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Shared clusters between phylogenetic trees for genomic segments of *Rotavirus A* with distinct genotype constellations

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ABSTRACT

Rotavirus A (RVA) possesses a genome of 11-segmented, double-stranded RNAs, each of which is classified into genotypes. A variety of genotype constellations have been generated through reassortment, but reassortment does not appear to occur randomly. Here genomic sequences for 281 RVA strains with distinct genotype constellations retrieved from the International Nucleotide Sequence Database were analyzed to identify shared clusters between phylogenetic trees for genomic segments. The largest numbers of shared clusters were observed between the genomic segments encoding viral protein 1 (VP1) and VP2 as well as VP2 and VP3, suggesting that not only VP1 but also VP3 may interact with VP2 inside the core. Larger numbers were also associated with the segment encoding non-structural protein 5/6 (NSP5/6), which may be a hub for bundling genomic segments. Although VP7 and VP4 segments were associated with smaller numbers, reassortments between these segments as well as VP7 and VP6 segments appeared to be restricted due to interactions in constructing the virion. For VP4 segment, the number with NSP1 segment was significantly greater than those with other segments, possibly reflecting that specificities in receptor binding and interferon antagonism may define host range restriction. Overall, no cluster was shared by all genomic segments, supporting that RVA constituted a single species.

1. Introduction

Rotaviruses (RVs) constitute the genus *Rotavirus* in the family *Sedoreoviridae* (Matthijnssens et al., 2022). The RV genome is divided into 11 segments of linear double-stranded RNAs (dsRNAs), with a total length of 18.0–18.5 kbp. Each genomic segment encodes structural viral protein 1 (VP1), VP2, VP3, VP4, VP6, VP7, non-structural protein 1 (NSP1), NSP2, NSP3, NSP4, or NSP5/6.

The RV virion is non-enveloped and has icosahedral symmetry with a size of ~100 nm in diameter (Desselberger, 2014). The innermost capsid composed of VP2 encloses dsRNAs associated with VP1 (RNA-directed RNA polymerase) and VP3 (capping enzyme) to form the core. The core is covered by the intermediate capsid composed of VP6 to form the double-layered particle (DLP), which is further surrounded by the outermost capsid composed of VP7 (surface glycoprotein) and VP4 (spike protein) to form the triple-layered particle (TLP). VP7 and VP4 are targets of adaptive immunity and contain neutralization epitopes.

During the RV replication, NSP1 antagonizes interferon (IFN) expression and suppresses innate immunity (Arnold, 2016). NSP2 forms viroplasms together with NSP5/6, and binds to RV single-stranded, positive-sense RNAs (+RNAs) to mediate conformational changes and facilitate interactions among +RNAs (Borodavka et al., 2017). NSP3 binds to the 3′-termini of RV +RNAs to promote circularization and enhance translation. NSP4, which exhibits enterotoxin activity, is expressed on rough endoplasmic reticulum as the receptor for DLP.

RVs are classified into species *Rotavirus A-D* and *F-J* (RVA-RVD and RVF-RVJ) as well as putative species *Rotavirus K* and *L* (RVK and RVL) based on the amino acid sequence identity of VP6 with the cut-off value of 53 % (Matthijnssens et al., 2012; Walker et al., 2022; Johne et al., 2022, 2023b). RVs are considered to exchange genetic information only within each species through reassortments and occasionally intra-segmental recombinations (Hoxie and Dennehy, 2020).

RVA is the most prevalent among RV species, and is an etiological agent of acute gastroenteritis in young mammals and birds, transmitted

Abbreviations: BTV, *Bluetongue virus*; DLP, double-layered particle; dsRNA, double-stranded RNA; Γ, gamma-distribution for rate heterogeneity among sites; GTR, general time reversible model; I, invariable sites; IFN, interferon; INSD, International Nucleotide Sequence Database; ML, maximum likelihood; NJ, neighbor-joining; NSP, non-structural protein; +RNA, positive-sense RNA; RV, rotavirus; RVA-RVD and RVF-RVL, *Rotavirus A-D* and *F-L*; S.D., standard deviation; TLP, triple-layered particle; VP, viral protein.

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through fecal-oral route (Crawford et al., 2017). Each genomic segment of RVA is classified into genotypes based on the nucleotide sequence identity with the cut-off values of 80 %, 80 %, 85 %, 83 %, 84 %, 81 %, 79 %, 85 %, 85 %, 85 %, and 91 % for VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6 segments, respectively. So far, 42 G, 58 P, 32 I, 28 R, 24C, 24 M, 39 A, 28 N, 28 T, 32 E, and 28H genotypes have been identified for VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6 segments, respectively (Matthijnssens et al., 2008; Johne et al., 2023a). RVA strains are characterized with genotype constellations designated as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where x's denote genotype numbers (Matthijnssens et al., 2011).

