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Identification of Group A Rotavirus Gene 4 Types by Polymerase Chain Reaction

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Five genetically distinct human rotavirus (HRV) gene 4 groups have been described on the basis of comparative nucleotide sequencing and the predicted amino acid sequences, and at least four of them represent distinct VP4 antigenic types. To identify each gene 4 type and investigate its distribution in HRV isolates from patients with diarrhea, we developed a polymerase chain reaction (PCR) typing method using sequence information available for four genetically distinct gene 4 types. Rotavirus double-stranded RNAs (dsRNAs) isolated from stool samples were first reverse transcribed and amplified by PCR by using two oligonucleotide primers that correspond to regions that are highly conserved among all known HRV gene 4 types. The 876-bp dsDNA products were then reamplified by PCR in the presence of a cocktail containing one conserved plus-sense primer and four type-specific minus-sense primers (selected from the hypervariable region of gene 4), resulting in products of 345, 483, 267, and 391 bp corresponding to gene 4 types 1, 2, 3, and 4, respectively. This method reliably identified the gene 4 types of 16 well-characterized HRV isolates. Our results were independently confirmed for all 16 strains by reverse transcription and PCR amplification of HRV dsRNA in the presence of alternate type-specific primer pairs. For direct gene 4 typing of HRV in stool samples, we developed a method to extract rotavirus dsRNA from stool specimens by using glass powder. Our results suggest that gene 4 typing will be useful in providing more a complete characterization of HRV strains of epidemiologic or vaccine-related interest.

The importance of group A human rotaviruses (HRVs) in diarrheal illnesses of infants and young children has resulted in efforts to develop a vaccine (7) and to characterize the antigens involved in immunity. At least seven serotypes of HRV have been described on the basis of cross-neutralization studies with hyperimmune sera containing neutralizing antibodies to both the VP7 and VP4 polypeptides (15, 30). Genetic and molecular experiments and studies with neutralizing, serotype-specific monoclonal antibodies (MAbs) have identified VP7 as a major type-specific neutralization protein (15). The availability of MAbs that specifically bind the VP7 polypeptides of HRV serotypes 1 to 4 led to the development of enzyme immunoassays (EIAs) for the rapid serotyping of HRV in stool samples (31). As a result, antigenic diversity in the VP7 polypeptide of HRV is well defined. These methods have been used in epidemiologic surveys of circulating HRVs to demonstrate that serotypes 1 to 4 are found worldwide, whereas the distribution serotypes 8 and 9 and the newly described serotype 12 have not been studied extensively (15, 30, 32).

Antigenic diversity within the VP4 neutralization antigen has not been clearly defined. The observation that rotaviruses isolated from humans or animals may possess dual serotype specificities and that the second specificity resides on VP4 suggests that a complete antigenic characterization of rotaviruses should include VP4 as well as VP7 (13, 25). Through comparative nucleotide sequencing, five genetically distinct gene 4 types have been identified among HRV strains on the basis of sequence and predicted amino acid conservation in strains that possess the same type and extensive diversity in strains with a different type (9). Strains that possess one or the other of the gene 4 types (referred to as P types 1 to 5) have been designated as belonging to VP4 genetic groups 1 to 5 (9). P type 1 is present in symptomatic strains of VP7 serotypes 1, 3, 4, and 9 (designated VP4 genetic group 1), P type 2 is present in members of VP7 serotype 2 (genetic group 2), P type 3 is present in strains with VP7 serotype 1 to 4 specificities (so far isolated only from newborn infants excreting rotavirus asymptomatically) (genetic group 3), P type 4 is present in the VP7 serotype 1 strain K8 (genetic group 4) (29), and P type 5 is present in the VP7 serotype 8 strain 69M (genetic group 5) (27). Recently, experimental evidence that members of VP4 genetic groups 1 to 4 can be divided into three antigenic groups, tentatively designated serotypes, and one subtype has been presented. The evidence was obtained on the basis of cross-neutralization tests with antisera to baculovirus-expressed VP4 polypeptides of prototype strains KU (group 1), DS1 (group 2), 1076 (group 3), and K8 (group 4) (10). Furthermore, all members of the same genetic group fell within the same antigenic group. Taken together, these results suggest that methods for identifying VP4 genetic groups at the nucleic acid level would be a valid proxy method to assess the diversity of gene 4 in circulating strains of HRV.

Recently, a hybridization method for identifying VP4 genetic groups has been described (17). In the present study we report the development of a polymerase chain reaction (PCR) method to identify (or type) gene 4 types 1 to 4 and demonstrate that 16 prototype strains from known VP4

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genetic groups could reliably be identified. Furthermore, to increase the sensitivity of PCR for clinical specimens, we developed a method for direct extraction of rotavirus double-stranded RNA (dsRNA) from stool specimens using glass powder. This method, in conjunction with gene 4 typing, can be used to study the molecular epidemiology of HRV gene 4 in nature. Along with the EIA (31), hybridization (6), and PCR (12) methods for identifying VP7 serotypes, gene 4 typing permits a more complete characterization of neutralization genes and antigens of epidemiologically important rotaviruses and may facilitate the discovery of new gene 4 types.

MATERIALS AND METHODS

Cells. MA104 cells were obtained from the Biologic Products Branch of the Centers for Disease Control and were grown in medium 199 (GIBCO, Long Island, N.Y.) with 5% fetal bovine serum at 37°C in a water-jacketed incubator with 5% CO_2 or in a 37°C room on a roller bottle apparatus.

Viruses. HRV strains were grown in MA104 cells and were purified by established methods (20). Virus particle and nucleic acid concentrations were estimated from the relationship that purified virions suspended at 185 μg of viral protein per ml contain a particle concentration of 2.1×10^{12}/ml and a dsRNA concentration of 45 μg/ml (23). Viral protein was estimated by using the Bio-Rad assay (Richmond, Calif.).

Stool samples were obtained from collections previously sent to the Centers for Disease Control for analysis. Samples from the United States were from patients with gastroenteritis and were submitted by members of the Rotavirus Study Group (19). Samples from Mexico and Costa Rica were also from patients with gastroenteritis or were from asymptomatic infants who secreted rotavirus and were submitted by G. Ruiz-Palacios and L. Mata for a collaborative serotyping project (32). Venezuelan samples were from asymptomatic newborn infants (in neonatal hospital wards) who excreted rotavirus (17). Samples for gene 4 typing were selected randomly from samples with known (G, VP7) serotypes, without regard to virus particle number.

