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Direct evidence for genome segment reassortment between concurrently-circulating human rotavirus strains

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Summary. Extensive heterogeneity in electropherotypes observed among group A human rotaviruses has been considered as a result of two major mechanisms; i.e., the accumulation of point mutations and genetic reassortment between concurrently-circulating strains. However, no evidence was reported thus far indicating that any one of field isolates of rotavirus was formed by direct reassortment of concurrently circulating two parental strains. Comparison of the genome of human rotavirus specimens collected over a six year period by electropherotyping and by the sequencing of selected gene segments identified two reassortants that were generated in nature between strains circulating co-dominantly in the same epidemic season. This is the first report directly showing that at least some part of electrophoretic diversity observed among rotavirus strains was explained by genetic reassortment between strains concurrently circulating in the human population. This supports the hypothesis that genetic reassortment among co-circulating strains operates as a key mechanism for the genetic variability of rotaviruses in nature.

Introduction

Group A human rotaviruses or *Rotavirus A*, members of genus *Rotavirus* within family *Reoviridae*, have been established as the single most important etiological agent of acute diarrhea worldwide [33]. The virion contains 11 segments of double-stranded RNA, which produce characteristic migration patterns, termed electropherotypes, upon polyacrylamide gel electrophoresis [6]. Since electropherotyping provides considerably more information about rotavirus strains than does any other diagnostic test [13] and an electropherotype is legitimately specific for identifying a single strain [3], this methodology has widely been used for epidemiological studies of human rotaviruses [6]. One of the key observations from such studies is that great diversity exists in electropherotypes observed among group A human rotaviruses obtained from children with acute diarrhea even in

geographically defined locations [11, 29, 30, 35, 36]. This reflects co-circulation of strains belonging to multiple lineages which resulted from the accumulation of point mutations and from genetic reassortment between concurrently-circulating strains. The fact that reassortants are easily generated upon co-infection in cell cultures [9, 22, 41, 42], that mixed infection of an individual with more than one rotavirus strain is a commonly observed phenomenon [24, 29, 30, 37], and that RNA-RNA hybridization provides evidence for reassortment between rotavirus strains belonging to different genogroups [14, 16, 21, 23, 25–27, 32, 43] are all indicative of the occurrence of frequent genetic reassortment among concurrently-circulating rotavirus strains. However, no direct evidence was thus far reported that shows a given strain being derived from reassortment of two parental strains.

In this study, we made an extensive comparison of the electropherotypes of human rotavirus specimens collected from Japanese children with acute diarrhea over a six year period, and selected candidates for the combination of two parent strains and their reassortant progeny. We sequenced some key gene segments from such candidate strains, and provide direct evidence that reassortants were generated between concurrently-circulating human rotavirus strains.

Materials and methods

Rotavirus-positive stool specimens used in this study were obtained from children hospitalized for acute diarrhea in a referral hospital in Honjo, Akita during the period between September 1991 and December 1996, and this stool panel is a part of the continuing collection of our rotavirus stool specimens published previously [12, 15, 18, 28, 29]. Rotavirus genomic RNAs were extracted by mixing approximately 10% stool suspension in 200 μl of phosphate-buffered saline, pH 7.2, and an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The RNAs were further extracted with chloroform, precipitated in ethanol, and suspended in 10 μl of deionized water. For those stool specimens that contain less amount of rotavirus particles, concentration by ultracentrifugation in a Beckman TLA100.4 rotor at 60,000 rpm for 1 h was carried out before RNA extraction. Because the representative of rotavirus specimens that had electropherotype LH37 was used many times for comparison, rotavirus was isolated in cell-culture from the stool specimen 94H121 essentially by the method of Kutsuzawa et al. [19].

The electrophoretic separation of genomic RNAs was performed on a 10% polyacrylamide gel (0.75 mm in thickness) with a 4% stacking gel in the buffer system of Laemmli [20] using an SE600 gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). After electrophoresis for 16 h at a constant current of 8 mA, the gels were stained with ethidium bromide and visualized under UV illumination.

In the final stage of identifying reassortant candidates, electrophoresis was performed under three different conditions; i.e., RNAs were run for 8.5 h at 10 mA on 7.5% gels, for 16 h at 8 mA on 10% gels, and for 18 h at 10 mA on 12.5% gels. After electrophoresis, the gels were fixed first in 10% trichloroacetate for 10 min, then in 50% methanol for 10 min, and stained with silver nitrate using the 2D-Silver stain II "Daiichi" kit (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) according to the manufacturer's instruction. The silver-stained gels that had been washed with deionized water were dried under vacuum at 60 °C for 1.5 h.

