When measles was introduced to the USA, extensive transmission was controlled within two generations of secondary transmission by maintaining high-level vaccine coverage, effective surveillance, and a prompt outbreak response [CDC, 2008a,b]. The two-dose strategy has eliminated measles without indigenous measles transmission chain [Janaszek et al., 2003; Meissner et al., 2004]. Thus, the proportion of countries offering children a second dose of measles vaccine is increasing, and 168 (88%) countries implement the two-dose strategy [WHO, 2006]. The new goal is a 90% reduction of measles mortality by 2010 compared with the mortality in 1999 [WHO, 2006].

As for the reporting system for measles surveillance in Japan, through 3,000 sentinel clinics or hospitals for pediatric infectious diseases and 450 clinics for adult measles surveillance, patients with clinically suspected measles are reported to the Regional Health Care Center mostly without any systematic virological studies. The last measles outbreak was observed in 2001 in Japan. Among 33,812 reported cases, most patients were under 5 years of age and had not been vaccinated. Through a vaccination campaign to increase immunization coverage at 1 year of age, the number of reported cases decreased to 545 in 2005. The Japanese Government implemented a two-dose, combined measles and rubella vaccine (MR) for children at 1 and 6 years of age in 2006 [National Institute of Infectious Diseases, 2007]. Therefore, elimination of measles would be expected. However, patients with measles were reported increasingly in March 2007, and this outbreak expanded subsequently throughout the Japanese districts, peaking in the middle of May. Further, several reports indicated measles transmission by Japanese travelers or participants in an international sporting event [Delaportel et al., 2007; CDC, 2008a].

This outbreak showed different characteristics, demonstrating that most patients were young adults or adolescents attending high school and university students, with a much lower proportion of young infants, at the early stage of the outbreak. Finally, cases of measles were reported in all age groups, and a total of 3,105 pediatric cases and 959 adult patients were reported in 2007 and the outbreak is still ongoing. The actual number of cases of measles was estimated to be 10 times higher than the number of reported cases for pediatric measles, and 50–60 times higher for measles in adults. Thus, the number of patients with measles was suspected to be approximately 31,000 for pediatric and 50,000 for measles in adults. The age distribution was quite different from the previous outbreak in 2001. The number of patients with measles was the highest at 1–4 years of age, accounting for 40–50% in 2001, which decreased to 22% in the outbreak in 2007. A significant shift in the age distribution of cases of measles in 2007 was observed to be 10–14 years or higher, accounting for 44% in 2007 [National Institute of Infectious Diseases, 2007]. A relatively large proportion of adult patients with measles had a previous immunization history and, thus, typical measles symptoms were not observed, with patients only with mild fever and skin eruptions. In this report, virus isolation and detection of the measles virus genome by the reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) were examined in clinical samples. The genotyping and antigenicity of current circulating viruses were also investigated.

MATERIALS AND METHODS

Materials

Nasopharyngeal swabs were obtained from 22 patients suspected of measles on day 1 or 2 of the onset of the rash. Four patients had typical symptoms of measles with post-pigmentation, and 18 had atypical symptoms with fever for less than 3 days and mild eruptions, which did not satisfy the criteria of clinical measles by WHO, [2006].

Virus Isolation

B95a cells were cultured in RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS) and 0.1 ml of clinical samples was used to inoculate a monolayer of B95a cells in a 24-well plate. After two passages, samples without cytopathic effect (CPE) were considered negative. Seven strains were isolated in this study. MVi/Aichi.JPN/44.06 [D9] was isolated from a sporadic case in Nagoya City, a central district of Japan, in 2006. MVi/Tokyo.JPN/17.07 [D5] and MVi/Tokyo.JPN/18.07 [D5] were isolated in Tokyo and MVi/Mie.JPN/19.07 [D5] and MVi/Mie.JPN/23.07 [D5] in Mie Prefecture, a central district of Japan, in 2007. In addition, MVi/Mie.JPN/41.07 [D5] was obtained in the middle of the outbreak in 2007 and MVi/Mie.JPN/03.08 [D5] was isolated in 2008.

RT-PCR and Sequence Analysis

Total RNA was extracted from 200 μl of clinical samples with a magnetic bead RNA extraction kit (TOYOBO Co., Ltd., Osaka, Japan), and the RNA pellet was suspended in 30 μl of distilled water. The pellet was subjected to RT-nested PCR and RT-LAMP targeting the C-terminus of the N protein region, known as the most variable region [WHO, 2001]. The measles virus genome was first converted to cDNA with the N-430 (+) primer (5′-ATTAGTAGTGATCAATCCAGG) with AMV reverse transcriptase (Life Technologies Inc., Gaithesburg, MD). The first PCR was performed with a set of N-850 (+) (5′-TAGAAACTATGTATCCTGCT-3′) and MPX (−) (5′-AGGCCTGATTGAACCATGAT-3′) and the nested PCR was conducted with N1200 (+) (5′-GATCCAGCATATTTTAGATTAG-3′) and NP-P2 (−) (5′-AGGGTAGGCGGATGTTGTTCT-3′). PCR was performed using 1.25 U of *Taq* DNA polymerase (TaKaRa Bio-Medicals, Tokyo, Japan) with a TaKaRa thermal cycler (TaKaRa BioMedicals), with 30 rounds of thermal cycling conditions: denaturing at 93°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2.5 min. PCR products were confirmed by

electrophoresis through 1.5% agar stained with ethidium bromide, as previously reported [Nakayama et al., 1995; Yamaguchi, 1997; Zhou et al., 2003]. PCR products were excised from the gel and applied to sequence analysis by the dye terminator method using ABI 3130 (Applied Biosystems, Tokyo, Japan).

Measles Virus Loop-Mediated Isothermal Amplification (LAMP)

The LAMP method is characterized by auto-cycling strand displacement DNA synthesis with *Bst* DNA polymerase (New England Biolabs, Ipswich, MA) and a specially designed set of primers. Six LAMP primers were synthesized, recognizing eight different regions: F3 (5′-ACATTGGCATCTGAACTC), B3 (5′-TCCTCGACTCTGTTTGAC), FIP (5′-TGTCCTCAGTAGTATGCATTGCAGGTATCACTGCCGAGGATG), BIP (5′-AGCCCAAGTGTCATTTCTACACGGTGTCCTATCTTCCTTGCCCCCC), F Loop (5′-ATCTCTGAAACAAG), and B Loop (5′-CAAAGTGAGAATGAGCT). For the LAMP reaction, the mixture was made up to a total of 25 μl of reaction mixture, containing 40 pmol (each) of FIP and BIP, 5 pmol (each) of F3 and B3, 20 pmol (each) of Loop F and Loop B, 1.4 mM each of dNTPs, 0.8 M betaine, 20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween-20, 0.5 U AMV reverse transcriptase (New England Biolabs), 8 U *Bst* DNA polymerase (New England Biolabs), and 5 μl of sample RNA. The reaction mixture was subjected to real-time turbidimeter LA200 (TERAMECS, Kyoto, Japan) [Mori et al., 2004; Fujino et al., 2005] and the LAMP reaction was carried out at 63°C for 60 min. The turbidity measurement was closely related to the amplification of DNA and the turbidity >0.1 was considered LAMP-positive [Mori et al., 2004].

Neutralization Test (NT) Antibody

A total of 32 serum samples were used, which were obtained in April 2007, just before the peak of the outbreak, as part of a sero-epidemiological study on entering primary school at the age of 6 years, having received two doses of measles vaccine. The AIK-C vaccine strain [A], MVi/Tokyo.JPN/31.00-K [D5], MVi/Tokyo.JPN/17.07 [D5], and MVi/Aichi.JPN/44.06 [D9] were used as the challenge virus. Sera were treated at 56°C for 30 min to inactivate the complement activity and serially diluted by twofold, starting from 1:4 dilutions. Diluted sera were mixed with approximately 100 TCID50 of challenge virus at 37 C for 90 min and the mixture was placed on a monolayer of B95a cells. NT antibody titers were expressed as the reciprocal of the serum dilutions that neutralized the appearance of the CPE of measles virus [Okafuji et al., 2006].

RESULTS

Laboratory Examination of Patients With Non-Typical Measles Illness

From March 2007, several patients with measles were observed in outpatient clinics and 22 clinical samples were examined for virus isolation and genome detection and the results are shown in Table I. Four patients had typical measles with a marked fever for more than 3 days and typical measles eruptions with post-pigmentation. Three patients were adults and one had a history of vaccination. One patient was 8 years old and complicated with Gitelman syndrome during the measles illness. Two measles strains (MVi/Tokyo.JPN/17.07 and MVi/Tokyo.JPN/18.07) were isolated and the measles genome was detected in all patients by RT-PCR and RT-LAMP (Table Ia).

During the measles outbreak, 18 clinical samples were obtained from patients with non-typical measles with mild febrile illness and eruptions for less than 3 days. They were over 15 years of age and had a past history of single-dose measles immunization at 1–3 years of age. Measles virus was not isolated but the measles genome was detected in 9 (50%) by RT-PCR and in 12 (67%) by RT-LAMP (Table Ib). All RT-PCR positive samples were also positive on RT-LAMP.

MVi/Aichi.JPN/44.06, MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, and MVi/Mie.JPN/41.07 were isolated from patients with modified measles and MVi/Mie.JPN/03.08 was isolated from typical pediatric measles patient. Detection of the measles genome was not examined directly from the clinical samples in these five cases.

Genotype Analysis

Seven measles strains were isolated and examined for sequence analysis of the C-terminal of the N protein region, as recommended by WHO, [2005a]. MVi/Aichi.JPN/44.06 was isolated from a sporadic case

TABLE I. Results of Laboratory Examinations

Case	Vac	V. Iso.	RT-PCR	LAMP
a. Four typical measles cases				
1 (23Y)	+	+	+	+
2 (19Y)	–	–	+	+
3 (18Y) Encephalitis	–	–	+	+
4 (8Y) Gitelman syndrome	–	+	+	+
	V. Iso. (+)	RT-PCR (+)	LAMP (+)	
b. 18 cases with mild fever and rash	0/18	9/18	12/18	

Vac: Past history of measles vaccination
V. Iso: Virus isolation

in Nagoya in 2006, a central district of Japan, before the nationwide outbreak, and this strain did not cause further transmission. MVi/Tokyo.JPN/17.07 and MVi/Tokyo.JPN/18.07 were isolated in Tokyo. MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, MVi/Mie.JPN/41.07, and MVi/Mie.JPN/03.08 were isolated in Mie Prefecture, a central area of Japan, during the outbreak 2007–2008. The results of phylogenetic analysis are shown in Figure 1. MVi/Aichi.JPN/44.06 was D9 and two stains isolated in Tokyo at the beginning of the outbreak in 2007 were identified as genotype D5. Four strains isolated in Mie Prefecture were also identified as genotype D5. MVi/Gunma.JPN/19.07 was isolated in Gunma Prefecture, located in the north direction of Tokyo, was also D5 [Morita et al., 2007]. Measles outbreaks last for more than a year and a relatively homologous strain has been circulating throughout Japan.

