Molecular Epidemiology of Enteric Viruses in Patients With Acute Gastroenteritis in Aichi Prefecture, Japan, 2008/09–2013/14

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Acute gastroenteritis is a critical infectious disease that affects infants and young children throughout the world, including Japan. This retrospective study was conducted from September 2008 to August 2014 (six seasons: 2008/09–2013/14) to investigate the incidence of enteric viruses responsible for 1,871 cases of acute gastroenteritis in Aichi prefecture, Japan. Of the 1,871 cases, 1,100 enteric viruses were detected in 978 samples, of which strains from norovirus (NoV) genogroup II (60.9%) were the most commonly detected, followed by strains of rotavirus A (RVA) (23.2%), adenovirus (AdV) type 41 (8.2%), sapovirus (SaV) (3.6%), human astrovirus (HAstV) (2.8%), and NoV genogroup I (1.3%). Sequencing of the NoV genogroup II (GII) strains revealed that GII.4 was the most common genotype, although four different GII.4 variants were also identified. The most common G-genotype of RVA was G1 (63.9%), followed by G3 (27.1%), G2 (4.7%) and G9 (4.3%). Three genogroups of SaV strains were found: GI (80.0%), GII (15.0%), and GV (5.0%). HAstV strains were genotyped as HAstV-1 (80.6%), HAstV-8 (16.1%), and HAstV-3 (3.2%). These results show that NoV GII was the leading cause of sporadic acute viral gastroenteritis, although a variety of enteric viruses were detected during the six-season surveillance period. ***J. Med. Virol.*** © 2015 Wiley Periodicals, Inc.

KEY WORDS: molecular epidemiology; pediatric; acute gastroenteritis; norovirus; enteric viruses

INTRODUCTION

Acute gastroenteritis remains a major cause of mortality among infants and young children in low- to middle-income countries and a cause of morbidity in developed countries [Wilhelmi et al., 2003]. Acute gastroenteritis, specifically diarrhea, is the second leading cause of death globally among children under 5 years of age, and the estimated annual incidence of childhood death from gastroenteritis is approximately 1.5 million [Wardlaw et al., 2010]. The major causative agents of acute gastroenteritis worldwide are enteric viruses, including norovirus (NoV), rotavirus A (RVA), sapovirus (SaV), human astrovirus (HAstV), and adenovirus (AdV) [Tam et al., 2012; Chhabra et al., 2013; Glass, 2013].

NoV and SaV are two of the five genera of the family *Caliciviridae* [Green, 2013]. NoV is genetically classified into at least five genogroups (GI–GV), of which GI, GII, and, rarely, GIV, have been detected in humans [Bruggink et al., 2015]. NoV GII is further divided into 22 genotypes based on the sequence of the capsid gene [Green, 2013]. Moreover, genotype four within NoV GII (GII.4), which is detected most frequently worldwide, is further divided into variants [Kroneman et al., 2013]. Several variants of NoV GII.4 have been associated with global or local epidemics [Bodhidatta et al., 2015; Zhirakovskaia et al., 2015]. SaV is genetically classified into five genogroups (GI–GV). The GI, GII, GIV, and GV genogroups contain human pathogens, whereas GIII is only known to contain porcine pathogens [Farkas et al., 2004]. NoV and SaV are associated with gastroenteritis at all ages and are responsible for outbreaks in various epidemiological settings,

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including restaurants, schools, daycare centers, hospitals, nursing homes, and cruise ships throughout the world, including Japan [Marks et al., 2000; Kobayashi et al., 2012; Green, 2013; Sakon et al., 2014; Oka et al., 2015]. RVA is the most common etiological agent of severe dehydrating diarrhea among infants and children worldwide [Parashar et al., 2009]. RVAs are divided into G-genotypes according to their VP7 gene sequences. Multiple RVA genotypes have been reported, of which five G-genotypes (G1–G4 and G9) are associated with global human rotavirus infection [Nguyen et al., 2007]. HAstV infection occurs mainly in infants and causes less severe gastroenteritis than other enteric viruses [Cunliffe et al., 2002]. AdV types 40 (AdV 40) and 41 (AdV 41) belong to sub-genus F and are referred to as enteric adenoviruses [Nguyen et al., 2007]; they cause acute sporadic gastroenteritis among children. These five major classes of viral pathogens have been difficult or impossible to cultivate. Therefore, polymerase chain reaction (PCR) has been increasingly used to identify these viruses in clinical specimens.

