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Rotavirus strains bearing the VP4P[14] genotype recovered from South African children with diarrhoea

Brief Report

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Summary. Human rotavirus VP4P[14] strains were previously recovered in South Africa [18]. The strains exhibited a long RNA pattern, VP6 subgroup II and VP7 serotype G1. Two of the VP8* genes were cloned and sequenced and demonstrated a high nucleotide homology with the prototype P[14] strains (PA169 and HAL1166).

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Human rotaviruses are the major viral agents of acute gastroenteritis in infants and young children worldwide [15]. The rotavirus particle consists of a double-layered capsid and an inner core which surrounds the 11 segments of double stranded (ds) RNA [4]. Separation of the RNA segments by polyacrylamide gel electrophoresis (PAGE) has become the most widely used technique in the study of rotavirus epidemiology as the RNA electropherotype is distinctive for each viral strain [4].

The inner capsid protein, VP6, determines the strain subgroup specificity [4] and human rotaviruses, in general, belong to either subgroup I or II [11, 14, 19, 24]. The outer capsid is composed of the VP4 protein (encoded by the fourth RNA segment) [17] and the VP7 protein (encoded by RNA segment 7, 8 or 9, depending on the strain) [15]. Both proteins are important as they are able to independently induce a protective neutralizing antibody response in the host [12, 20]. A binary system has been developed to record the VP4 or P type (for protease-sensitive) and the VP7 or G type (for glycoprotein) proteins [4, 13].

The VP4 protein is cleaved by proteolytic enzymes into VP5* and VP8* [3]. The VP8* gene contains a hyperdivergent region between nucleotides 205–554 (corresponding to amino acids 92–192) which is highly variable between strains

with different P genotypes, and is highly conserved between strains with the same P genotype [5, 9, 25]. Molecular techniques utilising this region of the gene constitute the basis for classification of the VP4 P genotypes [8, 16, 25, 28] and have been successfully used to investigate the distribution of the VP4 P genotypes in nature [8, 18, 21, 25].

The P[14] VP4 genotype has only been reported recently after the sequence analysis of the VP4 gene of human rotavirus strains PA169 and HAL1166 in Europe [8] and Mc35 in Thailand [28], and little is known of its distribution in nature. Strain PA169 (G6 serotype) was recovered from an infant with gastroenteritis in Italy [7] and strain HAL1166 (G8) was isolated from an infant with diarrhoea in Finland [6]. Mc35 (G10) was recovered from an ill child in Thailand [28].

During an epidemiological study to determine the frequency distribution of various P genotypes in South Africa, we identified the P[14] VP4 gene in six of 227 rotavirus field strains analysed, by dot-blot hybridisation with P type-specific probes [18]. In this study, we present the further characterisation of five of these P[14] genotype strains to establish their RNA electrophoretype, VP6 subgroup specificity, VP7 G serotype and partial nucleotide sequence of the VP8* gene. Four of the five original rotaviruses were recovered in 1987 (GR55/87, GR475/87, GR506/87 and GR951/87 and one in 1991 (GR67/91). The viral strains were recovered from infants (age range, 3–12 months) admitted to Ga-Rankuwa Hospital, South Africa for treatment for acute gastroenteritis.

Initially, the genomic ds RNA was extracted with phenol-chloroform and ethanol precipitation and subjected to polyacrylamide gel electrophoresis (PAGE) as described in detail previously [23]. Similarly, the VP6 subgroup specificity of the strains was determined using monoclonal antibodies to subgroup I or II as detailed elsewhere from this laboratory [11, 24]. The VP7 serotype was determined using a monoclonal antibody ELISA described in detail elsewhere [2], which was used in this laboratory.

The VP8* genes of two strains (GR67/91 and GR475/87) were amplified using a primer directed to the 5′ end of the gene (5′-GGCTATAAAATGGCTTC-3′) and a second primer based on the sequence of the VP4 gene of strain PA169 (5′-GGGGTTTAGGATACAAGT-3′). The second primer is targeted to nucleotides 862–880 of the VP4 gene. The clones were obtained by Dr Jorge Flores and Dr Duncan Steele at National Institutes of Health, Laboratory of Infectious Diseases, Maryland, USA using the methods described previously [25]. For this study, the plasmid clones were propagated to a high titre in terrific broth [22] and purified using a DNA purification assay (Magic Minipreps, Promega, Madison, WI, USA). The purified plasmid DNA was either sequenced directly, or PCR amplified first and then sequenced.