Enzymes. Super-reverse transcriptase was from Molecular Genetics Resources (Tampa, Fla.), *Taq* and Amplitaq polymerases were from Perkin-Elmer Cetus (Norwalk, Conn.), and proteinase K was from Boehringer Mannheim (Indianapolis, Ind.).

Oligonucleotide primers. Primers were synthesized in the CDC Biotechnology Core Facility, Centers for Disease Control (Brian Holloway), and were used without purification. One pair of primers, con 3 and con 2, made against the KU virus gene 4 sequence (accession number M21014) (Fig. 1) was chosen for the first amplification because the primers correspond to regions that are highly conserved among HRV strains from VP4 genetic groups 1 to 4, as determined by best fit analysis with the University of Wisconsin Genetics Computer Group sequence analysis program (3) (using sequences found in the GenEMBL data bank). Gene 4 sequences for HRV strains 1076, K8 (30) (accession number not available), RV5 (accession number M32559), and 69M (accession number M60600) were loaded into a VAX computer directory for use with the Genetics Computer Group program. The nucleotide sequence of the gene 4 of strain 1076 was provided by one of the authors (8). The typing primers were selected from the regions (the 5'-terminal 30%) of gene 4 known to be highly divergent between strains in different genetic groups and (when data were available) highly conserved in strains

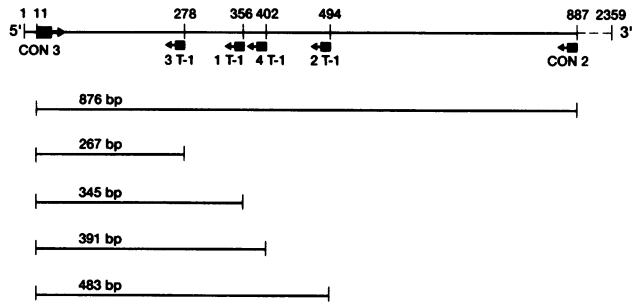

FIG. 1. HRV gene 4. The schematic shows the positions and directions of amplification relative to those for the plus (mRNA)-sense genomic strand for the consensus primers con 3 and con 2 and for the gene 4 type-specific primers 1T-1 to 4T-1. The sizes of the expected products of amplification from con 3, con 2 (first amplification), and con 3 plus 1T-1 through 4T-1 (second amplification, gene 4 typing PCR) are also shown.

from the same group (9). Five specific typing primers were prepared for gene 4 sequences of KU (primer 1T-1, VP4 genetic group 1), RV5 (primer 2T-1, group 2), 1076 (primer 3T-1, group 3), K8 (primer 4T-1, group 4), and 69M (primer 5T-1, group 5) (primer 5T-1 was selected after the sequence of the strain 69M gene 4 was published and does not appear in Fig. 1). The nucleotide (nt) positions and sequences of these primers (5' to 3') were as follows: con 3 (nt 11 to 32), TGGCTTCGCCATTTTATAGACA; con 2 (nt 868 to 887), ATTTCGGACCATTTATAACC; 1T-1 (nt 339 to 356), TCT ACTTGGATAACGTGC; 2T-1 (nt 474 to 494), CTATTGTT AGAGGTTAGAGTC; 3T-1 (nt 259 to 278), TGTTGATTAG TTGGATTCAA, 4T-1 (nt 385 to 402), TGAGACATGCAA TTGGAC; 5T-1 (nt 575 to 594), ATCATAGTTAGTAG TCGG.

A second set of genetic group-specific primer pairs was selected to confirm the results obtained with the typing primers. Their nucleotide positions on gene 4, polarities (plus or minus sense), and sequences (5' to 3') were as follows: 1C-1 (nt 314 to 331, plus sense), GGACTGCAGTA GTTGCTA; 1C-2 (nt 474 to 494, minus sense), TTAGTAT CAGAAGTTAGTGTA; 2C-1 (nt 1324 to 1344, plus sense), ATACGAACACGTACAATAAAC; 2C-2 (nt 1809 to 1828, minus sense), CATCATTTACTGAGTCAGTT; 3C-1 (nt 261 to 278, plus sense), GAATCCAACTAATCAACA; 3C-2 (nt 446 to 467, minus sense), TGTTGAAATTCGGCACTAACA; 3C-3 (nt 288 to 312, plus sense), AGAGGGTACCAA TAAAACTGATAT; 3C-4 (nt 589 to 606, minus sense), TGC AGTTTCTACTTCAGA; 4C-1 (nt 223 to 242, plus sense), ACCTCACTCAACTTAGT; and 4C-2 (nt 464 to 484, minus sense), ATAATGTTGAATATTGAGTGT.

RNA extraction. Rotavirus dsRNA was extracted from lysates of rotavirus-infected MA104 cells with phenol–chloroform–1% sodium dodecyl sulfate by a standard method (12). The RNA to be used for PCR was precipitated twice with 2 volumes of ethanol at −20°C overnight, dried under vacuum, and resuspended in H_2O. The concentration of rotavirus dsRNA from cell lysates was estimated by comparison with known amounts of dsRNA from purified virions that were analyzed by polyacrylamide gel electrophoresis (PAGE) and silver staining (16, 26).

HRV dsRNA was extracted from stool specimens with a commercial glass powder preparation (RNAID or Geneclean II; Bio 101, Inc., La Jolla, Calif.) by using a modification of a published procedure (33). ISOGENE (Perkin-Elmer Cetus,

Norwalk, Conn.) was also tested. Stool samples were suspended in 50 mM Tris-hydrochloride (pH 7.5) at approximately 10 to 20% (wt/vol) or 10% (vol/vol) and were clarified by centrifugation at 8,000 × *g* in a Beckman microcentrifuge with a bowl rotor for 7 min. For primer sensitivity determinations, purified virus particles were diluted serially in clarified, rotavirus-negative stool supernatants, and the dsRNA was extracted as described below. For some experiments, 200 to 400 μl of the supernatant was extracted with an equal volume of Freon and was clarified by centrifugation at 8,000 × *g* for 5 min, and 200 to 400 μl of this supernatant was mixed with sufficient 6 M guanidine thiocyanate to give a final concentration of 3.4 M; for other experiments, 200 to 400 μl of the clarified stool supernatant was mixed directly with guanidine thiocyanate (ultrapure grade; Boehringer Mannheim) to give the same final concentration. RNAID (10 or 12 μl) was added to this mixture, and the sample was vortexed and mixed on a Nutator rocker (Clay Adams Division, Becton-Dickinson, Parsippany, N.J.) for 10 min at room temperature. Each sample was then centrifuged for 30 s at 650 × *g* in a Beckman microcentrifuge, and the supernatant was removed by aspiration with separate Pasteur pipettes for each sample. The samples were then washed two times with 400 μl of the RNAID kit wash buffer and centrifuged at 850 × *g*. The supernatant was aspirated, and the samples were washed once more with the same buffer and were then finally centrifuged at 10,000 × *g* for 60 s. After aspiration of the supernatant, the samples were dried under vacuum for 5 min, resuspended in 17.5 to 25 μl of deionized H_2O, and incubated for 10 min at 65°C. The samples were centrifuged at 10,000 × *g* for 30 s, and the supernatant was transferred to microcentrifuge tubes (Lube Tube; Marsh Co., Rochester, N.Y.). The pellet was reextracted with the same volume of water (17.5 to 25 μl), and the combined supernatants were stored at −20°C until they were used. Immediately before use for PCR, the supernatants were incubated at 56°C for 5 min and were then centrifuged at 10,000 × *g* for 15 s to pellet the residual RNAID from the sample.

