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Complete genome analyses of G12P[8] rotavirus strains from hospitalized children in Surabaya, Indonesia, 2017–2018

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ABSTRACT

Rotavirus A (RVA) is a major viral cause of acute gastroenteritis worldwide. G12 RVA strains have emerged globally since 2007. There has been no report of the whole genome sequences of G12 RVAs in Indonesia. We performed the complete genome analysis by the next-generation sequencing of five G12 strains from hospitalized children with acute gastroenteritis in Surabaya from 2017–2018. All five G12 strains were Wa-like strains (G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) and were clustered into lineage-III of VP7 gene phylogenetic tree. STM430 sample was observed as a mixed-infection between G12 and G1 strains: G12/G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1. A phylogenetic tree analysis revealed that all five Indonesian G12 strains (SOEP379, STM371, STM413, STM430, and STM433) were genetically close to each other in all 11 genome segments with 98.0-100% nucleotide identities, except VP3 and NSP4 of STM430, suggesting that these strains have originated from a similar ancestral G12 RVA. The VP3 and NSP4 genome segments of STM430-G12P[8] were separated phylogenetically from those of the other four G12 strains, probably due to intra-genotype reassortment between the G12 and G1 Wa-like strains. The change from G12P[6] lineage-II in 2007 to G12P[8] lineage-III 2017–2018 suggests the evolution and diversity of G12 RVAs in Indonesia over the past approximately 10 years.

(200 words)

1. INTRODUCTION

Rotavirus A (RVA) is a major viral cause of acute gastroenteritis (AGE) worldwide, and RVAs continue to be the leading cause of diarrhea-related mortality among children <5 years of age which is estimated to be approximately 20% of the affected population. Low-income countries with no RVA vaccination programs are particularly affected by the RVA diarrhea.[1,2] The World Health Organization (WHO) has been recommending the use of RVA vaccines in all national immunization programs since 2009. To date, 114 countries have introduced RVA vaccines.[3] Systematic review reports revealed vaccine effectiveness with reductions of ≥32% in diarrhea-associated morbidity and mortality in all countries. Although the introduction of vaccines has reduced the number of RVA-associated deaths, the effectiveness of licensed vaccines in low-income countries at 30–40% lower than the effectiveness in high-income countries, is suboptimal[4,5] and showed that RVA gastroenteritis still results in >200,000 deaths annually.[6] These phenomenon remains incompletely understood.[7,8] In Asia, only a few countries have introduced the vaccine nationally or sub-nationally[9]. Indonesia has not yet implemented a RVA vaccine as a universal immunization program, but two RVA vaccines, Rotarix® (GSK, Belgium) and RotaTeq® (Merck & Co, USA) have been commercially available in the private market in Indonesia since 2011. In Indonesia, diarrhea is the leading cause of child mortality, and approximately 38–67% of hospitalized children in Indonesia have diarrhea due to RVA infection.[10,11] Indonesia is one of the countries with the greatest number of RVA deaths as a proportion of all global RVA deaths in children under 5 in 2017.[12]

RVA is a non-enveloped virus consisting of 11 gene segments of double-stranded RNA encoding six structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7genes) and five or six non-structural proteins (NSP1, NSP2, NSP3, NSP4, and NSP5/NSP6).[13] The RVA genotypes are determined based on the nucleotide sequence identity in the open reading frame of each gene and are designated using a letter and a number. Two genes encoding VP7 (a glycoprotein) and VP4 (a protease-sensitive protein) are referred to as G and P types, respectively, and both proteins play crucial roles in recognition and neutralization by the immune system. To date, RVAs have been classified into 42G and 58P genotypes by the RVA Classification Working Group (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg). Molecular epidemiological studies have identified the widespread circulation of various genotypes of RVAs, showing that five G-genotypes, i.e., G1–G4 and G9 and three P-genotypes, i.e., P[4], P[6], and P[8] are commonly associated with human infection.[14,15]

G12 rotaviruses have received little attention until recently. Since 1998, a decade after their first detection in the Philippines, G12 strains have been detected in Asia, Europe, South America, and North America, suggesting their possible emergence worldwide.[16] G12 genotype was the sixth major human G-genotype.[16,17] There have been reports of outbreaks in parts of West Africa such as Nigeria with G12 RVA strains[18,19] and some other countries identified G12 RVA as a prevalent genotype.[16,17] The vast majority of G12 strains have been isolated in Asia and more specifically in Southeast Asia[16]. G12 RVA has a very large genetic diversity caused mainly by genetic reassortments.[16,18]

The geographical region may contribute to the variation of G12 strains isolated around the world. Four different combinations of the G12 genotype and a P-genotype have been

103 detected: G12P[4], G12P[6], G12P[8] and G12P[9].[16,17] Thus far, G12 strains were clustered
104 into three lineages: I, II and III. The first G12 strain, L26 (G12P[4]), isolated from a diarrheic
105 child in the Philippines in 1987, was classified into lineage-I. More than 10 years after the
106 isolation of strain L26, the G12P[9] strain named T152 was isolated in Thailand and was
107 clustered into lineage-II. Many G12 strains isolated in the 2000's were clustered into lineage-
108 III.[16]

109 In Indonesia, the first detection of a G12 strain, G12P[6], was obtained from a 14-day-
110 old febrile infant with diarrhea, vomiting, moderate dehydration and malnutrition who was
111 brought to Sumber Waras Hospital in West Jakarta in December, 2007. The strain isolated
112 from this patient's stool was genotyped by semi-nested reverse transcription-polymerase
113 chain reaction (RT-PCR) targeting the VP7 and VP4 genes, which are two of the 11 segments
114 in the RVA genome.[20] There has been no other reports about G12 RVA strains in Indonesia,
115 and the whole genome sequence of G12 strains in Indonesia has not been reported.

116 A whole genome-based genotyping method using the analysis of all 11 gene segments
117 has been used for RVA epidemiology, with classification into genogroups based on the
118 overall genomic RNA homology. Three genogroups of human RVA have been defined: Wa-
119 like, DS-1-like, and AU-1-like. Of these three constellations, the Wa-like strains are the most
120 predominant followed by the DS-1-like strains, and the AU-1-like strains are the least
121 numerous strains in humans globally.[21] It is urgently necessary to fully sequence and
122 characterize G12 RVA strains, as this information will contribute to our understanding of the
123 evolutionary pattern and the diversity of emerging G12 strains. In this study, we performed
124 a whole genome analysis of five G12 RVA strains detected in Indonesia in 2017–2018 by
125 using the next-generation sequencing (NGS) technique.

