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Analysis of genetic factors related to preferential selection of the NSP1 gene segment observed in mixed infection and multiple passage of rotaviruses

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Summary. Reassortment is one of the major evolutionary mechanisms of the rotavirus genome. Preferential selection (assortment) of the NSP1 gene segment from either of the parental viruses after coinfection of these viruses has been reported as a notable finding in reassortment. To analyze genetic factors which are associated with preferential selection of the rotavirus NSP1 gene segment into progeny viruses, mixed infection and multiple passages were performed using two panels of rotaviruses, i.e., bovine rotavirus A5 clones, and simian rotavirus SA11 and five strains of SA11-based single NSP1 gene-substitution reassortants. In the first experiment, three A5 clones (A5-10, A5-13, and A5-16) that had genetically distinct NSP1 genes in the same genetic background were used. In coinfection of these A5 clones, it was noted that the A5-10 NSP1 gene, which encodes an incomplete protein product due to presence of a nonsense codon at an unusual position, was selected more preferentially than the A5-13 NSP1 gene with intact length and structure. The A5-16 NSP1 gene, with a deletion of 500 bp, was least efficiently selected. In the second experiment, we prepared two reassortants, SOF and SRF, which have NSP1 genes from rotavirus strains OSU and RRV, respectively, in the genetic background of SA11, which were used together with previously prepared reassortants SKF, SDF, and SNF, which had NSP1 genes from strains KU, DS1, and K9, respectively. Among the 6 NSP1 genes analyzed, the NSP1 gene from SKF was most preferentially selected, followed by SNF, SOF, SDF, SA11, and SRF, in that order. Although SOF exhibited less growth

efficacy than SA11, the growth rates of other reassortants were similar to that of SA11. These findings suggest that for the occurrence of preferential selection of the NSP1 gene, production of the intact NSP1 protein may not be involved, but the presence of intact length of the NSP1 gene may be required. Furthermore, it was also found that genetic similarity based on primary structure of this gene is not related to the selectivity of the NSP1 gene.

Introduction

Rotavirus (genus *Rotavirus*, family *Reoviridae*) is recognized as a major pathogen of acute infantile gastroenteritis in humans and is also widely distributed in mammals and birds [9]. The rotavirus genome is double-stranded (ds) RNA separated into 11 segments, each of which encodes one of 6 structural proteins (VP1–4, VP6, VP7) or 5 nonstructural proteins (NSP1–5). Due to the segmented nature of the genome, reassortment is one of the mechanisms of genomic evolution of rotaviruses [25]. In the rotavirus replication cycle, association of the replicated viral mRNA with nonstructural proteins NSP1 and NSP3 has been considered to be an initial process by which RNA segments are assorted into virions, although the precise mechanism is still unknown [21]. By coinfection experiments in vitro and in vivo, it has been observed that RNA segments from one of the parental rotavirus strains is sometimes preferentially selected in the progeny reassortant viruses [5, 11, 12]. We previously reported that in the genetic background of simian rotavirus SA11, the homologous VP7 gene (SA11-VP7 gene), which encodes major outer capsid protein, is preferentially selected over heterologous VP7 genes [13–15, 18]. This finding indicated that a VP7 gene might have best fitted the genetic background from which it was derived. However, we found also that the homologous NSP1 gene was not selected preferentially in the SA11 background, unlike the VP7 gene segment, through studies using SA11 and single NSP1 gene-substitution reassortants [19].

A nonstructural protein, NSP1 (486–495 amino acids), which is encoded by gene segment 5, has binding activity with mRNA at the 5′-end region [2, 8]. Although the NSP1 gene shows extremely low homology among strains, a cysteine-rich motif (putative zinc finger motif) at amino acid 42–72 in the N-terminal region is highly conserved in most strains and has been implicated in binding to viral mRNA [7, 8, 16, 17]. Moreover, a mutated NSP1 gene lacking the coding region for the cysteine-rich motif was detected in nondefective rotavirus clones [23, 24] and could be reassorted with RNA segments from heterologous rotavirus despite low frequency [20]. These findings indicated that this motif is not necessarily required for assortment of rotavirus RNA segments. Thus, the significance of the function of NSP1 in genome segment assortment is ambiguous, and the cause of the preferential selection of NSP1 gene has not been elucidated.