In this molecular epidemiological study, to gain a better understanding of enteric viruses circulating in Aichi prefecture, Japan, especially NoV GII, the prevalence of NoV, RVA, SaV, HAstV, and AdV in patients with acute gastroenteritis who consulted pediatric clinics, over six seasons (2008/09–2013/14), was determined. Furthermore, the detected NoV strains were characterized with molecular techniques.

MATERIALS AND METHODS

Sample Collection and Nucleic Acid Isolation

A total of 1,871 samples, including 1,644 fecal and 227 vomit samples, from 1,862 patients with suspected acute viral gastroenteritis at the pediatric sentinel hospitals in Aichi prefecture were collected from September 2008 to August 2014, over a period of six winter seasons (Table I). Samples were collected twice from nine patients. The ages of the patients ranged from 2 weeks to 84 years old (Table II).

In this study, 10% (wt/vol) of fecal suspensions and 50% of vomit suspensions were prepared with veal infusion broth. Samples were centrifuged at 10,000*g* for 20 min, from which 200 μl of supernatant was collected for analysis. Viral RNA/DNA were extracted from the supernatants using the High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the instructions recommended by the manufacturer. Viral RNA/DNA samples were eluted in 50 μl of nuclease-free water and stored at −80°C until use.

Detection and Sequencing of NoV, SaV, and AdV

NoV, SaV, and AdV were detected by reverse transcriptase polymerase chain reaction (RT-PCR) or PCR, and the viruses were classified by sequencing and genotyping. NoV GI and GII were detected separately. The primer sets COG1F/G1SKR and G1SKF/G1SKR were used for nested RT-PCR to detect the partial capsid genes of NoV GI [Kojima et al., 2002; Kageyama et al., 2003]. The primer sets COG2F/G2SKR and G2SKF/G2SKR were used for nested RT-PCR to detect the partial capsid genes of NoV GII [Kojima et al., 2002; Kageyama et al., 2003]. For the detection of SaV, the primer sets F13, F14/ R13, R14, and F22/R2 were used for nested RT-PCR to amplify the partial capsid genes [Okada et al., 2006]. For the detection of AdV, the primer set AdTU was used for the first PCR reaction, and the primer set AdnU-S/AdnU-A was used for the second PCR reaction to amplify the partial hexon genes [Saitoh-Inagawa et al., 1996]. The PCR products of NoV, SaV, and AdV were purified using the Wizard SV Gel and PCR Clean-up System (Promega Corp., Madison, WI). Then, the purified DNA was directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA) and an automated capillary sequencer, 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA). The obtained sequences were compared with the reference strains by BLAST search. All reactions were conducted following the instructions recommended by the manufacturers of the corresponding kits and reagents. Genotyping for NoV and variants of NoV GII was performed according to Fields Virology [Green, 2013] and a previous report [Kroneman et al., 2013], respectively.