2. MATERIALS AND METHODS

2.1. Specimens:

During the RVA surveillance in 2017–2018 among hospitalized children with acute gastroenteritis in Surabaya, Indonesia, 67 RVA-positive stool samples were detected by an immunochromatography assay and RT-PCR. We used these RVA-positive samples for the whole genome analyses by NGS. The most commonly observed RVA genotypes were G1 and G3, and 62 of the 67 samples were excluded from this study; G12 RVA strains were detected in only five samples, all of which were included in the present investigation.

We thus analyzed the whole genome sequences of G12 RVA strains isolated from five stool specimens of ≤5-year-old children with acute gastroenteritis treated as inpatients at Dr. Soetomo hospital and Soerya hospital in Surabaya, East Java, Indonesia in 2017–2018. SOEP379 was isolated from an inpatient child treated at Soerya hospital, and STM371, STM413, STM430, and STM433 were isolated from children treated at Dr. Soetomo hospital. Acute gastroenteritis was defined as the occurrence of three times looser than normal stools during a 24-hour period. This study was approved by the Research Ethics Board of both hospitals, Universitas Airlangga (ethics approval number 2054/UN3.14/LT/2015) in Indonesia and Kobe University (ethics approval number 1857) in Japan. Written informed consent was obtained from the children's parents or guardians.

2.2. Isolation of RNA virus and RT-PCR Genotyping

147 Stool specimens were 10-fold diluted with phosphate-buffered saline (PBS; pH 7.4) and
148 clarified by centrifugation at 10,000 × g for 10 min. Aliquots of all of the samples were then
149 stored at –80°C before use. Viral RNA was extracted using Trizol LS Reagent (Life
150 Technologies, Grand Island, NY, USA). In brief, 80 µl of the aliquot of a stool suspension in
151 PBS was mixed with 240 µl of Trizol LS reagent and incubated for 5 min at room temperature.
152 Then, 320 µl of 100% ethanol was added to the mixture. The mixture was placed into a Zymo-
153 Spin IIC column (Zymo Research, Irvine, CA) and was centrifugated at 12,000 × g for 1 min,
154 and washed with the pre-wash buffer and the wash buffer following the manufacturer's
155 instructions. The viral RNAs were eluted with DNase/RNase-free water and used for RT-
156 PCR genotyping and the whole genome analysis by NGS.
157 Viral extracted were subjected to genotyping in the VP7 (G typing) and VP4 genes (P
158 typing) by multiplex RT-PCR. The VP7 and VP4 primer sets used had been previously
159 described.[22] In particular, the VP7 primer set allowed us to correctly identify equine-like G3
160 among the other epidemic strains (G1, G2, typical human G3, G4, G8, G9, and G12).[23] The
161 RNA samples were initially incubated at 65°C for 5 min with the first PCR primers.
162 Subsequently, reverse transcription reaction was performed at 45°C for 10 min and at 94°C
163 for 2 min, followed by 40 cycles of amplification (at 98°C for 10 s, at 50°C for 15 s, and at
164 68°C for 40 s), with a final extension at 68°C for 3 min. One µl of diluted (50-fold) first PCR
165 products was then used for a second PCR. The initial denaturation step was conducted at
166 98°C for 10 min, followed by 20 cycles of amplification (at 98°C for 10 s, at 50°C for 15 s,
167 and at 68°C for 60 s), with a final extension at 68°C for 3 min. Both negative and positive
168 controls were included in each experiment. Then, RT-PCR products were run in the agarose

gel electrophoresis and D1000 ScreenTape assay on 4150 TapeStation (Agilent Technologies) was performed to visualize the size of PCR pruducts.

2.3. cDNA library building and Illumina Miseq sequencing for NGS Analaysis

The preparation of the cDNA library and the Illumina Miseq sequencing were performed as previously described.[24,25] Briefly, a 200-bp fragment library ligated with bar-coded adapters was constructed for individual strains using an NEBNext Ultra RNA library Prep Kit for Illumina ver. 1.2 (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Library purification was performed using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). The quality of the purified DNA libraries was assessed on a MultiNa MCE-202 bioanalyzer (Shimadzu Corporation, Kyoto, Japan). Nucleotide sequencing was performed on an Illumina Miseq sequencer (Illumina, San Diego, CA, United States) using the Miseq Reagent Kit ver. 2 (Illumina) to generate 151 paired-end reads.

2.4. Nucleotide analysis and determination of RVA genotypes detected by NGS Analysis

The sequence data were analyzed and assembled in the CLC Genomics Workbench Software ver. 7.0.3 (CLC Bio, Aarhus, Denmark). A *de novo* assembly was carried out for all samples and the resulting contigs were identified using the Nucleotide Basic Local Alignment Search Tool (NCBI BLAST). The mixed infection between RVAs was defined as when two or more sequences were present in the same genome segment and they were of the same or different genotypes. The different titers of RVA genomic RNA extracted from the stool specimen will reflect the number of copies in the library, and therefore, the number of

192 reads obtained for the genes of each strain. Thus, genome constellation of each RVA strain
193 in mixed infection sample was defined as all sequences for 11 genome segments have a
194 similar rate between total read count and length of obtained sequences.

195 The genotype of each of 11 gene segments of G12P[8] strains were determined using the
196 online database Virus pathogen Database and Analysis Resource (ViPR).[23] The sequence
197 results were deposited in the DDBJ/GenBank database with accession numbers LC581220
198 to LC581275.

