JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1996, p. 436–439 Vol. 34, No. 2
0095-1137/96/$04.00+0


# Unusual Diversity of Human Rotavirus G and P Genotypes in India

MADHUMATI RAMACHANDRAN,[1] BIMAL K. DAS,[1] AARTI VIJ,[1] RAMESH KUMAR,[1] S. S. BHAMBAL,[2] NIRMALA KESARI,[3] HARISH RAWAT,[4] LILITA BAHL,[5] SAVITRI THAKUR,[6] PATRICIA A. WOODS,[7] ROGER I. GLASS,[7] MAHARAJ K. BHAN,[1] AND JON R. GENTSCH[7]*

*Departments of Pediatrics and Microbiology, All India Institute of Medical Sciences, New Delhi,[1] Department of Pediatrics, Indira Gandhi Medical College, Bhopal,[2] Bapuji Child Health Institute and Research Center, J. J. Medical College, Davengere,[3] Department of Pediatrics, Government Medical College, Nagpur,[4] Indira Gandhi Medical College, Shimla,[5] and Department of Pediatrics, King George Medical College, Lucknow,[6] India, and Division of Viral and Rickettsial Diseases, Viral Gastroenteritis Section, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia[7]*

Received 8 August 1995/Returned for modification 13 October 1995/Accepted 20 November 1995

**Between April and December 1993, we determined P and G genotypes of group A rotavirus strains obtained from children admitted to diarrhea treatment centers in five Indian cities. From a total of 63 rotavirus-positive specimens, we identified 10 different strains with five different G genotypes and four distinct P types by using reverse transcription-PCR. The common worldwide strains $G_1P_8$, $G_2P_4$, $G_3P_8$, and $G_4P_8$ were underrepresented among Indian children (33%), whereas strains of P type 6 ($G_1P_6$, $G_2P_6$, $G_3P_6$, $G_4P_6$, and $G_9P_6$), which primarily infect asymptomatic newborns but are rare in children with diarrhea were common in India (43%). Of these, $G_9P_6$, a strain not previously reported to be found in children with diarrhea, was the most prevalent (22%). Eleven percent of the strains were nontypeable, and another 11% of the specimens had mixed infections. Using digoxigenin-labeled, genotype-specific hybridization probes, we confirmed all $G_9$ strains and mixed infections tested and identified three nontypeable strains (one $G_9$ and two $P_8$). The epidemiological significance of $G_9$ rotavirus strains, if confirmed in other settings, may have important implications for vaccine development.**

Studies in many countries suggest that $G_1P_8$, $G_2P_4$, $G_3P_8$, and $G_4P_8$ are the most common group A rotavirus (RV) strains found among children with diarrhea (2, 4, 16). Accordingly, the tetravalent reassortant RV vaccine currently under evaluation has been engineered to contain VP7 genes of serotypes $G_1$ to $G_4$, the most common G serotypes worldwide (6, 10). However, before these vaccines are tested in India, it is important to understand the epidemiology of RV strains currently circulating in the subcontinent. We report the results of our first year of surveillance of RV strains collected from children with diarrhea from five Indian cities. In this survey, we characterized the G and P types of the strains by reverse transcription-PCR (RT-PCR) and Southern hybridization.

The study was conducted between April and December 1993 in five diarrhea treatment centers at Shimla, Bhopal, Lucknow, Nagpur, and Davengere, India. Our goal was to obtain from each center about 20 RV isolates from children with acute diarrhea for further genotypic characterization. All subjects, aged between 6 months and 5 years, were admitted for treatment of acute dehydrating diarrhea. Chronic diarrhea was not observed. None of the infants were neonates or asymptomatic subjects. The stool specimens were transported frozen to the laboratory in the All India Institute of Medical Sciences, New Delhi, India, for determination of G and P types. Clarified 10% stool suspensions were tested for the presence of RV by a group A RV antigen enzyme immunoassay described previously (5). Genomic double-stranded RNA was extracted from RV-positive specimens by the glass powder method, and the P (VP4) and G (VP7) genotypes were determined by a one-step amplification RT-PCR method with type-specific primers as described previously (7, 8).

