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Characterization of human rotavirus genotype P[8]G5 from Brazil by probe-hybridization and sequence

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Summary. We report the molecular characterization of rotavirus genotype P[8]G5 strains found in fecal specimens collected in four different regions of Brazil, using digoxigenin (dig)-labeled oligonucleotide probes, sequence analysis, and RNA-RNA hybridization. The closest sequence relationships of the neutralization antigens of these strains were to the VP4 protein of P1A[8]G1 strain KU (93.3% identity in amino acids 11 to 282) and to the VP7 protein of G serotype 5 strain OSU (87.6% identity in amino acids 8 to 232). Based on VP7 sequence differences, we designed dig-probes that allowed us to discriminate porcine OSU-like strains from G5 strains isolated from Brazilian infants. The genetic relationships of two P[8]G5 isolates to other rotavirus genogroups were analyzed by RNA-RNA hybridization with [^{32}P]-GTP probes representative of serotypes P1A[8]G1 (Wa), P[8]G3 (AU17), and P9[7]G5 (OSU). The Brazilian P[8]G5 strains showed sequence homology with genes of Wa-like and OSU-like strains, suggesting that these two strains were naturally occurring reassortants between members of the Wa and porcine rotavirus genogroups. The identification of these strains in diverse geographic areas of Brazil underscores their stability and demonstrates the emergence of clinically important rotavirus diarrhea strains by reassortment.

Introduction

Rotaviruses are recognized as the most important cause of acute gastroenteritis in infants and young children, as well as the young of a variety of mammalian and

avian species [24]. Although all of the group A rotavirus proteins, both structural and nonstructural, act as antigens and are recognized by the immune system, the proteins considered most important for vaccine development are the outer capsid proteins VP4 and VP7, encoded by genome segments 4, and 7, 8 or 9, respectively. These viral antigens induce neutralizing antibodies, elicit a protective immune response, and form the basis for the current classification of group A rotaviruses into P (VP4) and G (VP7) serotypes [10, 11, 21].

To date 14 G (G1 to G14) and 10 P (P1 to P10) serotypes of group A rotaviruses have been identified [5, 11, 16, 26, 32, 36]. At least 9 other genetically distinct P genotypes are recognized that have not yet been classified into P serotypes by cross-neutralization tests [14, 20, 22]. While reassortment between the currently recognized G and P serotypes could lead to 140 possible subtypes, genotyping studies of the distribution of the genes encoding recognized P antigenic types, combined with G serotyping or genotyping of the same strains, demonstrate that only four G-P genotype combinations – designated P[8]G1, P[4]G2, P[8]G3 and P[8]G4 – are most commonly identified in children with diarrhea [2, 4, 10, 16, 23]. Neutralization antigen genes derived from the main serotypes from the basis for the current experimental reassortant live oral vaccines tested in field trails in several countries [15]. Numerous other rotavirus serotypes, including some previously isolated only in animals, have been identified, but so far, have not been shown to be epidemiologically important [3, 5, 9, 11, 13, 35, 38]. Recent reports suggest that the diversity of rotavirus strains circulating in Brazil [25, 37] and India [33] is larger than most other countries surveyed, indicating that perhaps antigens to some of these uncommon serotypes should be considered for inclusion in future vaccines for these countries.

We recently demonstrated that rotavirus genotype P[8]G5 was present as single (9%) and mixed (12%) infections in a collection of 130 rotavirus-positive fecal specimens from 8 of the 9 states and the Federal District of Brazil [25]. That study confirmed a similar study from the Sao Paulo district of Brazil. Since a molecular analysis of the genetic relationships of these strains to other rotaviruses had not previously been reported, we carried out sequence analysis, RNA-RNA hybridization, and Southern hybridization to characterize these strains in detail and determine if they were derived by reassortment. We report a partial sequence of the VP4 and VP7 genes of 5 P[8]G5 Brazilian strains. In addition, by RNA-RNA hybridization analysis, we studied the origin of the gene segments among human P1A[8], G1 (Wa), P[8], G3 (AU17), P[8]G5 (BR1054/H8), and porcine P9[7], G5 (OSU) strains. We also developed genotype-specific digoxigenin-labeled oligonucleotide-probes (dig-probes) to distinguish between the porcine serotype G5 strain OSU and G5 Brazilian strains.

