Original Article

Molecular Characteristics of Novel Mono-Reassortant G9P[8] Rotavirus A Strains Possessing the NSP4 Gene of the E2 Genotype Detected in Tokyo, Japan

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SUMMARY: Rotavirus A (RVA) has been detected in patients with gastroenteritis even after vaccine introduction in Japan. To investigate circulating RVA strains, RVA-positive stool specimens obtained in Tokyo in 2017 and 2018 were analyzed using next-generation sequencing. A total of 50 and 21 RVA samples were obtained in 2017 and 2018, respectively. In 2017, G2P[4] (40.0%) was the most prevalent strain, followed by G3P[8] (DS-1-like) (28.0%), G8P[8] (10.0%), G3P[8] (Wa-like) (8.0%), G9P[8]-E1 (8.0%), and mixed infection (6.0%). In 2018, G3P[8] (DS-1-like) (28.6%) and G9P[8]-E2 (28.6%) were the most prevalent strains, followed by G9P[8]-E1 (19.0%), G2P[4] (9.5%), G8P[8] (9.5%), and mixed infection (4.8%). Six G9P[8]-E2 strains detected in 2018 showed an atypical genotype constellation (G9P[8]-I1-R1-C1-M1-A1-N1-T1-E2-H1), which had not been reported previously. Phylogenetic analyses suggested that the RVA virus was generated by inter-genogroup reassortment between commonly circulating G9P[8] and G2P[4] strains in Japan. The G9P[8] strain seemed to be reassorted with only the NSP4 gene of the E2 genotype of the G2P[4] strain. Since this newly-emerged G9P[8]-E2 virus was detected in different locations in Tokyo, the virus appears to have already begun to spread to a wider area.

INTRODUCTION

Rotavirus A (RVA) is a major cause of gastroenteritis in infants and young children worldwide. In 2016, RVA caused 128,500 deaths in children under 5 years of age globally (1). Although most of these deaths occurred in developing countries, RVA imposes a huge burden even in developed countries, including Japan (2–4). Live attenuated RVA vaccines were introduced in Japan in 2011 (Rotarix[®], GlaxoSmithKline Biologicals, Rixensart, Belgium) and in 2012 (RotaTeq[®], Merck & Co., Inc., Kenilworth, NJ, USA), and while RVA vaccines have proven to be effective (5,6), the selective pressure of a vaccine may induce an epidemic strain shift (7–10).

The RVA genome contains 11 gene segments of double-stranded RNA that encode six structural (VPs) and six nonstructural proteins (NSPs) (11). To classify RVAs, a specific genotype is assigned to each of the 11 genome segments, according to predefined nucleotide sequence identity cutoff values (12–14). The classification system denotes the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes of an RVA strain as a descriptor Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x indicating genotype number),

respectively. Human RVAs have largely been classified into two genogroups based on genotype constellations (15). The Wa and DS-1 genogroups are described as G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 and G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, respectively (12–14). G1, G3, G4, G9, and G12 viruses are usually grouped into the Wa-like genotype constellation, and most G2 and G8 viruses are grouped into the DS-1-like genotype constellation (16). However, unusual RVAs such as DS-1-like G1P[8] viruses (G1-P[8]-I2-R2-C2-M2-A2-N2-T2-E2-H2) (17–21) and DS-1-like (equine-like) G3P[8] viruses (G3-P[8]-I2-R2-C2-M2-A2-N2-T2-E2-H2) (22–26) were recently detected, and have begun to spread worldwide. In Japan, DS-1-like G1P[8] viruses were circulating in 2012–15 (17–19), and DS-1-like G3P[8] viruses have been circulating since 2015 (25,27). These unusual strains cannot be determined by only genotyping or classical sequencing methods of the VP7 and VP4 genes. Next-generation sequencing (NGS) is effective for RVA surveillance because all gene sequences of many samples can be analyzed in a single assay. In the present study, RVA strains detected in Tokyo Metropolis in two consecutive seasons (2017–18) were investigated based on full-genome analysis using NGS.