TABLE 1. Digoxigenin-labeled oligonucleotide probes used to analyze G- and P-genotype-specific PCR products of RV strains

| Probe[a] | Strain | Nucleotide positions | GenBank accession no. | Sequence | Hybridization temp (°C)[b] |
|---|---|---|---|---|---|
| G type | | | | | |
| $G_9$-2 | 116E | 251–270 | L14072 | aaa ttc ata aca aga caa gag ac | 62 |
| P type[c] | | | | | |
| $P_8$-1 | Wa | 315–332 | M96825 | gac tgc agt cgt tgc tat | 60 |
| $P_4$-1 | RV5 | 339–360 | M32559 | aca tgt tag tca aac aaa tag g | 62 |
| $P_6$-2 | 1076 | 111–135 | M88480 | gat tat tcc cgg acc gtt tgc tca a | 70 |
| $P_{11}$-1 | 116E | 77–100 | L07934 | taa tat agg tgc cag aaa gaa a | 62 |

[a] All probes were positive sense.
[b] Temperatures for probe hybridization were determined experimentally.
[c] P genotypes were described by Estes and Cohen (76).

* Corresponding author. Mailing address: Viral Gastroenteritis Section, MS-G04, Centers for Disease Control and Prevention, 1600 Clifton Rd. N.E., Atlanta, GA 30333. Phone: (404) 639-3577. Fax: (404) 639-3645.

TABLE 2. P and G genotypes of RV strains from children with diarrhea detected in five cities of India

| City | No. tested | No. of specimens with: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Common serotypes | | | | Classical neonatal strains | | | | | Other strains ($G_4P_{11}$) | Mixed infections | Nontypeable strain[a] |
| | | $G_1P_8$ | $G_2P_4$ | $G_3P_8$ | $G_4P_8$ | $G_1P_6$ | $G_2P_6$ | $G_3P_6$ | $G_4P_6$ | $G_9P_6$ | | | |
| Bhopal | 25 | 0 | 1 | 5 | 0 | 5 | 0 | 0 | 2 | 7[b] | 0 | 2[c] | 3 |
| Davengere | 2 | 1[d] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 |
| Shimla | 7 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 5[b,e] | 0 | | |
| Lucknow | 15 | 0 | 8 | 0 | 1[d] | 1 | 1 | 1 | 1 | 1[b] | 0 | 1 | |
| Nagpur | 14 | 0 | 3 | 0 | 0 | 0 | 0 | 1 | 0 | 2[d] | 1 | 4[c] | 3 |
| Total | 63 | 1 | 13 | 5 | 2 | 6 | 1 | 2 | 3 | 15 | 1 | 7 | 7 |

[a] Strains that could not be identified by RT-PCR and probe analysis.
[b] Three of three, three of three, and one of one genotype $G_9$ strains from Bhopal, Shimla, Lucknow, and Nagpur, respectively, were confirmed by probe analysis.
[c] Two strains from Bhopal and three of three strains from Nagpur were confirmed to be mixed (Bhopal, $P_6$ plus $P_8$ plus $P_{11}$; Nagpur, $P_6$ plus $P_{11}$).
[d] One nontypeable strain each from Davengere, Lucknow, and Nagpur was identified as type $P_8$, $P_8$, and $G_9$, respectively, by probe analysis.
[e] Two of two strains were confirmed as $P_6$ by sequence analysis (VP4 amino acid residues 13 to 134 [strain L1, accession no. U32620] and 111 to 224 [strain L53, accession no. U32621] were >90% identical to the sequence of $P_6$ strain ST3) (9).