Materials and methods

Fecal specimens, cultivation of human rotavirus and plaque assay

Human fecal specimens were obtained from 1982 to 1994 in 9 states of Brazil and the Federal District of Brazil in a National Laboratory Surveillance Program, as well as in 1994 at the

Hospital of the State University of Londrina (Parana), from children under 5 years old with acute gastroenteritis and culture-adapted as described in the accompanying manuscript [25, 31]. Two P[8]G5 strains (BR1054/RJ-86; H8/PR-94) from different regions and with distinct electropherotypes, were subsequently plaque purified in MA-104 cells as described [34], followed by several additional passages in the same cell line.

Reverse transcription-polymerase chain reaction (RT-PCR) G and P genotyping

Double-stranded RNA extracted from cultured virus was examined by polyacrylamide gel electrophoresis (PAGE) and silver staining [30], and G- and P- genotyped by one amplification or half-nested RT-PCR as previously described [7, 12, 18, 19]. The nomenclature for P serotypes and P genotypes, which has recently been modified according to the recommendations of a Rotavirus Nomenclature Working Group, has been used in this study. The details of these changes can be found in a recent review of rotavirus replication and molecular biology by Estes [10].

Nucleotide sequencing

Portions of the VP7 and VP4 genes of two P[8]G5 strains were sequenced. For VP7, the consensus plus-sense primer 9con1 was used in conjuction with the G-genotyping minus-sense oligonucleotide FT-5 to generate a 742-bp PCR product by RT-PCR [7, 17]. The PCR product was purified on QIAquick columns (Qiagen Inc., Chatsworth, CA) and sequenced with the above two primers. Two internal primers – H89-1 (nucleotide [nt] 252–274, + sense): 5′ GAA TTC TAC GGC AAG TGA AAC AT and H89-2 (nt 605–628, – sense): 3′TAA TCG TAC ATG AAG AGC CCA TAG – were used to complete the sequence of the RT-PCR product described above. For VP4, the 345-bp RT-PCR product generated with the plus-sense primer Con3 and the minus-sense primer 1T1 (12) was purified and sequenced for these two strains. Two internal primers – H84-1 (nt 157–177): 5′ TGG GGT CAT GGA GAA ATA AAT and H84-2 (nt 625–646): 3′ ACT CTT GGG ACC TTG GAA TAA T – were used to complete the sequence of the genotype P[8] RT-PCR products. The sequence reaction was prepared with the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc., Foster City, CA) on an automated sequencer model 373A (Applied Biosystems, Inc). The data were compared with the other rotavirus sequences by using the University of Wisconsin, Genetics Computer Group package of programs [8].

Dig-probes, Southern hybridization, and chemiluminescent detection

Since the sequence of human G5 strains was distinct from the porcine reference strains, two 5′ digoxigenin-labeled probes were designed to distinguish them. The sequence of the OSU VP7 gene probe spanned nt 171 to 192 (5′AGT CGT ACT TGC ACC GCT CAT T), while the sequence of the probe from the corresponding region of the Brazilian human G5 strains is 5′GGT TAT ACT TGC GCC AAT TA. Both probes are of positive polarity. Southern blotting, hybridization, and chemiluminescent detection were performed as described previously [1], except that the temperature for probe hybridization was increased to 62 °C for both probes, based on experimentation [25].

RNA-RNA hybridization

The rotavirus strains Wa P1A[8], G1, AU17, P[8]G3, OSU, P9[7], G5, H8A P[8]G5, and BR1054 P[8]G5, were used for probe hybridization experiments after plaque-purification,

preparation of working stocks and preparation of purified virions in MA104 cells. Probes for hybridization were synthesized from purified single-shelled virus particles by in vitro transcription in the presence of [^{32}P]-GTP [29]. The hybridization and the PAGE analysis were performed as reported [29].

Accession numbers

The partial VP4 and VP7 gene sequences of Brazil P[8]G5 strains have been assigned the following accession numbers: U41003 and U41004 for strain BR1054, and U41005 and U41006 for strain H8, respectively.

Results

Nucleotide sequencing analysis and genotype-specific probe assay for P[8]G5 strains

In a previous report, we detected rotavirus genotype P[8]G5 by RT-PCR as single (9%) and mixed (12.3%) infections circulating in 8 of the 9 investigated states and in the Federal District of Brazil (25). Partial sequence analysis of the VP4 and VP7 neutralization antigen genes of two P[8]G5 strains showed that the highest predicted amino acid homology was 93.3% identity with P1A[8], G1 strain KU for the VP4 gene, and 87.6% aa identity with the P9[7], G5 strain OSU, for the VP7 gene (Tables 1 and 2). The VP7 gene was also relatively closely related serotype G11 strain YM (84.4% amino acid identity). Based on these data, these strains have been tentatively classified as P[8]G5. However, a VP7 sequence alignment showed that both serotype G5 OSU and G11 YM strains had relatively few substitutions in antigenic regions A (aa 87 to 101), and B (aa 141 to 151), compared to Brazilian G5 strains H8 and 1054, while as expected, strains of G serotypes 1 to 4 had numerous changes in these regions relative to the Brazilian isolates (Fig. 1). OSU and YM had 4 and 5 substitutions, respectively, in region C (aa 208 to 224), while G1 to G4 strains had 4 to 8 changes in these regions.