PCR was performed to amplify the cloned VP8* directly from the plasmid DNA [16, 25] using the two primers mentioned as well as primer 2706 described previously [25] and a VP4 P[14] specific primer 4943 (5′-GGTGTAGTTCCTG-CGTA-3′) which is complementary to nucleotides 538–554. All primers, except p2706 which is a common primer, were specific for the VP4 gene of strain PA169,

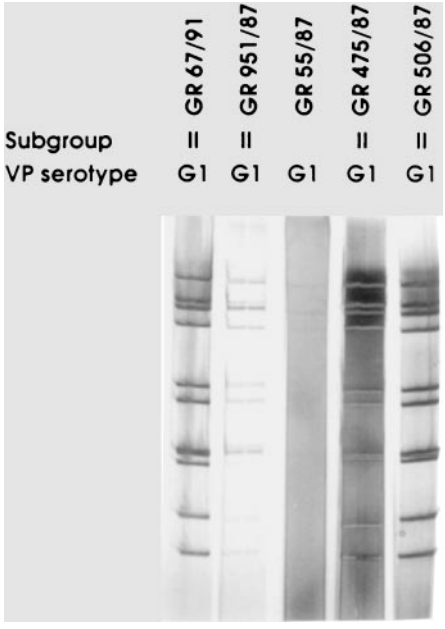

Fig. 1. Polyacrylamide gel electrophoresis of the P[14] genotype human rotavirus strains. Lane GR951/87 indicates the specimen with a dual infection

and were designed by Dr Jorge Flores and Dr Duncan Steele at the National Institutes of Health, Laboratory in Infectious Diseases, MD, USA.

For direct sequencing of VP8* clones, the T7 sequencing kit (Pharmacia Biotech, USA) was employed following the manufacturer's instructions. To confirm the sequences, PCR was performed to amplify the hyperdivergent region of the gene and the PCR products were then sequenced according to the protocols of Sequenase PCR product sequencing kit (United States Biochemicals, OH, USA). Sequencing was performed employing the same primers used in the PCR assay.

In four of the five strains, PAGE demonstrated a similar long RNA electrophoretype (Fig. 1). Four strains reacted strongly with the group A antibody and were subgrouped as VP6 SGII by monoclonal antibody analysis. Strain GR 55/87 could not be subgrouped unambiguously, although it gave a weak reactivity with subgroup II monoclonal antibodies; this strain also yielded faint RNA bands on PAGE (Fig. 1) and so the inability to unequivocally subgroup strain GR55/87 was probably due to an insufficient amount of the virus in the stool specimen. The combination of rotavirus strains with a long RNA pattern and subgroup II specificity has been reported previously from this laboratory and are not unusual in human rotaviruses [24, 25]. Both European strains with the P[14] genotype, (PA169 and HAL1166), were previously characterised as subgroup I and had a long RNA electropherotype [6, 7], as did the strain from Thailand [28]. This combination of VP6 subgroup specificity and RNA electropherotype is usually more typical of rotaviruses recovered from animal species [14].

One specimen, GR951/87, demonstrated more than 11 ds RNA segments on PAGE (Fig. 1), indicating that a dual infection with two rotavirus strains had occurred in the same host and demonstrating the potential for reassortment of the

gene segments. This specimen previously hybridised to both the P[14] and the P[8] VP4 genotype probes [18]. Rotaviruses undergo genetic reassortment at a higher frequency during mixed infection, further suggesting the importance of this mechanism in generating "new" viral genotypes [10, 13].