Southern hybridization with oligonucleotide probes was carried out to confirm the P and G genotypes according to the protocols of Boehringer Mannheim Corp. as described by Ando et al. (1). The probes, labeled at the 5′ end with digoxigenin and homologous to internal sequences of the different P (types $P_4$, $P_8$, $P_6$, and $P_{11}$)-genotype-specific PCR products, were selected with the aid of multiple sequence alignments to maximize homology within the members of the same genotype

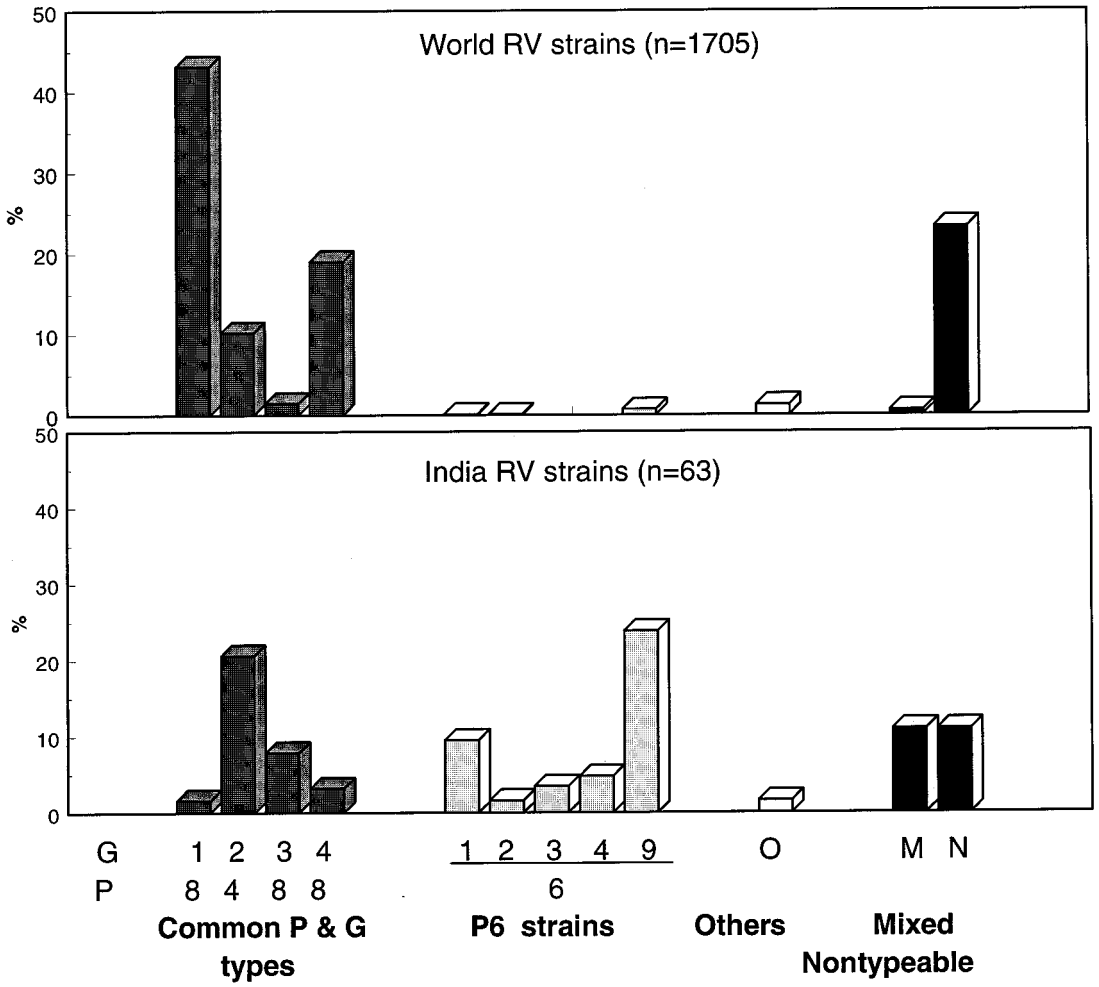


FIG. 1. Summary of RV strain types worldwide and comparison with those in India. (Top) Composite summary of the results of Bern et al. (3), Rasool et al. (11), Santos et al. (12), Silberstein et al. (13), and Steele et al. (14). Only studies in which both G and P genotypes were determined have been included. (Bottom) Genotypes of Indian strains from this study.