TABLE I. Positive Samples and Enteric Viruses From 2008/09 to 2013/14 Seasons

Season	No. of samples	No. (%) of positive samples	No. of detected viruses	No. (%) of enteric viruses					
				NoV GI	NoV GII	RVA	SaV	HAstV	AdV
2008/09	367	131 (35.7)	132	1 (0.8)	97 (73.5)	20 (15.2)	4 (3.0)	1 (0.8)	9 (6.8)
2009/10	275	158 (57.5)	166	5 (3.0)	119 (71.7)	11 (6.6)	2 (1.2)	3 (1.8)	26 (15.7)
2010/11	314	210 (66.9)	230	3 (1.3)	126 (54.8)	65 (28.3)	7 (3.0)	19 (8.3)	10 (4.3)
2011/12	308	182 (59.1)	226	2 (0.9)	130 (57.5)	67 (29.6)	9 (4.0)	6 (2.7)	12 (5.3)
2012/13	315	188 (59.7)	231	1 (0.4)	117 (50.6)	85 (36.8)	8 (3.5)	1 (0.4)	19 (8.2)
2013/14	292	109 (37.3)	115	2 (1.7)	81 (70.4)	7 (6.1)	10 (8.7)	1 (0.9)	14 (12.2)
Total	1,871	978 (52.3)	1,100	14 (1.3)	670 (60.9)	255 (23.2)	40 (3.6)	31 (2.8)	90 (8.2)

TABLE II. Age Distribution of Enteric Viruses From 2008/09 to 2013/14 Seasons

			No. (%) of enteric viruses					
Age (year)	No. of samples	No. of detected viruses	NoV GI	NoV GII	RVA	SaV	HAstV	AdV
0	450	235	0 (0.0)	149 (63.4)	58 (24.7)	5 (2.1)	4 (1.7)	19 (8.1)
1	457	323	1 (0.3)	200 (61.9)	74 (22.9)	12 (3.7)	8 (2.5)	28 (8.7)
2	203	141	0 (0.0)	90 (63.8)	25 (17.7)	5 (3.5)	6 (4.3)	15 (10.6)
3	155	94	1 (1.1)	48 (51.1)	26 (27.7)	5 (5.3)	3 (3.2)	11 (11.7)
4	146	75	2 (2.7)	41 (54.7)	21 (28.0)	2 (2.7)	3 (4.0)	6 (8.0)
5	102	47	2 (4.3)	26 (55.3)	9 (19.1)	4 (8.5)	1 (2.1)	5 (10.6)
6	66	32	3 (9.4)	17 (53.1)	6 (18.8)	1 (3.1)	1 (3.1)	4 (12.5)
7	52	30	3 (10.0)	18 (60.0)	8 (26.7)	1 (3.3)	0 (0.0)	0 (0.0)
8	43	14	0 (0.0)	10 (71.4)	3 (21.4)	1 (7.1)	0 (0.0)	0 (0.0)
9	35	13	0 (0.0)	9 (69.2)	4 (30.8)	0 (0.0)	0 (0.0)	0 (0.0)
10	28	13	0 (0.0)	7 (53.8)	4 (30.8)	2 (15.4)	0 (0.0)	0 (0.0)
11–15	59	31	1 (3.2)	21 (67.7)	5 (16.1)	2 (6.5)	2 (6.5)	0 (0.0)
16–	26	19	0 (0.0)	12 (63.2)	5 (26.3)	0 (0.0)	2 (10.5)	0 (0.0)
Unknown	49	33	1 (3.0)	22 (66.7)	7 (21.2)	0 (0.0)	1 (3.0)	2 (6.1)
Total	1,871	1,100	14 (1.3)	670 (60.9)	255 (23.2)	40 (3.6)	31 (2.8)	90 (8.2)

Detection of RVA and HAstV

For the detection of RVA, the primer sets Beg9/End9 and Beg9/VP7-1 were used for nested RT-PCR to amplify the partial VP7 genes [Gouvea et al., 1990; Ushijima et al., 1992]. RVA-positive samples were genotyped by PCR using genotype-specific primers (aBT1, aCT2, aET3, aDT4, aAT8, and aFT9) and an RVG9 primer [Gouvea et al., 1990]. For the detection of HAstV, the primer sets PreCAP/End and AC1/AC230 were used to amplify the partial capsid genes by RT-PCR [Saito et al., 1995; Matsui et al., 1998; Sakon et al., 2000]. Genotyping of HAstV-positive samples was performed by PCR using genotype-specific primers (AST-S1, AST-S2, AST-S3, AST-S4, AST-S5, AST-S6, AST-S7, and AST-S8) and an End primer [Matsui et al., 1998].