MATERIALS AND METHODS

Samples: RVA-positive samples detected by real-time qPCR at the Tokyo Metropolitan Institute of Public Health between January 2017 and December 2018 were used in this study. The samples were derived from several different hospitals in Tokyo Metropolis. In the case of group infection, a single sample in the case was selected for analysis. The study protocol was approved by

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the review board of each of the participating institutions.

cdNA library and nucleotide sequencing: Viral RNA was extracted from 10% stool suspensions in PBS using a QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. NGS was performed as previously described (18,28,29). Briefly, a 200-bp fragment library was constructed for each sample using an NEBNext Ultra RNA Library Prep Kit for Illumina v1.2 (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. A 151-cycle paired-end-read sequencing run was conducted using a MiSeq desktop sequencer (Illumina, San Diego, CA, USA) and a MiSeq Reagent Kit v2 (300 cycles). Sequence data were analyzed using CLC Genomics Workbench Software v7.0.3 (CLC Bio, Aarhus, Denmark), and data from representative strains were deposited into DDBJ/GenBank databases (LC477350–LC477679). Accession numbers of VP7 genes of G9P[8]-E2 strains are as follows: LC477367 (Tokyo18-27), LC477368 (Tokyo18-30), LC477372 (Tokyo18-38), LC477373 (Tokyo18-39), LC477374 (Tokyo18-40), and LC477377 (Tokyo18-43). Those of VP4 genes are LC477397, LC477398, LC477402, LC477403, LC477404, and LC477407. Those of NSP4 genes are as follows: LC477637, LC477638, LC477642, LC477643, LC477644, and LC477647.

Genotyping and phylogenetic analysis: Genotypes of the 11 genome segments were determined using the RotaC 2.0 automated genotyping tool for RVA (30). Near-full-length genome sequences were aligned with reference sequences using CLUSTAL W, which is included in the MEGA software package, version 7.0.18, and the MAFFT multiple sequence alignment software program, version 7.222 (31). Sequence alignments and phylogenetic tree analyses were performed using MEGA

7 software (32). Genetic distances were calculated using the Tamura three-parameter at a nucleotide level that was selected as the best substitution model based on the corrected Akaike information criterion value by MEGA software; phylogenetic trees were constructed using the maximum likelihood method with 1,000 bootstrap replicates. Reference sequences were retrieved from GenBank and lineage designations were defined based on previously reported G2 (33), G3 (34) and G9 trees (35).

RESULTS

Genotype distribution based on full-genome sequencing: A total of 50 and 21 RVA-positive stool specimens were obtained in Tokyo in 2017 and 2018, respectively; thus, 71 RVA samples were analyzed using NGS. The genotype constellations of these strains were classified into six patterns (Table 1): G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2 (G2P[4]), G3-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 (G3P[8] (Wa)), G3-P[8]-I2-R2-C2-M2-A2-N2-T2-E2-H2 (G3P[8] (DS-1)), G8-P[8]-I2-R2-C2-M2-A2-N2-T2-E2-H2 (G8P[8]), G9-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 (G9P[8]-E1), and G9-P[8]-I1-R1-C1-M1-A1-N1-T1-E2-H1 (G9P[8]-E2). In 2017, G2P[4] (40.0%) was the most prevalent strain, followed by G3P[8] (DS-1) (28.0%), G8P[8] (10.0%), G3P[8] (Wa), and G9P[8]-E1 (8.0%). Three samples were considered mixed infection cases because two different sequences for each gene segment were detected. In 2018, G3P[8] (DS-1) and G9P[8]-E2 (28.6%) were the most prevalent strains, followed by G9P[8]-E1 (19.0%), G2P[4], and G8P[8] (9.5%). One sample was considered a mixed infection case. All four mixed infection cases seemed to comprise a mix of G2P[4] and G9P[8] strains.

Table 1. Genotype constellations of RVAs detected in Tokyo, Japan (2017–2018)

Pattern	No. of samples	Rate (%)	Genotype										
			VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
2017													
G2P[4]	20	(40.0%)	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
G3P[8] (Wa)	4	(8.0%)	G3	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
G3P[8] (DS-1)	14	(28.0%)	G3	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
G8P[8]	5	(10.0%)	G8	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
G9P[8]-E1	4	(8.0%)	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Mixed	3	(6.0%)											
Total	50	(100.0%)											
2018													
G2P[4]	2	(9.5%)	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
G3P[8] (DS-1)	6	(28.6%)	G3	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
G8P[8]	2	(9.5%)	G8	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
G9P[8]-E1	4	(19.0%)	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
G9P[8]-E2	6	(28.6%)	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E2	H1
Mixed	1	(4.8%)											
Total	21	(100.0%)											

The gray color indicates a DS-1-like genotype.