To confirm the relationships between reassortant candidates and their presumed parents, partial gene segment 3 (the VP3 gene), segment 6 (the VP6 gene) and the entire gene segment 11 (the NSP5 gene) were sequenced. The VP3 genes corresponding to nt 404–928 were amplified with a pair of primers, VP3F404 (5′ CTACCTGGATGGAAATTAAC 3′) and

VP3R930 (5′ TATCCAATAAGATGGAGC 3′). The VP6 genes corresponding to nt 14–592 were amplified with a pair of primers VP6F14 (5′ AGTCTTCGACATGGAGGTTC 3′) and VP6R592 (5′ ACTTGAATTTCTGATCCAGC 3′). The full-length NSP5 genes were amplified with a pair of primers, Beg11 (5′ GGCTTTTAAAGCGCTACAGTGATG 3′) and End11 (5′ GGTCACAAAACGGGAGTGGG 3′). Rotavirus RNAs were heated at 95 °C for 5 min in the presence of 42.9% dimethyl sulfoxide, and then chilled on ice for 5 min. The reverse transcription (RT) reaction was carried out for 30 min at 42 °C in a 20 μl reaction mixture containing 1×Ampdirect (Shimadzu Co., Ltd., Kyoto, Japan), 0.8 μM of primers, 0.5 mM of dNTPs, 5 units of RAV-2 reverse transcriptase (Takara Shuzo Co., Ltd., Kusatsu, Shiga, Japan) and denatured rotavirus RNA. Prior to PCR, this RT reaction mixture was mixed with 30 μl of a solution containing 1×Ampdirect, 1×Ampaddition (Shimadzu), 0.67 μM of primers, 0.17 mM of dNTPs and 0.25 units of rTaq polymerase (Takara Shuzo). The PCR was carried out under the condition of 25 cycles of 90 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, followed by a final 8 min at 72 °C. The amplified PCR products were cloned into pCR 2.1 vector by using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). Three clones for each PCR product were sequenced by using the ABI PRISM Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Forster City, CA, USA) according to the manufacturer's instruction. M13 forward (−21) and M13 Reverse primers were used for sequencing. The sequencing data were collected by an ABI PRISM 310 genetic analyzer (Perkin-Elmer) and analyzed with the DNASIS software package (Hitachi software engineering Co., Ltd., Yokohama, Kanagawa, Japan).

Results

A total of 409 rotavirus stool specimens identified by positive latex agglutination assays were subjected to RNA polyacrylamide gel electrophoresis. Of these, 313 specimens (76.5%) were typed into 37 electropherotypes (data not shown). However, six specimens (1.5%) were untypeable due to mixed infection of more than one strain, 53 were untypeable because stool materials had been consumed during repeated electrophoresis before assignment of appropriate electropherotypes, and rotavirus RNAs were not visualized in 37 specimens even after concentration by ultracentrifugation.

Assuming that a reassortant was generated between two parental strains circulating concurrently in a given rotavirus season, we made an extensive comparison of electropherotypes to see whether any combination of two electropherotypes from concurrently-circulating strains would produce the electropherotype from another strain in the same epidemic season or in the following epidemic season. After side-by-side comparison of electropherotypes on 10% gels, seven sets of electropherotypes, each comprising one reassortant electropherotype and two parental electropherotypes, were left for further scrutiny (data not shown).

Since an electropherotype is not an absolute character inherent to a given strain but can be changed under different electrophoresis conditions [13], co-electrophoresis was carried out under three different conditions including acrylamide concentration, running times and electric current (for 8.5 h at 10 mA on 7.5% gel, for 16 h at 8 mA on 10% gel, and for 18 h at 10 mA on 12.5% gel) before establishing the relationships between putative parents and progeny reassortants. Furthermore, at this stage, silver staining was employed in place of ethidium bromide staining in order to achieve a higher resolution so as to be