Based on the sequence analysis of rotavirus genotype G5 strains from Brazil and the published sequence of the VP7 gene of strain OSU, two dig-probes were developed. We used these probes to test 12 of our G5 isolates and the G5 OSU prototype by Southern-blot hybridization and chemiluminescent detection. These two probes showed specific reactivity with the homologous RT-PCR products and allowed us to specifically detect Brazilian G5 strains and differentiate them from prototype serotype G5 strain rotavirus OSU (Fig. 2).

RNA-RNA hybridization

Sequence analysis suggested that these P[8]G5 strains were naturally occurring reassortants between a human and a porcine strain. To confirm this, we used RNA-RNA hybridization analysis. All Brazilian G5 strains analyzed by PAGE had a long electropherotype but one of two distinct migration patterns (unpubl. data). A representative strain of each pattern, BR1054 and H8, was plaque-purified in MA-104 cells and used to prepare probes by in vitro transcription, for

Table 1. Homology between a fragment of the VP4 gene (nt 40–858, aa residues 11–128) of Brazil strain 1054 and selected rotavirus VP4 genes or gene fragments

Strain	P [genotype]	% homology	
		nt	aa
Brazil H8	–	**93.2**	**92.3**
A5	1	63.8	58.1
SA11	2	64.2	57.34
RRV	3	61.8	56.6
RV5	4	84.0	84.6
L27	4	84.5	83.8
UK	5	59.8	57.3
1076	6	69.8	66.9
ST3	6	69.6	65.1
OSU	7	60.6	54.4
YM	7	60.8	55.5
P	8	NA[a]	**92.3**
VA70	8	NA	**88.6**
KU	8	**93.5**	**93.3**
Wa	8	**90.1**	**90.1**
K8	9	59.0	51.8
69M	10	63.1	58.5
116E	11	49.9	41.8
PA169	14	57.5	66.9

[a] Not available

Table 2. Homology between a fragment of the VP7 gene (nt 69–744, aa residues 8–232) of Brazil strain 1054 and other rotavirus VP7 genes

Strain	G serotype	% homology	
		nt	aa
Brazil H8	–	**98.2**	**99.6**
Wa	1	73.2	71.1
S2	2	69.6	68.4
107E1B	3	72.6	79.1
ST3	4	70.4	67.6
OSU	5	**86.2**	**87.6**
NCDV	6	73.6	77.3
Ty-1	7	68.1	58.7
B37	8	70.3	72.0
WI61	9	75.0	75.6
B223	10	72.5	73.3
YM	11	78.4	84.4
L27	12	71.9	76.4
L338	13	74.0	74.7
F123	14	73.9	74.7

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                8                                                      57

Brazil 1054  TILTLGISLVFIDYILKSVTRTMDFIIYRFLLVVVILAPIIKTQNYGINL
  Brazil H8  ------------------------------------------------------
        OSU  -V--FL-----VN--------------------I-V---L--A-------
         YM  ----XL---I--T-----I-----------F-I-V---FV----------
     107E1B  -V--CL--VILLN-V---L--I----------II---S-FLNA-------
         S2  ----IL--IILLN----TI-N---Y--F----LIALIS-FVR-----MY-
         Wa  ---IFL--IILLN--------I--Y-------IT-A-FALTRA----L--
        ST3  -V-FYL--F-LVS----TIIKI--Y----ITF-I-V-SVLSNA-------

                58                                        A.           107
Brazil 1054  PITGSMDTSFANSTASETFGAPTLCLYYP NEAATEIADDKWTD TLSQLFL
  Brazil H8  ---------------------------- ------------- -------
        OSU  --------PYM---T----LTS------- ---------T---E -------
         YM  --------PYM---M----LTS------- H----Q------K- -------
     107E1B  --------PYM---RE-V-LTS------- T------N-NS-K- -------
         S2  -----L-AVYT---SG-S-LTS------- A--KN--S--E-EN -------
         Wa  -------AVYT---QE-V-LTS------- T--S-Q-N-GD-K- S---M--
        ST3  --------AY----QDNN-LFS------- S--P-Q-S-TE-K- -------