Phylogenetic Analysis

Phylogenetic analysis was performed using the CLUSTALW and MEGA program (version 6.06). A phylogenetic tree was generated using the neighbor-joining method (distance calculation was conducted using the Kimura two-parameter correction and pairwise deletion) and was validated based on 1,000 bootstrap replicates.

Nucleotide Sequences and Accession Numbers

The nucleotide sequence information for virus strains determined in this study has been deposited in the DDBJ database under the following accession numbers: LC086683–LC086713 and LC089040–LC089704.

RESULTS

Prevalence and Distribution of Enteric Viruses

Among the 1,871 samples, 1,100 enteric viruses were detected by RT-PCR and/or PCR in 978 samples (978/1,871: 52.3%), including 903 fecal (903/1,644: 54.9%) and 75 vomit (75/227: 33.0%) samples. Co-infection was identified in 113 samples (113/978: 11.6%): 104 samples contained two viruses, and 9 samples contained three viruses. NoV was the most frequently detected virus (684/1,100: 62.2%), of which 670 samples were positive for GII and 14 were positive for GI, followed by RVA (255/1,100: 23.2%), AdV (90/1,100: 8.2%), SaV (40/1,100: 3.6%), and HAstV (31/1,100: 2.8%) (Table I). Over half (51.1–71.4%) of the detected enteric viruses in each season were NoV GII; therefore, NoV GII was the most commonly detected pathogen during the six seasons, independent of age (Table II). RVA was the second most commonly detected enteric virus, accounting for 16.1–30.8% of the detected strains. In addition to these two viruses, AdV showed a unique age distribution because it was found only in children less than 6 years of age. HAstV was also commonly detected in infants and preschool children; however, HAstV was also found in patients over 11 years of age. These data collectively suggest that NoV GII was the major causative pathogen of acute gastroenteritis for patients of all ages. Considering the seasonal detection patterns of enteric viruses, as shown in Figure 1, we observed a significant increase in the sample collection rate during the winter, from November to February, compared with the spring and summer. Moreover, Figure 1 shows that NoV, particularly NoV GII, is the major enteric virus encountered during all six winters included in our study. Interestingly, RVA was the predominant enteric virus encountered during the springs of 2011–2013 but not during the spring seasons for the other 3 years. The prevalence of the other three viruses, SaV, HAstV, and AdV, was low, and no specific seasonal patterns were observed for those viruses.

Phylogenetic Analysis of NoV, SaV, and AdV

Phylogenetic analyses of NoVs were performed using the 282-bp capsid gene sequences. According to reference sequences, 14 NoVs of GI strains were