PCR. Our strategy to develop a PCR typing method for gene 4 types was identical, in principle, to that used by Gouvea and coworkers (12) for gene 9 (VP7) typing by PCR. dsRNA was first reverse transcribed and amplified by PCR (designated the first amplification step) with a primer pair corresponding to gene 4 sequences that are highly conserved among strains from VP4 genetic groups 1 to 4 (Fig. 1, primers con 3 and con 2). Portions of the 876-bp product dsDNAs were then amplified by PCR by using a cocktail of primers that included the plus-sense consensus primer con 3 and the four minus-sense genetic group-specific primers 1T-1, 2T-1, 3T-1, and 4T-1. The nucleotide mismatch between the specific primers and the corresponding heterologous sequences ranged from 39 to 67%. Selection of specific primers that gave products of different sizes permitted identification of the VP4 genetic group by agarose gel analysis. The use of a cocktail of primers has the added advantage of identifying in one PCR the genetic group of any HRV isolate from VP4 genetic groups 1 to 4.

For PCR experiments, dsRNA (approximately 50 to 250 ng per reaction) was prepared from rotavirus-infected cell lysates or stool extracts. A two-amplification procedure was usually used. Briefly, 1 to 5 μl of dsRNA was added to 0.5 ml of low-bind microcentrifuge tubes containing 3.5 μl of dimethyl sulfoxide (Sigma, St. Louis, Mo.) in a final volume of 8.5 μl, and the samples were mixed and denatured at 97°C for 5 min in a heating block that contained mineral oil in its wells. The samples were then cooled on ice for 5 min and centrifuged at 10,000 × *g* for 10 s to remove the condensation from the walls of the tubes. A reverse transcription PCR mixture (41.5 μl) containing 12 to 13.5 μl of H_2O, 16 μl of deoxynucleoside triphosphate mixture (containing 1.25 mM [each] dATP, dGTP, dCTP, and dTTP; Pharmacia-LKB, Piscataway, N.J.), 5 μl of 10× buffer II (100 mM Tris-hydrochloride [pH 8.3], 500 mM KCl) (Perkin-Elmer Cetus), 3.5 to 5 μl of 25 mM $MgCl_2$, 2 μl of primer (containing 25 μM [each] con 3 and con 2), and 1.5 μl of RT reverse transcriptase-Amplitaq or reverse transcriptase-*Taq* mixture (containing 9 U of reverse transcriptase and 1.9 U of *Taq* or Amplitaq) (*Taq* or Amplitaq worked equally well) was then added to each denatured dsRNA sample tube (to give a final reaction volume of 50 μl). About 100 μl of mineral oil (catalog no. M-3516; Sigma) was then added. The samples were then mixed by gentle flicking, centrifuged at 10,000 × *g* for 5 s, and subjected to one cycle of reverse transcription (42°C, 30 min) and 30 cycles of PCR, both of which were done in thermal cycler (Ericomp, Inc., San Diego, Calif.). Each PCR cycle contained steps of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C. A cooling cycle was used to bring the samples to 17°C at the completion of the experiment.

More recently, we have used a separate reverse transcription reaction in which the composition of the master mix was the same as that described above, except that the final volume was 49 μl instead of 50 μl, *Taq* was left out, and mineral oil was not added. In this case, after denaturation of the RNA and addition of the master mix, the reverse transcription reaction was incubated for 60 min at 42°C in a circulating H_2O bath. One microliter (1.9 U) of *Taq* was then added; this was followed by the addition of 100 μl of mineral oil, and 30 cycles of PCR were carried out by using the same steps described above.

For the typing reaction (second amplification), 0.5 to 5.0 μl of the first amplification product (5 μl was used if there was no visible product and 0.5 μl was used if there was a large amount of product) was mixed with 45 μl of reaction mixture in a final volume of 50 μl. When less than 5 μl of the first amplification product was used, the volume was completed to 50 μl with 10 mM Tris-hydrochloride (pH 8.3)–2.5 mM $MgCl_2$. The components of the reaction mixture (per 45 μl) were 19.5 μl of water, 16 μl of deoxynucleoside triphosphate mixture, 5 μl of 10× buffer II, 3 μl of 25 mM $MgCl_2$ (including the contribution of the first-amplification DNA product; final $MgCl_2$ concentration, 1.75 mM), 1 μl of the typing primer cocktail (containing 20 μM [each] con 3, 1T-1, 2T-1, 3T-1, and 4T-1), and 0.5 μl (2.5 U) of Amplitaq. The samples were overlaid with mineral oil, mixed by gentle flicking, centrifuged, and subjected to 15 to 25 cycles of PCR by using the same steps and cycles described above. The samples were then analyzed by agarose gel electrophoresis.

Confirmation PCRs were carried out by using dsRNA as the template and the same conditions as described above for the first amplification of the typing reaction. The number of PCR cycles varied from 25 to 30. Magnesium chloride concentrations varied from 1.5 to 2.5 mM for the confirmation primer pairs.

Agarose gel analysis. Agarose gel analysis of PCR products was carried out by standard methods (11) by using 1.5 and 3.0% gels (and a 2:1 ratio of Nusieve GTG-Seaplaque [FMC Bioproducts, Rockland, Maine]) for the first and second PCR amplification products, respectively.

EIA serotyping. Strains were serotyped by using a MAb EIA (31).

