

## Relative Frequencies of Rotavirus Serotypes 1, 2, 3, and 4 in Venezuelan Infants with Gastroenteritis

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We have used a recently developed monoclonal antibody enzyme-linked immunosorbent assay (K. Taniguchi, T. Urasawa, Y. Morita, H. B. Greenberg, and S. Urasawa, *J. Infect. Dis.* 155:1159-1166, 1987) for serotyping rotaviruses recovered from 134 Venezuelan infants over a period of 15 months. One hundred and nine of the specimens were typed with the following distribution: serotype 1, 48%; serotype 2, 16%; serotype 3, 22%; and serotype 4, 14%. Three specimens reacted with two different monoclonal antibodies. In addition, 6 specimens (5%) containing enough outer capsid antigen could not be typed; partial RNA sequence analysis of the glycoprotein gene from three of these six strains failed to reveal sequence differences with prototype strains that could be serotyped with the monoclonal antibodies. Variations in the recovery rates of the different serotypes were observed. Serotypes 2, 3, and 4 predominated at the beginning of the study, and serotype 1 predominated at the end of the study. Diarrheal illness appeared to be more prolonged in infants shedding rotavirus serotypes 1 and 3 than in those shedding serotypes 2 and 4.

The development of a rotavirus vaccine should decrease the high diarrheal mortality rates observed in infants and young children in developing countries (14). A Jennerian approach to vaccine development by using heterologous bovine (23) or simian (7) strains to immunize the human host has been tested with some success. However, it appears from recent vaccine failures (3, 15) that more successful vaccines will require the introduction of strains antigenically similar to those rotaviruses commonly isolated from infants with disease. Both the planning and the evaluation of second-generation rotavirus vaccine candidates require the availability of a simple serotyping test that can be used in the field. In addition, widespread use of such a test will greatly enhance knowledge of the natural history and epidemiology of rotaviruses. The use of enzyme immunoassays based on the specific recognition of serotype-specific epitopes on VP7 (the major rotavirus neutralizing antigen) has been reported by several groups (2, 11, 20, 21). A serotyping test recently developed by Taniguchi et al. (21), which uses anti-VP7 neutralizing monoclonal antibodies against the four epidemiologically important human serotypes (i.e., serotypes 1, 2, 3, and 4), was used in this study to analyze the relative frequency and temporal distribution of those serotypes among infants hospitalized with acute diarrhea at a pediatric hospital in Caracas, Venezuela.

### MATERIALS AND METHODS

**Rotavirus detection.** Stool specimens from 134 infants (8 days to 24 months of age) known to be positive for rotavirus were studied. The children were admitted for acute diarrhea to the emergency ward at the Hospital de Niños J. M. De Los Ríos in Caracas, Venezuela, between December 1981 and February 1983. Rotavirus detection was performed by a confirmatory enzyme-linked immunosorbent assay (ELISA) as previously described (16). Most of the specimens had been previously subgrouped by ELISA (24), and some of

them were subjected to RNA extraction for electrophoretic and cross-hybridization analyses (7).

**Monoclonal antibodies.** Four serotype-specific monoclonal antibodies that recognize VP7 were used: KU-4 (serotype 1), S2-2G10 (serotype 2), YO-1E2 (serotype 3), and ST-2G7 (serotype 4). In addition, monoclonal antibody YO-2C2, which recognizes the VP3 outer capsid protein of all four serotypes in ELISAs, was used as a positive control to determine the presence of double-shelled virus particles. A monoclonal antibody (YO-156) that recognizes an inner capsid common epitope in all group A rotaviruses was used as a control to detect the presence of rotavirus antigen in the sample (22). The reactivity of these monoclonal antibodies with a series of laboratory strains has been previously described (21, 22).