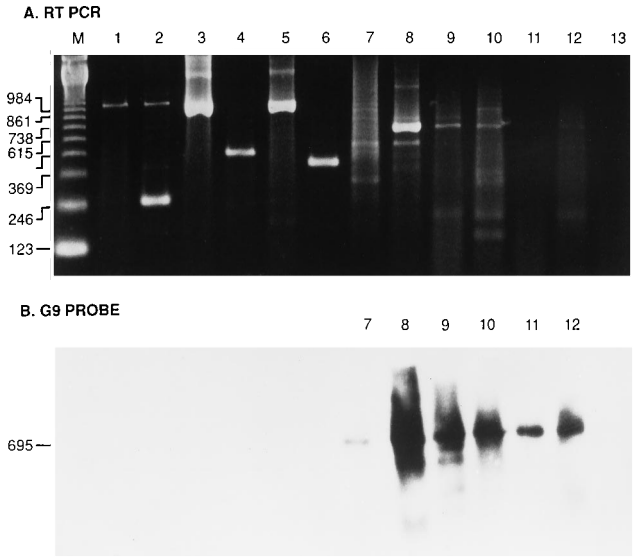


FIG. 2. Analysis of strain specificity of genotype $G_9$-specific probe by RT-PCR and Southern hybridization. An oligonucleotide probe (nucleotides 251 to 270 of the strain 116E VP7 gene) labeled at the 5′ end with digoxigenin was hybridized to VP7 gene PCR products of strains with different G genotypes. (A) Ethidium bromide-stained agarose gel showing PCR products. Lane M, 123-bp ladder molecular size markers; lane 1, $G_1$ strain Wa, full-length VP7 gene; lane 2, $G_2$ strain S2, VP7 gene typing product, nucleotides 37 to 281; lane 3, $G_3$ strain YO, full-length VP7 gene; lane 4, $G_3$ strain 157C, VP7 gene typing product, nucleotides 37 to 501; lane 5, $G_4$ strain Hochi, full-length VP7 gene; lane 6, $G_4$ strain Hochi VP7 gene typing product, nucleotides 37 to 440; lanes 7 to 11, genotype $G_9$ strains, including culture-adapted strains AC3/6 and 116E (lanes 7 and 8) and three $G_9$ clinical specimens (lanes 9 to 11); lanes 12 and 13, two previously nontypeable strains. The band in lane 7 is not visible by ethidium bromide staining. (B) Results of Southern hybridization of probe to PCR products immobilized on a nylon membrane. Lanes 7 to 12 are as described for panel A.

and minimize homology to members of distinct genotypes (Table 1) (7a). To confirm $G_9$ strains, we designed a primer pair based on the VP7 gene sequence of strain 116E (accession no. L14072) which amplified a 695-bp PCR product between nucleotides 131 and 826 and an internal probe in this region. All the primers and probes were synthesized in the Molecular Biology Core Facility of the Centers for Disease Control and Prevention, Atlanta, Ga.