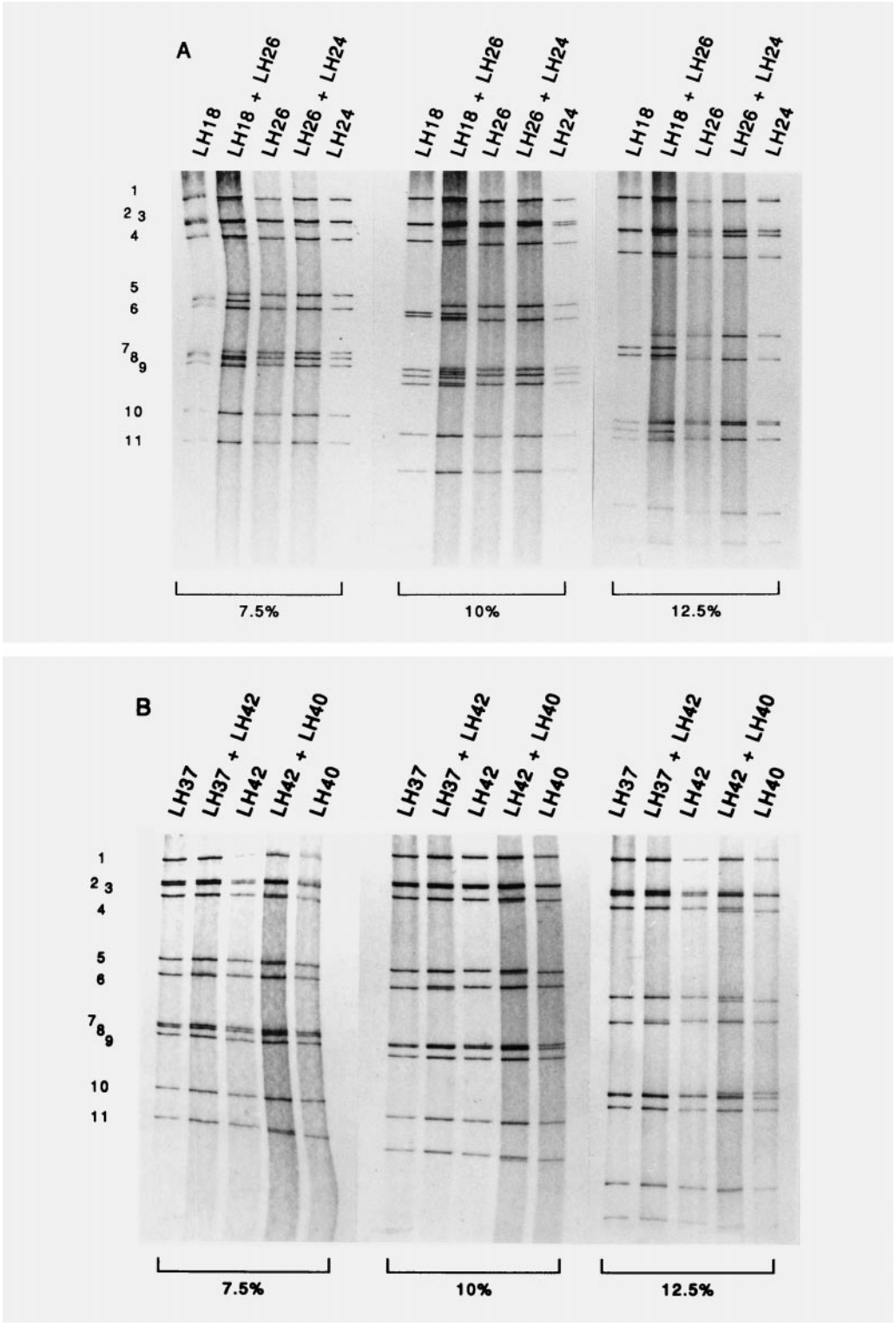

Fig. 1 (*continued*)