TABLE 1. HRV strains used to test gene 4 PCR typing method

Strain[a]	VP7 serotype	VP4 serotype[b]	VP4 genetic group[c]
Wa	1	1A	1
KU	1	1A	1
YO	3	1A	1
P	3	1A	1
VA70	4	1A	1
HOCHI	4	1A	1
F45	9	1A	1
WI61	9	1A	1
DS1	2	1B	2
S2	2	1B	2
M37	1	2	3
1076	2	2	3
McN13	3	2	3
ST3	4	2	3
K8	1	3	4
AU1	3	?	4(?)
69M	8	?	5

[a] Only the nucleotide sequences of strains Wa, KU, P, VA70, DS1, RV5 (not used in this study), M37, 1076, McN13, ST3, K8, and 69M have been completed (9, 14, 27, 29).
[b] Adapted from reference 10.
[c] Adapted from reference 9.

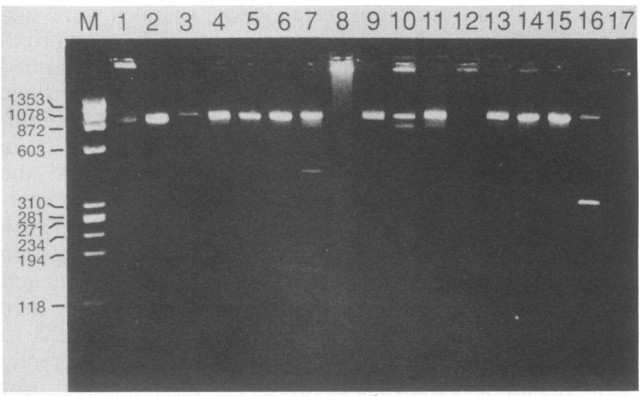

FIG. 2. Reverse transcription and PCR amplification (first amplification) of rotavirus dsRNAs with consensus primers con 3 and con 2. dsRNAs were prepared from lysates of infected cells by phenol-chloroform extraction and were amplified as described in Materials and Methods. Products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Markers (lane M) are ϕX174 and *Hae*III restriction fragments. Products were amplified from HRV strains possessing gene 4 type 1 (lane 1, Wa viral dsRNA; lane 2, KU; lane 3, YO; lane 4, P; lane 5, VA70; lane 6, HOCHI; lane 7, F45; lane 8, WI61), type 2 (lane 9, DS1; lane 10, S2), type 3 (lane 11, M37; lane 12, 1076; lane 13, ST3; lane 14, McN13), type 4 (lane 15, K8), and type 5 (lane 17, 69M). The sequence of strain AU1 gene 4 (lane 16) has not been published. Numbers on the right are expressed in base pairs.

Nucleotide sequence accession number. The GenBank accession number for the gene 4 sequence for HRV strain 1076 is M88480.

RESULTS

HRV strains used to test gene 4 typing method. To validate the typing method for gene 4, we assembled a panel of well-characterized strains (Table 1) from VP4 genetic groups 1 to 5. A direct correlation exists between genetic groups, as determined by nucleotide sequence conservation (9) and VP4 serotype (10), suggesting that methods for identifying gene 4 types at the nucleic acid level are a valid proxy for studying the gene 4 diversity of circulating HRV strains.

Gene 4 typing by PCR. Analysis of 17 strains was carried out by two PCR amplifications. In the first, use of the primers con 2 and con 3 yielded intense dsDNA products of the predicted size (876 bp) for 14 of the 17 strains tested (Fig. 2). With strain WI61 (Fig. 2, lane 8), the absence of a product band was probably an artifact caused by extraneous material, perhaps cellular nucleic acids (after PAGE and silver staining, this preparation had a very high background) in the RNA preparation. Likewise, the high-molecular-weight bands observed in strain WI61 and several other products (especially products from strains Wa and S2; Fig. 2, lanes 1 and 10, respectively) may be artifacts of amplification of complex nucleic acids from cells, since they were not primer specific (also see Fig. 4) and were not observed after amplification of rotavirus RNAs extracted from stools. We confirmed that WI61 dsRNA can be efficiently amplified with con 3 and con 2 by repeating this experiment with RNA from a partially purified virus preparation (data not shown). We also demonstrated, using other dsRNA preparations, that strain 1076 (Fig. 2, lane 12) can be reproducibly amplified to produce an 876-bp dsDNA, although at a somewhat lower yield (data not shown).

The nature of the nonspecific but discrete products that we and others have observed at various intensities from experiment to experiment (Fig. 2 and 3) (22) has not been characterized, but it probably results from mispriming events during amplification of complex nucleic acids (4). These products do not interfere with gene 4 typing.

When portions of the first amplification products were subjected to gene 4 typing PCR (Fig. 3), we observed that only the eight reactions primed with dsDNA derived from HRV strains from VP4 genetic group 1 produced a dsDNA product of about 345 bp (Fig. 3, lanes 1 to 8), the expected size of the product of con 3 and the genetic group 1-specific primer 1T-1 (Fig. 1). No significant products indicative of

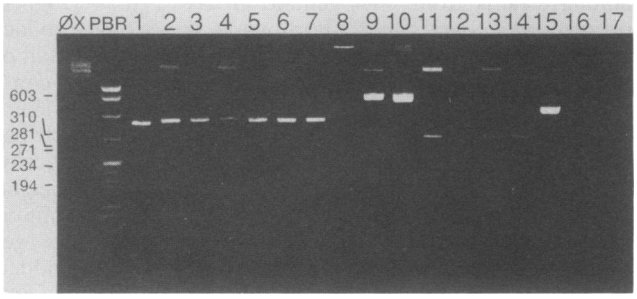

FIG. 3. Typing by PCR of gene 4 from HRV strains. Portions of the products from reactions used to generate Fig. 2 were amplified by PCR by using a cocktail of consensus primer con 3 and primers 1T-1 to 4T-1 and were analyzed by agarose gel electrophoresis and ethidium bromide staining. The lane designations are the same as those in Fig. 2. Because the ϕX174 (ϕX) markers were very faint, the positions of fragments of relevant sizes (603 to 194 bp) are indicated on the left. The relevant fragments of the pBR restriction fragments are (PBR lane, largest to smallest fragments) 622, 527, 404, 309, and 242 plus 238 bp, respectively.