199

200 2.5. Phylogenetic tree analyses

201 Sequence comparisons were carried out with the references retrieved from GenBank.
202 Alignment between the study and reference strains was performed using a Multiple Sequence
203 Comparison by Log Expectation (MUSCLE) alignment.[26] Phylogenetic trees were
204 constructed in MEGA version 10.0 software using Maximum likelihood method.[27] The best
205 substitution models were selected based on the corrected Akaike Information Criterion
206 (AICC) value as implemented in MEGA10. Models used in this study were Tamura 3-
207 parameter (T92)+G+I (VP7, NSP3, NSP4, and NSP5), (T92)+G+I (NSP2), Tamura-Nei
208 (TN93)+G+I (VP1, and VP3), General Time Reversible (GTR)+G+I (VP4, VP2, and NSP1).
209 The reliability of the branching order was estimated from 1000 bootstrap replicates. Lineages
210 of G12 RVA strains were assigned according to the literature.[16,28]

211

212 **3. RESULTS**

213 3.1. Genotype constellation of Indonesian G12 strains

Five G12 RVA strains isolated from stool samples of pediatric patients with acute gastroenteritis were used: SOEP379, STM371, STM413, STM430 and STM433. The patients were 6 -24 months old (average 12.6 months old), consisting of two boys and three girls. The clinical symptoms of the patients were diarrhea (5/5, 100%), waterly diarrhea (1/5, 20%), vomitting (2/5, 40%), fever (2/5, 40%), abdominal pain (1/5, 20%), some dehydration (3/5, 60%), and severe dehydration (1/5, 20%). We analyzed the whole genome of all five strains by NGS. The open reading frames of the 11 genome segments of SOEP379, STM371, STM413, STM430 and STM433 strains were successfully determined using the Illumina MiSeq technology. The genotype constellation of all five strains revealed to be G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1, i.e., typical Wa-like strains. Of these, STM430 sample was observed as mixed-infection sample between G12 and G1 strains with two genome constellations: G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 and G11-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1. No reassortment event with inter-genogroups or inter-species of RVA strains was detected (Table 1).

3.2. Phylogenetic tree analysis:

The analysis demonstrated that all of the Indonesian G12 strains have a close relationship with each other, as depicted by the single clade in the phylogenetic tree of VP7 gene (Fig. 1). They were clustered into lineage-III. The VP7 gene of these strains were closely associated with the VP7 gene of strain RVA/Human-wt/USA/2013774166/2013/G12P8 and shared 99.14%–99.24% nucleotide identity (Table 2). In the phylogenetic tree of VP7 genes, we also identified the mixed-genotype strain with RVA G1 (STM430-G1P[8]), which was clustered

236 together with RVA/Human-wt/IDN/STM457/2018/G1P[8] from Indonesia and near
237 RVA/Human-wt/IND/RV1326/2013/G1 from India (Fig. 1).

238 The VP4 genes of all five G12 strains were genotyped as P[8] (Fig. 2). On the
239 phylogenetic tree, all Indonesian G12 strains clustered closely to RVA/Human-
240 wt/BGD/Bang-065/2008/G9P8 in VP4 gene and shared 99.08%–99.35% nucleotide identity
241 (Table 2). STM430-G1 strain was also genotyped as P[8] but grouped as a different branch
242 from the other G12 strains; it was found to be closely related to two strains of G1P[8] isolated
243 from Indonesia, RVA/Human-wt/IDN/STM453/2018/G1P[8]. In addition, type P[8] of strain
244 STM430-G1 is also phylogenetically close to RVA/Human-wt/JPN/Tokyo17-21/2017/G3P8
245 (Fig. 2). The genetic backbone of all samples was Wa-like strain (Table 1, Fig. 2). The results
246 of the phylogenetic analysis of the VP6, VP1–VP3, and NSP1–NSP5 genes of the five study
247 strains are illustrated in Supplemental Figures S1-S9.

248 Five Indonesian G12 strains were closely related to each other in all 11 genome segments
249 with 98.0-100% nucleotide identities, except VP3, and NPS4 gene of STM430-G12 strain
250 (Figs. 1, 2 and Suppl. Fig. S1-S9). The VP3 and NSP4 genes of STM430-G12 strain were
251 separated into a different clade from the four other G12 study strains and clustered with other
252 Indonesian strains RVA G1P[8] (RVA/Human-wt/IDN/STM387/2017/G1P8; RVA/Human-
253 wt/IDN/STM453/2018/G1P8 or RVA/Human-wt/IDN/STM457/2018/G1P8) as described in
254 the phylogenetic tree.

255 The VP4, VP6, VP1–VP3, and NSP1–NSP3 genes of all these Indonesian G12 strains,
256 except VP3 and NSP4 of STM430-G12, were clustered with the closest reference G12 strains
257 outside Indonesia, including strains from Bangladesh, Hungary, USA, Thailand, and
258 Slovakia (Figs. 1, 2 and Suppl. Figs. S1-S9). Interestingly, NSP4 genes in all five study

strains were closest to RVA/Cow-wt/UGA/BUW-14-A035/2014/G12P8 isolated from a cow (i.e., a strain transmitted from a human to a cow) in Uganda (Suppl. Fig. S8).

The nucleotide sequence identities for the 11 genes between samples and closest references analyzed by NCBI homology BLAST are shown in Table 2. Within samples, some study strains exhibited 100% similarity in several genes; for example, STM413 and STM433 in VP7 gene, as well as STM371 and SOEP379 in NSP4 gene (data not shown). A homology analysis using NCBI BLAST showed no evidence of inter-species reassortment events in any of the gene segments. Nevertheless, some animal strains were included in the phylogenetic tree of several genes because they are highly related to those of human RVAs and have homology with the study strains, suggesting that human RVA strains were also potentially transmitted to an animal and vice versa. The RVA strains isolated from animals that were observed in the present phylogenetic tree were RVA/Cow-wt/UGA/BUW-14-A035/2014/G12P in VP6, VP7, NSP1, and NSP4 genes (Fig. 1, Suppl. Figs. S1, S5, and S8), RVA strain Rat-wt/ITA/Rat14/2015/G3P3 in VP6 (Suppl. Fig. S1), Porcine RV NSP3 (NS34) mRNA (strain PRICE) in NSP3 (Suppl. Fig. S7), RVA/Cow/B85/MP/India/2008 in NSP4 (Suppl. Fig. S8), and RVA/Donkey-wt/ZAF/MRC-DPRU1967/XXXX/GXPX in NSP5 (Suppl. Fig. S9). Their nucleotide sequence similarity compared to the study strains were around 96.9–99.6%.

3.3. Confirmation of mixed infection in STM430 sample

To confirm mixed infection for STM430 sample, the RNA was re-extracted from original clinical sample. RVA VP7 genotyping using the semi-nested multiplex PCR [23] were performed for both 1st and 2nd extracted RNA of STM430 together with other G1 (STM387)

and G12 (STM413) samples. The size of PCR products was determined by D1000 ScreenTape assay on 4150 TapeStation (Agilent Technologies). The result revealed that all G12 samples contain 264 bp-band of G12 RVA. However, both 1st and 2nd STM430 samples contain two bands, 264 bp-band of G12 RVA and 636 bp-band of G1 RVA, indicating mixed infection of G12 RVA and G1 RVA (Fig. 3).