A variety of genotype constellations have been generated in RVA mainly through reassortment. However, reassortment does not appear to occur randomly. For example, DS-1-like background G1P[8] strains (G1-P[8]-I2-R2-C2-M2-A2-N2-T2-E2-H2) emerged through reassortment between Wa-like strains (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) and DS-1-like strains (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2) only after introduction of RVA vaccines (Yamamoto et al., 2014; Fujii et al., 2014). Reassortment may be suppressed or enhanced if novel combinations of genotypes between genomic segments are deleterious or advantageous, respectively. Thus, reassortment patterns between genomic segments during evolution of RVA may reflect functional relationships among genomic segments.

Since reassortants may contain genomic segments that have followed different evolutionary histories, occurrences of reassortments during evolution of RVA may be detected as topological differences between phylogenetic trees for genomic segments. The extent of topological differences or similarities can be measured as the number of different or shared clusters between phylogenetic trees, respectively (Hoxie and Dennehy, 2021). In particular, shared clusters may represent monophyletic reassorting groups of RVA strains, which may exchange genetic information only within the clusters (Suzuki et al., 2023, 2024). The purpose of the present study was to identify shared clusters between phylogenetic trees for genomic segments of RVAs with distinct genotype constellations for obtaining insights into functional relationships among genomic segments.

2. Materials and methods

2.1. Sequence data

A total of 120,663 entries containing the term "rotavirus" in the source organism field were retrieved from the International Nucleotide Sequence Database on December 5, 2023. Each sequence was used as the query of the TBLASTX search against the database containing prototype sequences for genotypes of 11 genomic segments in RVA (supplementary Table S1) (Camacho et al., 2009). Annotation as a particular segment of RVA was made when the *E*-value for the top hit sequence was $< 10^{-10}$.

Annotated sequences were retained when they had $\geq$ 500 nucleotide sites as well as $\geq$ 50% of the lengths of cognate genomic segments for the prototype simian strain SA11, which were 1062, 2362, 1356, 3302, 2693, 2591, 1614, 1059, 1105, 751, and 667 sites for VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6 segments, respectively (Matthijnssens et al., 2008). Sequences with identical strain and isolate names were grouped. After eliminating laboratory strains, vaccine strains, and strains from mixed infections and environmental samples, there remained 4249 strains with sequence data for 11 genomic segments. The longest sequence was selected for each genomic segment in each strain.

For determining genotype constellations of 4249 strains, pairwise alignments were made for each genomic segment of each strain with prototype sequences for genotypes (supplementary Table S1) using MAFFT (version 7.427) (Katoh et al., 2002). Genotype was assigned based on the nucleotide sequence identity of $\geq$ 80%, $\geq$ 80%, $\geq$ 85%, $\geq$ 83%, $\geq$ 84%, $\geq$ 81%, $\geq$ 79%, $\geq$ 85%, $\geq$ 85%, $\geq$ 85%, and $\geq$ 91% for VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6 segments, respectively (Matthijnssens et al., 2008). A total of 281 genotype constellations were identified. The strain with the longest sequence over 11 genomic segments was selected for each genotype constellation (supplementary Table S2).

2.2. Data analysis

Multiple alignment of 281 nucleotide sequences was made for each genomic segment using MAFFT (version 7.427) (Katoh et al., 2002). After eliminating nucleotide sites containing gaps and ambiguous nucleotides, 575, 1661, 1143, 2751, 1875, 1257, 785, 685, 840, 422, and 491 sites were identified to be shared by all sequences for VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6 segments, respectively. Phylogenetic analyses were conducted using MEGA (version 10.2.6) (Kumar et al., 2018). The best fit model of nucleotide substitutions was selected for each genomic segment based on the corrected Akaike information criterion; the general time reversible model with gamma-distributed rate heterogeneity among sites including invariable sites (GTR + Γ + I) for VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, and NSP3 segments and GTR + Γ for NSP4 and NSP5/6 segments.

Phylogenetic tree was constructed for each genomic segment using the maximum likelihood (ML) method based on the best fit model, as well as the neighbor-joining (NJ) method based on the p distance, which has been reported to produce more reliable topologies than the ML method (Nei and Kumar, 2000; Yoshida and Nei, 2016). Reliabilities of interior branches in phylogenetic trees were assessed by the bootstrap method with 1000 re-samplings. Phylogenetic trees were visualized using FigTree (version 1.4.4) (Rambaut, 2018). Shared clusters were identified as the clusters consisting of the same sets of strains between phylogenetic trees for genomic segments (Suzuki et al., 2023, 2024).