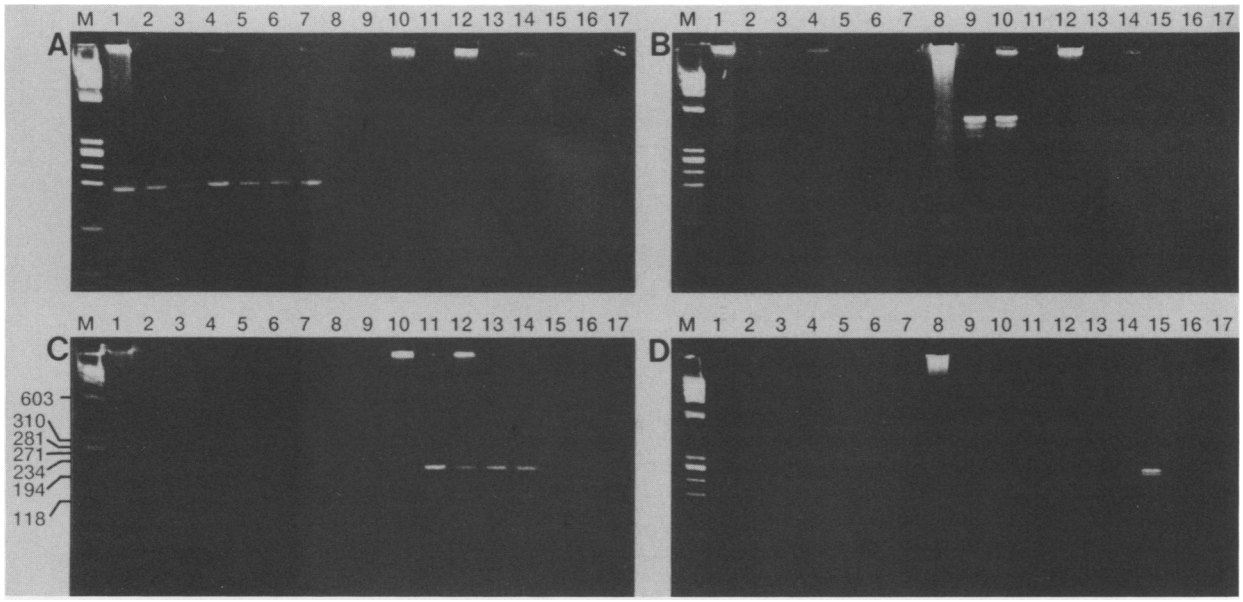

FIG. 4. Confirmation of HRV gene 4 types by PCR. Rotavirus dsRNAs were reverse transcribed and amplified by PCR by using a gene 4 type 1-specific primer pair (A), a type 2-specific primer pair (B), a type 3-specific primer pair (C), or a type 4-specific primer pair (D). The strains that were amplified and the lanes in which they tested are the same as those in Fig. 2.

hybridization of group 2-, 3-, or 4-specific primers to the dsDNAs from strains that possess genetic group 1 gene 4's were observed (i.e., products of 267, 391, or 483 bp). Likewise, only reactions primed with dsDNAs from strains that possess gene 4's from genetic group 2, 3, or 4 produced products of about 483 bp (genetic group 2; Fig. 3, lanes 9 and 10), 267 bp (group 3; Fig. 3, lanes 11 to 14), and 391 bp (group 4; Fig. 3, lane 15), which are the predicted lengths of the products of amplification of these DNAs with con 3 and the group-specific primers 2T-1, 3T-1, and 4T-1, respectively. The typing band for strain WI61 (Fig. 3, lane 8) was not visible, while the band for strain 1076 (Fig. 3, lane 12) was faintly visible. We subsequently demonstrated, using other RNA preparations, that both can be reliably typed (data not shown). Strain AU1 (Fig. 3, lane 16) was included in this experiment because the genes of this strain appear to be closely related to those of strain K8 (i.e., as a possible second member of VP4 genetic group 4), as determined by hybridization (5, 24). Strain AU1 gave a very weak band of 391 bp (comigrating with the product of strain K8) and several nonspecific bands, suggesting that it cannot be reliably typed. The reason that we cannot amplify strain AU1 efficiently will not be known until the AU1 gene 4 is sequenced. In the meantime, we are trying to obtain alternate K8-like and AU1-like isolates to determine whether this result is peculiar to the AU1 isolate in our laboratory.

The failure of strain 69M to give a detectable product (Fig. 3, lane 17) was not surprising, since it possesses a unique gene 4 whose sequence was not published when we carried out this study. After its sequence was published, we designed two typing primers for 69M and have shown that (when they were used in a primer cocktail with con 3 and 1T-1 to 4T-1) they produce 583- and 660-bp products from 69M DNA after the first amplification but not from comparable DNAs produced from VP4 genetic group 1 to 4 strains (data not shown). Taken together, these results suggest that the VP4 genetic groups of 16 of 16 well-characterized (excluding strain AU1) prototype HRV strains can be reliably identified on the basis of the size of the PCR products produced during the second (typing) PCR amplification.

PCR confirmation of gene 4 typing. Additional genetic group-specific primer pairs (one plus-sense and one minus-sense primer for each pair) were selected for each genetic group for cases in which confirmation of a typing reaction was needed. Since some of these primer pairs interfere with each other when they are used as a cocktail, they were used separately in PCRs, starting with HRV dsRNA as a template (i.e., confirmation is a one-step reverse transcription and PCR amplification procedure). To summarize these results, primer pairs 1C-1 and 1C-2, 2C-1 and 2C-2, 3C-1 and 3C-2, and 4C-1 and 4C-2 (specific for genetic groups 1, 2, 3, and 4, respectively) specifically reverse transcribe and amplify dsRNA of HRV strains from VP4 genetic groups 1 to 4, respectively, to yield 180-, 504-, 206-, and 261-bp products (Fig. 4, A to D, respectively). Although strain WI61 did not produce a specific product in the experiment (Fig. 4A, lane 8), we confirmed that primer pairs 1C-1 and 1C-2 can specifically reverse transcribe and amplify dsRNA from partially purified WI61 virus particles (data not shown).

Gene 4 typing of HRV in stool samples. To study the diversity of gene 4 types in circulating strains of HRV, we first developed a method to improve the extraction of dsRNA from stool samples. Recent experience with our previous methods demonstrated that some specimens amplified poorly; we believed that this was due to inhibitors of reverse transcriptase that were present in the stool samples (11). A recent report (33) described the use of glass powder for RNA extraction and suggested that inhibitors in stool could be reduced or eliminated by its use (33). Several types of glass powder, ISOGENE, GENECLEAN II, and RNAID, are commercially available (33, 34). Although our preliminary results showed that all three glass powders could extract dsRNA from stool samples, we chose to analyze RNAID in detail because of the better qualitative results obtained with that glass powder. The RNAID method was chosen over the hydroxyapatite method reported previously

