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VP4 Genotyping of Human Rotavirus in the United States

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The VP4 (P) and VP7 (G) types of 171 rotavirus isolates obtained from children with diarrhea in the United States were characterized by PCR typing assays. Strains P1G1 predominated (71%); this was followed by strains P1G3 (20%) and P2G2 and P1G4 (2% each). Mixed types were identified in five (3%) specimens. Two (1%) strains bearing the P3 genotype (P3G1 and P3G2) were found in children with severe dehydrating diarrhea, although the P3 genotype has been regarded as a possible marker for virus attenuation.

Rotaviruses possess a double-stranded RNA (dsRNA) genome fragmented into 11 segments and enclosed by a multiple-capsid shell. The outer capsid contains two proteins: VP7, encoded by gene segment 7, 8, or 9; and VP4, encoded by gene segment 4 (20). Studies of the antigenic relationships among rotaviruses have shown that both VP7 and VP4 proteins induce neutralizing antibodies (18, 24). Glycoprotein VP7 specifies the virus G neutralization antigen, and to date, nine G serotypes have been identified in humans (1, 7, 12, 20, 30). VP4, a minor constituent of the outer capsid, is associated with several biologically important functions. It has hemagglutination activity in many strains from animals and is responsible for restriction of human rotavirus growth in tissue culture; it is the site for protease activation of infectivity and appears to play a major role in rotavirus virulence (20). On the basis of nucleotide comparisons among VP4 sequences, five P (for protease-susceptible) human genotypes were initially described (13). For those, a correlation was observed between the P genotype and the G serotype of an isolate: the P1 genotype (Wa-like) was associated with G1, G3, G4, and G9 serotypes; the P2 genotype (DS1-like) was associated with G2 serotype in strains obtained from patients with symptomatic infections, and the P3 genotype (M37-like) was associated with the G1, G2, G3, or G4 serotype in strains obtained from infants in nurseries with asymptomatic infections. Unique P4 and P5 genotypes were found in strains K8 and 69M, respectively (27, 29). Recently, a sixth P genotype was identified in an asymptomatic child in the United States (22), and strains bearing P genotypes closely related to that of bovine strain B223 have been recovered from asymptomatic children in India (6, 10).

A set of 171 rotavirus isolates obtained from children with diarrhea in several locales in the United States were analyzed for their G and P genotypes by two-step PCR typing assays described by Gouvea et al. (14) and Gentsch et al. (11), respectively. The VP7 genes of some relevant rotavirus strains were further characterized by restriction endonuclease analysis (REA) of their PCR-generated cDNA copies as described previously (16). Samples included 145 stool specimens collected for an epidemiologic survey and previously characterized for their G types (15), 22 additional samples collected in the last 3 years at the Children's Hospitals of Buffalo and Philadelphia, and four cell-adapted strains used in our laboratory as reference G strains; G1 strain O, G2 strain SC2, and G4 strain CC4 were originally from Philadelphia, and G3 strain SK5 was from Atlanta.

The use of the two-step amplification-typing technique enabled us to identify the P types of 100% of the isolates, although only 65% gave positive results in the first amplification. In this step, viral dsRNA was subjected to coupled reverse transcription and PCR in the presence of primers designed to amplify an 876-bp DNA fragment of gene 4 (Fig. 1). This fragment was then used as a template in a second PCR with a pool of type-specific primers designed to generate type-specific fragment lengths (Fig. 2). The typing results are summarized in Table 1. Strains of P1G1 predominated (71%), followed by P1G3 (20%) and P2G2 and P1G4 (2% each). Two strains bearing a P3 genotype were found: P3G1 (strain VE7156) and P3G2 (strain SC2). Types P4 and P5 were not found, and mixed types, including a third strain bearing a P3 genotype, were present in the remaining five specimens. The presence of mixed G types had been suspected by RNA electropherotype analysis and confirmed by enzyme immunoassay (EIA) by using G-specific monoclonal antibodies in a previous study (15). The presence of mixed P types was confirmed by a one-step VP4 confirmatory assay described by Gentsch et al. (11).