Six G9P[8]-E2 strains detected in 2018 were atypical genotype constellations, which underwent reassortment with the NSP4 gene of the DS-1-like genotype. Of the 71 patients, 10 were RVA-vaccinated children. Four patients were Rotarix-vaccinated children (two G2P[4], one G3P[8] (DS-1) and one mixed infection cases), and four were RotaTeq-vaccinated children (one G3P[8] (Wa), one G3P[8] (DS-1) and two G9P[8]-E2 infection cases). For two children (one G2P[4] and one mixed infection cases), it was unclear which vaccine

they had received. There was no marked disproportion in genotype distribution between vaccinated and unvaccinated cases.

Phylogenetic analysis: Of the 71 samples detected in Tokyo in 2017–18, 56 strains (15 G2P[4], 4 G3P[8] (Wa), 17 G3P[8] (DS-1), 7 G8P[8], 7 G9P[8]-E1, and 6 G9P[8]-E2 strains) were used for phylogenetic analyses. The remaining samples were excluded because the sequence data were incomplete (less than 80% length of at least one segment).

Fig. 1. The VP7 phylogenetic tree of RVA detected in Tokyo (2017–2018). The phylogenetic trees of the VP7 gene of G2 (A), G3 (B), G8 (C), and G9 (D) genotypes are shown. The strains detected in this study are indicated in circles (G2P[4]), squares (G3P[8]), diamonds (G8P[8]), triangles (G9P[8]-E1), and inverted triangles (G9P[8]-E2). The open markers indicate strains detected in 2017, and the closed markers indicate strains detected in 2018. Some similar strains are compressed to a triangle to save space. The phylogenetic trees were constructed using the Maximum Likelihood Method with 1,000 bootstrap replicates. Percent bootstrap support is indicated by the value at each node when the value was 95% or larger.

Phylogenetic trees for the VP7 gene are shown in Fig. 1. The G2 strains in Tokyo were divided into lineages 4a-1 and 4a-3 (Fig. 1A). The G3 strains in Tokyo were divided into lineages 1 and 3 (Fig. 1B). The strains of lineage 3 were typical human G3P[8] with the Wa-like

genotype constellation, whereas the strains of lineage 1 were equine-like G3P[8] with the DS-1-like genotype constellation. The G8 strains in Tokyo were similar to Asian (including Japan) G8P[8] strains that were far from African G8 strains (Fig. 1C). The G9 strains in

Fig. 2. The VP4 phylogenetic tree of RVA detected in Tokyo (2017–2018). The phylogenetic trees of the VP4 gene of P[4] and P[8] genotypes are shown. The indicated markers and the method of tree construction are the same as in Fig. 1.

Tokyo were divided into lineages 3 and 6 (Fig. 1D). Only one strain was lineage 3, and the remainder were lineage 6. The G9P[8]-E2 strains were concentrated in one cluster, whose nucleotide identities were more than

99.8%.

In the phylogenetic tree for the VP4 gene (Fig. 2), all G2 strains in Tokyo were P[4] type, and the remainder were P[8] type. In the P[4] tree, the G2 strains in Tokyo

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Fig. 3. The NSP4 phylogenetic tree of RVA detected in Tokyo (2017–2018). The phylogenetic trees of the NSP4 gene of E1 and E2 genotypes are shown. The indicated markers and the method of tree construction are the same as in Fig. 1.

were divided into clusters as well as the VP7-G2 tree (Fig. 1A). In the P[8] tree, the G3 (DS-1), G8, and G9 strains in Tokyo were clustered together. The G9P[8]-E1 and G9P[8]-E2 strains belonged to the same cluster. The

nucleotide identities of the VP4 genes of G9P[8]-E2 strains were more than 99.6%.

To investigate the derivation of the G9P[8]-E2 strains, a phylogenetic tree was constructed for the NSP4 gene

(Fig. 3). The G3P[8] (Wa) and G9P[8]-E1 strains in Tokyo were shown in the E1 tree, and the G2P[4], G3P[8] (DS-1), G8P[8] and G9P[8]-E2 strains were shown in the E2 tree. Asian G8P[8] strains (including

Japan, this study) were clustered independently. The G3P[8] (DS-1) strains were also concentrated in one cluster. Furthermore, the G9P[8]-E2 and major G2P[4] strains were concentrated in one cluster, whose

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(continued)

Fig. 4. The phylogenetic trees of other genes of RVA detected in Tokyo (2017–2018). The phylogenetic trees of VP1 (R1), VP2 (C1), VP3 (M1), VP6 (I1), NSP1 (A1), NSP2 (N1), NSP3 (T1) and NSP5 (H1) are shown. The indicated markers and the method of tree construction are the same as in Fig. 1.

nucleotide identities were more than 99.6%.