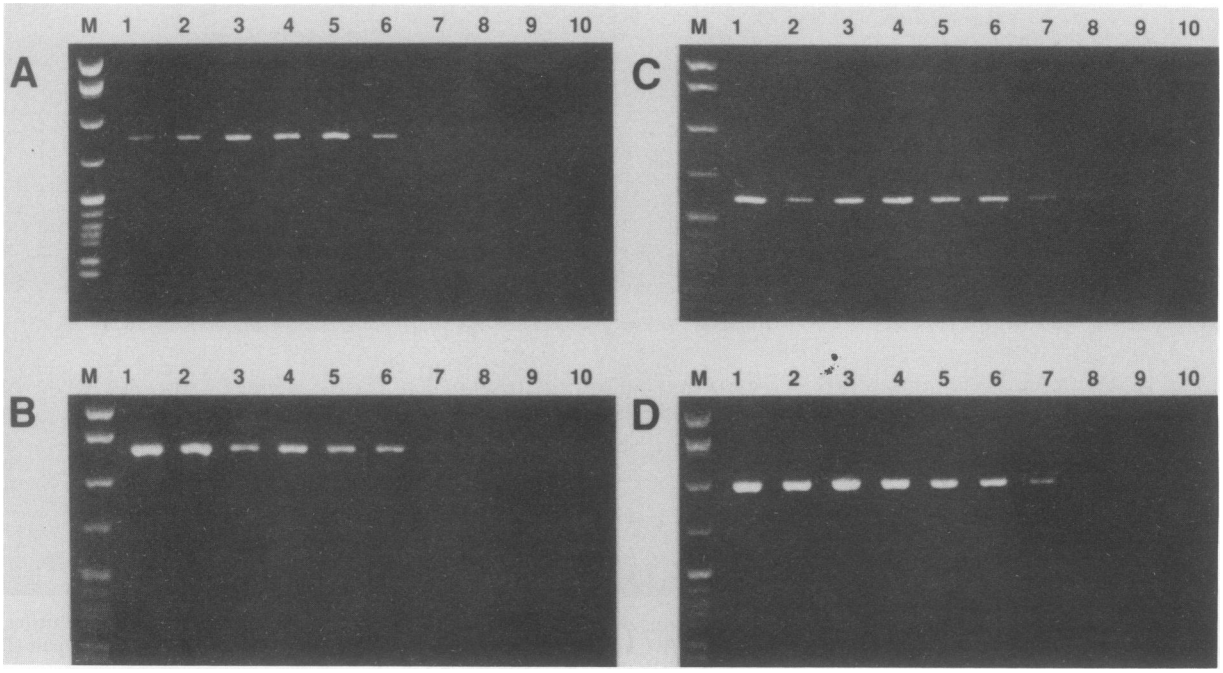

FIG. 5. Sensitivity of the two-amplification PCR for gene 4 typing. Tenfold serial dilutions of purified virus particles (10^{11} to 10^{1} particles) from strains Wa (gene 4 type 1), DS1 (type 2), M37 (type 3), and K8 (type 4) were carried out in clarified stool suspensions; and the dsRNA of each virus particle was extracted with RNAID glass powder. One-tenth of each sample was reverse transcribed and amplified by PCR with consensus primers con 3 and con 2, and 2.5 μl of each sample was subjected to typing by PCR as described in the legend to Fig. 3. (A) Gene 4 typing of the DNA product from Wa virus dilutions (lanes 1 to 10 correspond to the analysis of the dsRNA from 10^{9} to 10^{0} virus particles, respectively, assuming 100% recovery). (B) Gene 4 typing of DNA from DS1 virus dilutions (lanes 1 to 10 correspond to the analysis of the dsRNA from 10^{10} to 10^{1} virus particles, respectively). (C) Gene 4 typing of DNA from M37 virus dilutions (lanes 1 to 10 correspond to the analysis of the dsRNA from 10^{9} to 10^{0} virus particles, respectively). (D) Gene 4 typing of DNA from K8 virus dilutions (lanes 1 to 10 correspond to the analysis of the dsRNA from 10^{9} to 10^{0} virus particles, respectively). The molecular masses of the pBR restriction fragments (lane M) are given in lane PBR of Fig. 3.

(11) because phenol-chloroform extraction was not necessary and it was more sensitive. Using RNAID with the two-step gene 4 typing method, we could detect dsRNAs from as few as 1,000 Wa (VP4-genetic group 1), 1,000 DS1 (group 2), 10 M37 (group 3), or 1,000 K8 (group 4) virus particles pipetted into a stool specimen (Fig. 5).

To determine whether the stool extraction and gene 4 typing methods had utility for diverse strains of circulating HRV, we analyzed several sets of samples from the United States, Mexico, Costa Rica, and Venezuela. Of the eight VP7 serotype 2 samples from Costa Rica (Fig. 6) that were reverse transcribed and amplified with con 3 and con 2 (Fig. 6A, lanes 6 to 13), seven had visible products (Fig. 6A, lanes 6 to 10 and 12) (although they are not visible in Fig. 6A, a product was faintly visible in lane 11 of the original photograph [data not shown]). Products of 876 bp were found in all four positive control dsRNAs from VP4 genetic groups 1 to 4 (Fig. 6A, lanes 1 to 4), but not in a known rotavirus-negative stool sample (Fig. 6A, lane 5). When portions of these dsDNAs were subjected to gene 4 typing by PCR (Fig. 6B, lanes 6 to 13), seven of the eight Costa Rican samples produced dsDNA products (Fig. 6B, lanes 6 to 10, 12) (although they are not visible in Fig. 6B, a product was faintly visible in lane 11 of the original photograph [data not shown]), all of which comigrated with the genetic group 2-positive control (Fig. 6B, lane 2; 483-bp dsDNA). Of the eight samples that were positive for genetic group 2 in the typing reaction, five were confirmed by the production of a 504-bp product (Fig. 6C, lanes 7 to 10 and 12), and one additional genetic group 2-positive sample was obtained in a repeat experiment by using confirmation primers 2C-1 and 2C-2.

We also validated the method described here for stool samples containing rotaviruses with VP7 serotype 1 specificity (from ill children in the United States) and for specimens containing rotaviruses with VP7 serotype 1 or 4 specificity (from newborn infants in a Venezuelan hospital neonatal ward) (17) (gene 4 typing results for U.S. children and Venezuelan infants are shown in Fig. 7 and 8, respectively). In total (Table 2), we analyzed 50 samples, and 47 were positive by PCR. We found that, with one exception, strains with VP7 serotype 1 specificity were from VP4 genetic group 1, whereas all of the VP7 serotype 2 isolates were from genetic group 2. The lone exception to this pattern, excluding isolates from infants in neonatal wards (i.e., the Venezuelan isolates), was a VP7 serotype 1 isolate from Mexico, which contained a genetic group 3 gene 4. Among the 14 known (eight VP7 serotype 1 and 6 VP7 serotype 4) strains from Venezuela that caused asymptomatic infections, all but 1 (which was negative by PCR) were from VP4 genetic group 3, which is in agreement with the results of a hybridization study previously conducted with these isolates (17). The few samples (3 of 50) that were antigen positive but negative by PCR (using at least two different pairs of primers) were not analyzed further.