4. DISCUSSION

By using RT-PCR Genotyping and NGS analysis, we identified G12 RVAs as mono-infection in four samples (STM371, STM431, STM433, and SOEP379) and a co-infection of G12/G1 mixed-genotype in one sample (STM430). All strains were P[8] and showed a Wa-like genotype constellation, G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1. The single sample with the G12/G1 mixed-infection, exhibited two genotype constellations: G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 and G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1.

In Indonesia, the RVA genotypes G1P[8] and G3P[8] human strains are prevalent.[22,29,30] This may suggest co-infection between G1P[8]/G3P[8] and other G-types, such as the G12 isolated in this study. The finding of mixed-genotypes in this study showed that the NGS method is not only useful for whole genome-based analyses, but can also reveal the possibility of the occurrence of multiple isolates/strains/species. Other than, NGS analysis can also prove that there is no contamination on RT-PCR Genotyping results with more than one band in the gel image of electrophoresis.

The RVA strains with rare or uncommon G/P types have been detected after the introduction of RVA vaccine and became dominant genotypes. It was reported that the emergence of G12P[8] RVA as a predominant genotype was newly observed in countries

using RotaTeq,[31,32] whereas the dominance of G2P[4] and equine-like G3P[8] was found in countries using Rotarix.[33,34] These findings were obtained in countries with high vaccine coverage and effectiveness. However, emerging of previously rare or novel genotypes was observed in the vaccine era, indicating that the widespread vaccine use may accelerate strain diversities.[35] The RVA strains with new genotypes have also been found in countries with low coverage or no vaccination programs.[36,37]

Indonesia has not yet implemented a RVA vaccine national program. The use of Rotarix and RotaTeq in the private market at a relatively high price would have very limited coverage. We reported that uncommon equine-like G3 RVA strains were predominantly spread in Indonesia.[22,24] An isolate of an uncommon G12 RVA strain was firstly identified in Indonesia in December 2007 and was genotyped as G12P[6][20], but there was no subsequent report about G12 RVA detection in Indonesia until 2016. During the RVA surveillance in 2017–2018, we detected five G12 RVA strains from hospitalized children. In the present study, we conducted a complete molecular characterization of these five G12 RVA Indonesian strains by NGS.

With the present phylogenetic analyses using the full-genome sequence of the 11 gene segments, we obtained direct evidence of the five strains' relatedness to other strains, even within the same genotype. The shifting of RVA genotypes was demonstrated by the difference in VP7 gene of G12 RVA between strain detected in 2007 classified in lineage-II and the present study strains isolated in 2017–2018, classified as lineage-III. The findings suggest an evolution of RVA genes may occur after ≥10 years, particularly regarding changes in VP4 gene and the lineage of VP7. The majority of RNA viruses have been reported to have an evolutionary rate of between 1.0×10^{-3} to 1.0×10^{-6} substitutions/site/year.[38] The

evolutionary rate of the African G12 RVA sequences was 1.678×10^{-3}, (95% highest posterior density [HPD], 1.201×10^{-3} to 2.198×10^{-3}) substitutions/site/year.[39]

The nucleotide sequence divergence between lineage-II and lineage-III ranges from 2.6% to 3.2%.[16] However, lineage-II is a minority cluster when compared with lineage-III. G12 RVA lineage-I includes G12 strains from Japan, Argentina, Thailand, and Paraguay, whereas G12 RVA lineage-II consists of strains from the United States, India, Bangladesh, Germany, Uganda, and South Africa.[16,20] Hence, it was also suggested that the population mobility may contribute to genotype distribution.

The lineage-III G12 RVA strains in VP7 gene isolated in Indonesia were closely related to the USA strains, indicating that they might have originated in the USA. Indeed, each of the 11 genes of RVAs analyzed in this study showed the closest references from U.S., Bangladesh and Hungary (Table 2). This finding suggests that RVAs have genetically diverse population of segmented dsRNA viruses due to the genomic reassortant ability between human strains or inter-species with animal strains.[40] The RVAs strains might be transported across the globe by the increasing mobility of humans and animals. Examples are seen in the RVA genome in the Belgian strain B4633-03, which was nearly identical to the Dhaka25-02 strain and the United Kingdom strain MV404-02, which was closely related to the Dhaka12-03 strain.[16] We compared our five study strains to other G12 strains that are Wa-, DS-1-, AU-1-, and ST3-like strains observed in Asian countries, such as the Philippines, Thailand, and Bangladesh (Table 1); the results indicate that all five study strains belong to Wa-like genogroups without reassortment with inter-genogroups or inter-species.

The branch topology of the equivalent segments from all five Indonesian G12P[8] strains in this study fell within the same monophyletic lineage for all segments, except VP3 and

NSP4 of STM430/G12P[8] strain. Clustering within same monophyletic lineage indicate that these G12 strains may have originated from the same ancestor. G12P[8] virus seems to be undergoing further intra-genotype reassortment with G1P[8] virus in a mixed infection STM430 sample. The reassortment events have occurred in its VP3, and NSP4 genes (proved by average coverage in NGS results almost similar to other genes of STM430 G12). However, it is possible that the reassortments also are undergoing in the VP2, NSP2, NSP3, and NSP5 genes (data not shown). In the future, we will conduct other experiments to confirm the undergoing reassortment events in the RVA mixed infection samples. RVA G1P[8] was also found to be predominant in Indonesia.[22] Circulating RVA mixed-genotypes may thus contribute to intra-genogroups events. Our present findings revealed that gene segments of STM430 were closely related to those of RVA G1 strains that we previously isolated in Indonesia.[22] No reassortment for inter-genogroups was observed, even though an equine-like G3P[8]/[6] DS-1-like RVA strain was predominant at a hospital in the suburbs of Surabaya in 2015–2018.[22]

The NSP4 gene of all five G12 strains isolated in this study was closest to RVA/Cow-wt/UGA/BUW-14-A035/2014/G12P which is isolated from domestic animals in Uganda and is human-to-animal RVA transmission.[41] This phenomenon suggests that NSP4 gene reassortment event easily occurs between human and animal strains. Importantly, there is no human possessing an animal strain. Our present findings demonstrate that the genotype constellation of G12 RVA strains are similar. This may be because only a few G12 strains were detected in the study; the further isolation of G12 RVAs throughout Indonesia is necessary.