                108                                         B.          157
Brazil 1054  TKGWPTGSVYFKGYADIASFSVEPQLYCDYNIVLM KYDINLQL DMSELAD
  Brazil H8  ------------------------------------ -------- -------
        OSU  ------------------------------------ ---G---- ------G
         YM  ----S-------E-T-------D------------ ---G-S-- -------
     107E1B  I-----------D-T-------D--------L--- ---AT--- -------
         S2  ------------D-N--TT--MN--------V--- R--NTSE- -V-----
         Wa  ------------E-SN-VD---D--------L--- ---QS-E- -------
        ST3  -----------NE-SNVLE--ID-K------V--I RFVFGEE- -I-----

                158                                                    207
Brazil 1054  LILNEWLCNPMDITLYNYQQTDEANKWISMGSSCTIKVCPLNAQTLGIGC
  Brazil H8  ----------------Y----------------------------------
        OSU  -------------M--Y-------------T----------T-------
         YM  ----------------Y-------------N----------T-------
     107E1B  -L--------------Y-------------------------T-------
         S2  -------------S--Y---NS-S-------TD--V------T-------
         Wa  ------------V---Y---SG-S-----------V------T-------
        ST3  ----------------Y----G-------------V------T-------

                208     C.              232
BRAZIL 1054  LTTDTNSFETVASTEKL AITDVVDG
  BRAZIL H8  ----------------- --------
        OSU  S---I-------NA--- --------
         YM  ----PTT--E---A--- V-------
     107E1B  ------T--E--TA--- V-------
         S2  K---VDT--I---S--- V-----N-
         Wa  Q--NVD---MI-EN--- --V-----
        ST3  Q--N-AT-----DS--- --I----S
```

Fig. 1. Comparisons of Brazil genotype G5 strains (aa 8 to 232) BR1054 and H8 with other selected rotavirus VP7 proteins. The positions of the antigenic regions A (aa 87–100), B (aa 142–150), and C (aa 208–224) are underlined

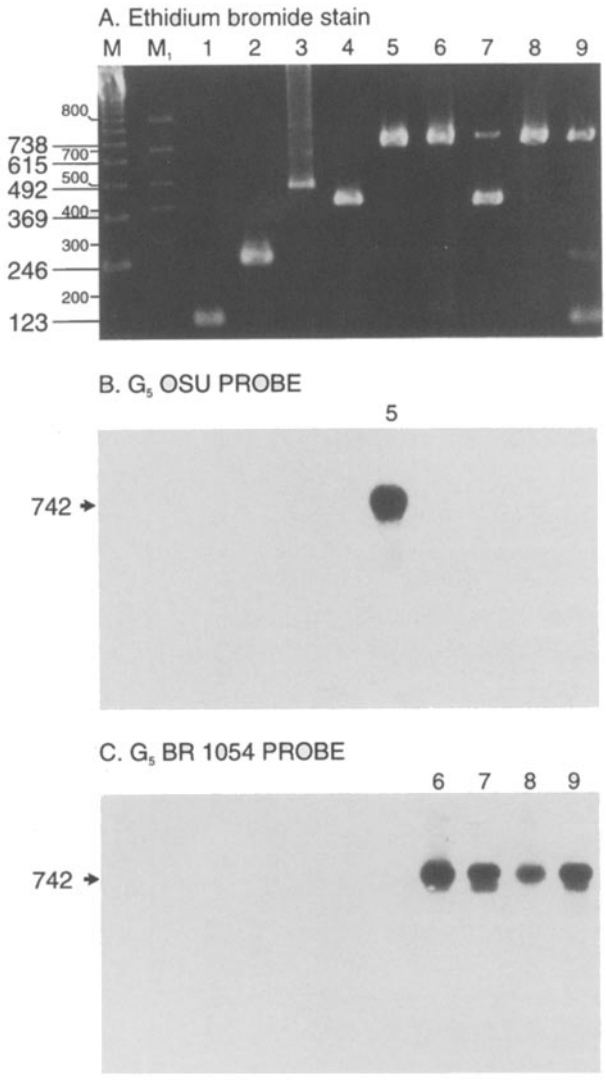

Fig. 2. Characterization of rotavirus G5 genotype-specific RT-PCR products with digoxigenin-labeled oligonucleotide probes. **A** Ethidium bromide stained agarose gel showing RT-PCR products: *M* 123 bp ladder molecular weight marker (GIBCO-BRL Laboratories Gaithersburg, MD); M_1 Molecular weight marker type XI, digoxigenin-labeled (Boehringer Mannheim Biochemicals, Indianapolis, IN); *1–4* Brazilian strains of genotypes G1 to G4, respectively; *5* prototype strain G5 OSU; *6–9* Brazilian strains of genotype G5. *7* and *9* show mixed infections of genotypes G4 + 5 and G1 + 5, respectively. **B** and **C** show the results of Southern hybridization and chemiluminescent detection with G5 OSU and G5 BR1054 probes, respectively