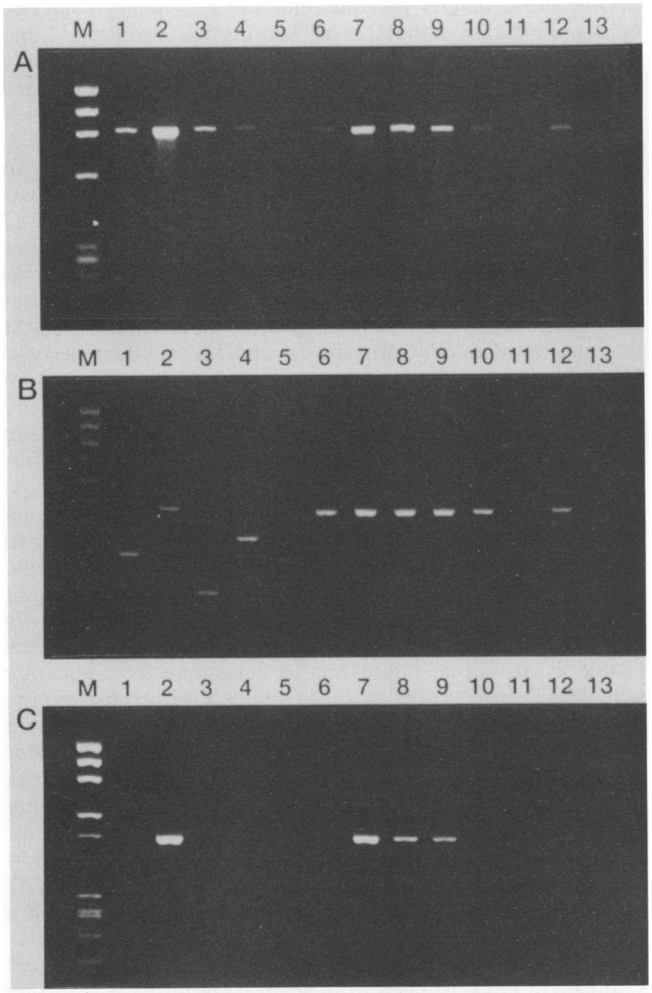

FIG. 6. PCR typing and confirmation of VP7 serotype 2 samples from Costa Rica. (A) Samples were reverse transcribed and amplified by PCR with HRV consensus primers con 3 and con 2; lanes 1 to 4, 876-bp DNAs generated from HRV strains possessing gene 4 types 1 (Wa), 2 (DS1), 3 (M37), and 4 (K8), respectively; lane 5, products amplified from a stool specimen negative for rotavirus; lanes 6 to 13, 876-bp products amplified from stool samples containing VP7 serotype 2 HRV strains from Costa Rica. (B) Portions of the products shown in panel A. The samples in lanes 1 to 13 were amplified by PCR by using the cocktail of gene 4 typing primers described in the legend to Fig. 3. The sizes of typing bands for gene 4 types 1, 2, 3, and 4 were 345, 483, 267, and 391 bp, respectively. (C) The dsRNAs used to generate the products shown in panel A. The samples in lanes 1 to 13 were reverse transcribed and amplified with a primer pair specific for gene 4 type 2, and the products were analyzed as described in the legend to Fig. 2. The extra marker band (fifth band from the top) in the ϕX174 marker (lane M) is a 500-bp dsDNA that was produced by using the control primers and template from the Perkin-Elmer Cetus PCR kit.

DISCUSSION

In this report we described a PCR method for identifying the gene 4 types of HRV strains. In the absence of MAbs that would permit discrimination of the known VP4 types by EIA, this method, along with a recently published hybridization technique for the same purpose (17), serves as a proxy for the determination of the VP4 genetic group of HRV strains directly from stool samples. To increase the sensitivity of the reaction (1, 12), we used a two-step (nested priming) method for gene 4 typing in which a primer pair corresponding to highly conserved regions was used to generate an 876-bp dsDNA from the 5'-terminal third of gene 4 by reverse transcription and PCR amplification of dsRNA (first amplification). The DNA was subsequently used as a template in the gene 4 typing PCR (second amplification) by using a cocktail of one plus-sense consensus primer and four (one for each gene 4 type) genetic group-specific minus-sense primers selected from sequences of gene 4 lying within the 876-bp region. It should be noted that, although false-positive results were not observed in the experiments described here, use of two amplification methods increases the chance of cross-contamination during the manipulation of samples, and therefore, appropriate negative controls should be included as monitors for this problem.

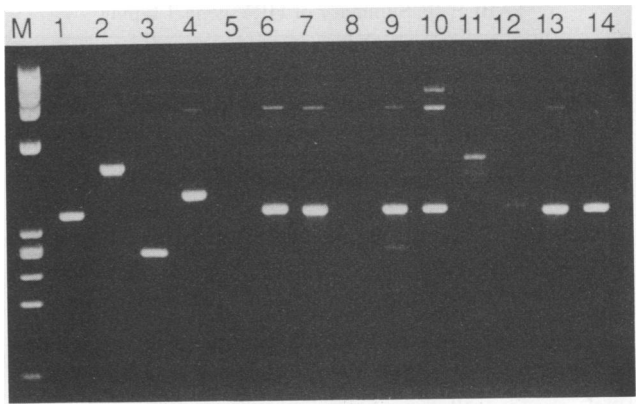

FIG. 7. Typing by PCR of gene 4 from a group of predominantly VP7 serotype 1 strains. Samples were extracted with RNAID glass powder, reverse transcribed, and amplified by PCR as described in the legend to Fig. 5. Portions of the products were subjected to typing by PCR as described in the legend to Fig. 5. Lanes 1 to 4, positive controls; lane 5, negative control; lanes 6 to 14, gene 4 typing products for a group of rotavirus samples from the United States.