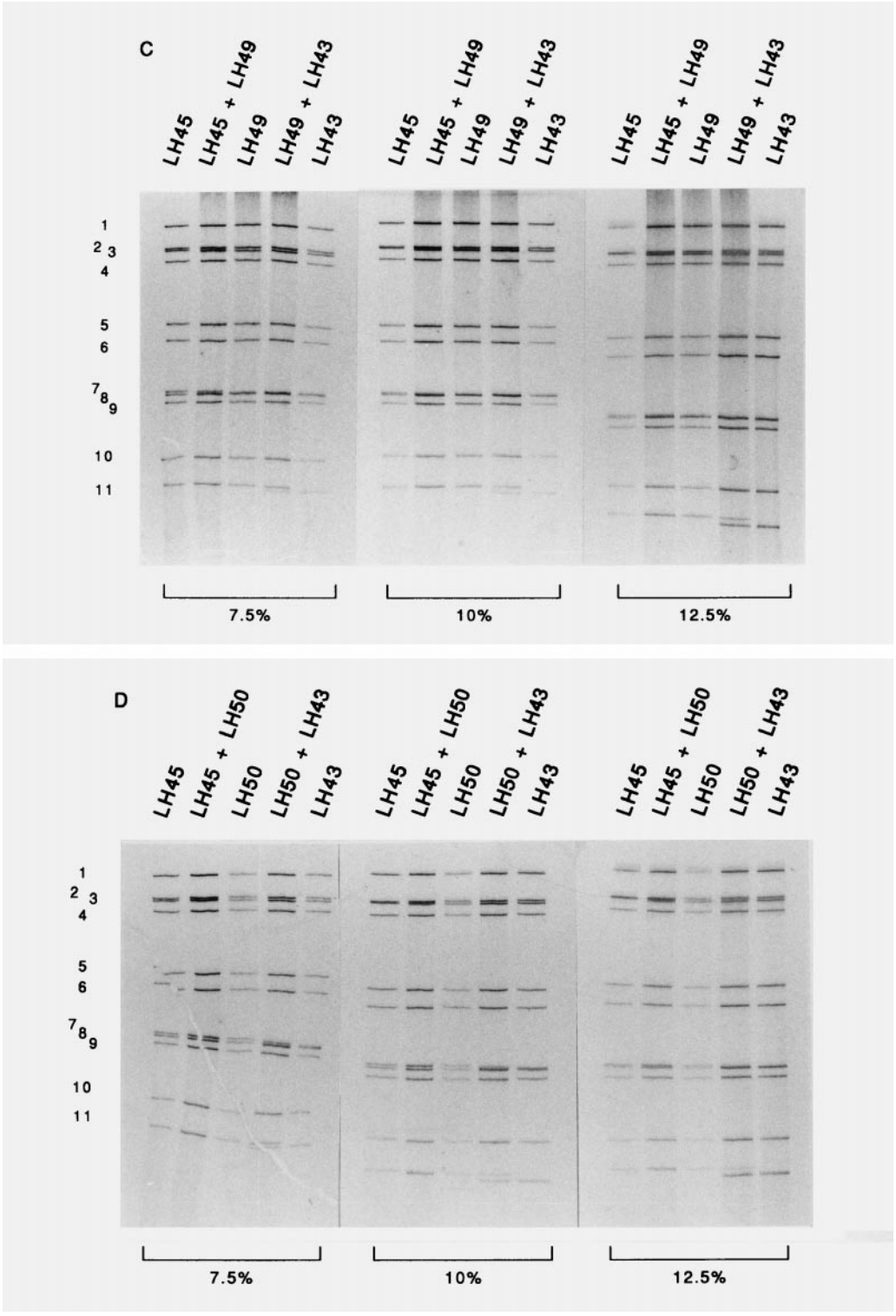

Fig. 1 A–D. Co-electrophoresis of genomic RNAs extracted from four sets of the combination of one reassortant and its two parental candidates. Electropherotypes are indicated on the top of each lane of the gels, and the acrylamide concentrations of the gels are indicated at the bottom of each panel. See text and Table 1 for interpretation

Table 1. Parental origin for each of 11 segments of reassortant candidates by co-electrophoresis

Season	Parental electropherotype	Reassortant	Parental origin of segment										
			1	2	3	4	5	6	7	8	9	10	11
91–92	LH24 × LH18 (co-D) (m)	LH26	D	D/m	D	D	D	D	D	D	D	D	m
94–95	LH37 × LH40 (D) (m)	LH42	D/m	D	D/m	D	D	m	D	D	D/m	D	D
95–96	LH43 × LH45 (co-D) (co-D)	LH49	A/B	A/B	A	A/B	A/B	A/B	A	A/B	A/B	A/B	B
	LH43 × LH45 (co-D) (co-D)	LH50	A/B	A/B	A	A/B	A/B	A/B	B	A/B	A/B	A/B	B

D Segment of LH24 or LH37 (co-dominant or dominant electropherotype)
m Segment of LH18 or LH40 (minor electropherotype)
A Segment of LH43
B Segment of LH45
D/m, *A/B* Segment consists with both parental electropherotypes

able to discriminate closely-migrating RNA bands. After this step, two candidates were excluded because they failed to establish the relationships at least on any one of the gels with different acrylamide concentrations. Another candidate was excluded as indeterminate because of ambiguity in the identification of closely migrating segments 2 and 3, and segments 7, 8 and 9 (data not shown).