use in RNA-RNA hybridization experiments with other reference human and animal strains from the human Wa genogroup and porcine OSU group (Fig. 3). The H8A and BR1054 probes were hybridized with their reciprocal RNA, as well as with dsRNAs from Wa P1A[8], G1, AU17 P[8]G3, and OSU P9[7], G5. Both the H8A and the BR1054 probes produced 11 hybrids with homologous genomic RNAs and each other, indicating that these two strains are genetically closely related (Fig. 3). The H8A probe produced 8 and 9 hybrids with dsRNAs from AU17 and Wa, respectively, and the BR1054 probe yielded 8 and 7 strong hybrids and one faint hybrid with dsRNAs from these strains, respectively. With genomic RNAs from OSU, the H8A probe formed 6 hybrids and the BR1054 probe produced two strong and four faint hybrids (Fig. 3), consistent with the possibility that both H8A and BR1054 strains showed sequence homology with the corresponding genes of Wa genogroup strains as well as of OSU.

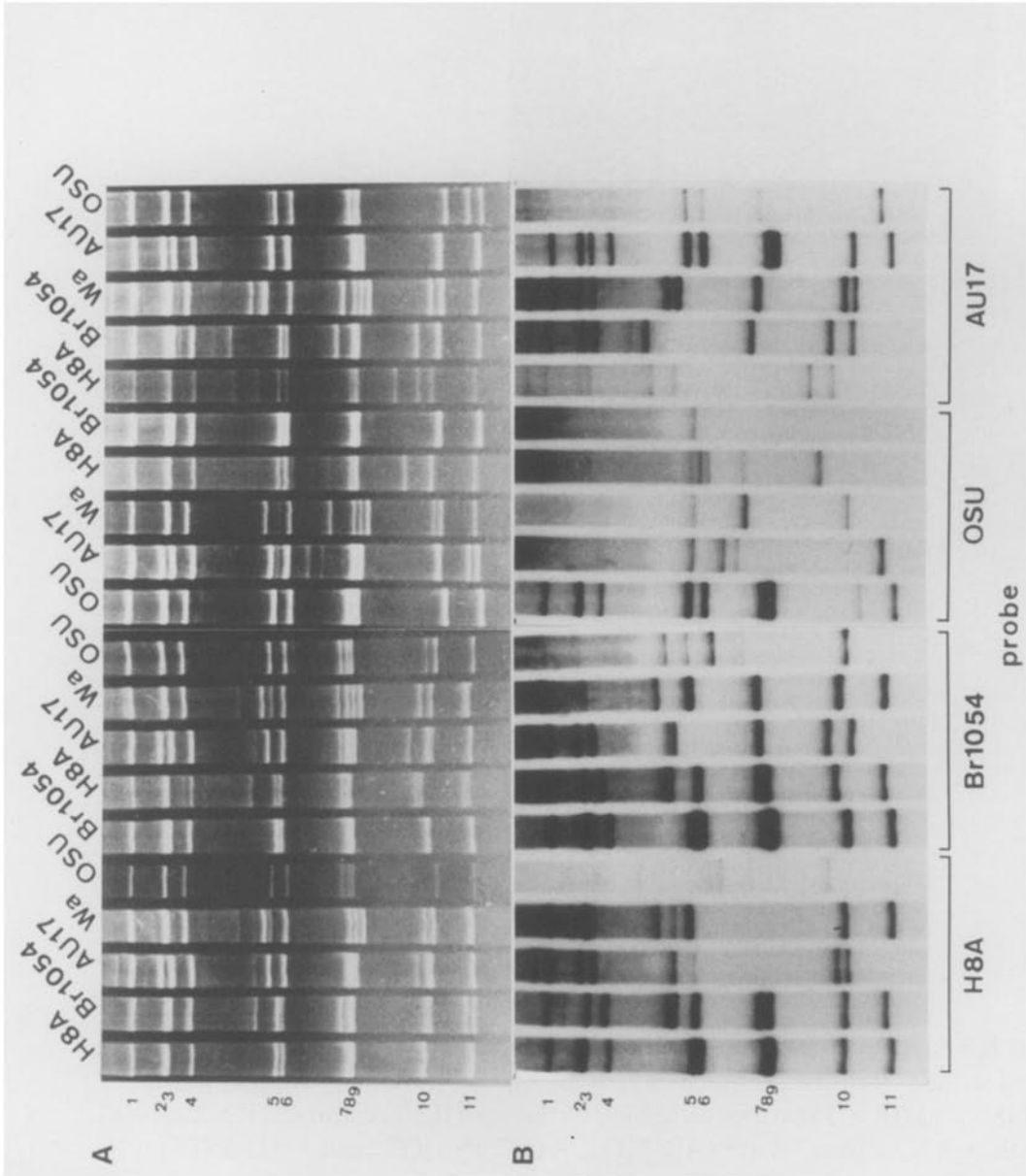

Fig. 3. RNA-RNA hybridization analysis of rotavirus prototype serotype G5 OSU strain and Brazilian genotype G5 isolates. **A** Ethidium bromide stained 10% PAGE showing the electrophoretic pattern of laboratory strains: Wa P1A[8], G1 Au17 P[8]G3, and OSU P9[7], G5, and two Brazilian isolates, H8A and BR1054 P[8]G5. **B** shows the autoradiograph of the hybrids obtained with the indicated [^{32}P]-labelled probes synthesized by in vitro transcription

Discussion

In this study we present data on the molecular characterization of Brazilian genotype P[8]G5 strains. To characterize these strains, we sequenced fragments of the VP4 and VP7 genes of 2 genotype P[8]G5 strains and showed that the closest deduced amino acid relationships for these strains were to the serotype G5 strain OSU, for the VP7 gene, and serotype P1A[8], strain KU, for the VP4 gene. The relatively high homology between these two strains suggests that the Brazilian genotype P[8] strains belong to the corresponding serotype P1A, but because of the profound influence of the G serotype on the antigenicity of the VP4 protein [6], this assignment must first be confirmed by serological analysis.