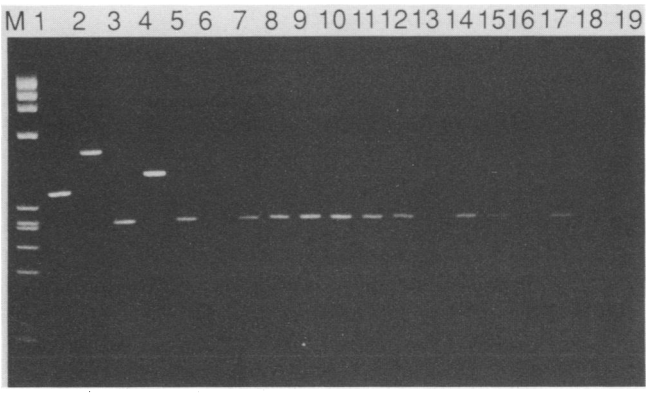

FIG. 8. Typing by PCR of gene 4 from a group of HRV strains of VP7 serotypes 1 and 4 that caused asymptomatic infections. Samples were extracted with RNAID glass powder, reverse transcribed, and amplified by PCR as described in the legend to Fig. 5. Portions of the products were subjected to gene 4 typing by PCR as described in the legend to Fig. 5. Lanes 1 to 4, positive controls; lanes 5 to 18, products for a group of rotavirus samples from Venezuela determined by PCR typing; lane 19, negative control.

TABLE 2. Summary of HRV gene 4 typing

Sample origin	Gene 4 PCR typing		No. of samples with the following VP4 genetic group:					No. of samples of VP7 serotype:			
	No. of samples tested	No. of samples positive	1	2	3	4	Mixed	1	2	3	4
United States	19	18	16	1	0	0	1	7	1	0	0
Venezuela	14[a]	13	0	0	13	0	0	8	0	0	6
Mexico	9	9	2	6	1	0	0	3	6	0	0
Costa Rica	8	7	0	7	0	0	0	0	8	0	0
Total	50	47									

[a] VP7 serotypes were determined by EIA and a serotype-specific hybridization method (6). The samples were also shown previously to have M37-like gene 4's (17).

The regions from which the typing primers were selected represent the most variable sequences of gene 4 among strains from different genetic groups, as demonstrated by comparative nucleotide sequencing and the predicted amino acid sequence (9). Evidence that this region of VP4 (the VP8 cleavage fragment) contains the antigenic sites involved in delineating VP4 serotypes has also been presented (10). As for the hypervariable regions of genes 7 to 9, which are responsible for delineating VP7 serotypes (15), the comparable regions of gene 4 are highly conserved among HRV strains in the same VP4 genetic group and, thus, are well-suited for selecting group-specific PCR primers.

The method described here is highly specific, in that the genetic groups of 16 well-characterized HRV strains could be reliably identified. Likewise, the sensitivity of the method is very high. With the typing primers for which we had sensitivity data, we could obtain a positive typing reaction with the dsRNA from as few as 10 to 1,000 virus particles (this sensitivity is 500 to 50,000 times more sensitive than that obtained by using ROTACLONE, a commercial EIA, and about 200 to 20,000 times greater than that of a recently reported hybridization method for gene 4 typing [17]). Thus, PCR typing may be advantageous in samples with $<10^8$ virus particles per ml of stool extract.

Gene 4 typing offers several other important benefits. First, on the basis of the observation that all members of a VP4 genetic group (from nucleotide and deduced amino acid sequence conservation) can be classified into the same VP4 antigenic group (10) and in the absence of immunologic methods (either MAb- or polyclonal antibody-based tests) to study the antigenic diversity of VP4, gene 4 typing by PCR and a recently reported hybridization method for the same purpose (17) serve as valid proxy methods to assess VP4 diversity. Although the VP4 proteins of genetic group 1 to 4 strains can be distinguished by cross-neutralization tests with antisera to VP4 or VP8 polypeptides that are expressed (10, 18), these tests are too cumbersome for studying VP4 diversity in field isolates, and MAbs that are able to distinguish the known VP4 antigenic types have yet to be isolated. Second, in conjunction with recently described PCR (12), hybridization (17) and EIA (31) methods for use in the identification of VP7 serotype specificity, gene 4 typing will permit us to more fully characterize the diversity of both HRV neutralization antigens before MAbs with new VP4 and VP7 specificities become available.

Our initial results indicate that HRV isolates from two epidemiologically important VP7 serotypes (serotypes 1 and 2) from several parts of the world possess almost exclusively gene 4 types 1 and 2. This finding was not unexpected, since gene 4 types 1 and 2 have been identified (by nucleotide sequencing) only in prototype strains with VP7 serotype 1, 3, and 4 (type 1) and VP7 serotype 2 (type 2) specificities. In another recent study in which a hybridization method was used, type 1 gene 4 was identified in strains with VP7 serotype 3, 4, and 9 specificities (>50% of the total number of samples with typeable gene 4's) and type 2 gene 4 was identified in strains with VP7 serotype 2 specificity (about 20% of the total) (28). Taken together, these results suggest that strains with VP4 serotype 1A and 1B specificities (type 1 and 2 gene 4's) are predominant strains in many areas of the world. A single isolate with a type 3 gene 4 and VP7 serotype 1 specificity was identified in an asymptomatic Mexican infant who excreted rotavirus. Such strains (frequently designated neonatal or asymptomatic rotaviruses) have been isolated almost exclusively from newborn infants in neonatal wards, are shed asymptomatically, can belong to VP7 serotypes 1 to 4, and share a highly conserved gene 4 that has been postulated to account for their avirulent phenotype (9). All other isolates containing type 3 gene 4's identified in this study were from infants in neonatal wards (Table 2). It will be interesting to determine the frequency with which these strains occur in subjects with symptomatic infections, and in this regard, several isolates with type 3 gene 4's have recently been identified in neonates or older infants with gastroenteritis (28). The other two gene 4 types (types 4 and 5) occur in strains that have been isolated only rarely (2, 21, 29). Gene 4 typing will permit us to better assess the frequency and distribution of gene 4 types and to determine whether they occur in strains with various VP7 serotype specificities. In a recent study (28), strains with a type 5 gene 4 and VP7 serotype 1, 3, 6, and 8 specificities have been identified at a low frequency (<10% of total typed samples) in HRV isolates from several countries, suggesting that reassortment may contribute to antigenic diversity in these strains, as observed previously for rotaviruses with VP4 serotype 1A and 2 specificities (10).

The need for a dual serotyping system for HRV VP4 and VP7 was suggested several years ago (13). Recent studies on the genetic and antigenic diversities of HRV VP4 have confirmed the need for a VP4 classification system (10, 18), but the several different terminologies which have arisen to describe the genetic and antigenic specificities attributed to VP4 and VP7 (9, 10, 30) have resulted in some confusion in this field. We support the adoption of the proposed binary nomenclature system for rotavirus serotyping. The system designates the antigenic specificities attributed to VP7 as G types and those attributed to VP4 as P types (30).

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