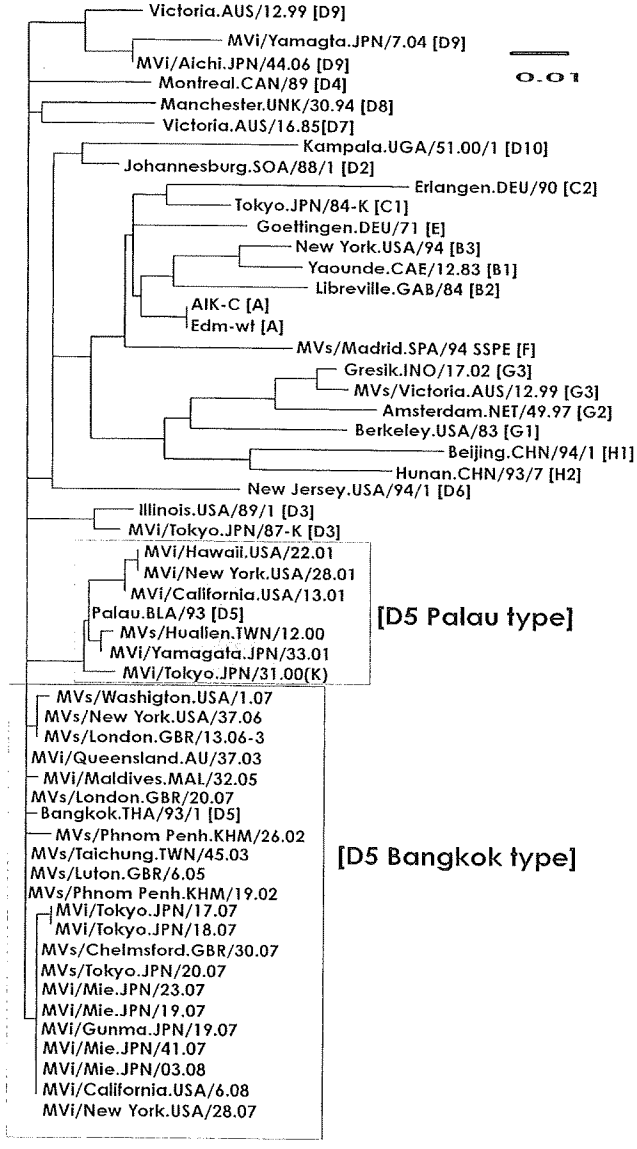

Fig. 1. Phylogenetic analysis of measles virus in the N gene. MVi/Aichi.JPN/44.06 was isolated in 2006 and MVi/Tokyo.JPN/17.07, MVi/Tokyo.JPN/18.07, MVi/Mie.JPN/19.07, MVi/Mie.JPN/23.07, MVi/Mie.JPN/41.07, and MVi/Mie.JPN/03.08 were isolated during the outbreak 2007–2008 in this study. MVi/Gunma.JPN/19.07 was reported by Morita et al. [2007] and other strains were registered in GeneBank. The Palau type D5 strains are shown in gray square and the Bangkok type D5 strains in open square.

Two different reference strains, MVi/Bangkok.THA/93/1 and MVi/Palau.BLA/93, were identified as genotype D5 and the Palau type D5 was transmitted via a Japanese traveler in 1993 [Rota et al., 1998]. The Palau type D5 was a major circulating genotype from 1990 to 1997 and from 2002 and afterward [Nakayama et al., 2003; Zhou et al., 2003; Okafuji et al., 2006]. However, the D5 strains in 2007–2008 belonged to the Bangkok type D5. Among D5 sequences obtained in 2007–2008, there was one nucleotide difference in the target of the N gene. Accession numbers of the partial N gene sequence of the isolates in this study are AB426895–99.

In Switzerland, 11 cases of measles were reported between March and April 2007 and, thereafter, a large nationwide outbreak was reported with further expansion to other European countries and the USA [Delaporte et al., 2007; Richard et al., 2008; CDC, 2008b]. MVi/New York.USA/28.07 and MVi/California.USA/6.08 were registered as imported from Switzerland and classified into the Bangkok type D5. In 456 nucleotides of partial N gene, there was no difference among isolates in outbreak in Japan, 2007–2008 and those prevalent in Europe and the USA. The epidemiological linkage was not identified but would be suspected by molecular epidemiological data.

The entire length of the H gene was sequenced and analyzed together with reference strains and data from the previous reports. Four strains isolated in 2007 were classified as the Bangkok type D5, not Palau type (Fig. 2). Differences in the nucleotide and amino acid sequences of the H gene are shown in Table II. Sequence variations of the H gene consisted of 52 (2.8%) to 59 (3.2%) nucleotide differences among genotypes A (AIK-C vaccine strain) and D5 strains, and 66 (3.6%) between genotypes A and D9. There were 16–19 (2.6–3.1%) amino acid changes in D5 or D9 in comparison with genotype A. In the same D5 strains, MVi/Tokyo.JPN/17.07 [Bangkok type] showed 35–43 (1.9–2.3%) nucleotide differences in comparison with the Palau type D5 strains, MVi/Palau.BLA/93 or MVi/Tokyo/JPN/31.00-K, but 16 (0.9%) in comparison with MVi/Bangkok.THA/93/1. Accession numbers for the entire H gene sequence of the isolates in this study are AB426900–04.

Antigenic Differences

MVi/Tokyo.JPN/18.07 [Bangkok type D5], MVi/Tokyo.JPN/31.00-K [Palau type D5], MVi/Aichi.JPN/44.06 [D9], and the AIK-C vaccine strain [A] were used for the analysis of antigenicity. Five or six serum samples for each NT antibody titer were selected, for which the NT titers against the AIK-C strain had already been checked, and a total of 32 sera were used for the analysis of antigenicity. The challenge viruses were

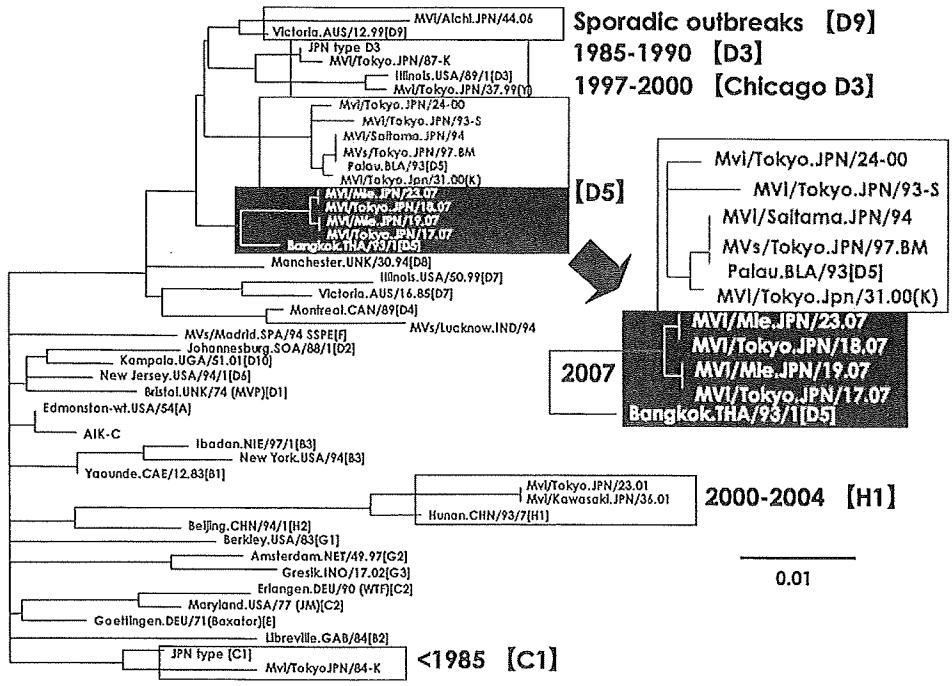

Fig. 2. Phylogenetic analysis of measles virus in the H gene. Strains in gray square are Palau type D5 and those in black square Bangkok type D5.

adjusted to 100 TCID50 and the results are shown in Figure 3. There was no significant difference in NT antibody titers within fourfold dilutions against the different genotypes of A, Palau type D5, Bangkok type D5, and D9.

Characteristics of Wild-Type Measles Virus

Seven wild-type measles virus strains were isolated and the current wild type did not induce cell fusion in Vero cells. D5 and D9 strains infected and replicated in Vero cells without demonstrating typical cell fusion. Infectious titers in Vero cell culture fluid detected 7 days after infection were similar or 1/2 or1/3 lower than those in the B95a cell culture (data not shown).

Measles virus grew well at 35–37°C and virus growth at 39°C differs from strain to strain [Nakayama et al., 2003]. Virus growth of MVi/Aichi.JPN/44.06 at different temperatures of 33, 35, 37, and 39°C was investigated in B95a cells, and the results are shown in Figure 4. It grew well at 33, 35, and 37°C, but the infective titers at 39°C decreased to approximately 1/100 in comparison with those observed at 33 or 35°C. Three D5 strains isolated in 2007 and MVi/Tokyo.JPN/93-S [Palau type D5] were cultured in B95a cells at 35°C and 39°C and the infective titers on day 7 of culture are also shown in Figure 4. As previously reported, MVi/Tokyo.JPN/93-S grew well at 39°C as it did at 35°C, but the infectivity of the Bangkok type D5 was lower at 39°C than at 35°C.

DISCUSSION

The WHO global measles and rubella laboratory network (LabNet) has been established and the Western Pacific Region adopted the goal of measles elimination by increasing the use of laboratory testing as an integral component of its surveillance. They recommended that effective surveillance comprised laboratory confirmation by the detection of IgM antibody in single serum, together with virus isolation for genotyping. However, the sensitivity of virus isolation is low and depends on the timing of sample collection and transporting conditions. The detection of measles virus-specific IgM antibodies in serum is a standard serological diagnostic

TABLE II. Nucleotide and Amino Acid Differences in D5 and D9 Strains

	AIK-C	Palau	Bangkok	Tokyo, 2000	Tokyo, 2007	Aichi, 2006
AIK-C		52 (2.8%)	53 (2.9%)	59 (3.2%)	57 (3.1%)	66 (3.6%)
Palau	16 (2.6%)		25 (1.3%)	10 (0.5%)	35 (1.9%)	45 (2.4%)
Bangkok	18 (2.9%)	8 (1.3%)		33 (1.8%)	16 (0.9%)	39 (2.1%)
Tokyo.JPN/2000	17 (2.8%)	5 (0.8%)	11 (1.8%)		43 (2.3%)	54 (2.9%)
Tokyo.JPN/17.07	16 (2.6%)	8 (1.3%)	4 (0.7%)	11 (1.8%)		50 (2.7%)
Aichi.JPN/44.06	19 (3.1%)	11 (1.8%)	10 (1.6%)	14 (2.3%)	10 (1.6%)	

Values present diagonally below in the table body indicate amino acid differences and the values present diagonally above in the table body indicate nucleotide differences.

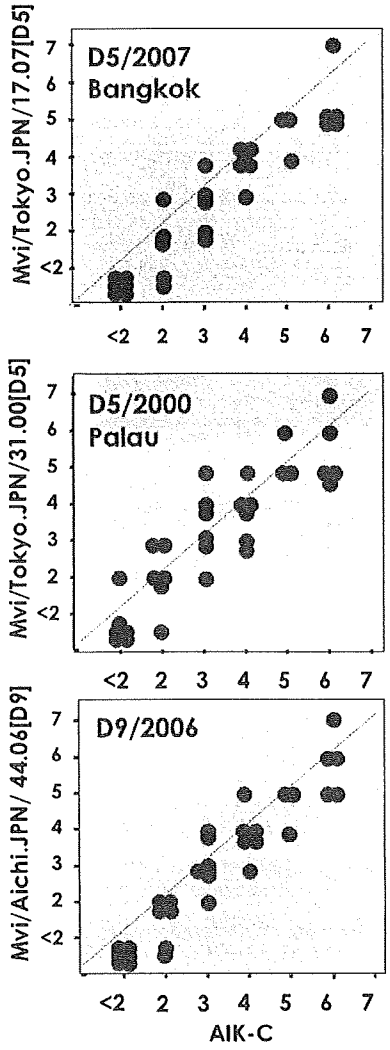

Fig. 3. Neutralization test antibodies against the AIK-C vaccine strain (genotype A), MVi/Tokyo.JPN/17.07 (genotype D5 Bangkok type), MVi/Tokyo.JPN/31.00 (genotype D5 Palau type), and MVi/Aichi. JPN/44.06 (genotype D9).