The VP7 genes show considerable homology to the corresponding gene of G serotype 11 strain YM, as well as to G5 OSU. The observation that the numbers of aa substitutions in antigenic regions A to C between Brazilian G5 strains and both serotypes G5 and G11 strains OSU and YM are similar, suggests that these strains could have antigenic relationships with both G5 and G11. Brazilian G5 strains 1054 and H8a could not be detected by a MAb against the G5 OSU strain, in contrast to previously described Brazilian G5 strains [17]. Nor did these two strains react with Mabs to common human rotavirus G1 to G4 strains. It should also be noted that Australian porcine G5 strains have been shown to have dual G5 and G3 serotype specificity [27]. Further investigations will be necessary to confirm the G serotype of these strains.

Two P[8]G5 strains (BR1054 and H8A), with distinct long electropherotypes, were compared to common human rotavirus strains P1A[8]G1 (Wa), P[8]G3 (AU17), and to porcine OSU (P9[7]G5) rotavirus by RNA-RNA hybridization. These results were consistent with our sequencing studies which demonstrated that Brazilian G5 strains are naturally occurring reassortants between human (P[8]Wa-like) and animal (G5, OSU-like) strains. Many previous reports have demonstrated that reassortment between human rotavirus and animal rotavirus strains is a probable mechanism for the generation of genetic diversity among rotavirus childhood diarrhea strains [28]. However, the current study is, to our knowledge, the first to show that such strains can be widely dispersed in a population, perpetuated over a long period of time in humans, and be of a genotype (G5) other than G1 to G4. It should be noted, however, that it cannot be excluded that some of these reassortants could have been generated during passage in cell culture.

The results of vaccine trials and epidemiologic studies suggest that a future vaccination campaign against rotavirus, using only the VP7 gene of the four most common serotypes in reassortant rotavirus vaccine strains, could be extremely useful to reduce the number of hospitalizations and doctor visits for rotavirus. However, our results and previous reports [17, 25] concerning the possibility of endemic distribution of G5 strains in Brazil's urban and rural areas, suggest that vaccine efficacy should be monitored carefully in Brazil, where strain diversity is much more extensive than other regions, before a national vaccination campaign is begun.

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