In general, our results support the reported correlation between the two antigenic specificities for each isolate; all P1 strains were type G1, G3, or G4 and all P2 strains were type G2. The only exception was the finding of both types P1 and P2 (Fig. 2, lane 2; Fig. 3, lanes 1 and 2) but only type G1 in specimen VE6911. Type G2 could not be identified by the PCR G-typing assay or EIA with a neutralizing monoclonal antibody (15). Failure to identify type G2 by PCR typing and EIA was reported for isolates from Japan (23, 31) and Bangladesh (33); however, this has not been our experience with specimens obtained in the United States. Electrophoretic analysis of the viral RNA showed a typical long pattern and no evidence for more than a single strain (15). Therefore, the presence of a strain with the unusual antigenic combination P2G1 in specimen VE6911 remains a possibility, and attempts to isolate such a strain in cell cultures are under way.

Strains of the four major human G types were recovered from healthy newborn infants in Venezuela (G1 strains or

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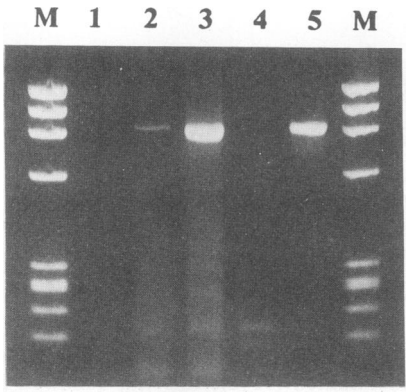

FIG. 1. First PCR amplification. Rotavirus dsRNA was reverse-transcribed and amplified with the conserved primer pair con2-con3 designed to amplify an 876-bp segment of rotavirus gene 4. Products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The products shown are from specimens SK77 (lane 1), VE6911 (lane 2), VE7366 (lane 3), VF9221 (lane 4), and VE7156 (lane 5). Markers (lanes M) are *Hae*III-digested φX174 DNA.

M37-like strains), Sweden (G2 strains or 1076-like strains), Australia (G3 strains or McN-like strains), and England (G4 strains or ST3-like strains) (19). All of these strains possessed the unique VP4 genotype P3, which was quite distinct from that of strains causing symptomatic infections (13). The P3 genotype is therefore regarded as a possible marker for rotavirus attenuation (8). Our results do not support its association with exclusively asymptomatic infections, because we identified the P3 genotype in isolates from specimens obtained from infants with severe diarrhea. The cell-adapted strain SC2 was originally isolated from the stool of an 11-day-old infant who was admitted to the Pennsylvania Hospital, Philadelphia, in 1981 with a 4-day history of watery diarrhea. He received intravenous rehydration and was discharged 5 days later with the diagnosis of rotavirus

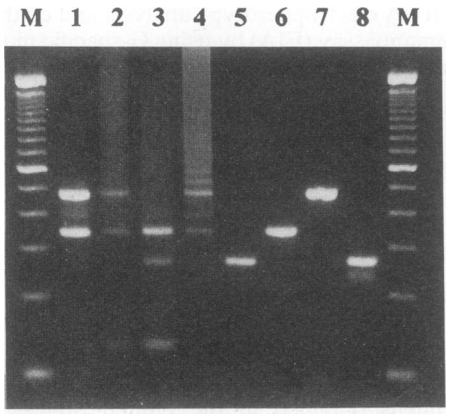

FIG. 2. PCR P typing (second PCR amplification). One microliter of each product obtained in the first PCR (Fig. 1) was amplified with a pool of nested P-specific primers and the common con3 primer. Products of specimens SK77 (lane 1), VE6911 (lane 2), VE7366 (lane 3), and VF9221 (lane 4) indicated infections with multiple strains, but that of specimen VE7156 did not (lane 5). Lanes 6 to 8, positive controls for P1 to P3, respectively; lanes M, 100-bp marker with highlighted 600-bp segment (Bethesda Research Laboratories, Gaithersburg, Md.).