Four sets of electropherotypes that resulted from this test are shown in Fig. 1, and the putative parental origin of each of the 11 genome segments for each reassortant candidates is presented in Table 1. Rotavirus strain possessing electropherotype LH26 (represented by RNAs extracted from stool specimen 92H083) that was found in the 91–92 season was presumed to be naturally-occurring reassortant between the strain possessing electropherotype LH18 (represented by RNAs extracted from stool specimen 91H249) and another strain possessing electropherotype LH24 (represented by RNAs extracted from stool specimen 92H069) that were also found in the same 91–92 season (Fig. 1A, Table 1). On all of the gels using the three different acrylamide concentrations, two parental candidates possessing electropherotypes LH24 and LH18 had one gene segment (segment 2) in common. On a 7.5% gel, segments 6 and 10 of LH26 co-migrated indistinguishably with those of LH18 as well as those of LH24, but these segments of LH26 co-migrated only with those of LH24 on other gels. Similarly, on a 10% gel, segment 10 of LH26 co-migrated with that of LH18 as well as that of LH24, but this segment of LH26 co-migrated only with that of LH24 on other gels. On the other hand, segments 11 appeared to derive from LH18. Thus, we posited that LH26 was a reassortant between LH24 and LH18 that shared gene segment 2 and that LH26 derived nine gene segments from LH24 and its gene segment 11 from LH18.

Similarly, rotavirus strain possessing electropherotype LH42 (represented by RNAs extracted from cell-culture adapted viruses from stool specimen 95H125)

that was found in the 94–95 season was likely to be naturally-occurring reassortant between rotavirus strains possessing electropherotype LH37 (represented by RNAs extracted from the cell-culture adapted virus from stool specimen 94H121) and another strain possessing electropherotype LH40 (represented by RNAs extracted from stool specimen 95H097) that were also found in the same season (Fig. 1B, Table 1). On gels with different acrylamide concentrations, LH37 and LH40 were shown to have segments 1, 3, and 9 in common. In addition, they appeared to have segments 7 and 10 in common on a 10% gel. Co-electrophoresis under different conditions revealed, however, that the gene segments 7 and 10 of only LH37 seemed to be transmitted to progeny LH42. As to the parental origin of the remaining six gene segments, it was postulated that LH42 derived five gene segments from LH37 and segment 6 from LH40.

In the 95–96 season, it appeared that two reassortants possessing electropherotypes LH49 (represented by RNAs extracted from stool specimen 96H063) and LH50 (represented by RNAs extracted from stool specimen 96H070) were generated between the strain possessing electropherotype LH43 (represented by RNAs extracted from stool specimen 96H026) and another strain possessing electropherotype LH45 (represented by RNAs extracted from stool specimen 96H001) both of which were prevalent during the 95–96 season (Fig. 1C–D, Table 1). The parental strains possessing electropherotypes LH43 and LH45 were shown to share eight gene segments on gels with different acrylamide concentrations. The origin of the remaining three gene segments of two progeny reassortants were unambiguously determined under all electrophoresis conditions that LH49 derived gene segments 3 and 7 from LH43 and gene segment 11 from LH45, while LH50 derived gene segment 3 from LH43 and segments 7 and 11 from LH45.

In order to finally confirm the parent-progeny relationship for each of the four sets of the electropherotypes shown in Fig. 1 and Table 1, sequencing studies were performed on selected gene segments. Of the four sets of candidates, two that appeared in the 95–96 season were confirmed as reassortants; i.e., both electropherotypes LH49 (96H063) and LH50 (96H070) were explained by reassortment between co-dominantly circulating electropherotypes LH43 (represented by 96H026) and LH45 (represented by 96H001) (Table 1). More specifically, the nucleotide sequence of gene segment 3 corresponding to nucleotide position from 424 to 910 of 96H063 (LH49) was identical with that of 96H026 (LH43), and differed from that of 96H001 (LH45) by two nucleotides out of the 487 nucleotides sequenced (Fig. 2A). In contrast, the entire gene segment 11 of 96H063 (LH49) was identical with that of 96H001 (LH45) but different from that of 96H026 (LH43) in that gene segment 11 of both 96H063 (LH49) and 96H001 (LH45) had nine nucleotide insertion starting at position 446. Thus, these gene segment 11 were 673 nucleotides in length (Fig. 2B). Exactly same sequencing results were obtained among gene segments 3 and among gene segments 11 of 96H070 (LH50), 96H026 (LH43) and 96H001 (LH45) (Fig. 2A and 2B). As clearly evident from co-electrophoresis experiment (Fig. 1C and 1D), two reassortant candidates LH49 and LH50 are different in their gene segments 7. Thus, the simplest explanation for the making of LH49 is that it is a single gene 11 substitution reassortant