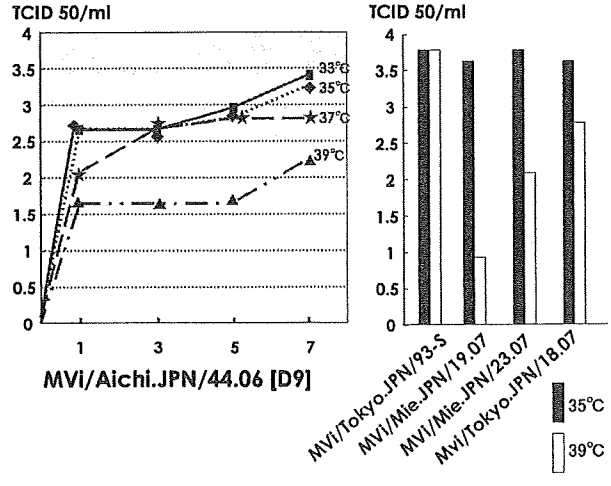

Fig. 4. Virus growth at different temperatures of 33, 35, 37, and 39°C.

method when serum samples are obtained on day 4 or 5 of rash onset and later [WHO, 2005b]. These methods were effective in the case of the primary infection causing typical measles illness. However, in the case of modified or subclinical measles, the detection rate of serum IgM antibodies was reported to be extremely low and the genomic detection by RT-PCR should be examined [Mosquera et al., 2005]. In this report, serum IgM antibody against measles virus was initially examined in several patients, but it was negative because all patients visited the clinics within day 1 or 2 after rash onset. In addition, the patients over 15 years of age having a single dose of measles immunization demonstrated very mild febrile illness with eruptions. Among 18 patients, 12 were diagnosed by RT-LAMP. The remaining six patients were not confirmed, because paired sera were obtained rarely from adults with mild symptoms. Detection of the measles genome is reliable and sensitive method and these six patients were supposed to have some other virus infections.

van den Hof et al. [2003] examined 791 serum samples from Dutch persons aged 2–49 years. The sero-prevalence and mean titers of measles EIA and NT were lower in the vaccinated populations than in older individuals infected naturally, and vaccine-acquired immunity weakened year by year. Glass and Grenfell [2004] developed a mathematical model to predict clinical and subclinical measles cases. Vaccination levels dropped from >90% in the 1990s to 84% in 2001/2002 in England, and they considered that clinical measles cases would remain fairly stable over time if vaccine coverage is maintained at 90%, but that there would be a pronounced increase in the numbers of clinical cases if vaccine coverage is around 84% or below. In any case, they predicted an increase in the number of subclinical measles. In the measles outbreak in Japan 2007–2008, a large proportion of cases of measles comprised adults or teenagers with very mild illness, and many cases went undiagnosed. These cases were confirmed by detection of the measles genome in this study and the diagnostic approach is now combining genome detection with virus isolation and the detection of IgM antibodies [CDC, 2008c].

Circulating wild-type measles virus genotypes have been investigated since 1984 and dynamic changes in the major circulating genotype have been reported [Nakayama et al., 1995; Yamaguchi, 1997; Zhou et al., 2003; Nakayama et al., 2004; Okafuji et al., 2006]. The dominant circulating genotypes changed drastically in large outbreaks in 1984, 1987–1988, 1991–1993, and 2001–2002 and each outbreak was caused by a different genotype in Japan, as summarized in Figure 5. Genotype C1 was an indigenous strain for a long period before 1985, D3 was involved in the 1987–1988 outbreak, and D5 in 1990–1993. The genotype of measles virus was studied in India from 1994 to 1997. The indigenous strain in India was D4, and a large outbreak was observed in 1997, caused by the Chicago type D3 strain [Nakayama et al., 2004]. In 1997, the Chicago type D3 was isolated in Japan, which was a different cluster from

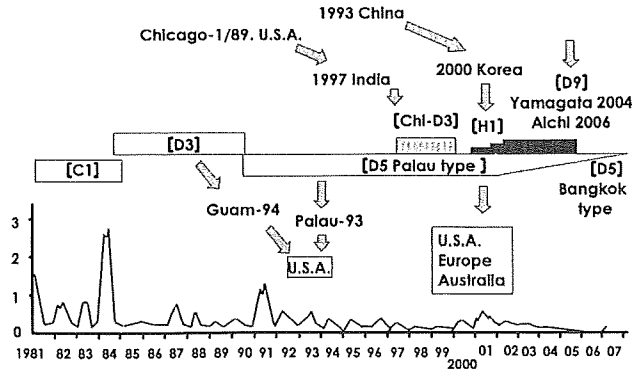

Fig. 5. Major circulating measles genotypes since 1984.

the D3 strains in 1987–1988. The Chicago-type D3 in 1997–1999 was supposed to have been imported from outside, and it was replaced by D5 in 2000, similar to the previous Palau type D5 before 1997. The H1 genotype was isolated from adult measles patients, and this genotype became a dominant strain after 2000 [Zhou et al., 2003]. In 2004, a sporadic outbreak was reported at a junior high school in Yamagata Prefecture and several measles strains were isolated. They were identified as genotype D9, which had not previously been reported in Japan [Mizuta et al., 2005]. In 2005, sporadic outbreaks were reported in Chiba and Ibaraki Prefectures, caused by the Palau type D5. In Asian countries, several different indigenous genotypes have been reported: H1 in China [Xu et al., 1998], G2 in Indonesia and Malaysia [Rota et al., 2000], D4 in India [Wairagkar et al., 2002], D3 in Papua New Guinea [Miki et al., 2002], and D7 in Australia [Chibo et al., 2003]. D8 in Nepal [Truong et al., 2001] and D9 in Australia [Chibo et al., 2003] were also reported. The sporadic outbreak caused by D9 in Yamagata in 2004 was suggested to have been imported from outside. In this study, D9 was also isolated in a sporadic outbreak in Aichi Prefecture in 2006, and seven nucleotide differences in the partial N gene (7/456: 1.5%) were observed between the two D9 strains of the MVi/Yamagata.JPN/7.04 and MVi/Aichi.JPN/44.06. There was no information on the isolation of D9 during 2004–2006 and no epidemiological linkage. Thus, they seemed to have been imported independently from other Asian counties, considering the narrow transmission chain in Japan.

The Palau type D5 was an indigenous strain since 1990 in Japan but was interrupted by outbreaks of the Chicago type D3 from 1997 to 2000 and of H1 from 2001 to 2004. Genotype D5 was detected in a sporadic outbreak in 2006 around Tokyo and transmitted to Okinawa, which was identified as the Bangkok type D5, which was different from the Palau type circulating during1990–2005 in Japan [National Institute of Infectious Diseases, 2007; Morita et al., 2007]. The Bangkok type D5 isolated in Japan in 2007 would have been imported from other Asian countries rather than being a result of the accumulation of mutations of the indigenous Palau type D5. Similar Bangkok type D5 strains (MVi/Queensland.AU/37.03, MVi/Maldives. MAL/32.05, MVs/Phnom Penh.KHM/19.02, MVs/Taichung. TWN/45.03) were reported in Asian countries and also in the UK (MVs/Chelmsford.GBR/30.07).

In 2007 and 2008, similar strains were reported in Europe and the USA and epidemiological linkage to the outbreak in Switzerland was identified [Delaportel et al., 2007; Richard et al., 2008; CDC, 2008b]. In this report they were thought to have been transmitted from Japan, demonstrating high-level sequence homology to Japanese isolates. Asia is the major reservoir of the wild measles virus and several Asian genotypes have been detected in the USA. and Europe. Global knowledge on the distribution of genotypes is imperative in identifying the geographical regions where more aggressive vaccination campaigns should be implemented in order to eliminate measles.

Minor antigenic changes in the Chicago type D3 and H1 strains were reported in comparison with the NT titers against the AIK-C vaccine strain [Zhou et al., 2003; Nakayama et al., 2004]. In this study, 1.3–1.8% amino acid differences were observed between the D5 Bangkok and Palau types. Therefore, the difference in the antigenicity was investigated and there were no significant differences in NT antibody titers against the Bangkok type D5, Palau type D5, and D9 strains in comparison with those against the AIK-C vaccine strain. No significant difference was demonstrated in terms of antigenicity. Some virus strains of the Chicago type D3 and Palau type D5 grew as well at 39°C as they did at 33, 35, and 37°C. However, the Bangkok type D5 and D9 showed poor growth at 39°C. The responsible genomic region(s) for virus growth at different temperatures is now under investigation.

Recommendations to prevent further transmission from the index case of importation noted that the outbreak investigation was conducted when the index case of a Japanese boy had a measles-like illness and only six additional cases were identified [CDC, 2008a]. Transmission was interrupted because of the high vaccination coverage rates of two-dose MMR among children and adolescents. The prompt response was sustained by strong and effective surveillance systems. The WHO has recommended that all children should be provided with a second opportunity for measles vaccination [WHO, 2006]. This second opportunity is scheduled just before entry to primary school in most countries, and a two-dose schedule of combined measles–rubella vaccine was launched in 2006 in Japan. The scheduled timing of the two doses is at the age of 12–24 months and 5–6 years. Supplemental immunization for the other populations was not considered, and most school children (over 8 years of age) did not have the benefit of the two-dose schedule. Okafuji et al. [2006] reported that NT antibodies decreased to undetectable levels in approximately 10% of vaccine recipients 6–7 years after vaccination when the measles outbreak was controlled. The outbreak in Japan 2007 highlighted the inadequacy of the immunization strategy, leading to a gap in immunization among teenagers without

supplemental immunization. Additionally, several had no immunization history because of a distrust of the vaccine due to the MMR scandal from 1989 to 1993 in Japan [Ueda et al., 1995]. All school children and young teenagers should have a second dose of measles vaccine to attain the goal of measles elimination. Thus, the Japanese government has decided to launch a catch-up campaign targeting young teenager at 13 and 18 years of age for the next 5 years, anticipating elimination of measles by 2012. The infrastructure for the surveillance system based on laboratory-based diagnosis is now in preparation.

ACKNOWLEDGMENTS

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A Long-Term Survey on the Distribution of the Human Rotavirus G Type in Thailand

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The distribution of the G type of human rotavirus was surveyed in Thailand between July 1993 and June 2007. A significant yearly change in the distribution of the G type distribution was found. From 1993–1994 to 1998–1999, the G1 type was the most dominant. In 1999–2000, G9 began to appear at a high frequency. In 2000–2001, 2001–2002, and 2002–2003, G9 was very common. In 2003–2004, G1 became the most prevalent type again, and since then it has been detected at the highest frequency. G12 strains, which were first detected in 1998–1999, were also found in 2004–2005 and 2006–2007. The G4 and G3 types were moderately prevalent in 2001–2002 and 2004–2005, respectively. Nucleotide sequence analysis of the VP7 genes of the G9 and G12 strains which reemerged in Thailand showed that they were each similar to the contemporary strains in other countries. ***J. Med. Virol. 82:157–163, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: rotavirus; G type; Thailand; sequence analysis; VP7

INTRODUCTION

Group A human rotavirus is the most common etiologic agent of severe gastroenteritis in infants and young children worldwide. It has been estimated that all children will be infected at least once by the age of 5 years, and that rotavirus is responsible globally for ~600,000 deaths each year, mostly in developing countries [Parashar et al., 2006]. In both developed and developing countries, rotavirus infection leads to a high rate of hospital admission related to dehydration.