In the present study, to analyze the factor associated with preferential NSP1 gene selection, we employed two experimental systems for coinfection and

multiple passages of rotaviruses that have genetically distinct NSP1 genes in identical genetic backgrounds.

Materials and Methods

Viruses and cells

In experiment 1, three clones of bovine rotavirus A5, i.e., A5-10, A5-13, and A5-16 were used [23]. These viruses have a genetically distinct NSP1 genes in the identical genetic background. While A5-13 has an intact 1579-bp-long NSP1 gene encoding a complete protein product (491 amino acids), the A5-10 NSP1 gene (1587 bp) has a nonsense codon at nucleotides 153 to 155, and the A5-16 NSP1 gene (1087 bp) lacks an internal 500-bp sequence. Both NSP1 genes of A5-10 and A5-16 are estimated to encode incomplete products comprising 40 amino acids and 50 amino acids, respectively, and lack the coding region of a cysteine-rich sequence (nucleotides 156 to 230) found in most intact NSP1 genes.

In experiment 2, a simian rotavirus SA11 (SA11-L2 clone) [22] and 5 single NSP1 gene-substitution reassortants were employed. The reassortants, designated SKF, SDF, SNF, SOF, and SRF, had an NSP1 gene from rotavirus strain KU (human, G1), DS1 (human, G2), K9 (canine, G3), OSU (porcine, G5), and RRV (simian, G3), respectively, in the genetic background of SA11. Three reassortants SKF, SDF, and SNF, had been prepared in the previous study [19]. Two reassortants, SOF and SRF, were isolated in the present study by repeated plaque purification from culture fluid from coinfection with SA11 and OSU, and SA11 and RRV, respectively. The NSP1 gene segments from these reassortants and SA11 exhibited different migration speeds in polyacrylamide gel electrophoresis (PAGE) (Fig. 1). NSP1 genes in the five reassortants and SA11 strain show nucleotide and amino acid sequence identities of 56–84%, and 32–83%, respectively (data not shown). Among NSP1 genes analyzed in our present study, NSP1 genes of human rotaviruses KU (SKF) and DS1 (SDF), and porcine rotavirus OSU (SOF) are genetically similar to each other, but distinct from those of RRV (SRF), SA11, and K9 (SNF), as shown in Fig. 2.

MA-104 cells were used to propagate rotaviruses, and CV-1 cells were used for plaque formation by rotaviruses.

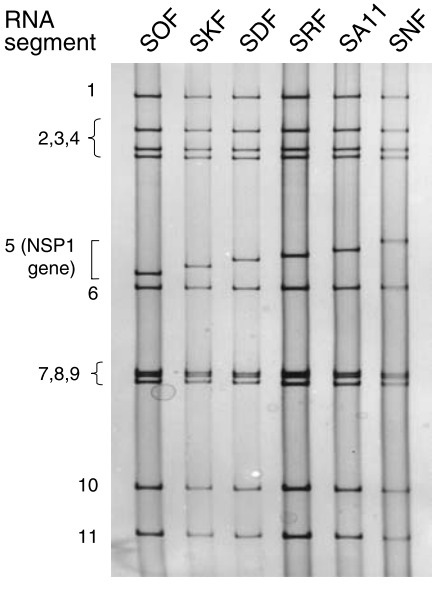

Fig. 1. RNA patterns in PAGE of single-NSP1 gene reassortants (SOF, SKF, SDF, SRF, and SNF) and SA11, which were used in experiment 2. RNA segments 1–11 are indicated on the left

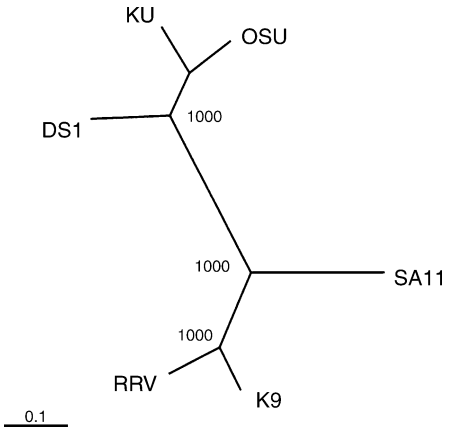

Fig. 2. Phylogenetic tree of NSP1 genes derived from 6 rotavirus strains, analyzed in this study, constructed by the neighbor joining method using the Clustal W program and visualized by Treeview software. The scale bar represents a genetic distance that is equivalent to 0.1 substitutions per site. Bootstrap probability of 1000 trials are indicated at diverging points of branches