Four of the strains were VP7 serotyped by monoclonal antibody as G1 strains, which was the most common G serotype found amongst circulating human rotaviruses in the Ga-Rankuwa region during 1988–1989 [26]. The fifth strain

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          91
PA169     AGA AAA ACA ACA AAT GTT ACA GTT AAT CCA GGT CCG TTC GCA CAG
HAL1166   --G --- --G --- --- --- --- --- --- --- --A --- --T --G --A
GR475/87  --G --- --G --- --- --- --- --- --- --- --A --- --T --G --A
GR 67/91  --G --- --G --- --- --- --- --- --- --- --A --- --T --G --A
Mc35      --G --- --- G-G --C --- --- --- --- --- --A --A --T --- --A

          136
PA169     ACT GGA TAT GCT CCA GTT AAT TGG GGA CAC GGT GAA TTG TCT GAT
HAL1166   --- --G --C --C --- --- --C --- --- --- --- --- --- --C ---
GR475/87  --- --G --C --C --- --- --C --- --- --- --- --- --- --C ---
GR 67/91  --- --G --C --C --- --- --C --- --- --- --- --- --- --C ---
Mc35      --- --A --- --- --- --- --C --- --G --T --- --- --- --- ---

          181                             205
PA169     TCA ACA TTA GTT CAA CCG ACG TTA GAC GGC CCA TAT CAG CCA ACC
HAL1166   --G --- --- --- --G --- --- C-- --T --A --- --- --A --T ---
GR475/87  --G --- --- --- --G --- --- C-- --T --A --- --- --A --T ---
GR 67/91  --G --- --- --- --G --- --- C-- --T --A --- --- --A --T ---
Mc35      --- --- --- --- --G --N --- C-N --T --A --G --- --- --- ---

          226
PA169     ACA TTT AAT TTA CCA ATT GAC TAT TGG ATG CTA ATT GCG CCT ACT
HAL1166   --G --- --C --- --- --C --- --- --- --- --- --- --- --C ---
GR475/87  --- --- --- --- --- --C --- --- --- --- --- --- --- --C ---
GR 67/91  --- --- --- --- --- --- --- --- --- --- --- --- --- --C --C
Mc35      --G --- --C --- --T --- --T --- --- --- T-G --- --A --A ---

          271
PA169     CAA ATA GGT AGA GTA GCA GAG GGT ACG AAC ACA ACT GAT AGA TGG
HAL1166   --- --- --- --- --G --G --- --- --- --- --G --- --- --- ---
GR475/87  --- --- --- --- --G --G --- --- --T --- --G --- --- --- ---
GR 67/91  --- --- --- --- --- --- --- --- --- --- --- --- -TA --- ---
Mc35      --- --- --A --G --G --G --A --- --A --T --- --- A-- --- ---

          316
PA169     TTC GCT TGT GTA CTT GTT GAA CCG AAC GTA TCA AAT ACG CAG AGA
HAL1166   --- --- --- --T --A --- --- --A --T --G CA- --- --- --- ---
GR475/87  --- --- --- --T --A --- --- --A --T --G CA- --- --- --- ---
GR 67/91  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mc35      --- --C --- --- --- --- --- TT- --- --- CA- --- --- --A ---

          361
PA169     GAG TAC GTT TTA GAT GGA CAG ACG GTG CAG CTA CAA GTT TCA AAC
HAL1166   --A --T --- --- --- --- --- --- --- --- --G --- --- --- --T
GR475/87  --A --T --- --- --- --- --- --- --- --- --G --- --- --- --T
GR 67/91  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mc35      --A --T --- --- --- --- --- --- --A --A --- --- --- --- ---
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            406
PA169       AAC TCA AGT ACT CTT TGG AAA TTC ATA CTA TTT ATC AAA CTA GAA
HAL1166     --T --- --- --C --- --- --- --- --- --- --C --T --- --- ---
GR475/87    --T --- --- --C --- --- --- --- --- --- --C --T --- --- ---
GR 67/91    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mc35        G-T --- --- --C --- --- --- --T --- T-- --- --T --- --G ---