TABLE 1. VP4 (P) and VP7 (G) typing of human rotavirus from 167 fecal specimens and four cell-adapted strains

Type	No. (%) of strains of type:				
	P1	P2	P3	P1+P2	P1+P3
G1	122 (71)		1 (0.5)	1	1
G2		4 (2)	1 (0.5)		
G3	34 (20)				
G4	4 (2)				
G1+G2				2	
G1+G3	1				

gastroenteritis because no other pathogen was found. Strain VE7156 was recovered from a 17-day-old girl who was delivered prematurely at the Children's Hospital of Buffalo. She developed severe, watery diarrhea the day after she was transferred from the intensive care unit to the ward for premature neonates. Rotavirus was diagnosed by electron microscopy and EIA, and no other enteropathogen was found. An isolate of the P3 genotype was identified by the two-step P-typing assay (Fig. 2, lane 5) and confirmed by the one-step confirmatory assay in which a distinct set of type-specific primers was used (Fig. 3, lanes 8 to 11). Both assays gave unequivocal and exclusive P3-positive results, thus implicating this P3G1 or M37-like strain as the causative agent of the severe diarrheal illness.

We recently observed that the VP7 gene of strain M37 could be distinguished from those of other G1 strains by its unique profile obtained by digestion with *Sau*96I endonuclease (16). REA of VE7156 and three P1G1 strains recovered from children with diarrhea in the same hospital within 1 month is shown in Fig. 4. Strain VE7156 shared the typical S1 profile with the other "local" G1 strains and not the S5 profile of the M37-like strain. Also, the REA profile for the P3 strain SC2, which has been described previously (16), showed the same profile as the "local" G2 strain. This finding is consistent with the hypothesis that P3 strains from infants in a nursery are most likely natural reassortants bearing the VP7 gene of a concurrent "virulent" strain (8).

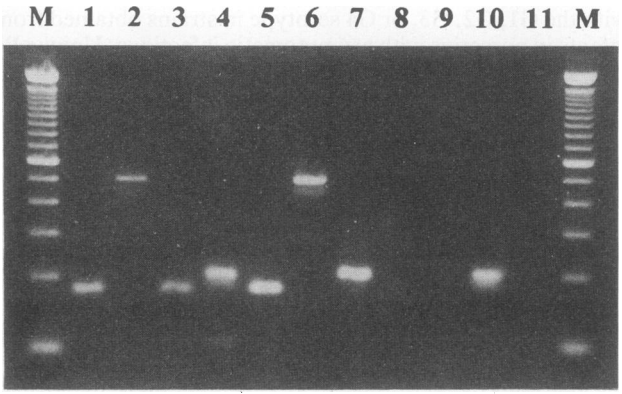

FIG. 3. Confirmation of the P types of specimens VE6911 (lanes 1 and 2), VE7366 (lanes 3 and 4), and VE7156 (lanes 8 to 11). Rotavirus dsRNA was reverse-transcribed and amplified by PCR by using a P1-specific primer (lanes 1, 3, 5, and 8), a P2-specific primer (lanes 2, 6, and 9), a P3-specific primer (lanes 4, 7, and 10), or a P4-specific primer (lane 11). Lanes 5 to 7, positive controls for P1 to P3, respectively; lanes M, 100-bp marker with highlighted 600-bp segment.

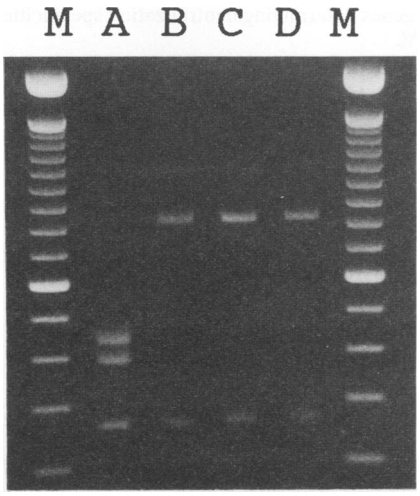

FIG. 4. Restriction profiles of VP7 gene copies of strain M37 (lane A) and fecal isolates VE7156 (lane B), VE7050 (lane C), and VE7351 (lane D) digested with *Sau*96I. Lanes M, 100-bp marker ladder with highlighted 600-bp segment.