A

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LH45   424 GTATGTAGGC TATAATGGAA AGAATACACG AGGTCTCTAT AATTTTTCAT 473
LH43   424 ********** ********** ********** *****A**** ********** 473
LH49   424 ********** ********** ********** *****A**** ********** 473
LH50   424 ********** ********** ********** *****A**** ********** 473

LH45   474 TTATATGTCA AAATGCAGCT ACAGATGATG ATGTAATAAT TGAATATATA 523
LH43   474 ********** ********** ********** ********** ********** 523
LH49   474 ********** ********** ********** ********** ********** 523
LH50   474 ********** ********** ********** ********** ********** 523

LH45   524 TACTCCAATG AGTTAGACTT TCAAAATTTT CTGTTGAGAA AAATTAAAGA 573
LH43   524 ********** ********** ********** ********** ********** 573
LH49   524 ********** ********** ********** ********** ********** 573
LH50   524 ********** ********** ********** ********** ********** 573

LH45   574 GAGAATGACC ACATCTCTTC CAATTGCTAG ATTATCAAAT CGTGTGTTTA 623
LH43   574 ********** ********** ********** ********** ********** 623
LH49   574 ********** ********** ********** ********** ********** 623
LH50   574 ********** ********** ********** ********** ********** 623

LH45   624 GAGATAAATT ATTTCCATCT ATTGTAAACA CACATAAAAA AGTGATAAAT 673
LH43   624 ********** ********** ********** T********* ********** 673
LH49   624 ********** ********** ********** T********* ********** 673
LH50   624 ********** ********** ********** T********* ********** 673

LH45   674 GTTGGGCCGA GGAATGAATC TATGTTCACA TTCCTAAATT TTCCAACTAT 723
LH43   674 ********** ********** ********** ********** ********** 723
LH49   674 ********** ********** ********** ********** ********** 723
LH50   674 ********** ********** ********** ********** ********** 723

LH45   724 TAAGCAATTT TCAAACGGTG CGTATATTGT GAAGCATACT ATTAAATTGA 773
LH43   724 ********** ********** ********** ********** ********** 773
LH49   724 ********** ********** ********** ********** ********** 773
LH50   724 ********** ********** ********** ********** ********** 773

LH45   774 AGCAGGAGAA ATGGTTGGGT AAAAGAGTAT CACAATTTGA TATCGGACAA 823
LH43   774 ********** ********** ********** ********** ********** 823
LH49   774 ********** ********** ********** ********** ********** 823
LH50   774 ********** ********** ********** ********** ********** 823

LH45   824 TATAAAAACA TGCTAAATGT GATCACTACT ATTTACTATT ACTATAATTT 873
LH43   824 ********** ********** ********** ********** ********** 873
LH49   824 ********** ********** ********** ********** ********** 873
LH50   824 ********** ********** ********** ********** ********** 873

LH45   874 ATACTATTCA AAACCTATAA TATACATGCT TGGTTCA 910
LH43   874 ********** ********** ********** ******* 910
LH49   874 ********** ********** ********** ******* 910
LH50   874 ********** ********** ********** ******* 910
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Fig. 2. **A** Nucleotide sequence of gene segment 3 (nt 424–910) of LH45 (96H001). The corresponding sequences of LH43 (96H026), LH49 (96H063) and LH50 (96H070) are shown below that of LH45 only where they differ. The sequence data have been deposited in the DDBJ and given accession numbers AB045213 (96H001), AB045214 (96H026), AB045215 (96H063) and AB045216 (96H070). **B** Nucleotide sequence of the entire gene segment 11 of LH45 (96H001). The corresponding sequences of LH43 (96H026), LH49 (96H063) and LH50 (96H070) are shown below that of LH45 only where they differ. Nine nucleotide insertions in LH45, LH49 and LH50 are underlined. The sequence data have been deposited in the DDBJ and given accession numbers AB045217 (96H001), AB045218 (96H026), AB045219 (96H063) and AB045220 (96H070)