Of the total of 458 stool specimens collected from children with diarrhea at treatment centers in five cities of India, 63 were positive for RV and were present in sufficient volumes for further testing. Using the one-step RT-PCR method without probe analysis, we could genotype 56 (89%) specimens (Table 2; Fig. 1). These included 10 distinct RV strains with five G genotypes and four P genotypes. Only one-third (21 of 63) of these were the four strains commonly associated with childhood diarrhea—$G_1P_8$, $G_1P_4$, $G_3P_8$, and $G_4P_8$. Forty-three percent were RVs previously called neonatal strains because they possessed $P_6$ specificity and were associated with G genotypes $G_1$, $G_2$, $G_3$, $G_4$, or $G_9$ (i.e., $G_1P_6$, $G_2P_6$, etc.). The most common strain, $G_9P_6$ (14 of 63), has not been reported previously to occur in children with diarrhea, and the second most common strain (13 of 63) was $G_2P_4$. Additionally, from a child with diarrhea in Nagpur, we identified a $P_{11}$ genotype that is usually found in bovine RVs or in those obtained from asymptomatic neonates. Some specimens had mixed infections (11%), and others had strains that were nontypeable (11%). Even within some cities, strain diversity was great; for example, the 15 RV-positive children from Lucknow had infections with seven distinct strains and the 25 positive children from Bhopal were infected with five distinct strains.

For selective confirmation of genotype assignments and to attempt to type viruses in samples that were negative by RT-PCR and ethidium bromide staining (i.e., nontypeable strains), we used hybridization with digoxigenin-labeled oligonucleotide probes. The strain specificities of the $G_9$ probe and of those homologous to internal sequences of the $P_4$-, $P_6$-, $P_8$-, and $P_{11}$-genotype-specific PCR products are shown (Fig. 2 and 3). The $G_9$ probe hybridized with the 695-bp VP7 gene product of strain 116E ($G_9P_6$) and genotype $G_9$ community strains but not to comparable products from strains of genotypes $G_1$ to $G_4$