A third P3 genotype strain was identified in the present study (specimen VE7366), but it was found in combination with a P1 genotype strain (Fig. 2, lane 3; Fig. 3, lanes 3 and 4). This specimen was from an 11-month-old child who developed mild diarrhea 2 days after being admitted for otitis media and respiratory symptoms to the pediatric ward of the Buffalo Children's Hospital. In this case, either rotavirus strain (P1G1 or P3G1) could have been responsible for the child's diarrhea.

Similar to the situation found in other countries, the present study shows that P3 rotavirus strains might also be endemic in hospital nurseries in the United States, because the two infections with rotavirus of the P3 genotype found in children in distinct wards of the Buffalo Children's Hospital were clearly nosocomial. Our study, however, consisted exclusively of infants with symptomatic infections, thus precluding an assessment of the frequency of P3 genotype and its possible association with widespread asymptomatic shedding of rotavirus by newborns. In studies conducted in Australia, Venezuela, England, and Sweden (3, 4, 19, 26), a single but distinct rotavirus strain had been endemic for years in nurseries for newborns, even though the strains circulating in the community had changed. This was also the case in a hospital in South Africa (28). In other studies, however, no unique "nursery strain" could be exclusively associated with asymptomatic infections; in fact, the strains shed by symptom-free children in hospital nurseries in Chile and Baltimore, Md., appeared to be the same as those that produced diarrheal illnesses among individuals in the communities (9, 32). The strains, however, were characterized by their electropherotypes; their P specificities remain to be determined. The recent development of genotyping assays by hybridization with P-specific probes (21) or by PCR with P-specific primers (11) and of serotyping by EIAs with monoclonal antibodies capable of discriminating distinct P types (5, 25) should rapidly generate this needed information.

The reasons for the symptomless nature of most neonatal infections are not understood. Host factors, such as age susceptibility, passive maternal protection, and the existence of strains naturally attenuated for newborns, have been suggested (8, 17, 19). The last hypothesis is favored by the fact that "asymptomatic" strains, which are endemic in nurseries for newborns in geographically distant places, bore a remarkably conserved fourth gene, thus strongly associating the P3 genotype with virus attenuation. However, the finding of P3 strains in patients with severe diarrhea indicates that, although a possible major virulence factor, the P3 phenotype is not in itself a guarantee for silent infections in normal children. Moreover, other surveys have implicated the P3 genotype in diarrheal illness (2, 5, 21). The incidence of P3-related diarrhea was very low, but its occurrence in both neonates and older children in the community or in hospital settings is nevertheless intriguing. Further clinical and epidemiologic investigations, including a search for asymptomatic shedding of rotaviruses by older children and adults, are needed to ascertain the frequency and virulence of distinct P types.