B

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LH45    1 GGCTTTTAAA GCGCTACAGT GATGTCTCTC AGCATTGACG TGACGAGTCT  50
LH43    1 •••••••••• •••••••••• •••••••••• •••••••••• ••••••••••  50
LH49    1 •••••••••• •••••••••• •••••••••• •••••••••• ••••••••••  50
LH50    1 •••••••••• •••••••••• •••••••••• •••••••••• ••••••••••  50

LH45   51 TCCCTCAATT TCTTCTAGTA TCTTTAAAAA TGAATCGTCT TCTACAACGT 100
LH43   51 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 100
LH49   51 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 100
LH50   51 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 100

LH45  101 CAACTCTTTC TGGAAAATCT ATTGGTAGGA ACGAACAGTA TGTTTCATCA 150
LH43  101 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 150
LH49  101 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 150
LH50  101 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 150

LH45  151 GATATCGAAG CATTCAATAA ATACATGTTG TCGAAGTCTC CAGAGGATAT 200
LH43  151 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 200
LH49  151 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 200
LH50  151 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 200

LH45  201 TGGACCATCT GATTCTGCTT CAAACGATCC ACTCACCAGT TTTTCGATTA 250
LH43  201 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 250
LH49  201 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 250
LH50  201 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 250

LH45  251 GATCGAATGC AGTTAAGACA AATGCAGATG CTGGCGTGTC TATGGATTCA 300
LH43  251 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 300
LH49  251 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 300
LH50  251 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 300

LH45  301 TCAACACAAT CACGACCTTC AAGCAACGTT GGGTGCGATC AAATGGATTT 350
LH43  301 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 350
LH49  301 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 350
LH50  301 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 350

LH45  351 CTCCTTAACT AAAGGTATTA ATGTTAGTGC TAGTCTTGAT TCATGTGTAT 400
LH43  351 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 400
LH49  351 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 400
LH50  351 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 400

LH45  401 CAATTTCAAC TAACCATAAA AAGGAGAAAT CTAAAAAGGA TAAAAAGGAT 450
LH43  401 •••••••••• •••••••••• •••••••••• •••••••••• •••••      445
LH49  401 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 450
LH50  401 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 450

LH45  451 AAAAGTAGGA AACACTACCC AAGAATTGAA GCAGATTCTG ATTCTGAAGA 500
LH43  446     •••••• •••••••••• •••••••••• •••••••••• •••••••••• 491
LH49  451 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 500
LH50  451 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 500

LH45  501 TTATGTTTTG GATGATTCAG ATAGTGATGA CGGTAAATGT AAAAATTGCA 550
LH43  492 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 541
LH49  501 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 550
LH50  501 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 550

LH45  551 AATACAAAAA GAAATATTTT GCACTAAGAA TGAGGATGAA ACAAGTCGCA 600
LH43  542 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 591
LH49  551 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 600
LH50  551 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 600

LH45  601 ATGCAATTGA TAGAAGATTT GTAATGTCGA CCTGAGGACA CACTAGGGAG 650
LH43  592 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 641
LH49  601 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 650
LH50  601 •••••••••• •••••••••• •••••••••• •••••••••• •••••••••• 650

LH45  651 CTCCCCACTC CCGTTTTGTG ACC 673
LH43  642 •••••••••• •••••••••• ••• 664
LH49  651 •••••••••• •••••••••• ••• 673
LH50  651 •••••••••• •••••••••• ••• 673
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between LH45 and LH43, i.e., LH49 derives its gene segment 11 from LH45 and the remaining 10 genes from LH43. Similarly, the simplest explanation for LH50 is that it is a single gene 3 substitution reassortant between LH45 and LH43, i.e., LH50 derives its gene 3 from LH43 and the remaining 10 genes from LH45.

On the contrary, sequencing studies on selected gene segments failed to establish parent-progeny relationships for other two sets of reassortant candidates (data not shown).

Discussion

The existence of genetic diversity among co-circulating rotavirus strains captured attention as soon as the electrophoretic analysis of the genomic RNAs from field rotavirus specimens disclosed extensive variability in their electropherotypes [11, 15, 24, 29, 30, 35, 40]. Two alternative mechanisms for the generation of such genetic diversity were proposed [3, 27, 38]; i.e., (i) changes in mobility of the individual RNA segment due to the gradual accumulation of point mutations and (ii) reassortment of genomic RNA segments upon coinfection of two different strains.