Rotaviruses possess a genome comprising 11 double-stranded RNA (dsRNA) segments, enclosed in a triple-layered protein capsid [Estes and Kapikian, 2007]. The outermost layer is composed of two proteins, VP7 and VP4, which are associated with the G type and P type, respectively. At least 15 different G types (G1–G15) and 26 P types (P[1]–P[26]) have been found in humans and animals. Among them, 11 G types (G1–G6, G8–G11, and G12) and 10 P types (P[3]–P[6], P[8]–P[11], P[14], and P[19]) have been isolated from humans. The common G types worldwide are G1–G4, and G9 [Gentsch et al., 1996; Santos and Hoshino, 2005]. However, the distribution of the G type varies each year, and a distinct G type distribution has been found in different countries. Knowledge of the prevalence of the G type in each country has become more relevant, since two types of human rotavirus vaccine, RotaTeq (Merck & Co., Inc., Whitehouse Station, NJ) and Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium), were developed in 2006 [Ruiz-Palacios et al., 2006; Vesikari et al., 2006]. The efficacy of both vaccines as to the heterotypic human rotaviruses in circulation is a serious concern. With this background, fundamental data on the distribution of the G type worldwide is important for the prevention of infection with human rotavirus. In many studies, however, surveys on the distribution of the G type have been carried out for a short-term or on small numbers of samples.

In this study, the yearly change of the distribution of the G type of human rotavirus was surveyed long-term and on a large-scale in Thailand between July 1993 and June 2007. Nucleotide sequence analyses of rotavirus strains with the G9 or G12 specificity, which were detected in this study are also described.

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MATERIALS AND METHODS

Stool Specimens and Study Sites

A total of 7,452 stool samples were collected from the same or different study sites in each period between July 1993 and June 2007 (1993–2007) in Thailand (Table I). In 1993–1994, 1,150 samples were collected from four hospitals: the Nakorn Ping Hospital in Chiang Mai, the Nakhon Phanom Hospital in Nakhon Panom, the Children's Hospital in Bangkok, and the Hadyai Hospital in the Songkhla Province (near Malaysia), which are located in the northern, northeastern, central, and southern parts of Thailand, respectively. In 1994–1997 and 1999–2000, 359 samples were collected only from the Queen Sirikit National Institute of Child Health in Bangkok. In 1997–1999 and 2000–2001, 593 samples were collected from Nakhon Panom. In 2001–2003, 2,153 samples were collected from six hospitals located throughout Thailand: the Maesod Hospital, Tak Province on the Myanmar border, the Nong Khai Hospital, Nong Khai Province on the Laos border, Chantaburi Hospital, Chanthaburi Province near the Cambodia border, Sa Kaeo Hospital, Sa Kaeo Province on the Cambodia border, and Hadyai Hospital. Between July 2003 and June 2007, 3,197 stool specimens were examined at three or four study sites: the Maesod Hospital, the Nong Khai Hospital, the Chanthaburi Hospital, and the Hadyai Hospital.

Detection of Rotavirus

All stool specimens were screened for rotavirus by polyacrylamide gel electrophoresis (PAGE) of the segmented rotaviral genome as described previously [Pongsuwanna et al., 1996], since non-group A rotaviruses and picobirnaviruses can also be detected by this method, although PAGE of RNA exhibits relatively low sensitivity. In brief, rotaviral RNA was extracted from stool specimens with a disruption solution comprising sodium dodecyl sulfate (SDS), 2-mercaptoethanol, and EDTA, and then with phenol–chloroform. The RNA was electrophoresed on 10% acrylamide gels (2-mm thick) for 16 hr at 20 mA at room temperature. RNA segments were visualized by silver staining.

RT-PCR

PCR-typing was undertaken in two steps (first and second amplifications) as described previously [Gouvea et al., 1990; Taniguchi et al., 1992]. In the first amplification, complementary DNA corresponding to the full-length VP7 gene was amplified with a pair of primers for the 3′ and 5′ ends of VP7 genes. The second amplification was carried out using a mixture of primers that are specific to each of six variable regions of the VP7 genes of G1–4, G8, and G9 paired with a primer for the 3′ end of the VP7 gene.

Nucleotide Sequence Determination

Full-length cDNAs of the VP7 genes of 16 G9 strains and 7 G12 strains were prepared by RT-PCR. Direct sequencing was carried out using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kits (AB Applied Biosystems, Foster City, CA) with an automated sequencer, an ABI Prism 3100 Genetic Analyzer (AB Applied Biosystems). Nucleotide sequences were analyzed for the construction of phylogenic trees using Clustal W 1.8.

Nucleotide Sequence Accession Numbers

The nucleotide sequence data described in this article for the VP7 genes of Thai rotavirus strains were submitted to the GenBank database and have been assigned the accession numbers: AB436813 (G12 strain MS064-05), AB436814 (G12 strain MS310-06), AB436815 (G12 strain MS329-06), AB436816 (G12 strain MS038-07), AB436817 (G12 strain MS040-07), AB436818 (G12 strain MS041-07), AB436819 (G12 strain MS051-07), AB436820 (G9 strain HRV-205-99), AB436821 (G9 strain HRV00-01), AB436822 (G9 strain HY006-01), AB436823 (G9 strain CHB010-01), AB436824 (G9 strain NK002-01), AB436825 (G9 strain MS057-02), AB436826 (G9 strain HY070-02), AB436827 (G9 strain SK066-02), AB436828 (G9 strain NK018-02), AB436829 (G9 strain CHB058-02), AB436830 (G9 strain HY095-03), AB436831 (G9 strain MS037-03), AB436832 (G9 strain SK041-03), AB436833 (G9 strain NK022-03), AB436834 (G9 strain CHB002-04), and AB436835 (G9 strain NK008-04).

RESULTS

Detection of Rotavirus

Of the 7,452 stool specimens screened for rotavirus RNA by PAGE, 2,560 (34.4%) were positive for rotavirus (Table I). The survey of the G type distribution in Thailand since 1988 showed that the incidence of rotavirus was high between September and February, particularly in November, December, and January, the coldest months of the year in Thailand, and that there were few incidences of rotavirus infection in June and July (data not shown), and therefore the year between July of a given year and the next June was considered as one rotavirus season. For example, 1993–1994 means the year between July 1993 and June 1994.

Distribution of the G Type

In the survey between July 1993 and June 2007, G1 (47.0%) was overall the most dominant G type in Thailand, followed by G9 (22.6%), G2 (9.1%), and G4 (3.5%) (Table I). G1 was the most prevalent from 1993–1994 to 1998–1999 and from 2003–2004 to 2006–2007. G9 appeared in 1999–2000 at a high frequency, and was the most prevalent in 2000–2001, 2001–2002, and 2002–2003, the prevalence rates being 79.2%, 82.6%, and 49.1%, respectively. In 2002–2003, G2 was highly prevalent type (35.8%). G3 was rare in Thailand throughout the years studied, but G3 was a highly prevalent (22.0%) in 2004–2005. G4 showed a high prevalence only in 2001–2002. A very unusual G8 strain was

TABLE I. G Type Distribution of Human Rotavirus in Thailand Between July 1993 and July 2007

Collection year	Collection sites	No. of specimens collected	No. of specimens positive for rotavirus (positive rate %)	G type (%)							
				G1	G2	G3	G4	G8	G9	G12	Not determined
1993–1994	**Total**	**1,150**	**368 (32.0)**	**316 (85.9)**	**7 (1.9)**	**0**	**11 (3.0)**	**0**	**0**	**0**	**34 (9.2)**
	Bangkok	414	125 (30.2)	116 (92.8)	0	0	2 (1.8)	0	0	0	7 (5.6)
	Chiang Mai	200	54 (27.0)	44 (81.5)	5 (9.3)	0	0	0	0	0	5 (9.3)
	Nakorn Panom	119	37 (31.1)	35 (94.6)	0	0	2 (5.4)	0	0	0	0
	Songkhla	417	152 (36.5)	121 (79.6)	2 (1.3)	0	7 (4.6)	0	0	0	22 (14.5)
1994–1995	**Bangkok**	**163**	**99 (60.7)**	**79 (79.8)**	**4 (4.0)**	**0**	**4 (4.0)**	**0**	**1 (1.0)**	**0**	**11 (11.1)**
1995–1996	**Bangkok**	**47**	**31 (66.0)**	**26 (83.9)**	**4 (12.9)**	**0**	**1 (3.2)**	**0**	**0**	**0**	**0**
1996–1997	**Bangkok**	**113**	**51 (45.1)**	**32 (62.8)**	**9 (17.6)**	**0**	**3 (5.9)**	**0**	**0**	**0**	**7 (13.7)**
1997–1998	**Nakorn Panom**	**147**	**29 (19.7)**	**26 (89.7)**	**1 (3.4)**	**0**	**0**	**0**	**0**	**0**	**2 (6.9)**
1998–1999	**Nakorn Panom**	**262**	**101 (38.5)**	**91 (90.1)**	**2 (2.0)**	**0**	**0**	**0**	**0**	**1 (1.0)**	**7 (6.9)**
1999–2000	**Bangkok**	**36**	**14 (38.9)**	**1 (7.1)**	**6 (42.9)**	**0**	**0**	**0**	**5 (35.7)**	**0**	**2 (14.3)**
2000–2001	**Nakorn Panom**	**184**	**53 (28.8)**	**3 (5.7)**	**0**	**1 (1.9)**	**3 (5.7)**	**0**	**42 (79.2)**	**0**	**4 (7.6)**
2001–2002	**Total**	**1,061**	**356 (33.6)**	**4 (1.1)**	**0**	**1 (0.3)**	**57 (16.0)**	**0**	**294 (82.6)**	**0**	**0**
	Nong Khai	156	62 (39.7)	0	0	0	1 (0.6)	0	61 (98.4)	0	0
	Chanthaburi	242	73 (30.2)	2 (2.7)	0	0	9 (12.3)	0	62 (84.9)	0	0
	Tak	271	84 (31.0)	2 (2.4)	0	1 (1.2)	18 (21.4)	0	63 (75.0)	0	0
	Songkhla	235	80 (34.0)	0	0	0	29 (36.3)	0	51 (63.8)	0	0
	Sa Kaeo	157	57 (36.3)	0	0	0	0	0	57 (100.0)	0	0
2002–2003	**Total**	**1,092**	**428 (39.2)**	**2 (0.5)**	**153 (35.8)**	**0**	**2 (0.5)**	**0**	**210 (49.1)**	**0**	**61 (14.3)**
	Nong Khai	250	107 (42.8)	0	9 (8.4)	0	2 (1.9)	0	78 (72.9)	0	18 (16.8)
	Chanthaburi	164	59 (36.0)	1 (1.7)	22 (37.3)	0	0	0	21 (35.6)	0	15 (25.4)
	Tak	143	50 (35.0)	1 (2.0)	36 (72.0)	0	0	0	5 (10.0)	0	8 (16.0)
	Songkhla	343	131 (38.2)	0	16 (12.2)	0	0	0	105 (80.2)	0	10 (7.6)
	Sa Kaeo	192	81 (42.2)	0	70 (86.4)	0	0	0	1 (1.2)	0	10 (12.3)
2003–2004	**Total**	**543**	**174 (32.0)**	**107 (61.5)**	**14 (8.1)**	**0**	**6 (3.5)**	**1 (0.6)**	**18 (10.3)**	**0**	**28 (16.1)**
	Nong Khai	156	47 (30.1)	30 (63.8)	4 (8.5)	0	1 (2.1)	0	6 (12.8)	0	6 (12.8)
	Chanthaburi	207	72 (34.8)	43 (59.7)	8 (18.6)	0	5 (11.6)	0	8 (18.6)	0	8 (18.6)
	Tak	155	47 (30.3)	34 (72.3)	0	0	0	0	0	0	13 (27.7)
	Songkhla	25	8 (32.0)	0	2 (25.0)	0	0	1 (12.5)	4 (50.0)	0	1 (12.5)
2004–2005	**Total**	**422**	**109 (25.8)**	**36 (33.0)**	**5 (4.6)**	**24 (22.0)**	**2 (1.8)**	**0**	**2 (1.8)**	**2 (1.8)**	**38 (34.9)**
	Nong Khai	119	49 (41.2)	13 (26.5)	0	8 (16.3)	0	0	1 (2.0)	0	27 (55.1)
	Chanthaburi	164	21 (12.8)	18 (85.7)	3 (14.3)	0	0	0	0	0	0
	Tak	139	39 (28.1)	5 (12.8)	2 (5.1)	16 (41.0)	2 (5.1)	0	1 (2.6)	2 (5.1)	11 (28.2)
2005–2006	**Total**	**1,105**	**428 (38.7)**	**361 (84.4)**	**6 (1.4)**	**6 (1.4)**	**0**	**0**	**5 (1.2)**	**0**	**50 (11.7)**
	Nong Khai	141	55 (39.0)	52 (94.5)	0	0	0	0	0	0	3 (5.5)
	Chanthaburi	569	273 (48.0)	233 (85.3)	5 (1.8)	1 (0.4)	0	0	3 (1.1)	0	31 (11.4)
	Tak	202	63 (31.2)	45 (71.4)	1 (1.6)	5 (7.9)	0	0	0	0	12 (19.0)
	Songkhla	193	37 (19.2)	31 (83.8)	0	0	0	0	2 (5.4)	0	4 (10.8)
2006–2007	**Total**	**1,127**	**289 (25.6)**	**120 (41.5)**	**21 (7.2)**	**6 (2.1)**	**0**	**0**	**2 (0.1)**	**6 (2.1)**	**134 (46.4)**
	Nong Khai	136	16 (11.8)	14 (87.5)	0	0	0	0	0	0	2 (12.5)
	Chantaburi	520	127 (24.4)	61 (48.0)	16 (12.6)	0	0	0	0	0	50 (39.4)
	Tak	343	128 (37.3)	34 (26.6)	5 (3.9)	6 (4.7)	0	0	2 (1.6)	6 (4.7)	75 (58.6)
	Songkhla	128	18 (14.1)	11 (61.1)	0	0	0	0	0	0	7 (38.9)
Total		**7,452**	**2,560 (34.4)**	**1,204 (47.0)**	**232 (9.1)**	**38 (1.5)**	**89 (3.5)**	**1 (0.04)**	**579 (22.6)**	**9 (0.4)**	**378 (14.8)**