Phylogenetic trees for other gene segments (Fig. 4) showed that the G9P[8]-E2 strains were concentrated in a single cluster, close to at least one of the G9P[8]-E1 strains. The nucleotide identity of the G9P[8]-E2 strains was more than 99.6% in each gene segment.

DISCUSSION

The present study identified various patterns of RVA strains in Tokyo, Japan in 2017–18 (Table 1). Recently-emerging equine-like G3P[8] and G8P[8] strains were also detected, while no G1P[8] strain was detected, which was the major genotype in Japan until a few years

ago (18). The number of RVA-positive samples in 2017 was approximately 2.5 times higher than in 2018, the reason for which is unclear. At least, the major G2P[4] strains in 2017 were not found to have any specific antigenic variations in epitope regions (data not shown). After phylogenetic analyses of VP7, the G2 strains in Tokyo could be divided into multiple clusters. The major G2 strains were lineage 4a-1, but two strains in 2018 were lineage 4a-3. This is the first study to report the detection of strains belonging to lineage 4a-3 in Japan, and these are similar to other Asian and Australian strains.

Most G3 strains in Tokyo were the equine-like G3P[8] with the DS-1-like genotype constellation, which have been detected across the world (including Japan) in recent years (22–26). Typical human G3P[8] with a Wa-like genotype constellation resulted in a small epidemic. The equine-like G3P[8] and G8P[8] strains have been detected in several regions in Japan recently (25,27,36,37), suggesting they are beginning to circulate across the country.

The G9P[8]-E2 virus is a newly-emerged RVA strain. Phylogenetic analyses suggest that this virus was generated by inter-genogroup reassortment between G9P[8] and G2P[4] strains that commonly circulate in Japan. The G9P[8] strain seemed to be reassorted with only the NSP4 gene of the E2 genotype of the G2P[4] strain. These six G9P[8]-E2 strains have very similar sequences and are considered to be derived from a common ancestor virus that emerged in recent years. Furthermore, since this newly-emerged G9P[8]-E2 virus was detected in different locations in Tokyo, this virus seems to be already widespread, at least within Tokyo.

The current study also identified some new strains with intra-genogroup reassortment. Two G8P[8] strains (Tokyo16-4476 and Tokyo17-20) were reassorted with VP1 of DS-1-like G1P[8] strains (data not shown). These strains were similar to the SO1162 strain detected in Chiba, Japan, in 2017 (37). Three DS-1-like G3P[8] strains (Tokyo16-3861, Tokyo17-09, and Tokyo17-119) were reassorted with VP6 of G2P[4] strains (data not shown). These strains were similar to SS61720845 and SS96217158 strains that were detected in Spain in 2015 (23). However, the G9P[8]-E2 virus is a unique newly-detected strain with inter-genogroup reassortment.

It is known that the evolutionary rate of the NSP4 gene is higher than that of other RVA genes, and reassortment events seem to occur more frequently in the NSP4 gene segment (38,39). The G9P[8]-E2 strains described in the current study were generated by inter-genogroup reassortment of NSP4, which may reflect the variability of the NSP4 gene. The NSP4 protein is known to be a multifunctional protein that can bind other viral proteins (VP4 and VP6) and host proteins such as integrin, caveolin, and tubulin (40). However, little is known about differences in activities among NSP4 genotypes. The predicted amino acid sequences of RVA strains identified in the present study were compared with other reference strains, but no characteristic substitution was found in G9P[8]-E2 strains. Additionally, no marked change in virulence was observed in cases infected with G9P[8]-E2 strains, and no central nervous system manifestations or severe complicating disorders were reported in these cases.

Further studies need to be conducted to understand the relationship between the genotype and function of RVA proteins.