Mixed infection analysis

Two rotavirus clones among A5-10, A5-13, and A5-16, or two strains among SA11 and the 5 reassortants were simultaneously inoculated onto an MA104 cell monolayer in a six-well plate at a multiplicity of infection (m.o.i.) of five plaque forming units (p.f.u.)/cell for each virus, as described previously [13, 19]. In combination of mixed infection of A5-10 and A5-13, different titers of these viruses were also employed. Viruses pretreated with acetylated trypsin (20 μg/ml) at 37 °C for 1 h were inoculated onto MA104 cells washed with Eagle's minimum essential medium (E-MEM). After 1 h adsorption, 1 ml of maintenance medium, E-MEM containing acetylated trypsin (2 μg/ml), was added, and the cells were incubated until a significant cytopathic effect appeared (1 or 2 days postinfection). The harvested culture fluid, after freezing and thawing three times, was treated with acetylated trypsin and inoculated onto an MA104 cell monolayer at an approximate m.o.i. of 5 p.f.u./cell. Similarly, ten sequential passages of viruses were performed.

Characterization of virus clones

From mixed culture at passage numbers 2, 4, 6, 8, or 10, rotavirus dsRNA was extracted from 300 μl of coinfection culture fluid, and its RNA pattern was analyzed by PAGE as described previously [10]. From the 10^{th} passage of coinfection culture fluid of SA11 and reassortants, virus clones forming plaques on CV-1 cells were randomly picked and propagated in MA104 cells. The derivation of the NSP1 gene in each virus clone was determined by observation of RNA patterns in PAGE.

Single-step growth curve

A single-step growth curve was examined for SOF and SRF, as described previously [3, 22]. MA104 cells in a 6-well plate were infected with each virus at high m.o.i.(5 p.f.u./cell), and incubated at 37 °C. Culture fluid taken at several incubation times (0–36 h) after infection was employed for plaque count.

Results

Experiment 1

In the mixed infection of A5-13 and A5-16, the NSP1 gene segment from A5-13 was detected clearly in PAGE at all passage numbers, while the A5-16 NSP1 gene segment was faint even at the 2nd passage and undetectable at the 10th passage (data not shown). Similarly, in the coinfection of A5-10 and A5-16, the A5-10 NSP1 gene was selected more efficiently than that of A5-16 (data not shown). When the same titers of A5-10 and A5-13 were coinfected initially, the A5-10 NSP1 gene segment became predominant after the 5th passage in PAGE, although the NSP1 gene segments from these two clones were equally observed at the 2nd passage (Fig. 3). Even when a five-times-higher titer of A5-13 was employed for in initial coinfection with A5-10, the A5-10 NSP1 gene segment became predominant