Rotavirus was detected by PAGE analysis of RNA, and the G type was determined by RT-PCR. Data as a total in each collection year are shown in bold.

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detected in 2003–2004. G12 (strain T152) was first detected in Thailand in 1998–1999 [Pongsuwanna et al., 2002]. G12 strains were also found in 2004–2005 and 2006–2007, although the incidence was low. Although strain T152 was detected in the Children's Hospital in Bangkok, the G12 strains detected after 2004 were all in the Maesod Hospital, Tak Province (Myanmar border). Thus, a drastic yearly change in the G type distribution was observed in Thailand in 1993–2007. It was of note that G9 and G12 rotaviruses had reemerged in Thailand.

The trend of the changes in the distribution of the G type in different regions of Thailand was almost the same. However, there were also some differences in the distribution of the G type depending on the regions where collections were undertaken, for example, G2 was highly prevalent in the Tak and Sa Kaeo regions in 2002–2003 compared to the other regions, and the prevalence of G3 was much higher in the Tak region than in the other regions in 2004–2005 (Table I).

Sequence Analysis of G9 Human Rotaviruses

In order to characterize the G9 strains that reemerged in Thailand, the complete nucleotide sequences of the VP7 genes of 16 representative G9 strains collected in four different districts and different years in this study were determined, the sequences being compared with each other and with those of the representative G9 strains detected in Thailand and other countries. The identity was very high among the 16 Thai G9 strains detected between 1999 and 2004 in this study: 99.1–99.9% at the nucleotide level and 98.2–100.0% at the amino acid level. On comparison with Thai G9 strains in other studies, the 16 present Thai G9 strains showed identities of 99.4–99.6%, 98.0–98.2%, and 95.0–95.2% at the nucleotide level with strains CMH319Thai and CMH045Thai detected in 2000–2001, 97CM86 in 1997, and Mc345 in 1988, respectively. It has been shown that there are three lineages (I–III) of G9 strains on phylogenetic analysis [Hoshino et al., 2004]: I comprises prototypes WI61, F45, and AU32; II comprises strain 116E; and III comprises the G9 strains that reemerged. The Thai 16 G9 strains that reemerged in Thailand are also included in lineage III (Fig. 1).

Sequence Analysis of G12 Human Rotavirus

The RNA profiles of the seven Thai G12 strains were examined by PAGE. They all exhibited similar RNA patterns (Fig. 2). The complete VP7 nucleotide sequences of the seven G12 strains detected in 2004–2005 and 2006–2007 were also determined. The VP7 nucleotide sequences of the seven Thai G12 strains showed very high identities (99.3–100%). In contrast, they exhibited 90.2–90.4% identity to prototype strain L26, 90–90.2% to porcine RU172 strain, 97.2–97.6% to a Thai strain, T152, detected in 1998–1999, and 97.7–98.5% to the G12 strains that reemerged in other countries. On phylogenetic analysis, the seven Thai G12 strains were found to be closely related to the strains detected after 2000 and thus were included in lineage III (Fig. 3).

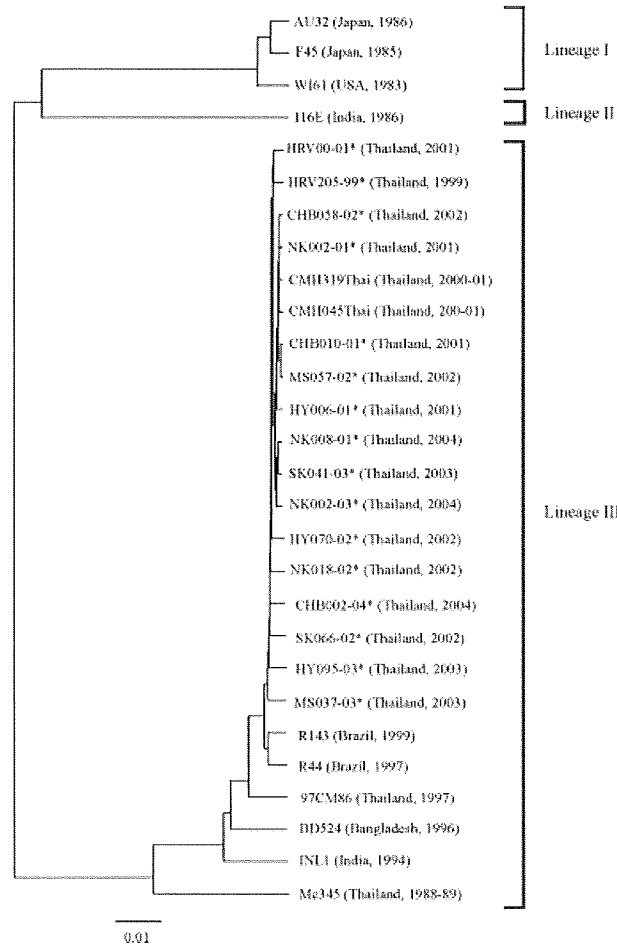

Fig. 1. Phylogenetic analysis of nucleotide sequences of the full VP7 genes of human G9 rotavirus strains detected in Thailand. The strain names with asterisks are those detected in this study. Reference sequences were obtained from the GenBank and EMBL databases. The bar indicates the variation scale.

DISCUSSION

In Thailand, diarrhea remains an important cause of morbidity and mortality among infants and young children, and rotavirus infection is a common cause of hospital admission [Pipittajan et al., 1991; Maneekarn and Ushijima, 2000; Bresee et al., 2004; Veeravigrom et al., 2004; Jiraphongsa et al., 2005; Sungkapalee et al., 2006]. It has been estimated that in Thai patients with diarrhea, the risks of rotavirus diarrhea, of a health care visit, and of hospital admission are 1 in 8, 1 in 36, and 1 in 85, respectively [Jiraphongsa et al., 2005]. The occurrence of rotavirus diarrhea in Thailand has a unimodal distribution pattern with a peak in October through to February [Jiraphongsa et al., 2005; data not shown], and rotavirus infection was found most frequently in children aged 6–11 months up to 2 years (data not shown).

In Thailand, a number of interesting epidemiological features were revealed by continuous surveys of

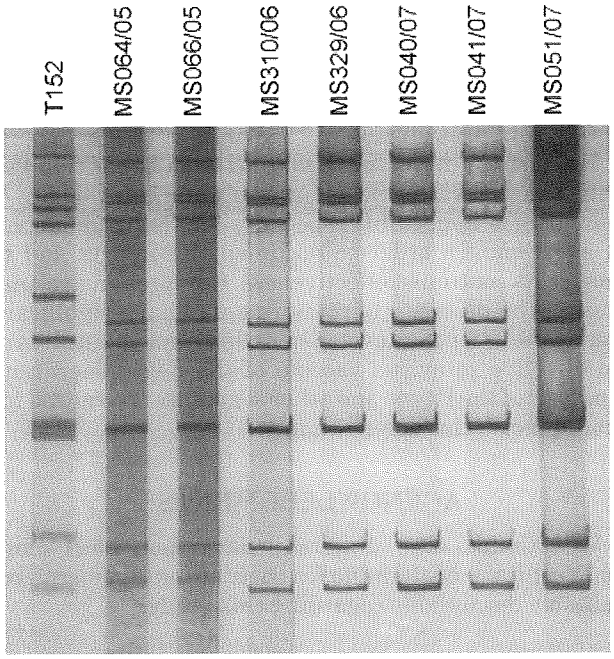

Fig. 2. RNA profiles of human G12 rotavirus strains detected in Thailand.

gastroenteritis virus among humans and animals: (1) unusual G10 human and porcine rotaviruses were detected [Pongsuwanna et al., 1996], (2) various combinations of G type and P type of bovine rotaviruses were found [Taniguchi et al., 1991], (3) a G12 human rotavirus with P[9] specificity was detected [Pongsuwanna et al., 2002], (4) group B and C porcine rotaviruses were detected [Pongsuwanna et al., 1996; unpublished data], and (5) picobirnaviruses were detected in pigs and humans [Pongsuwanna et al., 1996; Wakuda et al., 2005].