**Rotavirus serotyping.** ELISA serotyping was performed as previously described (21). Briefly, the wells of polyvinyl microtiter plates were coated with a 1:10,000 dilution of monoclonal antibody (ascitic fluid) in phosphate-buffered saline (PBS). After overnight incubation at 4°C, the plates were washed twice with PBST (PBS containing 0.05% Tween 20 and 0.02% sodium azide). Bovine serum albumin (1%) was added to the wells, and the plates were again incubated overnight at 4°C. After the plates were washed twice with PBST, 50 µl of sample (5 to 10% stool suspensions in PBS or tissue culture fluid) was placed in each well. After overnight incubation, the plates were washed three times with PBST and 50 µl of anti-rotavirus rabbit antiserum was added per well and allowed to react at 37°C for 1 h. The wells were washed three times with PBST and incubated with 50 µl of anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals). Finally, after three additional washes, the substrate (*para*-nitrophenyl phosphate diluted in 0.1 M sodium carbonate buffer [pH 9.6]) was added. The A<sub>410</sub> was read in a Molecular Devices ELISA reader. A rotavirus specimen was considered to be a specific serotype when its reactivity with the monoclonal antibody corresponding to that serotype was at

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TABLE 1. Frequency of detection of rotavirus serotypes 1, 2, 3, and 4 in Venezuelan infants with gastroenteritis

Time period	No. of samples tested	No. of samples typed	No. of samples with serotype				
			1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>a</sup>	4 <sup>a</sup>	Nontypable <sup>b</sup>
December 1981–February 1982	47	43	4 (9)	13 (30)	14 (33)	12 (28)	4 (9)
March 1982–July 1982	12	6	3 (50)	1 (17)	0 (0)	2 (33)	6 <sup>c</sup> (50)
August 1982–February 1983	75	60	45 (75)	4 (7)	10 (17)	1 (2)	15 (20)
Total	134	109	52 (48)	18 (16)	24 (22)	15 (14)	25 (19)

<sup>a</sup> Percentage of samples typed is indicated in parentheses.

<sup>b</sup> Percentage of samples tested is indicated in parentheses.

<sup>c</sup> Includes 3 specimens with mixed serotypes.

least twice as high as those with each of the three other serotyping monoclonal antibodies.

**Rotavirus cultivation.** Some of the specimens were adapted to grow in tissue culture (MA-104 or African green monkey kidney cells) by using the methods of Sato et al. (19). Stool suspensions were preincubated for 1 h at 37°C with trypsin (10 µg/ml) and adsorbed onto cells in roller tubes. After 1 h, the inocula were washed and the cells were fed with minimal essential medium with Eagle salts containing antibiotics, glutamine, and trypsin (0.5 µg/ml). The tubes were rotated at 37°C for 7 to 10 days or until cytopathic effect was apparent. Positive control rotavirus strains Wa (serotype 1), DS-1 (serotype 2), P (serotype 3), and ST 3 (serotype 4) were grown in stationary cultures of MA-104 cells and included in every assay.

**RNA sequencing.** In some instances, when the monoclonal antibodies failed to detect the serotype specificity of certain isolates, the viruses were amplified in tissue culture, partially purified, and subjected to in vitro transcription (6) to generate single-stranded RNAs. Two selected areas of the RNA species encoding VP7 in those isolates were sequenced. RNA was sequenced by the primer extension method by using two synthetic oligonucleotides targeted to nucleotides 532 to 552 and 801 to 819 of the VP7 gene as previously described (10).

RESULTS

**Relative recovery rates of rotavirus serotypes 1, 2, 3, and 4.** A total of 134 rotavirus-positive specimens were examined by the monoclonal antibody assay. As judged by the reactivity of the specimens in the wells precoated with cross-reactive monoclonal antibody directed to VP3, 118 specimens contained a sufficient number of double-capsid particles to enable serotyping. The remaining specimens reacted positively (albeit some of them reacted weakly) in the VP6 monoclonal antibody-coated wells but did not react in wells coated with VP3 cross-reactive monoclonal anti-

body. Table 1 shows the number of specimens typed and their time of isolation. Three of the specimens reacted with more than one monoclonal antibody; two of them reacted with monoclonal antibodies to serotypes 1 and 3 and one reacted with monoclonal antibodies to serotypes 1 and 2. Previous RNA analysis of two of these specimens had shown the presence of more than one rotavirus in them. Not enough material was available from the third specimen to analyze its RNA pattern, but attempts to recover it in tissue culture yielded a strain which reacted with a single serotyping monoclonal antibody (serotype 1). Eleven specimens which reacted positively with the common VP3 antibody could not be serotyped initially. However, after tissue culture adaptation, 5 of the 11 were successfully serotyped.