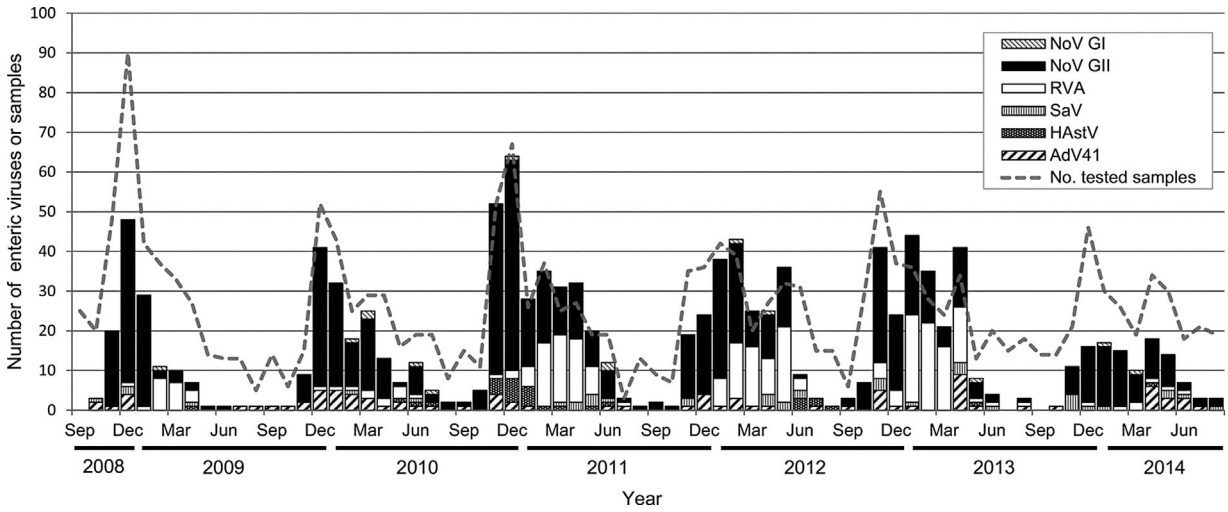

Fig. 1. Monthly trends in enteric viruses from the 2008/09 to the 2013/14 season in Aichi prefecture. The x-axis indicates the month. The y-axis indicates the number of detected enteric viruses (bars) and the number of samples tested in Aichi prefecture (line).

genotyped, of which 5 were GI.4, 5 were GI.7, 2 were GI.6, 1 was GI.1, and 1 was GI.3 (Table III). Of the 670 NoV GII strains, 397 were genotyped as GII.4, 131 as GII.3, 47 as GII.2, 45 as GII.14, 39 as GII.6, 6 as GII.12, 4 as GII.13, and 1 as GII.1 (Table III). The results also suggest that NoV GII.4 was the most predominant genotype in five of the six seasons studied, and NoV GII.3 was the most predominant genotype in the 2010/11 season. To further assess the emergence of variants of the GII.4 lineage and their persistence in Aichi prefecture, the sequences of 397 GII.4 strains were compared with those of the reference strains. Phylogenetic analysis showed that the GII.4 variants identified in Aichi prefecture clustered with four previously identified GII.4 variants: 202 (202/397: 50.9%) clustered with Den Haag_2006b; 125 (31.5%) with Sydney_2012; 52 (13.1%) with New Orleans_2009; and 18 (4.5%) with Asia_2003 (Fig. 2). As shown in Figure 2, dynamic exchange of the major NoV GII.4 variant was observed during the study period. The Den Haag_2006b GII.4 variant was predominant from the 2008/09 to the 2010/11 season, and owing to a small outbreak of New Orleans_2009 during the 2011/12 season; Sydney_2012 became the predominant strain in 2012/13.

The phylogenetic analysis showed that 40 SaV strains were divided into three genogroups: 32 as GI (32/40: 80.0%), 6 as GII (15.0%), and 2 as GV (5.0%). All 90 AdVs were classified as type 41 according to the phylogenetic analysis.

Genotyping of RVA and HAstV

G-genotyping based on the VP7 gene was performed for 255 RVA strains. Of the VP7 genotypes detected, RVA G1 (163/255: 63.9%) was the most prevalent, followed by G3 (69, 27.1%), G2 (12, 4.7%), and G9 (11, 4.3%) during the interval in this study. The most

TABLE III. Distribution of NoVs From 2008/09 to 2013/14 Seasons

	No. of enteric viruses in each season						
NoVs	2008/09	2009/10	2010/11	2011/12	2012/13	2013/14	Total
NVGI.1	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.8)	0 (0.0)	0 (0.0)	1 (0.1)
NVGI.3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.2)	1 (0.1)
NVGI.4	0 (0.0)	4 (3.2)	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)	5 (0.7)
NVGI.6	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.8)	1 (1.2)	2 (0.3)
NVGI.7	1 (1.0)	1 (0.8)	2 (1.6)	1 (0.8)	0 (0.0)	0 (0.0)	5 (0.7)
NVGII.1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.2)	1 (0.1)
NVGII.2	0 (0.0)	19 (15.3)	4 (3.1)	8 (6.1)	12 (10.2)	4 (4.8)	47 (6.9)
NVGII.3	17 (17.3)	8 (6.5)	78 (60.5)	14 (10.6)	8 (6.8)	6 (7.2)	131 (19.2)
NVGII.4	58 (59.2)	86 (69.4)	39 (30.2)	87 (65.9)	80 (67.8)	47 (56.6)	397 (58.0)
NVGII.6	19 (19.4)	1 (0.8)	1 (0.8)	1 (0.8)	0 (0.0)	17 (20.5)	39 (5.7)
NVGII.12	2 (2.0)	2 (1.6)	1 (0.8)	1 (0.8)	0 (0.0)	0 (0.0)	6 (0.9)
NVGII.13	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.8)	2 (1.7)	1 (1.2)	4 (0.6)
NVGII.14	1 (1.0)	3 (2.4)	3 (2.3)	18 (13.6)	15 (12.7)	5 (6.0)	45 (6.6)
Total	98	124	129	132	118	83	684