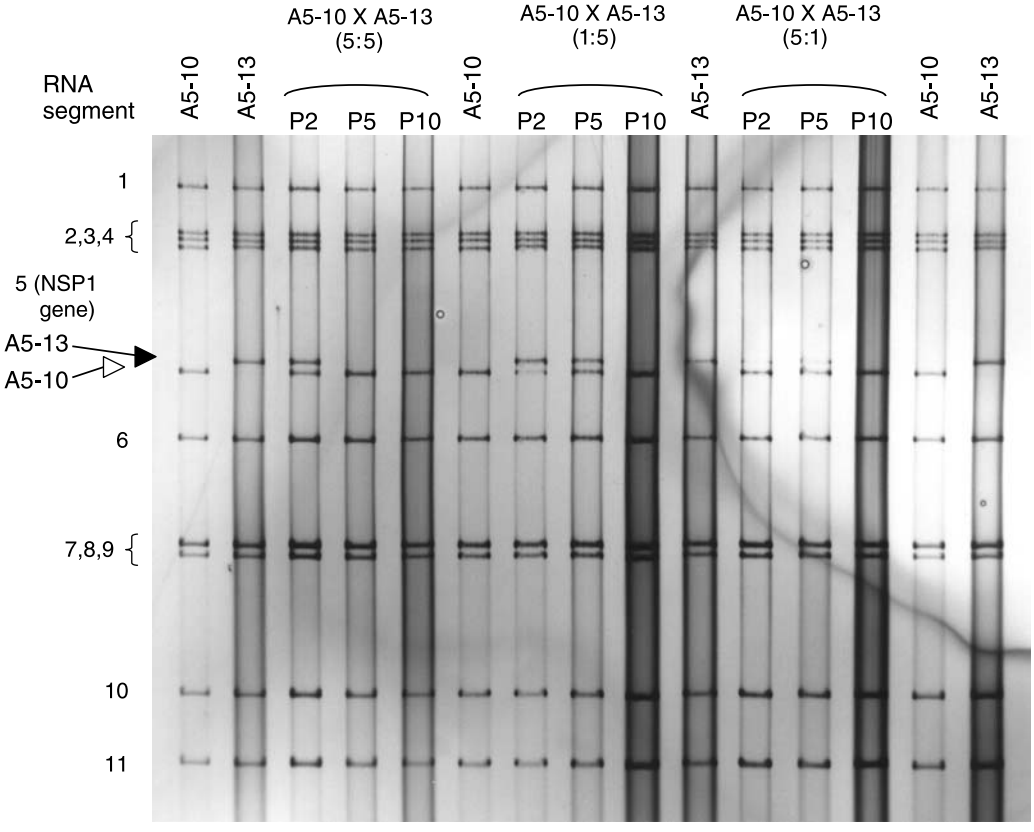

Fig. 3. Migration patterns in PAGE of rotavirus dsRNA extracted from culture fluid of mixed infection of A5-10 and A5-13, performed by three series of coinfection culture with different titers of each viruses at the initial infection; same titers (m.o.i. of 5 PFU/cell) (5:5), five-times-higher titer of A5-13 (m.o.i. of 5 PFU/cell) than that of A5-10 (m.o.i. of 1 PFU/cell) (1:5), five-times-higher titer of A5-10 (m.o.i. of 5 PFU/cell) than that of A5-13 (m.o.i. of 1 PFU/cell) (5:1). Migrating positions of NSP1 gene segments from A5-10 or A5-13 are indicated by arrows

after the 5th passage. The A5-10 NSP1 gene segment totally surpassed its A5-13 counterpart in mixed infection of these viruses using a higher titer of A5-10.

Experiment 2

Preferential selection of NSP1 genes among strains SA11, SKF, SDF, and SNF have been characterized previously [19]. In the present study, selection of the NSP1 gene was analyzed in coinfection of viruses including either SOF or SRF. As shown in Fig. 4, at the 10th passage of mixed infection of SRF with SA11, SKF, or SOF, the SRF (RRV) NSP1 gene segment was not visible, overwhelmed by the NSP1 genes from its counterparts. Similarly, the NSP1 gene segments from SDF and SNF surpassed the SRF NSP1 gene (data not shown). In coinfections SOF × SA11 and SOF × SKF, two NSP1 gene segments from both viruses were visible in PAGE in the 10th passage culture fluid, although the band of the SOF (OSU) NSP1 gene segment was stronger than that of SA11, and the SKF (KU) NSP1 gene segment was slightly stronger than that of SOF(OSU) (Fig. 4). From 9 different coinfections, more than 30 virus clones were isolated from 10th passage culture fluid and the derivation of their NSP1 gene was analyzed (Table 1). SA11,