Since vaccines were introduced in Japan, the dominant genotype constellation of RVA has changed from Wa-like to DS-1-like, such as DS-1-like G1P[8], G2P[4], G8P[8], and DS-1-like G3P[8] (17,18,25,36). Some of these strains were newly generated by inter-genogroup reassortment. A genotype shift before and after vaccine introduction has also been reported in several other countries (7–10). Considering the timing, vaccine-induced selective pressure could affect the generation and spread of these strains with DS-1-like genotype constellations. Monitoring all RVA strains that include an unusual reassortment will require continuous surveillance based on full-genome analysis.

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Conflict of interest None to declare.

REFERENCES

1. Troeger C, Khalil IA, Rao PC, et al. Rotavirus vaccination and the global burden of rotavirus diarrhea among children younger than 5 years. *JAMA Pediatr.* 2018;172:958–65.
2. Nakagomi T, Kato K, Tsutsumi H, et al. The burden of rotavirus gastroenteritis among Japanese children during its peak months: an internet survey. *Jpn J Infect Dis.* 2013;66:269–75.
3. Bányai K, László B, Duque J, et al. Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre rotavirus vaccine era: insights for understanding the impact of rotavirus vaccination programs. *Vaccine.* 2012;30 Suppl 1:A122–30.
4. Lanata CF, Fischer-Walker CL, Olascoaga AC, et al. Global causes of diarrheal disease mortality in children < 5 years of age: a systematic review. *PLoS One.* 2013;8:e72788.
5. Leshem E, Moritz RE, Curns AT, et al. Rotavirus vaccines and health care utilization for diarrhea in the United States (2007–2011). *Pediatrics.* 2014;134:15–23.
6. Karafillakis E, Hassounah S, Atchison C. Effectiveness and impact of rotavirus vaccines in Europe, 2006–2014. *Vaccine.* 2015;33:2097–107.
7. Mukhopadhyaya I, Murdoch H, Berry S, et al. Changing molecular epidemiology of rotavirus infection after introduction of monovalent rotavirus vaccination in Scotland. *Vaccine.* 2017;35:156–63.
8. Hungerford D, Allen DJ, Nawaz S, et al. Impact of rotavirus vaccination on rotavirus genotype distribution and diversity in England, September 2006 to August 2016. *Euro Surveill.* 2019 Feb 7;24(6):1700774.
9. Carvalho-Costa FA, de Assis RMS, Fialho AM, et al. The evolving epidemiology of rotavirus A infection in Brazil a decade after the introduction of universal vaccination with Rotarix®. *BMC Pediatrics.* 2019;19:42.
10. Lartey BL, Damanka S, Dennis FE, et al. Rotavirus strain distribution in Ghana pre- and post- rotavirus vaccine introduction. *Vaccine.* 2018;36:7238–42.
11. Estes MK, Greenberg HB. Rotaviruses. In: Knipe DM, Howley PM, editors. *Fields Virology*. 6th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2013. p. 1347–401.
12. Matthijnssens J, Ciarlet M, Heiman E, et al. Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. *J Virol.* 2008;82:3204–19.
13. Matthijnssens J, Ciarlet M, Rahman M, et al. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. *Arch Virol.* 2008;153:1621–9.
14. Matthijnssens J, Ciarlet M, McDonald SM, et al. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). *Arch Virol.* 2011;156:1397–413.