3. Results and discussion

3.1. Reassortment as a major evolutionary mechanism in RVA

Genomic sequences were available for 281 genotype constellations of RVAs in the INSD, as described above (supplementary Table S2). ML trees (supplementary Figs. S1–S11) and NJ trees (supplementary Figs. S12–S22) were constructed for 11 genomic segments of 281 RVA strains with distinct genotype constellations, and shared clusters were identified between phylogenetic trees for genomic segments (Table 1). Similar results were obtained from the analyses of ML and NJ trees. Overall, the observed numbers of shared clusters (11–49) were relatively small considering the possible numbers (0–278), suggesting that reassortment was a major evolutionary mechanism in RVA. Nevertheless, comparison of relative numbers of shared clusters may give insights into functional relationships among genomic segments.

3.2. Larger numbers of shared clusters associated with VP1, VP2, and NSP5/6 segments

Relatively large numbers of shared clusters ($\geq$ 30) were mostly associated with VP1, VP2, and NSP5/6 segments (Table 1). Notably, there was a tendency that the phylogenetic trees constructed using greater numbers of nucleotide sites were more reliable than those constructed using smaller numbers of nucleotide sites; the correlation coefficients between the numbers of nucleotide sites and the average bootstrap probabilities were 0.566 for ML trees ($P = 0.0695$ by *t*-test) and 0.642 for NJ trees ($P = 0.0333$ by *t*-test). Since the greatest numbers of nucleotide sites were available for VP1 and VP2 segments, phylogenetic trees for these segments may be less error-prone, which may result in association with larger numbers of shared clusters than other segments. Particularly large numbers ($\geq$ 45) of shared clusters were

Table 1
Numbers of shared clusters between ML trees (above the diagonal) and NJ trees (below the diagonal) for genomic segments.

Segment (Average ± S.D.)[a]	VP7 (0.667 ± 0.310)	VP4 (0.762 ± 0.251)	VP6 (0.676 ± 0.306)	VP1 (0.735 ± 0.294)	VP2 (0.670 ± 0.324)	VP3 (0.719 ± 0.296)	NSP1 (0.723 ± 0.279)	NSP2 (0.643 ± 0.295)	NSP3 (0.624 ± 0.306)	NSP4 (0.571 ± 0.308)	NSP5/6 (0.382 ± 0.346)
VP7 (0.680 ± 0.301)	-	*18*	27	26	29	*20*	*19*	*19*	*16*	*20*	*16*
VP4 (0.796 ± 0.236)	*20*[b]	-	*13*	*19*	*18*	*15*	24	*17*	*12*	*11*	*12*
VP6 (0.698 ± 0.305)	27	*13*	-	**40**	**35**	29	29	26	29	27	**30**
VP1 (0.758 ± 0.287)	26	*20*	**38**	-	**47**	**38**	**30**	**36**	**33**	**32**	**36**
VP2 (0.728 ± 0.302)	**30**[c]	*18*	**31**	**45**	-	**49**	**33**	**35**	**31**	25	**36**
VP3 (0.733 ± 0.285)	*20*	*16*	27	**38**	**48**	-	27	**30**	25	22	27
NSP1 (0.728 ± 0.278)	*19*	25	26	**30**	**33**	27	-	21	21	24	26
NSP2 (0.648 ± 0.297)	21	*16*	29	**38**	**38**	**33**	21	-	27	23	**31**
NSP3 (0.629 ± 0.308)	*17*	*12*	26	**30**	**30**	24	21	29	-	*20*	**32**
NSP4 (0.620 ± 0.302)	24	*14*	28	**35**	**33**	26	25	28	23	-	28
NSP5/6 (0.440 ± 0.345)	*17*	*13*	29	**34**	**34**	**31**	**31**	**32**	**31**	**36**	-

[a]Average ± standard deviation (S.D.) of bootstrap probabilities over 278 interior branches.

[b]Numbers of shared clusters ≤ 20 are italicized and gray-shaded.

[c]Numbers of shared clusters ≥ 30 are bold-faced and underlined.

observed between VP1 and VP2 segments and VP2 and VP3 segments (Table 1). It is possible that VP1, VP2, and VP3 segments may interact at the RNA level, especially when +RNAs are bundled into a supramolecular complex upon packaging into the innermost capsid (Borodavka et al., 2017; Oshima et al., 2022). However, it has been reported that at the protein level VP1 may be attached to VP2 at the five-fold symmetry axes inside the core (Kumar et al., 2020), and compatible and incompatible combinations of genotypes may exist between VP1 and VP2 upon dsRNA synthesis (Steger et al., 2019), which appeared to be consistent with the results obtained above. By contrast, VP3 has not been reported to be firmly anchored to either VP1 or VP2 (Kumar et al., 2020). However, the larger number of shared clusters observed between VP2 and VP3 segments compared with those between VP1 and VP2 segments and VP1 and VP3 segments suggested that VP3 may interact more strongly with VP2 than VP1.