Regarding the G type distribution in Thailand, a yearly change in the distribution of the G type was also found in previous studies [Pongsuwanna et al., 1989, 1993]. In 1988–1989, 1989–1990, and 1991–1992, G1 was most prevalent. In 1990–1991 and 1992–1993, G3 and G2 were predominant, respectively. Thus, following the previous studies, a 19-year survey of the distribution of the G type in Thailand was carried out. Such a long-term survey in the same country will be useful for understanding the epidemiology of human rotavirus, and will provide fundamental data useful for future introduction of rotavirus vaccines.

Other studies on the distribution of G type studies have been performed in Thailand. In Chiang Mai between 1995 and 1997, G1, G2, G4, and G9 were detected at frequencies of 47%, 40%, 3%, and 6%, respectively, and G9 was found to have reemerged in 1996–1997 in Thailand [Zhou et al., 2001]. In the present study, interestingly, G9 rotavirus was detected earlier and in 1994–1995. However, the genome could not be characterized because the stool sample is not available now. In Bangkok, Thailand, between November 2002 and March 2004, the G types of 36 rotavirus-positive specimens

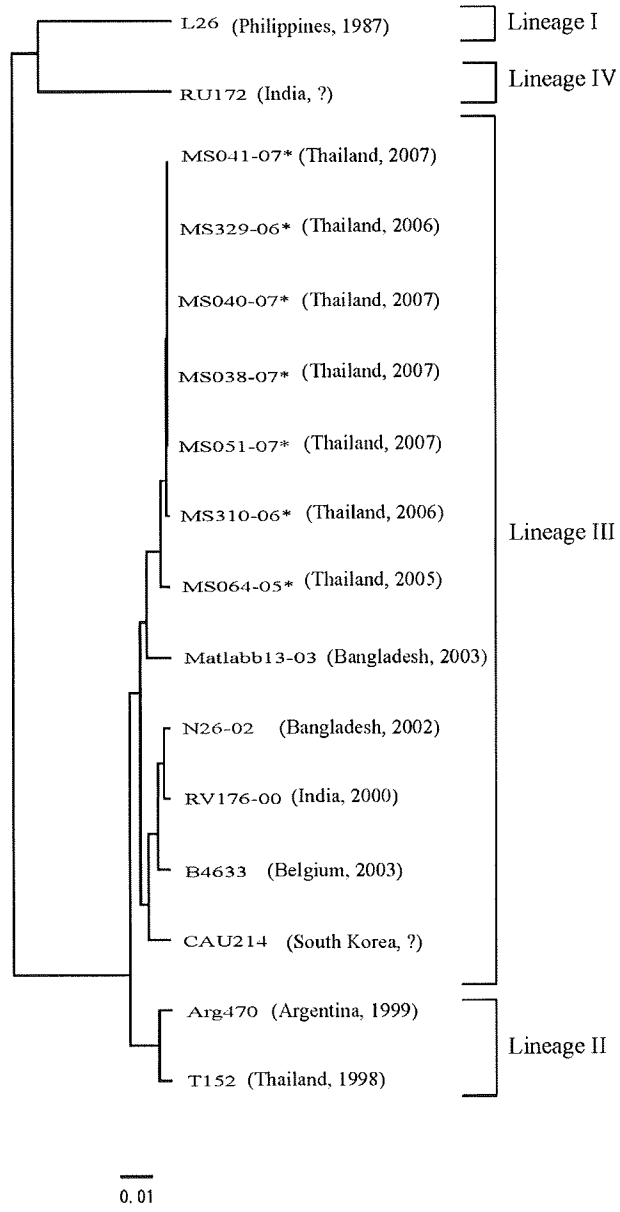

Fig. 3. Phylogenentic analysis of nucleotide sequences of the full VP7 genes of human G12 rotavirus strains detected in Thailand. The strain names with asterisks are those detected in this study. Reference sequences were obtained from the GenBank and EMBL databases. The bar indicates the variation scale.

were determined. Types G1, G2, and G9 were detected in 5.6%, 69.4%, and 25.0%, respectively [Theamboonlers et al., 2005]. In 2000–2001, 107 samples were subjected to G type determination: G9 was the most prevalent (91.6%), followed by G3 (5.6%) and G2 (2.8%) in Chiang Mai [Khamrin et al., 2006]. In 2003 and 2004, G2 and G1 were identified as the most dominant types, respectively [Khamrin et al., 2007]. The present long-term and large-scale study extended these small-scale studies and strengthened the data.

A changing distribution of rotavirus G types has been reported in other countries, although some differences

were observed. In Australia, G1 was the most prevalent in 1999–2001, G9 was highly prevalent in 2001–2003, and G1 was again the most prevalent type after 2004 [O'Ryan, 2009]. In Italy, G1 was highly prevalent in 2001, 2002, and 2004, and G9 was the most common type in 2005 [De Grazia et al., 2007]. In Ireland, G9 was the most prevalent type in 2001–2002, and G1 was identified as the most common G type in 2002–2004 [Reidy et al., 2005].

G9 rotavirus was first isolated in the United States in 1983 and then in Japan in 1985. After that, G9 rotaviruses were not detected for about a decade but reemerged in the mid-1990s. The G9 strains that reemerged were distinct genetically from those in the 1980s. At present, G9 rotaviruses have emerged as the fifth most common G type worldwide [Ramachandran et al., 2000; Santos and Hoshino, 2005]. Therefore, it has been suggested that G9 should be incorporated into candidate rotavirus vaccines [Montenegro et al., 2007]. At least three phylogenetic sequence lineages have been reported among the VP7 nucleotides of G9 rotaviruses [Hoshino et al., 2004]. Lineage 1 includes those isolated in the 1980s, lineage 2 G9 strains have been detected only in asymptomatic neonates in India, and the majority of G9 strains that are prevalent around the world today belong to lineage 3. The VP7 genes of the Thai G9 strains detected in this study were found to have very similar nucleotide and amino acid sequences, and were closely related to lineage 3 G9 strains.

G12 was first identified and characterized in 1990 among rotaviruses causing diarrhea in children in the Philippines [Taniguchi et al., 1990; Urasawa et al., 1990]. After a long period, the G12 strains have reemerged in Thailand [Pongsuwanna et al., 2002; Wakuda et al., 2003], the United States [Griffin et al., 2002], Japan [Shinozaki et al., 2004], Argentina [Castello et al., 2006], Nepal [Uchida et al., 2006], India [Ray et al., 2007], Bangladesh [Rahman et al., 2007], Belgium [Rahman et al., 2007], Slovenia [Steyer et al., 2007], South Korea [Le et al., 2008], Saudi Arabia [Kheyami et al., 2008], South Africa [Page et al., 2009], Hungary [Banyai et al., 2009], and Malawi [Cunliffe et al., 2009]. One porcine G12 virus was also detected in India [Ghosh et al., 2006]. A high incidence has been reported in several countries such as Nepal. There is a possibility that extensive spread of the G12 type at high frequency has occurred, as found for G9. Using phylogenetic analysis, G12 strains are grouped into four lineages: lineage I includes prototype strain L26; lineage II consists of strains from Thailand (strain T152), Japan and Argentina; lineage III includes the US strain, and most Indian and Bangladesh strains; and lineage IV comprises only a porcine strain, RU172 [Rahman et al., 2007]. Following the detection of G12 strain T152 in Thailand in 1998–1999, the G12 rotavirus was detected in this Thai study in 2004–2005 and 2006–2007. Differing from strain T152 detected in 1998–1999, the seven Thai G12 strains detected in 2004–2005 and 2006–2007 are included in lineage III. These results indicate that G12 rotavirus is now endemic in Thailand. However, the G12 strains were detected in the same district in different years, and they showed almost the same RNA profiles. This indicates that the G12 strains have not spread throughout Thailand. It is of interest to follow the spread of G12 rotavirus strains in Thailand.

The distribution of the G type in Thailand has changed with time. Continuous monitoring of epidemiology of rotavirus is important, especially for the introduction of a vaccine, in order to document its impact and to ensure its continued effectiveness. Comprehensive analysis of the diversity of rotavirus may have significant implications for the development and implementation of an effective rotavirus vaccine of the next generation. Analysis of the P type and other segments is required for more precise characterization.

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SUPPLEMENT ARTICLE

A Retrospective Evaluation of Hospitalizations for Acute Gastroenteritis at 2 Sentinel Hospitals in Central Japan to Estimate the Health Burden of Rotavirus

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Background. Two rotavirus vaccines have recently been licensed for use in >80 countries worldwide but not in Japan. To assess the value of introducing rotavirus vaccination in Japan, data on the burden of rotavirus disease are needed.

Methods. To describe the epidemiology of severe rotavirus disease among Japanese children aged <5 years, we examined retrospective demographic, clinical, and laboratory data from the period 2003–2007 for children hospitalized with acute gastroenteritis (AGE) at 2 sentinel hospitals in Japan.

Results. At each of the 2 hospitals, 17%–21% of all pediatric hospitalizations were for AGE. Three-fourths of all AGE-related admissions occurred during the winter (December–May). Rotavirus testing was performed for approximately three-fourths of patients admitted with AGE in the winter, of which 55% at one hospital and 59% at the other tested positive. By extrapolating the test results to those patients with AGE admitted in the winter who were not tested, we estimated that 39%–44% of year-round and 52%–57% of winter hospitalizations were attributable to rotavirus. The annual incidence of hospitalization for rotavirus AGE in the 2 cities served by the hospitals was estimated to be 3.8 and 4.9 per 1000 person-years.

Conclusions. The burden of severe rotavirus disease among Japanese children is substantial and warrants consideration of vaccination as a prevention strategy.

Acute gastroenteritis (AGE) is among the top causes of childhood morbidity and mortality worldwide, accounting for an estimated 1.8 million deaths among children aged <5 years [1]. Rotavirus is estimated to account for more than one-half a million of these diarrhea-related deaths each year [2–4]. Although deaths due to rotavirus are uncommon in industrialized countries, rotavirus remains an important cause of morbidity in young children. For example, among children aged <5 years in the United States, it is estimated that rotavirus infection causes 20–60 deaths, 55,000–70,000 hospital admissions, and 600,000 outpatient visits annually [5].