The detection of G12P[8] RVA has also increased recently in countries with and without high vaccine coverage.[42,43] The G12 genotype was first detected in 1987 in the Philippines[44], then remained undetected until it re-emerged in Thailand in 1998.[45] Although G12 was considered a rare genotype in the 2000's, G12P[8] is now common genotype in many countries including in Asian countries such as Bangladesh[46], India[47], Japan[48], Korea[49], Thailand[45], and Vietnam[50]. The emergence of G12 RVA strains may also have an impact on the efficacy of RVA vaccines. The increasing frequency of G12 RVAs raises questions about their origin and evolution and how they can spread all over the world. Characterizing the complete genomes may increase our understanding of the relatedness and the evolution of emerging G12 RVA strains, which could be the result of point mutations, genetic rearrangements or reassortment events, and inter-species transmission. Whole genome-based analyses are thus useful to understand the evolutionary dynamics of G12 strains.

5. CONCLUSIONS

To our knowledge, this is the first research article of the complete genome analyses of the G12 strains that have re-emerged in Indonesia. Five G12 RVA strains were obtained in the samples collected in Indonesia in 2017–2018. The results of our analyses demonstrated that G12P[8] is a newly emerging genotype in Indonesia, with genetic backbone of all strains Wa-like; no reassortment event was observed in inter-genogroups or animal strains. Further surveillance studies are necessary to gain more information about the circulating genotypes of human RVA in Indonesia, especially new emerging strains, such as G12 RVA strains.

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Data Availability

The data that support the findings of this study will be available at Kernel-Kobe University Repository (https://da.lib.kobe-u.ac.jp/da/kernel/). The sequence results were deposited in the DDBJ/GenBank database with accession numbers LC581220 to LC581275.

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Author Contribution

Yamani LN, Utsumi T, Doan YH, Lusida MI, and Shoji I designed the study and wrote the manuscript. Soegijanto S, Athiyyah AF, Sudarmo SM, Rahuh RG, Soetjipto, Juniastuti collected samples. Dinana Z, Wahyuni RM, and Gunawan E carried out RVA detection and genome analysis. Yamani LN, Doan YH, Katayama K, and Matsui C performed NGS sequencing. Yamani LN, Doan YH, Matsui C, Bawono RG conducted sequence data analysis. Deng L, Abe T, Shimizu H, Ishii K, Katayama K, Lusida MI, Shoji I gave critical revision of the article. All the authors contributed to the interpretation of the data, writing of the manuscript, and approved the final manuscript.

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419 **Conflict of interest**

420 The authors declare no conflict of interest regarding this study.

421

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Figure Legends

Fig. 1. Phylogenetic tree analysis of VP7 genes for G12 RVA strains detected in five samples collected in Surabaya, Indonesia. *Black inverted triangles and Bullets:* The RVA strains sequenced in this study. The reference strains obtained from the GenBank database are represented by the accession number, strain name, country, and year of isolation. Scale bar: 0.050 substitutions per nucleotide.

Fig. 2. Phylogenetic tree analysis of VP4 genes detected in five samples collected in Surabaya, Indonesia. *Black inverted triangles and Bullets:* The G12 and G1 RVA strains sequenced in this study, respectively. The reference strains obtained from the GenBank database are represented by the accession number, strain name, country, and year of isolation. Scale bar: 0.020 substitutions per nucleotide.

Fig. 3. The VP7 genotyping of STM430.

To confirm the mixed infection for STM430 sample, the RNA was re-extracted from original clinical sample. RVA genotyping using the semi-nested multiplex PCR were performed for both 1st and 2nd extracted RNA of STM430 together with other G1 (STM387) and G12 (STM413) samples. The size of PCR products was determined by D1000 ScreenTape assay on 4150 TapeStation (Agilent Technologies).

Table 1. RVA genotype constellations of five human G12 RVA study strains compared to the published human G12 RVA strains

G12 strain	Year	Genogroup	VP7 (Lineage)	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Reference
L26	1987	DS-1-like	G12 (I)	P[4]	I2	R2	C2	M1/M2	A2	N1	T2	E2	H1	Previous study[16]
T152	1998	AU-1-like	G12 (II)	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6	
RV161-00	2000–2002	DS-1-like	G12 (III)	P[6]	I2	R2	C2	M2	A2	N2	T2	E1	H2	
RV176-00	2000–2002	DS-1-like	G12 (III)	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2	
N26-02	2000–2002	DS-1-like	G12 (III)	P[6]	I2	R2	C2	M2	A2	N1	T2	E6	H2	
Dhaka25-02	2002–2005	Wa-like	G12 (III)	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1	
Dhaka12-03	2002–2005	Wa-like	G12 (III)	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1	
Matlab13-03	2002–2005	Wa-like	G12 (III)	P[6]	I1	R1	C1	M1	A1	N1	T2	E1	H1	
SOEP379-18	2017–2018	Wa-like	G12 (III)	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1	Present study
STM371-17	2017–2018	Wa-like	G12 (III)	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1	
STM413-17	2017–2018	Wa-like	G12 (III)	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1	
STM433-17	2017–2018	Wa-like	G12 (III)	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1	
STM430-17	2017–2018	Wa-like	G1/G12 (III)	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1	