While the molecular basis of this electropherotypic diversity is not completely understood [5], the observations in favor of either hypothesis are available in the literature. Under the selective pressure of neutralizing monoclonal antibodies against VP7, Dunn et al. [5] isolated an escape mutant that had an identical electropherotype except the migration of gene segment 8 (the VP7 gene) with a single nucleotide substitution from the parental sequence, indicating that changes in only one nucleotide altered the migration of a gene segment. Follett et al. [7, 8] analyzed by RNA finger-printing of the rotaviruses having almost identical electropherotypes except a few gene segments, and obtained the evidence for the occurrence of sequential point mutations. Palombo et al. [31] made a similar observation from an epidemiological study that two co-circulating strains possessing very similar electropherotypes with differences only in segments 7, 8, and 9 had only four nucleotide differences in the VP7 genes, and speculated that the difference in electropherotype was resulted from sequential point mutations.

On the other hand, it has been shown that rotaviruses of different animal origin or of the same host species readily reassort under cell-culture conditions [9, 22, 41, 42]. Characterization of unusual rotavirus isolates from epidemiological studies provided ample evidence for genetic reassortment mostly between strains of different genogroups [14, 16, 21, 23, 25–27, 32, 43]. Thus, reassortment between co-circulating strains is considered to operate as a mechanism of generating strain divergence. No direct evidence has been reported, however, that shows a given strain resulting from reassortment of two parental strains.

In order to provide direct evidence, if not the final proof, that can advance the argument in favor of the reassortant formation between co-circulating human rotavirus strains, we made an extensive comparison of the electropherotypes of the stool rotavirus specimens obtained from children with diarrhea in a defined area of Japan over a period of six years. Throughout this study, great emphasis was placed

on the electropherotype; i.e., a rotavirus strain was defined by a virus specimen whose genome showed a single distinct electropherotype. Conversely, two or more rotavirus specimens whose genomic RNAs showed an identical electropherotype were considered to derive from a single strain. For initial screening, this definition was applied to the results obtained by side-by-side comparisons on 10% gels with ethidium bromide staining, since the method has been the standard in our laboratory and is in consistent with any publications from our laboratory in the past. We are well aware of the precedence in the literature that described the cases in which two human rotavirus strains with different G serotypes had an identical electropherotype [1, 10], although Nakagomi et al. [29], as well as Coulson [4] and Pipittajan et al. [34] did not encounter such cases after extensive comparison of RNA electropherotypes from epidemiological specimens. Thus, in order to achieve the best possible accuracy of electropherotyping, we used the gels with three different acrylamide concentrations and the silver staining method for the co-electrophoresis experiments in which the relationships between the presumed reassortant and parents were established for each of seven sets of cases that had been chosen on the basis of side-by-side comparisons under the standard electrophoresis conditions.

Konno et al. [17] postulated for the first time the hypothesis that frequent reassortment was occurring between co-circulating strains based on the observation that a single dominant electropherotype was found at the start of an epidemic season followed by variety of minor electropherotypes towards the end of the season. Similar observations were also made by other investigators [2, 39], while Nakagomi et al. [29] failed to confirm this observation in the same location but in different epidemic seasons. None of these investigators, however, tried to interpret one electropherotype as a combination of two concurrently-circulating electropherotypes. Although it needs nucleotide sequencing to accurately establish the relationships between reassortants and their parent strains, comparison by electropherotypes is the prerequisite for the selection of possible candidates for reassortants. Thus, our vigorous selection process including confirmation by sequencing revealed that two out of 37 electropherotypes (5.4%) observed during a six year period were reassortants between co-circulating strains. To our best knowledge, this is the first direct evidence that will advance the argument in favor of the reassortant hypothesis.

Interpretation of the relative frequency of the possible reassortants between co-circulating strains may require caution. While the figure of 5.4% suggests that such events are not likely to be frequent, the number of specimens we analyzed is still limited and certainly far less from all strains circulating in the community. In this study both reassortants (LH49 and LH50) were found in the 95–96 season in which their parent strains (LH43 and LH45) were circulating co-dominantly [18]. This suggests that detecting reassortants involving minor strains is difficult, taken together the fact that no reassortant was identified in seasons in which there were no co-dominantly circulating strains.

In summary, this study shows that at least some part of electrophoretic diversity observed among rotavirus strains in a defined geographic location was

explained by genetic reassortment between strains concurrently circulating in the human population. These observations lend further support for the hypothesis that frequent genetic reassortment among co-circulating strains operates as a key mechanism for the genetic variability of human rotaviruses in nature.

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