strains are, by definition, less important from the epidemiological point of view. It was also taken into consideration that dominant strains accounted for more than 70% of rotaviruses detected from all strains identified during the 10-year study period [11]. In addition, to evaluate the virulence of each strain (as identified by electropherotype), a case control study was performed for each strain in which the presence or absence of exposure to the particular strain (electropherotype) was compared in inpatients (cases) and outpatients (controls).

The ratio of the number of subjects in each rotavirus season to the total number of subjects was not constant between the inpatient and the outpatient groups. Therefore, the percentage of G-serotype and RNA pattern (long or short) was standardized for each rotavirus season to minimize the possibility of confounding, using as weight the proportion of the number of inpatients and outpatients for each rotavirus season amongst the total of 763 patients. Multiple logistic regression analysis, with or without adjustment for rotavirus season, was also applied to evaluate whether there was any association between either the G-serotype or the RNA pattern and hospitalization. All statistical analyses were performed using the SAS software package [18], and all *P*-values presented are two-tailed.

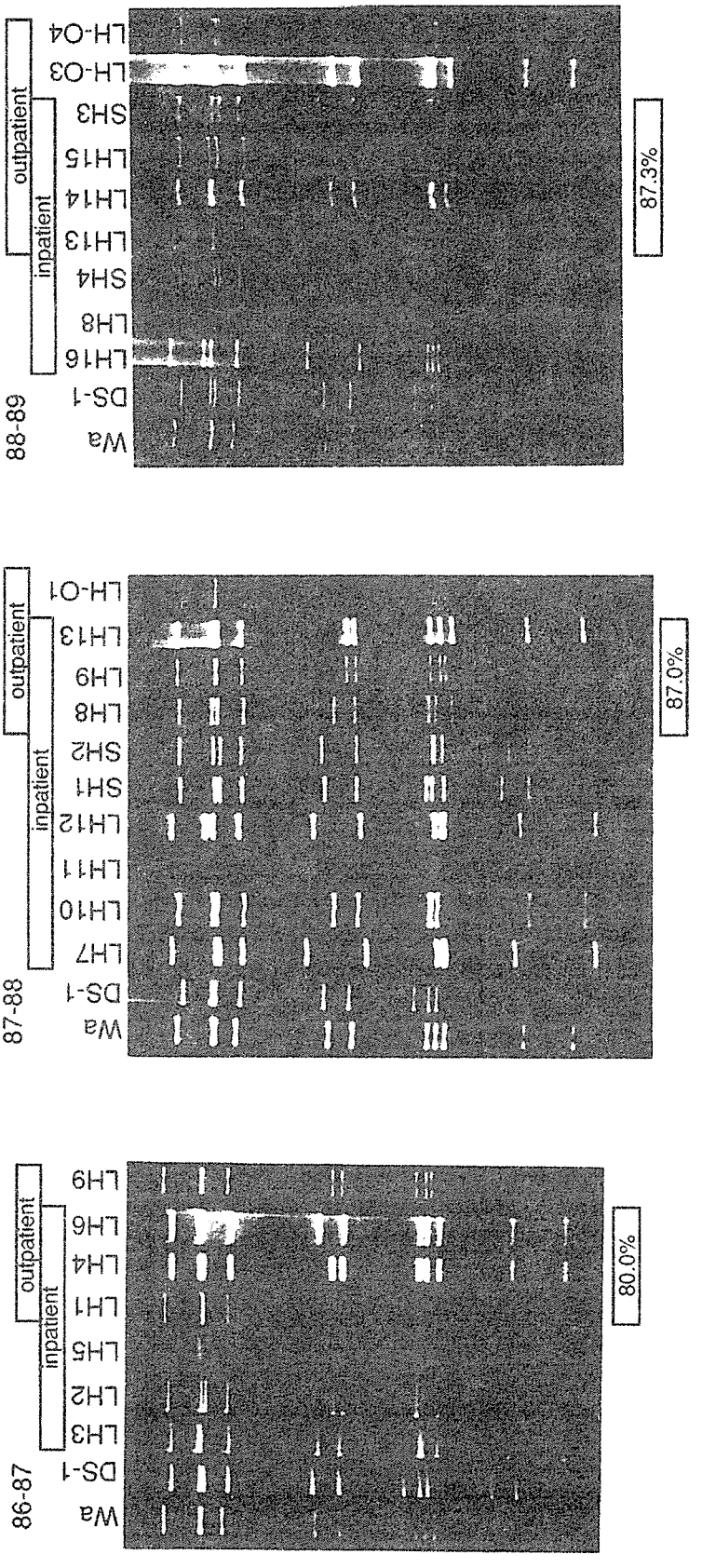

Fig. 1 (continued)