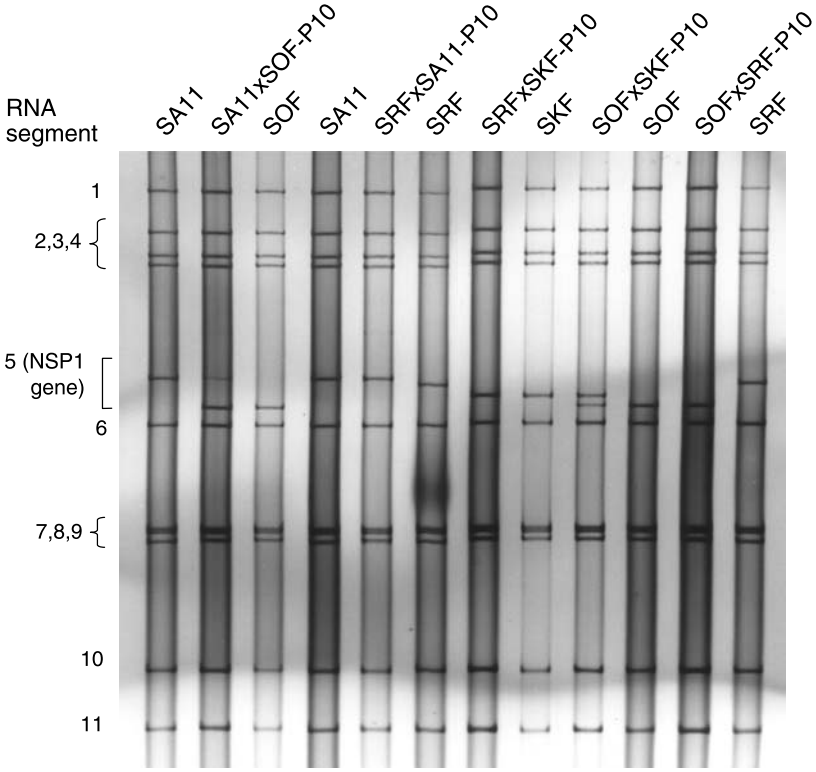

Fig. 4. Migration patterns in PAGE of rotavirus dsRNA from mixed infection culture (10th passage) of SA11 and SOF, SA11 and SRF, SRF and SKF, SOF and SKF, and SOF and SRF. RNA segments 1–11 are indicated on the left

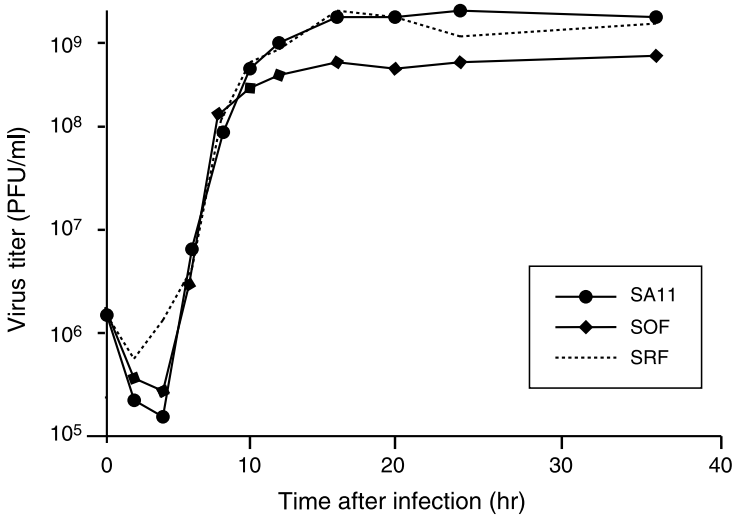

Fig. 5. Single-step growth curves of SA11, SOF, and SRF. Virus titers were determined by plaque assay using CV-1 cells

Table 1. Frequency of rotavirus clones selected from the 10^{th} passage of mixed infection among single-NSP1 gene-reassortants and SA11

Mixed infection	Number of clones examined	Number of single-NSP1 gene-reassortants or SA11 (%)
SA11 × SRF	36	SA11:36(100) SRF:0(0)
SA11 × SOF	50	SA11:26(52) SOF:24(48)
SOF × SDF	36	SOF:36(100) SDF:0(0)
SOF × SKF	44	SKF:36(81.8) SOF:8(18.2)
SOF × SRF	36	SOF:36(100) SRF:0(0)
SRF × SDF	36	SDF:36(100) SRF:0(0)
SRF × SKF	36	SKF:35(97.2) SRF:1(2.78)
SRF × SNF	36	SNF:36(100) SRF:0(0)
SOF × SNF	50	SNF:36(72) SOF:14(28)

SOF, SDF, SKF, and SNF accounted for the majority of isolates from coinfection culture of these viruses with SRF. The SOF strain was more frequently detected in coinfection of SOF and SDF, but less frequent in isolates from coinfection with

SNF or SKF. From each of the mixed infections SA11 × SOF and SA11 × SRF, a virus clone having a rearranged gene segment was detected (data not shown) and was not included for calculation of the selection frequency of the NSP1 gene.

Figure 5 shows single-step growth curves of SA11, SOF, and SRF. SRF exhibited an almost identical growth curve to SA11. However, growth efficacy of SOF was lower than SA11 after 10 h postinfection, and the maximum titer of SA11 was approximately 3 times higher than that of SOF.