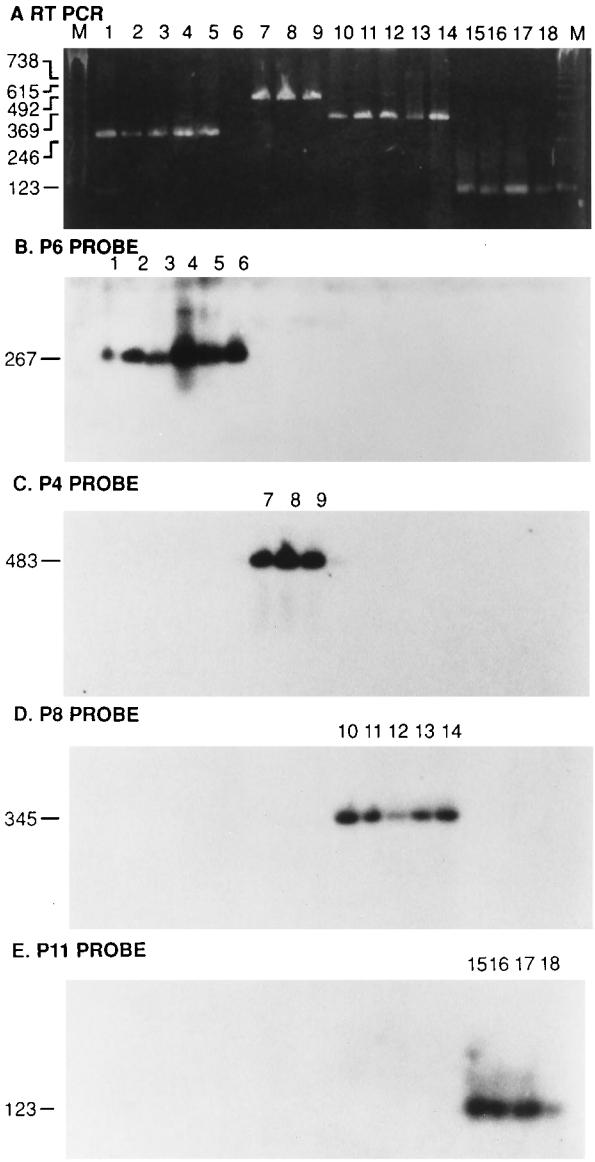


FIG. 3. Analysis of strain specificity of genotype $P_6$, $P_4$, $P_8$, and $P_{11}$ probes by RT-PCR and Southern hybridization. (A) Ethidium bromide-stained agarose gel showing PCR products. Lanes M, 123-bp ladder molecular size markers; lanes 1 to 18, genotype-specific PCR products of culture-adapted (lanes 1 to 4) or fecal (lanes 5 and 6) $P_6$ strains, from culture-adapted genotype $P_4$ (lanes 7 to 9) or $P_8$ (lanes 10 to 14) isolates, or from culture-adapted (lane 15) or fecal (lanes 16 to 18) $P_{11}$ strains. (B to E) Results of Southern hybridization of probes to PCR products immobilized on a nylon membrane. Relevant lanes are as described for panel A.

(Fig. 2). Altogether, we confirmed eight of eight genotype $G_9$-specific PCR products and classified one nontypeable strain (Fig. 2, lane 12) as genotype $G_9$.

The P-genotype-specific probes hybridized exclusively to the homologous PCR products as determined by control experiments done with standard RV strains (Fig. 3). Using these probes, we confirmed that five of five specimens had mixed infections with $P_6$ and $P_{11}$ genotypes and we identified two of eight nontypeable strains as genotype $P_8$.

This pilot study documents the extraordinary diversity of RV strains with distinct G and P genotypes identified among children with acute diarrhea. The 63 isolates in this study were of 10 distinct strains, and in two cities sampled—Bhopal and Lucknow—5 or more strains were circulating at the same time. Studies in most regions of the world have often identified only four serotypes to be common; unusual strains are rare and limited to geographically restricted areas (3, 11–14). Furthermore, the $G_1P_8$ strain, which is the most common in the rest of the world, was nearly absent in our small sample whereas $G_9$, a rare serotype worldwide, was the most common in India during the period of the study. Neonatal strain P type 6, which previously had been identified to be found primarily in asymptomatic neonates and was considered to be avirulent, was the most common P genotype among Indian patients with diarrhea. Finally, $P_{11}$, a strain found in animals and in Indian neonates, was also present in one child with diarrhea.

In this study, we used genotyping methods combined with Southern blot hybridization to fully characterize the strains that previously would have been considered nontypeable. It is conceivable that if these methods were applied to field isolates from other areas where a large proportion of strains remain untypeable, other unusual strains might be found to be present. A recent study done in Brazil has also identified several uncommon RV strains with distinct G and P types, including P genotype 6 in 15% of the typeable single infections, similar to our findings in India (15). Further, on the basis of genotyping methods, investigators from many countries have recently identified new P and G types circulating in their respective settings (12–15).

The main limitation of this study has been the small number of samples collected during this first year of surveillance. As a result, we can neither characterize the full diversity of strains in circulation nor explain trends in strain distribution by time or place within India.

The current study has important implications for the development of an effective RV vaccine for developing countries. Since RV strains in India include an additional G serotype, $G_9$, along with serotypes $G_1$ to $G_4$, it is possible that the tetravalent reassortant RV vaccine might not confer desired levels of protection in this setting. The epidemiological importance of serotype $G_9$ needs to be determined in other developing countries. It is also important to determine whether the tetravalent vaccine will provide cross-protection against $G_9$ infections, in which case the four G serotypes in the vaccine would suffice.

This study was supported in part by a grant from the Indo-U.S. Vaccine Action Program.