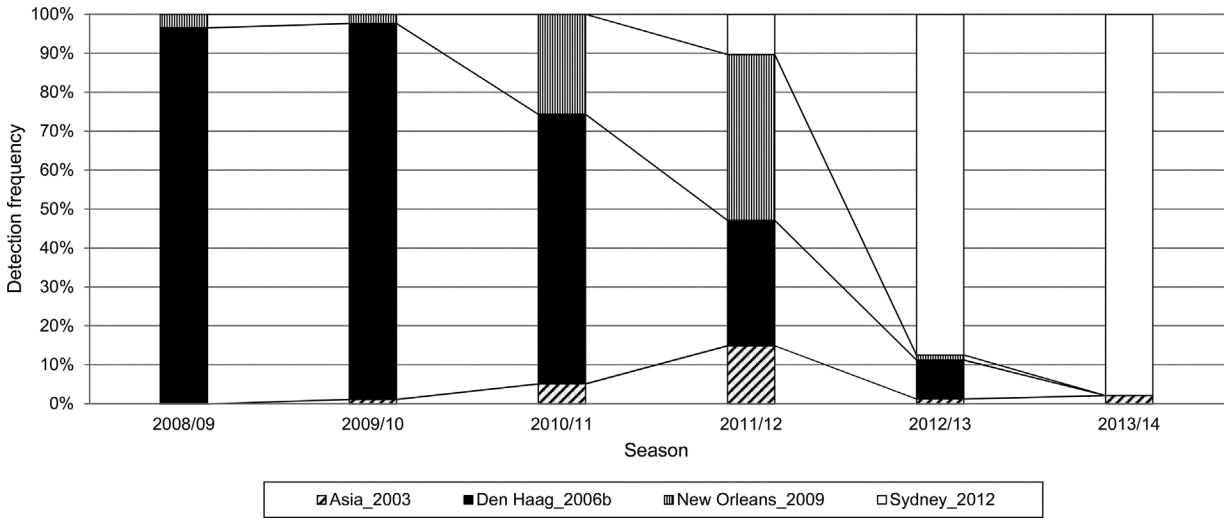

Fig. 2. Dynamic exchange of major NoV GII.4 variants from the 2008/09 to the 2013/14 season in Aichi prefecture. The x-axis indicates the season. The y-axis indicates the detection frequency of the NoV GII.4 variants. The reference strains of NoV GII.4 pandemic variants and the GenBank accession numbers used in this study were as follows: US95_96 (AJ004864), Farmington Hills_2002 (AY485642), Asia_2003 (AB220921), Hunter_2004 (AY883096), Yerseke_2006a (EF126963), Den Haag_2006b (EF126965), New Orleans_2009 (GU445325), and Sydney_2012 (JX459908).

frequent G-genotypes in 2010/11 and 2011/12 were G1 and G3, respectively, and G1 was the most prevalent genotype in 2012/13.

HAstV played a limited role in gastroenteritis in this study. Among 31 HAstV strains, 25/31 (80.6%) were identified as HAstV-1, 5 (16.1%) were identified as HAstV-8, and 1 (3.2%) was identified as HAstV-3. A previous study [Jeong et al., 2012] also reported that HAstV-1 was the most predominant HAstV serotype in Japan.