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REFERENCES

1. **Beards, G., L. Xu, A. Ballard, U. Desselberger, and M. A. McCrae.** 1992. A serotype 10 human rotavirus. J. Clin. Microbiol. **30**:1432–1435.
2. **Bern, C., L. Unicomb, J. R. Gentsch, N. Banul, M. Yunus, R. B. Sack, and R. I. Glass.** 1992. Rotavirus diarrhea in Bangladeshi children: correlation of disease severity with serotypes. J. Clin. Microbiol. **30**:3234–3238.
3. **Bishop, R. F., G. L. Barnes, E. Cipriani, and J. S. Lund.** 1983. Clinical immunity after neonatal rotavirus infection. A prospective longitudinal study in young children. N. Engl. J. Med. **309**:72–76.
4. **Chrystie, I. L., B. Totterdell, M. J. Baker, J. W. Scopes, and J. E. Banatvala.** 1975. Rotavirus infections in a maternity unit. Lancet **ii**:79.
5. **Coulson, B.** 1993. Typing of human rotavirus VP4 by an enzyme immunoassay using monoclonal antibodies. J. Clin. Microbiol. **31**:1–8.
6. **Das, M., S. J. Dunn, G. N. Woode, H. B. Greenberg, and C. D. Rao.** 1993. Both surface proteins VP4 and VP7 of an asymptomatic neonatal rotavirus strain (I321) have high levels of sequence identity with the homologous proteins of a serotype 10 bovine rotavirus. Virology **194**:374–379.
7. **Dunn, S. J., H. B. Greenberg, R. L. Ward, O. Nakagomi, J. W. Burns, P. T. Vo, K. A. Pax, M. Das, K. Gowda, and C. D. Rao.** 1993. Serotypic and genotypic characterization of human serotype 10 rotavirus from asymptomatic neonates. J. Clin. Microbiol. **31**:165–169.
8. **Flores, J., K. Midthun, Y. Hoshino, K. Green, M. Gorziglia, A. Z. Kapikian, and R. M. Chanock.** 1986. Conservation of the fourth gene among rotaviruses recovered from asymptomatic newborn infants and its possible role in attenuation. J. Virol. **60**:972–979.
9. **Gaggero, A., L. F. Avedano, J. Fernandez, and E. Spencer.** 1992. Nosocomial transmission of rotavirus from patients admitted with diarrhea. J. Clin. Microbiol. **30**:3294–3297.
10. **Gentsch, J. R., B. K. Das, B. Jiang, M. K. Bhan, and R. I. Glass.** 1993. Similarity of the VP4 protein of human rotavirus strain 116E to that of the bovine B223 strain. Virology **194**:424–430.
11. **Gentsch, J. R., R. I. Glass, P. Woods, V. Gouvea, M. Gorziglia, J. Flores, B. K. Das, and M. K. Bahn.** 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. J. Clin. Microbiol. **30**:1365–1373.
12. **Gerna, G., A. Sarasini, M. Parea, S. Arista, P. Miranda, H. Brussow, Y. Hoshino, and J. Flores.** 1992. Isolation and characterization of two distinct human rotavirus strains with G6 specificity. J. Clin. Microbiol. **30**:9–16.
13. **Gorziglia, M., K. Green, K. Nishikawa, K. Taniguchi, R. Jones,**

A. Kapikian, and R. Chanock. 1988. Sequence of the fourth gene of human rotaviruses recovered from asymptomatic or symptomatic infections. J. Virol. **62**:2978–2984.