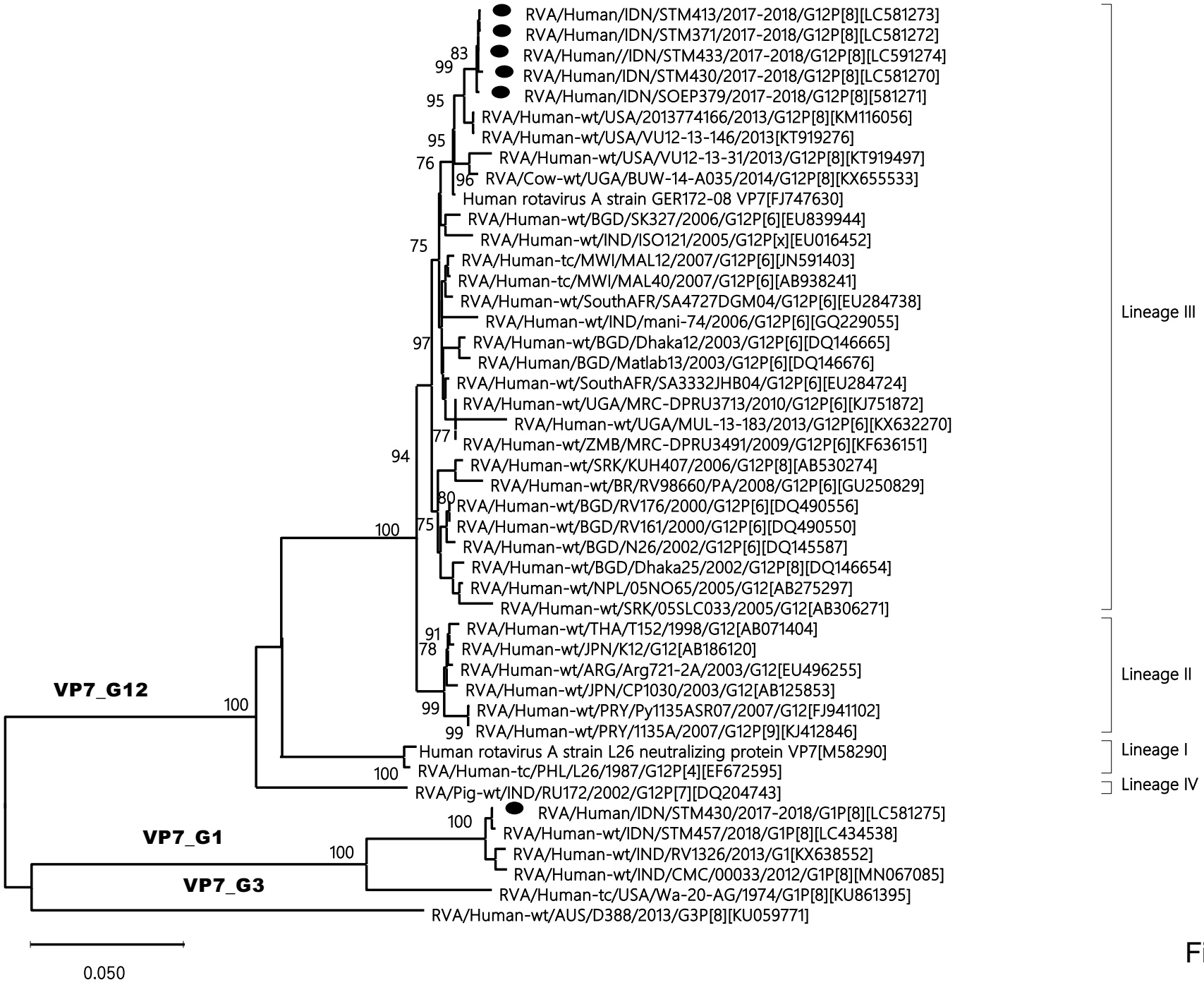
The Wa-, DS-1-, and AU-1-like genogroups were assigned to human strains if at least seven gene segments belonged to the Wa-, DS-1, and AU-1-like genotype, respectively. Colors were added to visualize patterns or gene constellations. *Green:* Wa-like, *red:* DS-1-like, and *yellow:* AU-like gene segments. *Gray:* Subtype 4A, and *blue:* RVA gene from animals.

*Green is Wa-like strain. *Red is DS-1-like strain. *Yellow is AU-1-like strain. *Gray is an ST3-like strain. *Blue is a RVA gene from an animal.

Table 2. Nucleotide sequence identity between G12 RVA genes obtained from Illumina MiSeq sequencing and the closest strains isolated from references based on NCBI homology BLAST

Gene	Accession no.	Genotype	Samples	Closest strain of NCBI homology (% nt identity)		Gene	Accession no.	Genotype	Samples	Closest strain of NCBI homology (% nt identity)	
VP7	LC581270 LC581271 LC581272 LC581273 LC581274	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	99.14% 99.24% 99.22% 99.24% 99.24%	RVA/Human-wt/USA/2013774166/2013/G12P[8]	NSP1	LC721867 LC581221 LC581222 LC581223 LC581224	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	99.45% 99.38% 99.52% 99.59% 99.52%	Hungary human strain ERN5009 isolated 2012
	LC581275	G1P[8]	STM430	99.81%	RVA/Human-wt/IDN/STM457/2018/G1P[8]		LC722500	G1P[8]	STM430	99.60%	RVA/Human-wt/IDN/STM457/2018/G1P[8]
VP4	LC721862 LC581261 LC581262 LC581263 LC721864	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	99.22% 99.08% 99.35% 99.26% 99.26%	RVA/Human-wt/BGD/Bang-065/2008/G9P[8]	NSP2	LC721868 LC581226 LC581227 LC581228 LC581229	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	98.80% 98.84% 98.88% 98.94% 98.94%	RVA/Human-wt/USA/2013774165/2013/G12P[8]
	LC722498	G1P[8]	STM430	99.83%	RVA/Human-wt/IDN/STM457/2017/G3P[8]		LC722501	G1P[8]	STM430	100%	RVA/Human-wt/IDN/STM387/2017/G1P8 and RVA/Human-wt/IDN/STM453/2017/G1P[8]
VP6	LC721863 LC581266 LC581267 LC581268 LC581269	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	99.76% 99.76% 99.76% 99.76% 99.82%	RVA/human/SVK/2451/I1	NSP3	LC721869 LC581231 LC581232 LC581233 LC581234	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	98.93% 98.77% 99.28% 98.88% 98.71%	RVA/Human-wt/THA/DBM2018-111/2018/G9P[8]
	LC722499	G1P[8]	STM430	99.77%	MN304727.1 RVA/Human-wt/NGR/2017		LC722502	G1P[8]	STM430	99.91%	RVA/Human-wt/IDN/STM457/2017/G1P[8]