NSP5/6 segment was also associated with larger numbers of shared clusters, even though the number of nucleotide sites available for constructing phylogenetic trees was relatively small (Table 1). It is possible that NSP5/6 may interact with other RVA proteins particularly in viroplasms (Papa et al., 2021). However, it has been reported in RVA that bundling of genomic segments may be initiated by short segments and proceed sequentially, as is the case for *Bluetongue virus* (BTV), which is another species of the family *Sedoreoviridae* with a genome of 10-segmented linear dsRNAs (Fajardo Jr. et al., 2017). In BTV, the shortest segment (S10) appears to interact with other small segments (S6-S9) in the early stage of bundling (Sung and Roy, 2021). These observations raise the possibility that NSP5/6 segment, which is the shortest among all genomic segments, may be a hub for bundling at least short genomic segments in RVA (Fajardo Jr. et al., 2017).

3.3. Smaller numbers of shared clusters associated with VP7 and VP4 segments

VP7 and VP4 segments were associated with relatively small numbers (≤ 20) of shared clusters (Table 1) (Hoxie and Dennehy, 2021). Acquisition of VP7 and VP4 segments with novel genotypes may be advantageous to other segments for escaping from adaptive immunity (Santos and Hoshino, 2005; Yinda et al., 2016). However, the number of shared clusters between VP7 and VP4 segments was not the smallest among all combinations of genomic segments (Table 1). These observations were analogous with those in BTV, where phylogenetic trees for S6 encoding VP5 and S2 encoding VP2, which were the counterparts of VP7 and VP4 in RVA, respectively, were distantly related to those for other segments but closely related to each other (Nomikou et al., 2015). There may thus be compatible and incompatible combinations between G types of VP7 and P types of VP4 in constructing the outermost capsid in RVA (Settembre et al., 2011; Valusenko-Mehrkens et al., 2023). The number of shared clusters with VP7 segment was also not small for VP6 segment (Table 1), which may reflect interactions between VP6 and VP7 in the intermediate and outermost capsids, respectively (Mathieu et al., 2001).

Interestingly, for NSP1 segment, the number of shared clusters with VP4 segment was not small, and for VP4 segment, the number of shared clusters with NSP1 segment was greater than those with other segments (Table 1). In fact, excluding VP1, VP2, and VP7 segments, which tended to have relatively large numbers of shared clusters with VP4 segment due to the reasons as described above, VP4 segment had a significantly greater number of shared clusters with NSP1 segment than the remaining segments ($P = 0.0121$ for ML trees and $P = 0.0497$ for NJ trees by Smirnoff-Grubbs test). Reportedly, specificities in both the receptor binding of VP4 (Huang et al., 2012) and the IFN antagonism of NSP1 (Lopez et al., 2016) may be the primary determinants of host range restriction in RVA (Feng et al., 2011, 2013). Thus, co-segregation of VP4 and NSP1 segments may be caused as a spurious correlation.

3.4. RVA as a single species

In RVA, strains with specific genotype constellations are largely responsible for infection of particular host species (Martella et al., 2010). However, inter-species and intra-species reassortments have

greatly contributed to evolution of RVA, as indicated above (Nakagomi and Nakagomi, 2002). Although mammalian and avian RVAs are distantly related, avian RVA appears to have acquired VP4 segment from mammalian RVA through reassortment (Trojnar et al., 2013). Indeed, the cluster consisting of avian and avian-like mammalian strains was shared by all genomic segments except for VP4 segment in both ML trees (supplementary Figs. S1–S11) and NJ trees (supplementary Figs. S12–S22). In addition, in the reverse-genetics system of SA11, mono-reassortants containing not only VP4 segment but also VP3 segment of chicken strain 02V0002G3 could be rescued (Patzina-Mehling et al., 2020). Furthermore, it turned out that no cluster was shared by all genomic segments. These observations supported that RVA was not divided into mono-phyletic reassorting groups of strains, and thus constituted a single species (Bobay and Ochman, 2018).

A limitation of the present study was that identification of shared clusters from the analysis of topologies in phylogenetic trees may be useful only for dividing RVA strains into mono-phyletic reassorting groups, each of which should consist of multiple strains. Thus, this approach may not be suitable for examining whether single divergent RVA strains such as those isolated from common gulls and common shrews have reassorted with other strains (Falkenhagen et al., 2022; Fujii et al., 2022; Johne et al., 2023a). For this purpose, it may be useful to analyze not only topologies but also branch lengths in phylogenetic trees for genomic segments of RVAs.

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CRediT authorship contribution statement

Yoshiyuki Suzuki: Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Masaya Yaeshiro:** Writing – review & editing, Validation, Investigation. **Daiki Uehara:** Writing – review & editing, Validation, Investigation. **Ren Ishihara:** Writing – review & editing, Validation, Investigation.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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