To prevent the high morbidity and mortality associated with rotavirus infection, 2 new vaccines—RotaTeq (Merck) and Rotarix (GlaxoSmithKline)—have been licensed for use in >80 countries worldwide. In prelicensure trials, each of these vaccines demonstrated high efficacy (85%–98%) against severe rotavirus disease [6, 7]. Currently, neither vaccine is licensed for use in Japan. Although rotavirus appears to

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The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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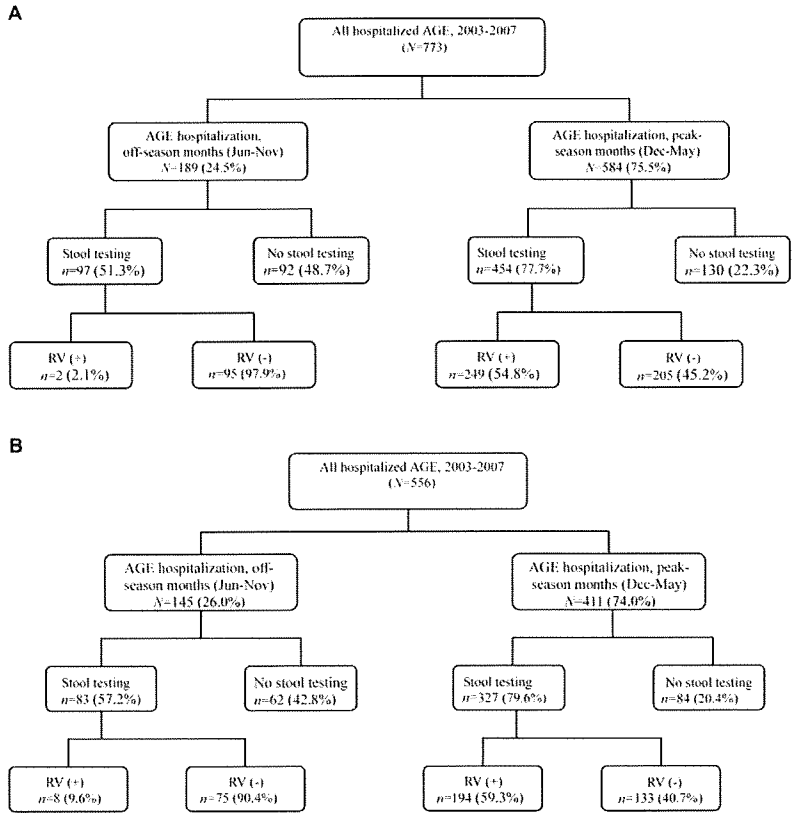

Figure 1. *A,* Distribution of hospitalizations for acute gastroenteritis (AGE), stratified by fecal testing for rotavirus (RV), in Mie National Hospital, Japan, 2003–2007. *B,* Distribution of hospitalizations for AGE, stratified by fecal testing for RV, in Yamada Red Cross Hospital, Japan, 2003–2007.

be an important cause of severe AGE among children in Japan [8–10], limited information is available on the full burden of rotavirus disease.

To estimate the health burden and to understand the epidemiology of rotavirus disease in Japanese children, we examined retrospective data for a 5-year period on rotavirus laboratory testing among children hospitalized with AGE at 2 sentinel hospitals in central Japan. We estimated the incidence of hospitalizations for rotavirus in the population served by these hospitals and then extrapolated data from our study to generate national estimates of hospitalizations for rotavirus in Japanese children. Our data will help physicians and policy makers to assess the potential value of introducing rotavirus vaccination in Japan.

MATERIALS AND METHODS

Study hospitals. We retrospectively reviewed all hospitalizations for AGE at 2 hospitals in Mie Prefecture, Japan: Mie National Hospital (MNH) in Tsu city and Yamada Red Cross Hospital (YRCH) in Ise city. We chose these hospitals because they are the main facilities for treatment of infectious disease for pediatric patients in these 2 cities, and most patients from these cities with severe diarrhea who need to be hospitalized are admitted to these hospitals. Institutional review boards at MNH, YRCH, and Emory University (Atlanta, GA) approved the study.

Hospital discharge data. For each hospital, we reviewed discharge logbooks or discharge summaries to collect information on children aged <5 years who were hospitalized with AGE during the 5-year period from 1 January 2003 through 31 December 2007. We additionally reviewed laboratory logbooks or medical charts, to confirm the diagnosis, and rapid test results for rotavirus infection. For all children hospitalized with AGE, we obtained information from their discharge summary and/or their medical chart on age, sex, month of admission, duration of hospital stay, city where they lived, symptoms at hospitalization, and whether fecal testing for rotavirus was performed.

Laboratory data. By reviewing either the medical record or the laboratory testing logbook, we determined whether fecal testing was performed for children with AGE, and if so, we abstracted the test results. At MNH, stool samples were tested using a commercially available enzyme immunoassay (Rapidtesta; Daiichi Pharmaceutical [currently Sekisui Medical]); sensitivity was 92% and specificity was 100%, compared with using an electron microscope [11]. At YRCH, samples were tested

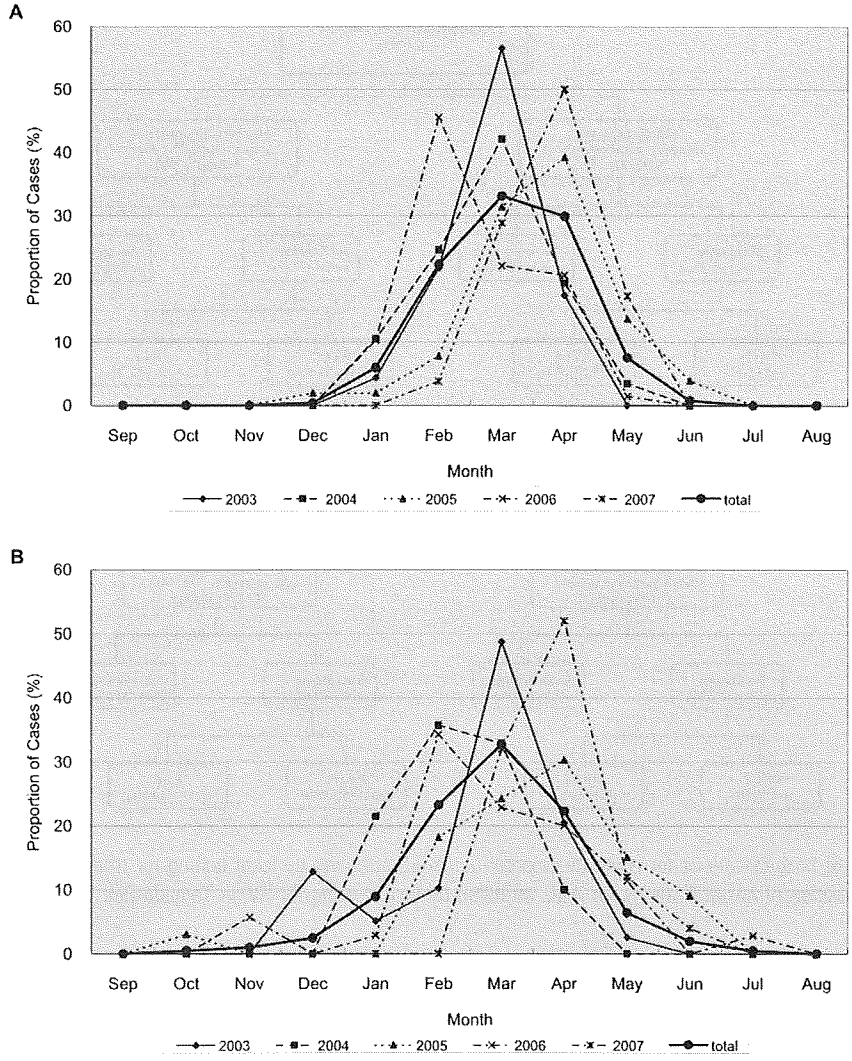

Figure 2. *A*, Distribution of rotavirus-positive acute gastroenteritis cases in Mie National Hospital, Japan, by month of admission, 2003–2007. *B*, Distribution of rotavirus-positive acute gastroenteritis cases in Yamada Red Cross Hospital, Japan, by month of admission, 2003–2007.

using a commercial latex agglutination test (Rota-Adeno Dry; Daiichi Pharmaceutical [currently Sekisui Medical]); sensitivity was 93.5% and specificity was 98.9%, compared with using an electron microscope [12].

Population census data. From the 2005 census, we obtained data on the population of children aged <5 years for the 2 cities that are served by the 2 sentinel hospitals (Tsu city, 12,549; Ise city, 5755) and for the entire Japanese national population of children aged <5 years (5,578,087).

Data analysis. We compared the demographic characteristics of children hospitalized for AGE who were tested for rotavirus and those who were not tested. Then, we estimated the number of rotavirus AGE cases among the group that was not tested for rotavirus by extrapolating results from the tested group to the untested group, adjusting for the month of the year and for age. We added the number of rotavirus AGE cases estimated in the untested group to the number of laboratory-confirmed rotavirus AGE cases in the tested group to calculate the total number of rotavirus AGE cases. This estimate of total rotavirus AGE cases was used to determine the proportion of all hospitalizations of patients with AGE attributable to rotavirus and to calculate the incidence of hospitalizations for rotavirus AGE in the 2 cities. Finally, incidence in these 2 cities was applied to national population data to estimate the number of hospitalizations for rotavirus AGE in Japan. Annual incidence was calculated using the total number of rotavirus-positive AGE cases during the study period as the numerator and using the population aged <5 years times 5 person-years as the denominator. Similarly, we used the total number of rotavirus-positive AGE cases during the study period as the numerator and the population aged <5 years as the denominator to calculate the risk of hospitalization per child by age 5 years.

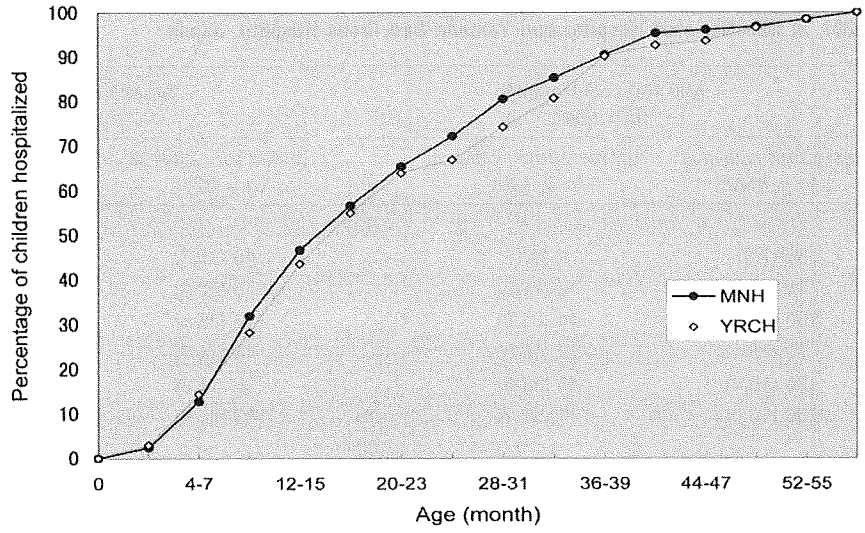

Figure 3. Cumulative age distribution of children aged <5 years hospitalized for rotavirus acute gastroenteritis; Mie National Hospital (MNH) and Yamada Red Cross Hospital (YRCH) in Mie Prefecture, Japan, 2003–2007.

RESULTS

AGE admissions and rotavirus testing. At MNH, specific age data on all patients were available for only 2 study years (January 2006–December 2007). A total of 1784 patients aged <5 years were discharged from the hospital during this period, and 21% of these discharges were for AGE. At YRCH, from January 2003 through December 2007, 3330 patients aged <5 years were discharged, and 17% of discharges were for AGE.

At MNH, 584 (76%) of the 773 patients with AGE during the 5-year study period were admitted from December through May (ie, rotavirus "peak-season" months), and 189 (24%) were admitted from June through November (ie, rotavirus "off-season" months) (Figure 1*A*). Of the 584 patients with AGE who were admitted during the peak season, 454 (78%) had a stool specimen tested for rotavirus, and of those tested, 249 (55%) were positive for rotavirus. By contrast, of the 189 patients admitted during the off-season, 97 (51%) had a stool specimen tested for rotavirus, but only 2 (2%) tested positive. The mean age of the 251 patients with rotavirus AGE was 21 months (median age, 17 months), and 200 (80%) were hospitalized for 2–4 days.