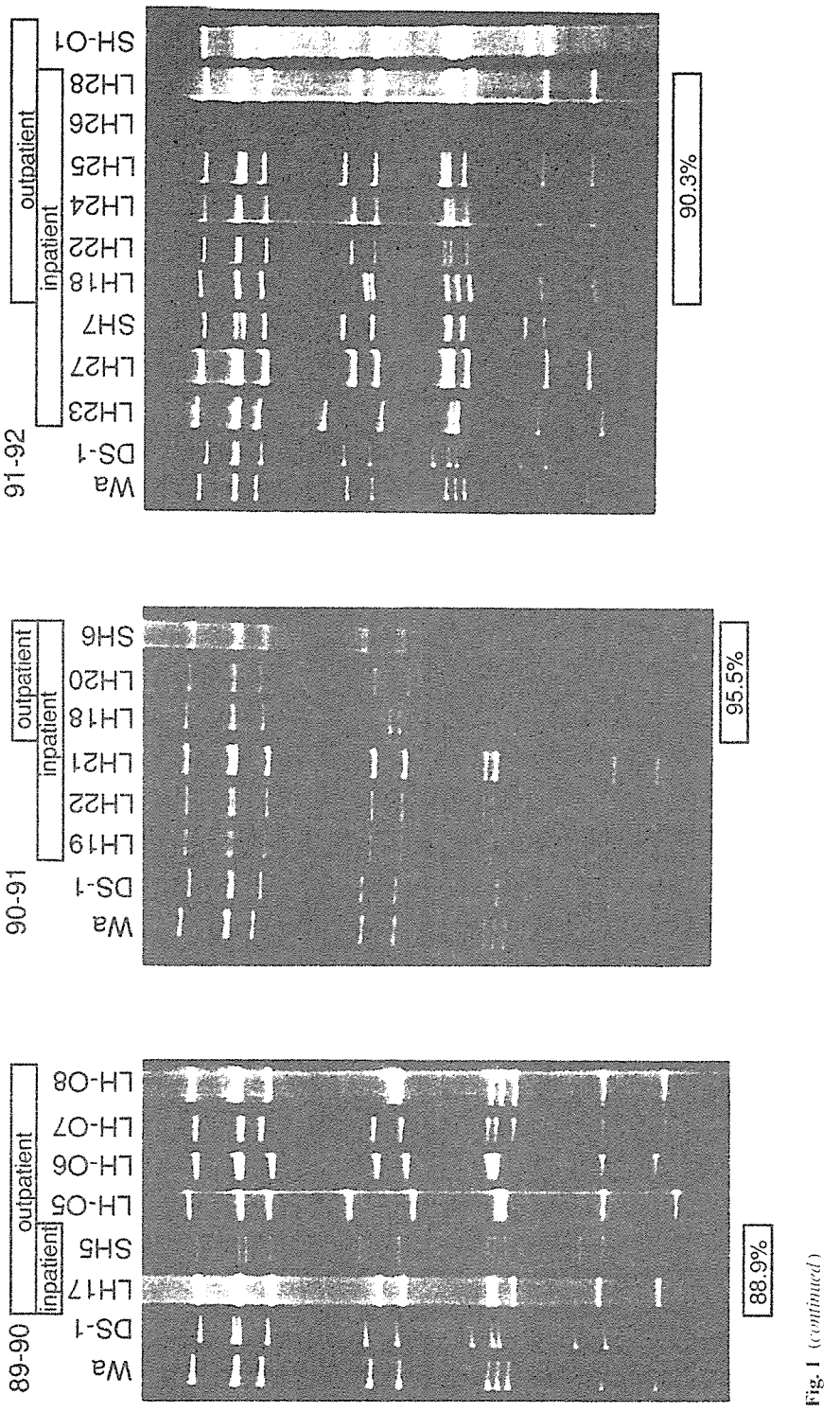

Fig. 1 (*continued*)