Discussion

Rotavirus NSP1, a nonstructural protein of 55 kDa, is expressed at lowest levels in rotavirus-infected cells and accumulates in the cytoplasm, possibly associated with the cytoskeleton [7]. Besides its function as an RNA-binding protein, NSP1 has been recognized as one of the virulence factors by the recent remarkable finding that this protein induces degradation of interferon regulatory factor 3 (IRF-3) in infected cells, causing subversion of innate immunity [1]. NSP1 has affinity specifically for the rotavirus mRNA and binds to the region near the 5′-end of the mRNA (within the first 34 nucleotides) [21]. Although NSP1 is variable in length and its primary sequence is poorly conserved, the cysteine-rich sequence, which may form zinc finger in its amino terminus is essential for RNA-binding activity [4, 6, 16]. However, the RNA-binding activity of NSP1 is not essential for replication of rotavirus, although NSP1 plays a beneficial role in the replication of rotavirus based on phenotypic observations (i.e., plaque size, viral growth rate) from NSP1 variants or deletion mutants [20, 23].

In our present study, it was notable that the A5-10 NSP1 gene was more preferentially selected than the A5-13 NSP1 gene in every case of initial coinfection with different relative titers of the two virus clones. As reported previously, viral growth efficacy of A5-10 was lower than that of A5-13 [20]. Therefore, the predominance of the A5-10 NSP1 gene is considered to be due to efficient assortment of this gene segment. It was also documented that intact NSP1 is not expressed in A5-10, in contrast to A5-13, which produces NSP1 of normal size and contains the cysteine-rich region [23]. These findings indicate that preferential selection of the NSP1 gene in this coinfection system is not defined by the production of intact NSP1 protein, which is consistent with our previous observation on preferential selection of NSP1 gene and difference in expression level of NSP1 in the genetic background of SA11 [19]. Regarding the less efficient selection of A5-16 NSP1 gene during coinfection with A5-10 or A5-13, it is not determinable whether it was due to the lower growth rate or lower efficiency in assortment into the virion.

In our previous study using coinfection among SA11, SKF, SDF, and SNF, the SKF (KU)-NSP1 gene was the most preferentially selected, followed by the NSP1 genes of SNF (K9), SDF (DS1), and SA11, in that order, although SA11 and three reassortants exhibited almost same growth efficacy [19]. To extend this study, our present study was designed employing two more reassortants, SOF and SRF. In the present study, almost the same numbers of SA11 and SOF clones were detected at the 10^{th} passage of mixed infection culture of

SA11 and SOF, although the SOF (OSU) NSP1 gene segment was more strongly observed than that of SA11 in PAGE. The density of the band representing the RNA segment in PAGE is considered to generally reflect the relative amount of rotavirus gene segment because the dsRNA sample is extracted from virus culture fluid containing a number of viruses. In contrast, in analysis of individual clones, unexpected deviation due to clone isolation may sometimes occur by manual selection of plaques. Thus, the SOF (OSU)-NSP1 gene segment was considered to be more preferentially selected than the SA11 NSP1 gene segment. Taken together with our previous findings, the most preferentially selected NSP1 gene was that from SKF (KU), followed by those from SNF (K9), SOF (OSU), SDF (DS1), SA11, and SRF (RRV), in that order. Except for slightly lower growth efficacy found in SOF, growth of other reassortants was mostly the same as that of SA11.

While NSP1 is genetically highly divergent, and a genetic typing system has not yet been established, species-specific and interspecies relatedness have been found among rotavirus strains from different animal species [4, 16]. Among the six NSP1 gene sequences analyzed in the present study, NSP1 gene sequences between KU and OSU, and RRV and K9 were genetically close, although nucleotide differences among these strains are located throughout the gene sequences (data not shown). However, preferential selection of the NSP1 gene does not seem to be related to the overall sequence identity, for example, the SRF (RRV)-NSP1 gene was the least efficiently segregated among NSP1 genes of the 6 strains, but the SNF (K9)-NSP1 gene segment surpassed those from 3 other reassortants and SA11. Despite the overall sequence diversity among strains, the 5′-end and 3′-end regions of the NSP1 gene, which are probably involved in assortment of RNA segments, were highly conserved, as in the case of other gene segments. Therefore, it seems that the specific genetic group defined by the overall primary structure of the NSP1 gene sequence may not be involved in its preferential selection. Considering the results from the A5 clones, it is possible that certain genetic structure (e.g., secondary structure) formed in the full-length of NSP1 gene may be critical for determining its functional adaptability to a given genetic background and accordingly, related to preferential selection.

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