Similar to MNH, 411 (74%) of the 556 patients with AGE were admitted to YRCH during the rotavirus peak season. Of the 411 patients, 327 (80%) had a stool specimen tested for rotavirus, and 194 (59%) had a positive test result (Figure 1*B*). Of all admissions for AGE at YRCH, 145 (26%) occurred during the off-season. Of these patients, 83 (57%) had samples tested for rotavirus, and only 8 (10%) tested positive. The mean age of the 202 patients with rotavirus AGE was 21 months (median age, 17 months), and 171 (85%) were hospitalized for 2–4 days.

Seasonality of hospitalizations for rotavirus AGE. The seasonal trends in hospitalizations for rotavirus-positive AGE at the 2 hospitals are shown in Figure 2. Overall, the number of rotavirus AGE cases at both hospitals started to increase in December, most often peaked in March, and returned to baseline during May–June. Except for in 2004, the peak months of hospitalization for rotavirus AGE were similar between the hospitals.

Age distribution of patients hospitalized for rotavirus AGE. The cumulative age distribution of children aged <5 years who were hospitalized for rotavirus AGE at MNH and YRCH is shown in Figure 3. Of all rotavirus AGE cases in this group, <10% occurred in children aged <6 months. Approximately 30% were hospitalized by age 1 year, and 60% were hospitalized by age 2 years.

Evaluation of testing practices and adjusted estimates of rotavirus disease burden. At both hospitals, >70% (MNH, 551 [71.3%] of 773; YRCH, 410 [73.7%] of 556) of children aged <5 years who were admitted with AGE were tested for rotavirus (Figure 1). However, ~30% were not tested, and we were unable to assess the burden of rotavirus disease among these patients. Therefore, we examined demographic characteristics of patients who were tested for rotavirus and those who were not tested, to determine whether these groups were similar, so that the testing results could be extrapolated to hospitalizations without rotavirus testing. Because ~80% of the testing was performed during the peak season (MNH, 454 [82.4%] of 551; YRCH, 327 [79.8%] of 410) and very few cases were detected during the off-season (MNH, 2 cases; YRCH, 8 cases), we limited our comparison of the 2 groups to children admitted to the hospitals during the peak season.

Table 1. Characteristics of Children Hospitalized for Acute Gastroenteritis, by Hospital and Status of Fecal Testing for Rotavirus, December–May, 2003–2007, at Mie National Hospital and Yamada Red Cross Hospital, Japan

Characteristic	Mie National Hospital (n = 584)			Yamada Red Cross Hospital (n = 411)		
	Tested for rotavirus (n = 454)	Not tested for rotavirus (n = 130)	P	Tested for rotavirus (n = 327)	Not tested for rotavirus (n = 84)	P
Age group, months			<.001			<.001
0–5	36 (7.9)	4 (3.1)		40 (12.2)	7 (8.3)	
6–11	115 (25.3)	16 (12.3)		68 (20.8)	9 (10.7)	
12–23	163 (35.9)	44 (33.8)		116 (35.5)	27 (32.1)	
24–35	70 (15.4)	22 (16.9)		48 (14.7)	7 (8.3)	
36–59	70 (15.4)	44 (33.8)		55 (16.8)	34 (40.5)	
Sex: male	256 (56.4)	75 (57.7)	.79	183 (56.0)	47 (56.0)	.998
Month of admission			<.001			<.001
January	48 (10.6)	14 (10.8)		46 (14.1)	25 (29.8)	
February	80 (17.6)	18 (13.8)		75 (22.9)	14 (16.7)	
March	104 (22.9)	22 (16.9)		86 (26.3)	5 (6.0)	
April	106 (23.3)	20 (15.4)		59 (18.0)	12 (14.3)	
May	42 (9.3)	7 (5.4)		31 (9.5)	9 (10.7)	
December	74 (16.3)	49 (37.7)		31 (9.5)	19 (22.6)	

NOTE. Data are no. (%) of children, unless otherwise indicated.

In both hospitals, children hospitalized with AGE who were tested for rotavirus were similar in sex to those who were not tested, but the 2 groups differed in age and the month of admission (Table 1). To extrapolate testing results to the untested group at both hospitals, we applied age-specific and admission month–specific proportions of rotavirus-positive tests to the corresponding number of untested children hospitalized at the hospitals in each age group and each month.

Using this method, we estimated an additional 53 cases of rotavirus AGE at MNH and 42 cases at YRCH among patients with AGE who were not tested for rotavirus. Thus, even under the assumption of no case occurrence of rotavirus AGE among the untested children during the off-season, we estimated that, at MNH, 304 (39.3%) of 773 hospitalizations for AGE year-round and 302 (51.7%) of 584 hospitalizations for AGE from December through May were caused by rotavirus. Similarly, we estimated that, at YRCH, 244 (43.9%) of 556 hospitalizations for AGE year-round and 236 (57.4%) of 411 hospitalizations for AGE from December through May were caused by rotavirus. Even without extrapolation, the actual measured burden of rotavirus disease was substantial. Among laboratory-confirmed cases alone, 251 (32.5%) of 773 AGE cases for which a patient was admitted throughout the year at MNH and 202 (36.3%) of 556 AGE cases for which a patient was admitted at YRCH were attributable to rotavirus.

Estimation of incidence of hospitalizations for rotavirus AGE in Tsu and Ise. To determine the incidence of hospitalizations for rotavirus AGE in Tsu and Ise cities, we restricted our analysis to the 449 patients with AGE who resided in Tsu and were hospitalized at MNH and the 250 patients with AGE who resided in Ise and were hospitalized at YRCH. We extrapolated the test results to the children who were not tested for rotavirus, to estimate the total number of rotavirus AGE cases that occurred in those patients residing in Tsu and Ise (data not shown). Using stool testing, we identified 196 hospitalizations for rotavirus-positive AGE among children living in Tsu, and by extrapolation to the untested group, we estimated an additional 44 hospitalizations, for a total of 240 hospitalizations for rotavirus AGE. Among children living in Ise, we identified 118 hospitalizations for rotavirus-positive AGE, and by extrapolation to the untested group, we estimated an additional 22 hospitalizations, for a total of 140 hospitalizations for rotavirus AGE. The testing-unadjusted and -adjusted incidence rates for hospitalization for rotavirus AGE in Tsu were 3.1 hospitalizations per 1000 person-years (95% confidence interval [CI], 2.3–4.2 hospitalizations per 1000 person-years) and 3.8 hospitalizations per 1000 person-years (95% CI, 2.8–5.1 hospitalizations per 1000 person-years), respectively. In Ise, testing-unadjusted and -adjusted rates were 4.1 hospitalizations per 1000 person-years (95% CI, 2.7–6.0 hospitalizations per 1000 person-years) and 4.9 hospitalizations per 1000 person-years (95% CI, 3.4–7.0 hospitalizations per 1000 person-years), respectively (Table 2).

Finally, we calculated the adjusted incidence of hospitalization for rotavirus AGE by age group (Figure 4). In both cities, the incidence of hospitalization for rotavirus AGE was greatest among children aged 6–23 months.

Table 2. Population-Based Figures for Hospitalizations for Rotavirus-Positive Acute Gastroenteritis in Tsu and Ise Cities, Mie Prefecture, Japan, 2003–2007

City	Population[a] aged <5 years	Crude (unadjusted) incidence		Adjusted incidence		Risk of hospitalization per child by age 5 years
		No. of cases	OR (95% CI) per 1000 person-years[b]	No. of cases	OR (95% CI) per 1000 person-years[b]	
Tsu	12,549	196	3.1 (2.3–4.2)	240.0	3.8 (2.8–5.1)	1 in 50
Ise	5755	118	4.1 (2.7–6.0)	140.8	4.9 (3.4–7.0)	1 in 36

NOTE. CI, confidence interval; OR, odds ratio.

[a] Numbers are from 2005 Japanese census data.

[b] By Fisher's exact test.

DISCUSSION

Testing for rotavirus is not routinely performed for patients hospitalized with AGE, because it adds cost without substantially altering treatment. At the 2 study hospitals, high rates of routine rotavirus testing allowed us to demonstrate clearly the substantial burden of rotavirus disease among children hospitalized with AGE. At these hospitals, rotavirus-associated AGE caused 6.1%–6.8% of all hospitalizations among children aged <5 years. Implementation of the new rotavirus vaccines, which demonstrated an efficacy of 59% against all-cause AGE and 85%–95% against severe rotavirus disease, could prove to be a potentially useful strategy to improve children's health in Japan.

Among children who were tested for rotavirus during the winter months, 55%–59% tested positive for rotavirus. By extrapolating laboratory testing results to the untested group and by adjusting for differences between the groups, we estimated that 39%–44% of year-round hospitalizations for AGE and 52%–57% of hospitalizations for AGE during the peak winter months were associated with rotavirus disease. These data translated into incidence rates of hospitalization for severe rotavirus disease in Tsu city and Ise city in Mie Prefecture, Japan, of 3.8 and 4.9 hospitalizations per 1000 person-years, respectively. In other words, we estimated that, by age 5 years, 1 in 50 children born in Tsu and 1 in 36 children born in Ise would be hospitalized with rotavirus AGE. Extrapolation of this incidence to national population data yielded an estimate of 25,000 hospitalizations for rotavirus AGE among Japanese children aged <5 years. From previously reported average direct medical costs of a single hospitalization for rotavirus AGE in Japan (¥136,000 [$1236]) [10], the direct cost of hospitalizations for rotavirus would be ~¥3.4 billion ($31 million) annually.

The observed rate of detection of rotavirus among children hospitalized with AGE is consistent with rates in 2 reviews of the scientific literature from 1986–1999 and 2000–2004 that reported a median rotavirus detection rate of 38%–40% among studies in high-income countries [3, 4]. The adjusted incidence of hospitalization for rotavirus AGE in Tsu and Ise is also comparable to rates reported from other industrialized countries, such as Sweden (3.7 hospitalizations per 1000 person-years) [13], Denmark (2.4 hospitalizations per 1000 person-years) [14], and the United States (2.7 hospitalizations per 1000 person-years) [15]. However, compared with a prospective active surveillance study conducted at 3 sentinel hospitals in Akita prefecture in Japan during 2001–2002 [10], our study showed lower rates of both overall rotavirus detection among children hospitalized with AGE (39%–44% vs. 55%) and the incidence of hospitalizations for rotavirus (3.8–4.9 vs. 7.9–17.6 hospitalizations per 1000 person-years). These differences could be attributable to several factors, including variation in practices of hospitalization and emergency department care; better quality or timeliness of specimen collection in prospective active surveillance, compared with in our retrospective review; differences in the sensitivity of the enzyme immunoassays used to detect rotavirus; or real differences in the epidemiology of disease during the 2 study periods. Of note, rotavirus detection rates among children hospitalized with AGE in the active surveillance study in Akita Prefecture were 25%, 64%, and 74% at the 3 study hospitals, highlighting the potential for variability among sites.

Some limitations should be considered when our results are interpreted. First, although we do not believe this practice is common, it is possible that some children living in these 2 cities, especially Tsu, sought care for AGE at other hospitals in adjacent cities, instead of at MNH and YRCH. If so, this would have led us to underestimate the incidence of hospitalization for rotavirus AGE. Second, because of substantial variation in recording practices at these hospitals, we were unable to collect detailed data on the clinical features and severity of AGE cases. Third, data from these 2 sites may not be representative of Japan, which could limit the generalizability of our results to the national population. Finally, because of the retrospective nature of our study, we could not ascertain the quality and timing of collection of stool specimens from patients with AGE, which may have led to somewhat lower detection rates of ro-