            451
PA169       AAA AAT GGA ACT TAC TCT CAA TAT TCA ACA TTG TCT ACG TCA AAT
HAL1166     --- --C --- G-- --- --C --- --- --- --- --A --T --- --- ---
GR475/87    --- --C --- G-- --- --C --- --- --- --- --- --- --- --- ---
GR 67/91    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mc35        --- --- --- --- --- A-- --- --- --- --- --A --- --A --- ---


            496
PA169       AAG TTG TGT GCA TGG ATG AAA AGA GAA GGG AGA GTA TAC TGG TAC
HAL1166     --A --A --- --- --- --- --- --- --- --A --- --- --- --- ---
GR475/87    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
GR 67/91    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mc35        --A C-- --- --- --- --- --- --- --- --- --- --- --T -CC ---

            541             554
PA169       GCA GGA ACT ACA CCG AAT GCC TCA GAA AGC TAT TAT CTA ACA ATA
HAL1166     --G --- --- --- --A --- --A --- --- --T --- --- T-- --- ---
GR475/87    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
GR 67/91    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mc35        --- --- GT- --- --- --- --A --G --- --- --- --- --G --G ---
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Fig. 2. Comparison of the nucleotide sequence in the hyperdivergent region (indicated in boldface) of the VP8* fragment of the VP4 gene of the prototype European strains (PA169 and HAL1166), the Thai strain (Mc35) and South African strains (GR475/87 and GR67/91). Differences in the nucleotide sequence are noted. Dashes denote identical nucleotides

could not be serotyped due to a lack of sufficient material. Other than strains PA169 (G6 serotype) and HAL1166 (G8), the P[14] genotype was also suggested on the basis of hybridisation studies to be present in two other human rotaviruses: PA710 (G3 serotype) and HAL1271 (G8 serotype) [7]. The presence of the P[14] genotype in serotypically unrelated human rotaviruses – G3, G6 and G8 in Europe and G10 in Thailand and including G1 in this study, clearly indicates that this genotype is circulating in nature, despite its apparent recent discovery in humans.

In this study, partial nucleotide sequence of the VP8* gene of two South African strains GR475/87 and GR67/91 was performed to confirm the P[14] genotype by comparison with the VP8* of the prototype strains PA169 and HAL1166 (Fig. 2). Comparison was made only in the genotype-specific hyperdivergent region (which lies between nucleotides 205–554), since much of our current knowledge on rotavirus VP4 genotypes is based on the nucleotide or amino acid sequence differences in this region [5, 9, 16, 25] and sequence analysis of the hyperdivergent region provides an alternative technique to examine the VP4 genotype present. The two South African strains indicated a remarkable nucleotide sequence conservation (92.6% identity) in the hyperdivergent region and also demonstrated a high degree of nucleic acid sequence homology with PA169 and

Table 1. Percent nucleotide sequence homology in the hyperdivergent region (nt 205–554) of the VP8* gene between South African strains GR475/87 and GR67/91, European strains PA169 and HAL1166 and Thai strain Mc35

Strains	GR475/87	GR67/91	PA169	HAL1166	Mc35
GR475/87	–	92.6%	92.0%	97.1%	85.7%
GR67/91		–	97.7%	90.3%	86.0%
PA169			–	89.7%	86.9%
HAL1166				–	85.7%
Mc35					–

HAL1166 (Fig. 2 and Table 1). Strain GR475/87 shares a sequence homology of 97.1% and 92% with strains HAL1166 and PA169, respectively. In contrast, GR67/91 appears to be more related to the hyperdivergent region of strain PA169 (97.7% sequence homology), than strain HAL1166 (90.3%).

Partial nucleotide sequence of the end of the VP8* gene was also obtained (data not shown). This region spanned 195 base pairs (nt 631–825) which encompasses the trypsin cleavage site of the VP4 gene (nucleotides 732–750, corresponding to amino acids 241–247). The partial sequences were analysed and compared with the corresponding sequences of strains PA169 and HAL1166. Complete nucleotide homology was observed between the two South African strains, which also showed high nucleotide homology with PA169, except for two base changes at position 716 (T-C) and 816 (A-C) in both local strains. However, the nucleotide sequence of the trypsin cleavage site was conserved between the South African strains and PA169. The PA169 VP4 protein is cleaved at amino acid position 241 and 247, and there is also a potential cleavage site at position 244 [8]. Cleavage of rotavirus VP4 protein has been shown to enhance viral infectivity [3]. The presence of a potential cleavage site at position 244 is thought to facilitate proteolytic processing of virulent strains [9].