VP1	LC721864 LC581246 LC581247 LC581248 LC581249	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	99.25% 99.26% 99.33% 99.23% 99.26%	RVA/Human-wt/USA/VU12-13-39/2013/G12P[8]	NSP4	LC721870 LC581236 LC581237 LC581238 LC581239	G12P[8]	STM430 (like G1) SOEP379 STM371 STM413 STM433	99.86% 99.46% 99.47% 99.31% 99.18%	RVA/Human-wt/IDN/STM457/2018/G1P[8] RVA/Cow-wt/UGA/BUW-14-A035/2014/G12P[8]
	LC722495	G1P[8]	STM430	99.88%	RVA/Human-wt/IDN/STM453/2018/G1P[8]		LC722503	G1P[8]	STM430 (like G12)	99.44%	RVA/Cow-wt/UGA/BUW-14-A035/2014/G12P8
VP2	LC721865 LC581251 LC581252 LC581253 LC581254	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	99.10% 99.19% 99.44% 99.40% 99.33%	RVA/Human-wt/BGD/Bang-015/2008/G12P[6]	NSP5	LC722504 LC581241 LC581242 LC581243 LC581244	G12P[8]	STM430 SOEP379 STM371 STM413 STM433	99.39% 99.54% 99.52% 99.38% 99.54%	RVA/Human-tc/USA/DC5685-40-AG/1991/G1P[8]
	LC722496	G1P[8]	STM430	99.89%	RVA/Human-wt/IDN/STM453/2018/G1P8		LC721871	G1P[8]	STM430	100%	RVA/Human-wt/IDN/STM457/2018/G1P[8]
VP3	LC721866 LC581256 LC581257 LC581258 LC581259	G12P[8]	STM430 (like G1) SOEP379 STM371 STM431 STM433	99.81% 98.44% 98.33% 98.48% 98.48%	RVA/Human-wt/IDN/STM387/2018/G1P[8] RVA/Human-wt/USA/VU08-09-6/2008/G12P[8]						
	LC722497	G1P[8]	STM430 (like G12)	97.59%	RVA/Human-wt/USA/VU08-09-6/2008/G12P[8]						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RVA/Cow-wt/UGA/BUW-14-A035/2014/G12P[8][KX655533]
Human rotavirus A strain GER172-08 VP7[FJ747630]
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RVA/Human-tc/MWI/MAL12/2007/G12P[6][JN591403]
RVA/Human-tc/MWI/MAL40/2007/G12P[6][AB938241]
RVA/Human-wt/SouthAFR/SA4727DGM04/G12P[6][EU284738]
RVA/Human-wt/IND/mani-74/2006/G12P[6][GQ229055]
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RVA/Human/IDN/STM430/2017-2018/G1P[8][LC581275]
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RVA/Human-wt/AUS/D388/2013/G3P[8][KU059771]
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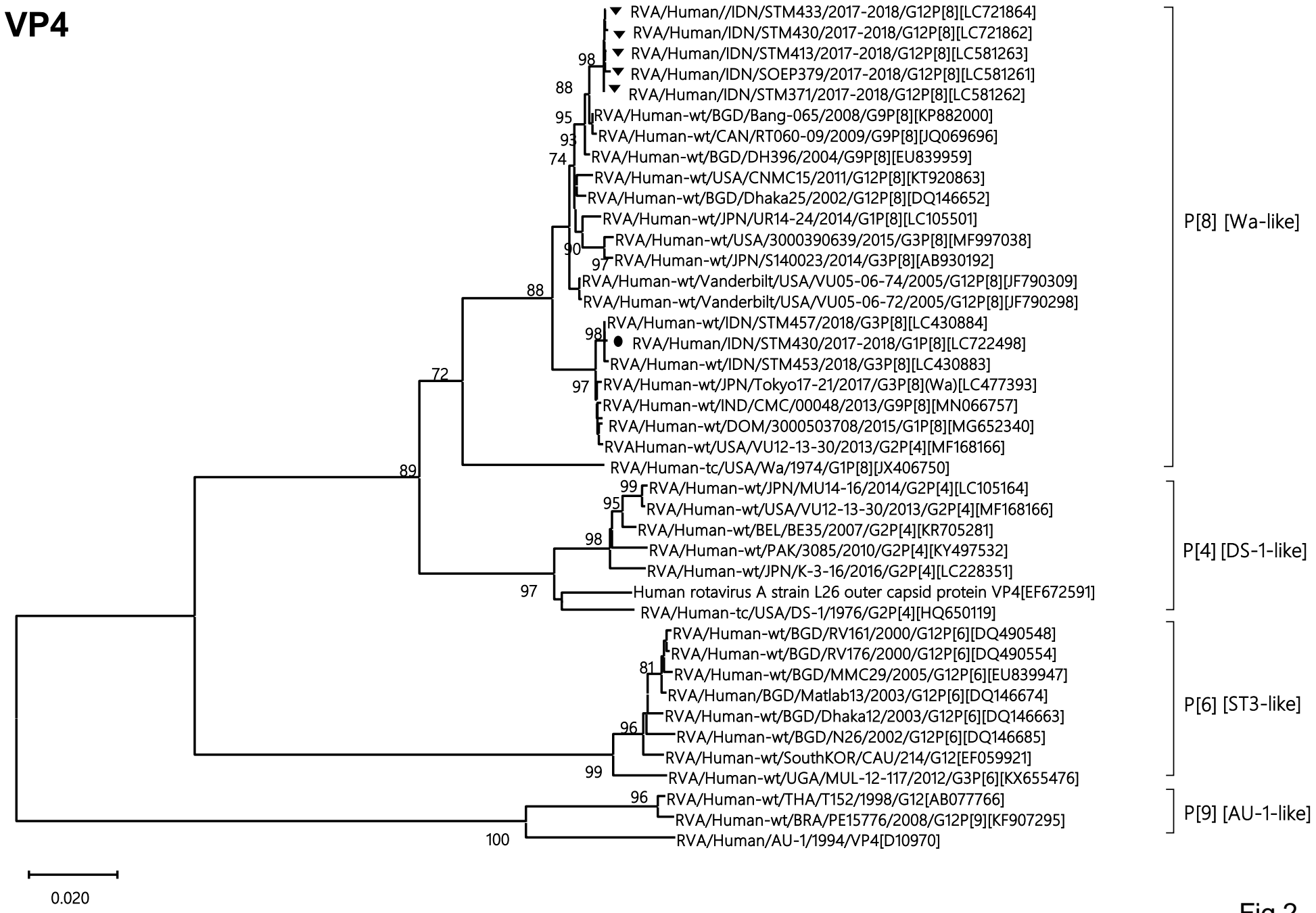
VP7_G1
VP7_G3
Lineage III
Lineage II
Lineage I
Lineage IV
0.050