## REFERENCES


1. **Ando, T., S. S. Monroe, J. R. Gentsch, Q. Jin, D. C. Lewis, and R. I. Glass.** 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridization. J. Clin. Microbiol. **33:**64–71.
2. **Beards, G., U. Desselberger, and T. Flewett.** 1989. Temporal and geographical distributions of human rotavirus serotypes, 1983 to 1988. J. Clin. Microbiol. **27:**2827–2833.
3. **Bern, C., L. Unicomb, J. Gentsch, N. Banul, R. B. Sack, R. I. Glass, and M. Yunus.** 1992. Rotavirus diarrhea in Bangladeshi children: correlation of disease severity with serotypes. J. Clin. Microbiol. **30:**3234–3238.
4. **Bishop, R., L. Unicomb, and G. Barnes.** 1991. Epidemiology of rotavirus serotypes in Melbourne, Australia, from 1973 to 1989. J. Clin. Microbiol. **29:** 862–868.
5. **Cicirello, H. G., B. K. Das, A. Gupta, M. K. Bhan, J. R. Gentsch, R. Kumar, and R. I. Glass.** 1994. High prevalence of rotavirus infection among neonates born at hospitals in Delhi, India: predisposition of newborns for infection with unusual rotavirus. Pediatr. Infect. Dis. J. **13:**720–724.
6. **Clark, H. F., D. Welsko, and P. A. Offit.** 1992. Infant responses to rotavirus WC3 reassortants containing human rotavirus VP7, VP4 or VP7+VP4, abstr. 1394. *In* Program and abstracts of the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
7. **Das, B. K., J. R. Gentsch, H. G. Cicirello, P. A. Woods, M. Ramachandran, A. Gupta, M. Ramachandran, R. Kumar, M. K. Bhan, and R. I. Glass.** 1994. Characterization of rotavirus strains from newborns in New Delhi, India. J. Clin. Microbiol. **32:**1820–1822.

7a. **Devereux, J., P. Haberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.

7b. **Estes, M. K., and J. Cohen.** 1989. Rotavirus gene structure and function. Microbiol. Rev. **53:**410–449.

8. **Gentsch, J. R., R. I. Glass, P. Woods, V. Gouvea, M. Gorziglia, J. Flores, B. K. Das, and M. K. Bhan.** 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. J. Clin. Microbiol. **30:**1365–1373.
9. **Glass, R. I., J. Keith, O. Nakagomi, T. Nakagomi, J. Askaa, A. Z. Kapikian, R. M. Chanock, and J. Flores.** 1985. Nucleotide sequence of the structural glycoprotein VP7 gene of Nebraska calf diarrhea virus rotavirus: comparison with homologous genes from four strains of human and animal rotaviruses. Virology **141:**292–298.
10. **Kapikian, A. Z.** 1994. Jennerian and modified Jennerian approach to vaccination against rotavirus diarrhea in infants and young children: an introduction, p. 409–417. *In* A. Z. Kapikian (ed.), Viral infections of the gastrointestinal tract, 2nd ed. Marcel Dekker, Inc., New York.
11. **Rasool, N. B. G., G. Larralde, and M. I. Gorziglia.** 1993. Determination of human rotavirus VP4 using serotype-specific cDNA probes. Arch. Virol. **133:** 275–282.
12. **Santos, N., M. Riepenhoff-Talty, H. F. Clark, P. Offit, and V. Gouvea.** 1994. VP4 genotyping of human rotavirus in the United States. J. Clin. Microbiol. **32:**205–208.
13. **Silberstein, I., L. M. Shulman, E. Mendelson, and I. Shif.** 1995. Distribution of both rotavirus VP4 genotypes and VP7 serotypes among hospitalized and nonhospitalized Israeli children. J. Clin. Microbiol. **33:**1421–1422.
14. **Steele, A. D., M. C. van Niekerk, and M. J. Mphahlele.** 1995. Geographic distribution of human rotavirus VP4 genotypes and VP7 serotypes in five South African regions. J. Clin. Microbiol. **33:**1516–1519.
15. **Timenetsky, M. D. S. T., N. Santos, and V. Gouvea.** 1994. Survey of rotavirus G and P types associated with human gastroenteritis in Sao Paulo, Brazil, from 1986 to 1992. J. Clin. Microbiol. **32:**2622–2624.
16. **Woods, P. A., J. Gentsch, V. Gouvea, L. Mata, A. Simhon, M. Santosham, Z.-S. Bai, S. Urasawa, and R. I. Glass.** 1992. Distribution of serotypes of human rotavirus in different populations. J. Clin. Microbiol. **30:**781–785.