P[14] rotaviruses have also been isolated from rabbits and compared to the earlier human rotaviruses with this VP4 genotype [1]. The observation is made that it is likely that the human P[14] rotaviruses, which were virtually all associated with a G-type found in cattle (G6, G8 and G10), may have arisen as reassortant events in cattle or humans [1]. This is suggested by the conservation of the VP7 and VP4 genes studied to date in lapine rotaviruses [1]. Furthermore, the complete nucleotide sequence of the VP4 genes of PA169, HAL1166 and Mc35 have been reported [8, 28]. The P[14] genes consist of 2362 bases encoding a protein of 776 amino acids, more typical of animal rather than human rotavirus strains [8, 28]. In addition, the P[14] genes from human rotaviruses, including the strains identified in this study, have the second arginine at amino acid position 247, which is also more typical of animal rotaviruses.

There is limited epidemiological data on the prevalence of the P[14] genotype in nature at present. These strains apparently persisted for four years in this region, yet they remained at a lower frequency [18] which may suggest that these strains were not successfully competitive with other human rotaviruses in the

whole viral pool. This would be supported by their rarity in other epidemiological studies [8, 28]. However, these results do suggest the global distribution and potential epidemiological significance associated with this genotype. Four of the babies infected with P[14] genotype strains in this study presented clinically with severe dehydrating diarrhoea. Interestingly, PA169, HAL1166 and Mc35 were also recovered from infants with diarrhoea [6, 7, 28]. The identification of the unusual rotavirus P[14] genotype in geographically diverse regions indicates that these, and possibly other unusual rotaviruses, need to be considered when designing a successful rotavirus vaccine for this sub-continent.

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References

1. Ciarlet M, Estes MK, Connor ME (1997) Comparative amino acid sequence analysis of the outer capsid protein VP4 from four lapine rotavirus strains reveals identity with genotype P[14] human rotaviruses. Arch Virol 142: 1 059–1 069
2. Coulson B, Unicomb LE, Pitson GA, Bishop RF (1987) Simple and specific enzyme immunoassay using monoclonal antibodies for serotyping human rotaviruses. J Clin Microbiol 25: 509–516
3. Estes MK, Graham DY, Mason BB (1981) Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J Virol 39: 879–888
4. Estes MK, Cohen J (1989) Rotavirus gene structure and function. Microbiol Rev 53: 410–449
5. Flores J, Midthun K, Hoshino Y, Green K, Gorziglia M, Kapikian AZ, Chanock RM (1986) Conservation of the fourth gene among rotaviruses recovered from asymptomatic newborn infants and its possible role in attenuation. J Virol 60: 972–979
6. Gerna G, Sarasini A, Zentilin L, Di Matteo A, Miranda P, Parea M, Battaglia M, Milanesi G (1990) Isolation in Europe of 69M-like (serotype 8) human rotaviruses with either subgroup I or II specificity and a long RNA electrophoretype. Arch Virol 112: 27–40
7. Gerna G, Sarasini A, Parea M, Arista S, Miranda P, Brussow H, Hoshino Y, Flores (1992) Isolation and characterization of two distinct human rotavirus strains with G6 specificity. J Clin Microbiol 30: 9–16
8. Gerna G, Sears J, Hoshino Y, Steele AD, Nakagomi O, Sarasini A, Flores J (1994) Identification of a new VP4 serotype of human rotaviruses. Virology 200: 66–71
9. Gorziglia M, Hoshino Y, Buckler-White A, Blumentals I, Glass RI, Flores J, Kapikian AZ, Chanock RM (1986) Conservation of amino acid sequence of VP8 and cleavage region of 84-kDa outer capsid protein among rotaviruses rocovered from asymptomatic neonatal infection. Proc Natl Acad Sci USA 83: 7039–7043
10. Greenberg HB, Kalica AR, Wyatt RG, Jones RW, Kapikian AZ, Chanock RM (1981) Rescue of non-cultivatable human rotavirus by gene reassortment during mixed infection with ts mutants of a cultivatable bovine rotavirus. Proc Natl Acad Sci USA 78: 420–424
11. Greenberg HB, McAuliffe V, Valdesuso J, Wyatt RG, Flores J, Kalica AR, Hoshino Y, Singh N (1983) Serological analysis of the subgroup protein of rotavirus, using monoclonal antibodies. Infect Immun 31: 91–99