DISCUSSION

The present study reports a six-season surveillance of acute viral gastroenteritis in Aichi prefecture to determine the epidemiological features of five major enteric viruses: NoV, RVA, SaV, HAstV, and AdV. One or more enteric viruses in 52.3% of the tested samples were identified. The detection rate of enteric viruses in this study was similar to those of previous studies that examined the same five enteric viruses [Fabiana et al., 2007; Li et al., 2009]. Another study that analyzed eight target viruses (Aichi virus, human parechovirus, and enterovirus in addition to the five enteric viruses analyzed in our study) reported a higher detection rate (70.4%) than that of our study [Thongprachum et al., 2015].

Here, the results show that NoV GII was the most commonly observed virus. By contrast, NoV GI was detected in only a few samples throughout the six-season surveillance period of our study. In Aichi prefecture, NoV GI was often detected in samples from sewage water during this study period (data not shown). Previous studies also showed that NoV GI is pervasive in environmental samples [Iwai et al., 2009; Pérez-Sautu et al., 2012]. Further study of NoV under variable conditions, such as viral load at the time of infection and route of infection, may be necessary to explain the gap in the detection rate of NoV GI in sporadic gastroenteritis cases and its reported prevalence in environmental water sources.

Phylogenetic analysis of the capsid region allowed for the identification of four different variants of NoV GII.4 present in Aichi prefecture patient samples during our study period. In most seasons, two or three different NoV GII.4 variants were detected. Early in the study period, the most prevalent variant was Den Haag_2006b. In Aichi prefecture, the first sample positive for the Sydney_2012 variant was collected in December 2011. After its initial identification, Sydney_2012 became a major NoV strain. Many studies have similarly reported the detection of Sydney_2012 in both Japan and worldwide during the same period [CDC, 2013; van Beek et al., 2013; Fioretti et al., 2014; Thongprachum et al., 2014]. On average, new variants of NoV GII.4 have appeared every two to three years [Robilotti et al., 2015].

In Japan, two rotavirus vaccines—Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq (Merck & Co., Inc., Whitehouse Station, NJ)—were introduced as voluntary vaccinations in November 2011 and July 2012, respectively. The detection rates of RVA were increased in three seasons (2010/11–2012/13) and decreased in the 2013/14 season over the course of this study (Table I). This epidemic trend is similar to the data from the Infectious Agents Surveillance Report (IASR) from the National Institute of Infectious Diseases (NIID), Japan. Currently, the epidemiological mechanisms of the increased and decreased rates during the 2010/11–2013/14 seasons remain unclear. However, rotavirus vaccinations might have helped

suppress the topical pandemics in the 2013/14 season. For these reasons, RVA may have been predominant in the 2010/11–2012/13 seasons but not in later seasons. These results will provide important information to develop effective prevention strategies for RVA.

In January 2010, a large-scale SaV-associated food poisoning incident involving catered lunchboxes occurred in Aichi prefecture [Kobayashi et al., 2012]. However, the overall prevalence of SaV was not high during the 2009/10 season. These results indicate that SaV played a limited role in pediatric gastroenteritis in this study.

Enteric AdV infections commonly occur in autumn and early winter, and enteric AdV primarily affects infants less than 2 years of age worldwide [Shinozaki et al., 1991; Bon et al., 1999; Modarres et al., 2006; Dey et al., 2011]. By contrast, in the present study, AdV was detected only in children under 6 years of age. Moreover, AdV prevalence lacked seasonality.

In many countries, the incidence of HAstV infection peaks in the winter [Qiao et al., 1999; Mounts et al., 2000; Phan et al., 2005; Malasao et al., 2012]. Although the detection rate of HAstV was very low, these results showed a similar trend. HAstV played a minor role as a causative agent of gastroenteritis during the winter in Aichi prefecture.

In conclusion, this study demonstrates that NoV was the leading cause of acute gastroenteritis in infants and young children in Aichi prefecture, Japan, from September 2008 to August 2014, although many different viral pathogens were detected. The systematic surveillance of pathogens that cause gastroenteritis is important for the preservation of public health. In addition, continuous monitoring of genotypes and novel GII.4 variants is necessary to control and predict NoV gastroenteritis outbreaks.

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