Fig. 1.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RVA/Human-wt/BGD/Dhaka25/2002/G12P[8][DQ146652]
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RVA/Human-wt/USA/3000390639/2015/G3P[8][MF997038]
RVA/Human-wt/JPN/S140023/2014/G3P[8][AB930192]
RVA/Human-wt/Vanderbilt/USA/VU05-06-74/2005/G12P[8][JF790309]
RVA/Human-wt/Vanderbilt/USA/VU05-06-72/2005/G12P[8][JF790298]
RVA/Human-wt/IDN/STM457/2018/G3P[8][LC430884]
RVA/Human/IDN/STM430/2017-2018/G1P[8][LC722498]
RVA/Human-wt/IDN/STM453/2018/G3P[8][LC430883]
RVA/Human-wt/JPN/Tokyo17-21/2017/G3P[8](Wa)[LC477393]
RVA/Human-wt/IND/CMC/00048/2013/G9P[8][MN066757]
RVA/Human-wt/DOM/3000503708/2015/G1P[8][MG652340]
RVAHuman-wt/USA/VU12-13-30/2013/G2P[4][MF168166]
RVA/Human-tc/USA/Wa/1974/G1P[8][JX406750]
RVA/Human-wt/JPN/MU14-16/2014/G2P[4][LC105164]
RVA/Human-wt/USA/VU12-13-30/2013/G2P[4][MF168166]
RVA/Human-wt/BEL/BE35/2007/G2P[4][KR705281]
RVA/Human-wt/PAK/3085/2010/G2P[4][KY497532]
RVA/Human-wt/JPN/K-3-16/2016/G2P[4][LC228351]
Human rotavirus A strain L26 outer capsid protein VP4[EF672591]
RVA/Human-tc/USA/DS-1/1976/G2P[4][HQ650119]
RVA/Human-wt/BGD/RV161/2000/G12P[6][DQ490548]
RVA/Human-wt/BGD/RV176/2000/G12P[6][DQ490554]
RVA/Human-wt/BGD/MMC29/2005/G12P[6][EU839947]
RVA/Human/BGD/Matlab13/2003/G12P[6][DQ146674]
RVA/Human-wt/BGD/Dhaka12/2003/G12P[6][DQ146663]
RVA/Human-wt/BGD/N26/2002/G12P[6][DQ146685]
RVA/Human-wt/SouthKOR/CAU/214/G12[EF059921]
RVA/Human-wt/UGA/MUL-12-117/2012/G3P[6][KX655476]
RVA/Human-wt/THA/T152/1998/G12[AB077766]
RVA/Human-wt/BRA/PE15776/2008/G12P[9][KF907295]
RVA/Human/AU-1/1994/VP4[D10970]
98
88
95
93
74
90
97
88
98
72
97
89
99
95
98
97
81
96
99
96
100
P[8] [Wa-like]
P[4] [DS-1-like]
P[6] [ST3-like]
P[9] [AU-1-like]
0.020

Fig.2.

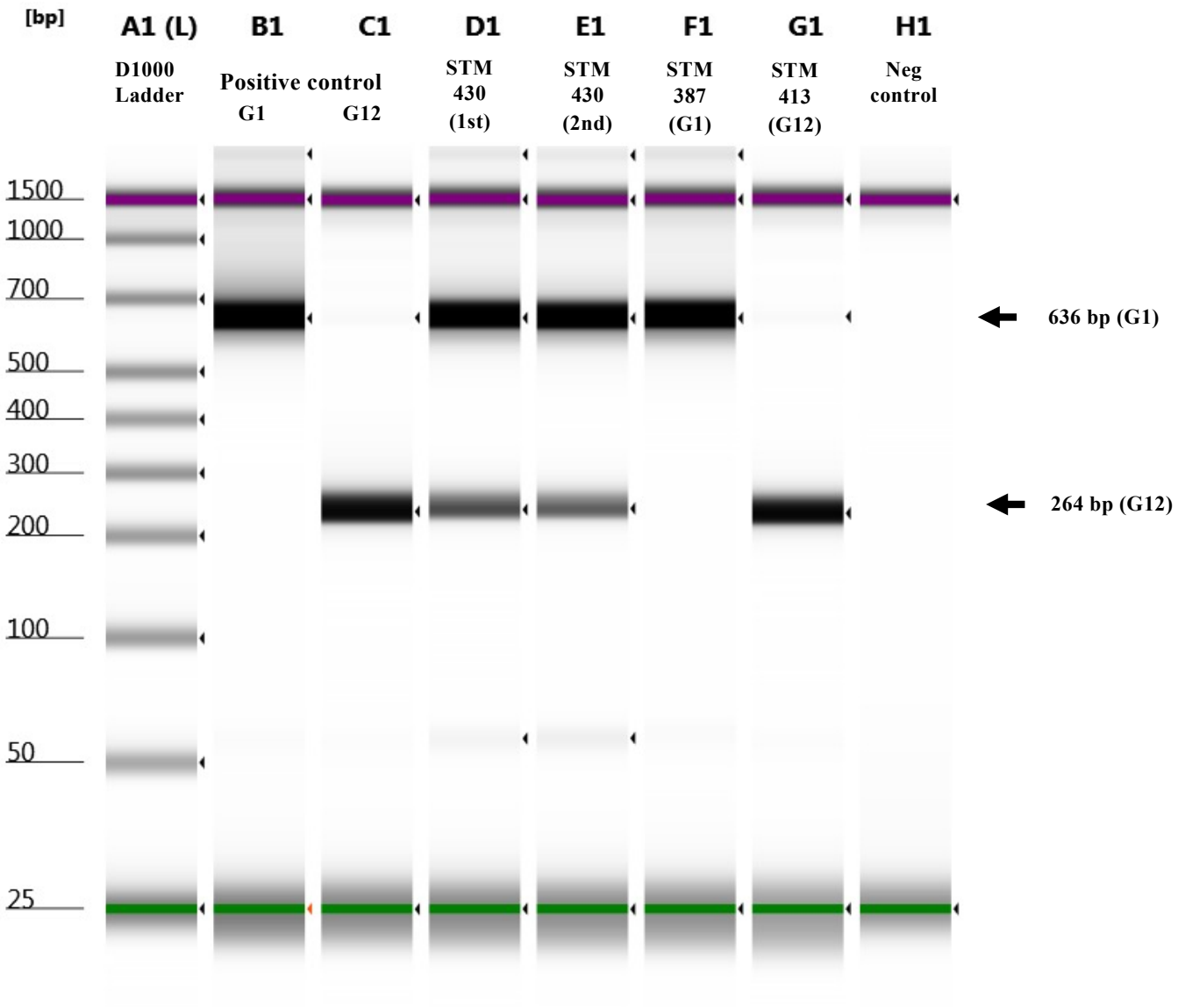
[bp]
A1 (L)
D1000 Ladder
B1
Positive control
G1
C1
G12
D1
STM 430 (1st)
E1
STM 430 (2nd)
F1
STM 387 (G1)
G1
STM 413 (G12)
H1
Neg control
1500
1000
700
500
400
300
200
100
50
25
636 bp (G1)
264 bp (G12)

Fig. 3.