12. Hoshino Y, Sereno M, Midthun K, Flores J, Kapikian AZ, Chanock RM (1985) Independent segregation of two antigenic of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc Natl Acad Sci USA 82: 8 701–8 704
13. Hoshino Y, Kapikian AZ (1994) Rotavirus vaccine development for the prevention of severe diarrhoea in infants and young children. Trends Microbiol 2: 242–249
14. Kalica AR, Greenberg HB, Espejo RT, Flores J, Wyatt RG, Kapikian AZ, Chanock RM (1981) Distinctive ribonucleic acid patterns of human rotavirus subgroups I and II. Infect Immun 33: 958–961
15. Kapikian AZ, Chanock RM (1990). Rotaviruses. In: Fields BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, Roizman B (eds) Virology. Raven Press, New York, pp 1 353–1 404
16. Larralde G, Flores J (1990) Identification of gene 4 alleles among human rotaviruses by polymerase chain reaction derived probes. Virology 179: 469–473
17. Liu M, Offit PA, Estes MK (1998) Identification of the simian rotavirus SA11 genome segment 3 product. Virology 163: 26–32
18. Mphalele MJ, Steele AD (1995) Relative frequency of human rotavirus VP4 (P) genotypes recovered over a ten-year period from South African children with diarrhea. J Med Virol 47: 1–5
19. Nakagomi O, Nakagomi T, Hoshino Y, Flores J, Kapikian AZ (1987) Genetic analysis of a human rotavirus that belongs to subgroup I but has an RNA pattern typical of subgroup II human rotaviruses. J Clin Microbiol 25: 1 159–1 164
20. Offit PA, Blavat G (1986) Identification of the two rotavirus genes determining neutralization specificities. J Virol 57: 376–378
21. Rasool NBG, Larralde G, Gorziglia M (1993) Determination of human rotavirus VP4 using serotype-specific CDNA probes. Arch Virol 133: 275–282
22. Sambrook J, Fritsch EF, Maniatis T (eds) (1989) Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press, New York
23. Steele AD, Alexander JJ (1987) Molecular epidemiology of rotavirus in Black infants in South Africa. J Clin Microbiol 25: 2 384–2 387
24. Steele AD, Alexander JJ (1988) The relative frequency of subgroup I and II rotaviruses in Black infants in South Africa. J Med Virol 24: 321–327
25. Steele AD, Garcia D, Sears JF, Gerna G, Nakagomi O, Flores J (1993) Distribution of VP4 gene alleles in human rotaviruses by using probes to the hyperdivergent region of the VP4 gene. J Clin Microbiol 31: 1 735–1 740
26. Steele AD, van Niekerk MC, Mphahlele MJ (1995) Geographic distribution of human rotavirus VP4 genotypes and VP7 serotypes in five South African Regions. J Clin Microbiol 33: 1 516–1 519
27. Taniguchi K, Nishikawa K, Urasawa T, Midthun K, Kapikian AZ, Gorziglia M (1989) Complete nucleotide sequence of the gene encoding VP4 of a human rotavirus (strain K8) which has unique VP4 neutralization epitopes. J Virol 63: 4 101–4 106
28. Urasawa T, Taniguchi K, Koyabashi N, Mise K, Hasewaga A, Yamazi Y, Urasawa S (1993) Nucleotide sequence of VP4 and VP7 genes of a unique human rotavirus strain Mc35 with subgroup I and serotype 10 specificity. Virology 195: 766–771

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