15. Nakagomi O, Nakagomi T, Akatani K, et al. Identification of rotavirus genogroups by RNA-RNA hybridization. *Mol Cell Probes*. 1989;3:251-61.
16. Heiman EM, McDonald SM, Barro M, et al. Group A human rotavirus genomics: evidence that gene constellations are influenced by viral protein interactions. *J Virol*. 2008;82:11106-16.
17. Fujii Y, Nakagomi T, Nishimura N, et al. Spread and predominance in Japan of novel G1P[8] double-reassortant rotavirus strains possessing a DS-1-like genotype constellation typical of G2P[4] strains. *Infect Genet Evol*. 2014;28:426-33.
18. Fujii Y, Doan YH, Suzuki Y, et al. Study of complete genome sequences of rotavirus A epidemics and evolution in Japan in 2012-2014. *Front Microbiol*. 2019;10:38.
19. Sugimoto D, Nakano M, Inada M, et al. Distribution of rotavirus genotypes from the 2008/2009 to the 2015/2016 season in Nara Prefecture, Japan. *Jpn J Infect Dis*. 2017;70:593-4.
20. Komoto S, Tacharoenmuang R, Guntapong R, et al. Emergence and characterization of unusual DS-1-like G1P[8] rotavirus strains in children with diarrhea in Thailand. *PloS One*. 2015;10:e0141739.
21. Luchs A, da Costa AC, Cilli A, et al. First detection of DS-1-like G1P[8] double-gene reassortant rotavirus strains on the American Continent, Brazil, 2013. *Sci Rep*. 2019;9:2210.
22. Cowley D, Donato CM, Roczo-Farkas S, et al. Emergence of a novel equine-like G3P[8] inter-genogroup reassortant rotavirus strain associated with gastroenteritis in Australian children. *J Gen Virol*. 2016;97:403-10.
23. Arana A, Montes M, Jere KC, et al. Emergence and spread of G3P[8] rotaviruses possessing an equine-like VP7 and a DS-1-like genetic backbone in the Basque Country (North of Spain), 2015. *Infect Genet Evol*. 2016;44:137-44.
24. Guerra SF, Soares LS, Lobo PS, et al. Detection of a novel equine-like G3 rotavirus associated with acute gastroenteritis in Brazil. *J Gen Virol*. 2016;97:3131-8.
25. Komoto S, Ide T, Negoro M, et al. Characterization of unusual DS-1-like G3P[8] rotavirus strains in children with diarrhea in Japan. *J Med Virol*. 2018;90:890-8.
26. Utsumi T, Wahyuni RM, Doan YH, et al. Equine-like G3 rotavirus strains as predominant strains among children in Indonesia in 2015-2016. *Infect Genet Evol*. 2018;61:224-8.
27. Fujii Y, Doan YH, Wahyuni RM, et al. Improvement of rotavirus genotyping method by using the semi-nested multiplex-PCR with new primer set. *Front Microbiol*. 2019;10:647.
28. Dennis FE, Fujii Y, Haga K, et al. Identification of novel Ghanaian G8P[6] human-bovine reassortant rotavirus strain by next generation sequencing. *PloS One*. 2014;9:e100699.
29. Doan YH, Haga K, Fujimoto A, et al. Genetic analysis of human rotavirus C: The appearance of Indian-Bangladeshi strain in Far East Asian countries. *Infect Genet Evol*. 2016;41:160-73.
30. Maes P, Matthijnsens J, Rahman M, et al. RotaC: a web-based tool for the complete genome classification of group A rotaviruses. *BMC Microbiol*. 2009;9:238.
31. Katoh K, Asimenos G, Toh H. Multiple alignment of DNA sequences with MAFFT. *Methods Mol Biol*. 2009;537:39-64.
32. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*. 2016;33:1870-4.
33. Doan YH, Nakagomi T, Agbemabiese CA, et al. Changes in the distribution of lineage constellations of G2P[4] rotavirus A strains detected in Japan over 32 years (1980-2011). *Infect Genet Evol*. 2015;34:423-33.
34. Wang YH, Pang BB, Ghosh S, et al. Molecular epidemiology and genetic evolution of the whole genome of G3P[8] human rotavirus in Wuhan, China, from 2000 through 2013. *PloS One*. 2014;9:e88850.
35. Phan TG, Okitsu S, Maneekarn N, et al. Genetic heterogeneity, evolution and recombination in emerging G9 rotaviruses. *Infect Genet Evol*. 2007;7:656-63.
36. Kondo K, Tsugawa T, Ono M, et al. Clinical and molecular characteristics of human rotavirus G8P[8] outbreak strain, Japan, 2014. *Emerg Infect Dis*. 2017;23:968-72.
37. Kamiya H, Tacharoenmuang R, Ide T, et al. Characterization of an unusual DS-1-like G8P[8] rotavirus strain from Japan in 2017: Evolution of emerging DS-1-like G8P[8] strains through reassortment. *Jpn J Infect Dis*. 2019;72:256-60.
38. Zeller M, Donato C, Trovão NS, et al. Genome-wide evolutionary analyses of G1P[8] strains isolated before and after rotavirus vaccine introduction. *Genome Biol Evol*. 2015;7:2473-83.
39. Tatsumi M, Nagaoka Y, Tsugawa T, et al. Characterization of the NSP4 gene of group A human rotavirus G1P[8] strains circulating in Sapporo, Japan from 1987 to 2000. *J Med Virol*. 2014;86:354-9.
40. Hu L, Crawford SE, Hyser JM, et al. Rotavirus non-structural proteins: structure and function. *Curr Opin Virol*. 2012;2:380-8.