14. **Gouvea, V., R. I. Glass, P. Woods, K. Taniguchi, H. F. Clark, B. Forrester, and Z.-Y. Fang.** 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J. Clin. Microbiol. **28**:276–282.
15. **Gouvea, V., M.-S. Ho, R. I. Glass, P. Woods, B. Forrester, C. Robinson, R. Ashley, M. Riepenhoff-Talty, H. F. Clark, K. Taniguchi, E. Meddix, B. McKellar, and L. Pickering.** 1990. Serotypes and electropherotypes of human rotavirus in the USA: 1987–1989. J. Infect. Dis. **162**:362–367.
16. **Gouvea, V., C. Ramirez, B. Li, N. Santos, L. Saif, H. F. Clark, and Y. Hoshino.** 1993. Restriction endonuclease analysis of the vp7 genes of human and animal rotaviruses. J. Clin. Microbiol. **31**:917–923.
17. **Haffegee, I. E.** 1991. Neonatal rotavirus infections. Rev. Infect. Dis. **13**:957–962.
18. **Hoshino, Y., M. M. Sereno, K. Midthun, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc. Natl. Acad. Sci. USA **82**:8701–8704.
19. **Hoshino, Y., R. G. Wyatt, J. Flores, K. Midthun, and A. Z. Kapikian.** 1985. Serotypic characterization of rotaviruses derived from asymptomatic human neonatal infections. J. Clin. Microbiol. **21**:425–430.
20. **Kapikian, A. Z., and R. M. Chanock.** 1990. Rotaviruses, p. 1353–1403. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
21. **Larralde, G., and J. Flores.** 1990. Identification of gene 4 alleles among human rotaviruses by polymerase chain reaction-derived probes. Virology **179**:469–473.
22. **Li, B., H F. Clark, and V. Gouvea.** 1993. Nucleotide sequence of the VP4-encoding gene of an unusual human rotavirus (HCR3). Virology **196**:825–830.
23. **Nakagomi, O., H. Oyamada, and T. Nakagomi.** 1991. Experience with serotyping rotavirus strains by reverse transcription and two-step polymerase chain reaction with generic and type-specific primers. Mol. Cell. Probes **5**:285–289.
24. **Offit, P. A., and G. Blavat.** 1986. Identification of the two rotavirus genes determining neutralization specificities. J. Virol. **57**:376–378.
25. **Padilla-Noriega, L., R. Werner-Eckert, E. R. Mackow, M. Gorziglia, G. Larralde, K. Taniguchi, and H. B. Greenberg.** 1993. Serologic analysis of human rotavirus serotypes P1A and P2 by using monoclonal antibodies. J. Clin. Microbiol. **31**:622–628.
26. **Perez-Schael, I., G. Daoud, L. White, G. Urbina, N. Daoud, M. Perez, and J. Flores.** 1984. Rotavirus shedding by newborn children. J. Med. Virol. **14**:127–136.
27. **Qian, Y., and K. Y. Green.** 1991. Human rotavirus strain 69M has a unique VP4 as determined by amino acid sequence analysis. Virology **182**:407–412.
28. **Steele, A. D., M. C. Niekerk, A. Geyer, P. Bos, and J. J. Alexander.** 1992. Further characterization of human rotaviruses isolated from asymptomatically infected neonates in South Africa. J. Med. Virol. **38**:22–26.
29. **Taniguchi, K., K. Nishikawa, T. Urasawa, S. Urasawa, K. Midthun, A. Z. Kapikian, and M. Gorziglia.** 1989. Complete nucleotide sequence of the gene encoding VP4 of a human rotavirus (strain K8) which has unique VP4 neutralization epitopes. J. Virol. **63**:4101–4106.
30. **Urasawa, S., A. Hasegawa, T. Urasawa, K. Taniguchi, F. Wakasugi, H. Suzuki, S. Inouye, B. Pongprot, J. Supawadee, S. Suprasert, P. Rangsiyanond, S. Tonusin, and Y. Yamazi.** 1991. Antigenic and genetic analyses of human rotaviruses in Chiang Mai, Thailand: evidence for a close relationship between human and animal rotaviruses. J. Infect. Dis. **166**:227–234.
31. **Ushijima, H., H. Koike, A. Mukoyama, A. Hasegawa, S. Nishimura, and J. Gentsch.** 1992. Detection and serotyping of rotaviruses in stool specimens by using reverse transcription and polymerase chain reaction amplification. J. Med. Virol. **38**:292–297.
32. **Vial, P. A., K. L. Kotloff, and G. A. Losonsky.** 1988. Molecular epidemiology of rotavirus infection in a room for convalescing newborns. J. Infect. Dis. **157**:668–673.
33. **Ward, R. L., M. McNeal, J. D. Clemens, D. A. Sack, M. Rao, N. Huda, K. Green, A. Z. Kapikian, B. S. Coulson, R. F. Bishop, H. B. Greenberg, G. Gerna, and G. M. Schiff.** 1991. Reactivities of serotyping monoclonal antibodies with culture-adapted human rotaviruses. J. Clin. Microbiol. **29**:449–456.