A temporal variation was observed in the distribution of the different rotavirus serotypes (Table 1). Thus, serotypes 2, 3, and 4 were predominant at the beginning (December 1981 to February 1982) whereas serotype 1 strains were predominant towards the second half of the study (August 1982 to February 1983). It should be noted that the rate and the absolute number of rotaviruses isolated were lower in the period March 1982 to July 1982 (15 to 22%) than in the rest of the observed period (37 to 94%).

Table 2 shows the age distribution of the infants and the duration of illness according to serotype. The distribution of serotypes did not appear to vary with age. Although the children included in this study were not monitored from the beginning of their illness, information was available on the duration of illness for most of them. Infants infected with serotypes 1 and 3 appeared to have a more prolonged illness than those infected with serotypes 2 and 4. Thus, the mean duration of illness was 6.0 ± 0.5 (mean plus standard error of the mean) days for infants shedding serotype 1 whereas it was 3.0 ± 0.4 and 2.8 ± 0.4 for those infected with serotypes 2 and 4, respectively (*P* < 0.001 in each case; Student's *t* test). Likewise, the duration of illness was significantly longer in infants shedding serotype 3 rotavirus (5.6 ± 0.7

TABLE 2. Age distribution and duration of diarrheal illness by rotavirus serotypes recovered from Venezuelan infants with gastroenteritis

Serotype	No. of infected infants at age (mo):						Age <sup>a</sup> (mo) of infected infants			Duration <sup>a</sup> (days) of illness		
	0-3	4-6	7-9	10-12	13-18	19-24	Range	Mean	Median	Range	Mean	SEM
1	12	12	16	8	1	1	0-24	6.9	7	1-16	6.02	0.49
2	3	3	0	7	2	1	1-22	9.0	10	1-8	3.06	0.42
3	2	10	5	3	1	0	3-13	6.8	5	1-14	4.95	0.74
4	1	5	6	2	1	0	3-14	7.5	7	1-5	2.77	0.39
Total or avg for all serotypes	18	30	27	20	5	2	0-24	7.3	7	1-15	5.01	0.3

<sup>a</sup> Records of age and illness duration were not available for 7 infants.

REGION 307-351		AA:	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101
strain type		nt: 307	THR	GLU	ALA	SER	THR	GLN	ILE	ASN	ASP	GLY	ASP	TRP	LYS	ASP	SER
D *	1		ACU	GAA	GCA	AGU	ACU	CAA	AUC	AAU	GAU	GGU	GAC	UGG	AAA	GAC	UCA
M37 *	1		ACU	GAA	GCA	AGU	ACU	CAA	AUC	<u>SER</u>	<u>AGU</u>	GAU	GGU	<u>GLU</u>	UGG	AAA	GAC
HN 226	NT		ACU	GAA	GCA	AGU	ACU	CAA	AUC	AAU	GAU	GGU	GAC	UGG	AAA	GAC	UCA
HN 254	NT		ACU	GAA	GCA	AGU	ACU	CAA	AUC	<u>SER</u>	<u>AGU</u>	GAU	GGU	<u>GLU</u>	UGG	AAA	GAC
HN 384	NT		ACU	GAA	GCA	AGU	ACU	CAA	AUC	AAU	GAU	GGU	GAC	UGG	AAA	GAC	UCA

  

REGION 670-711		AA	208	209	210	211	212	213	214	215	216	217	218	219	220	221
D *	1		GLN	THR	THR	ASN	VAL	ASP	SER	PHE	GLU	MET	ILE	ALA	GLU	ASN
M37 *	1		CAA	ACA	ACA	AAC	GUA	GAC	UCA	UUU	GAA	AUG	AUU	GCU	GAG	AAU
HN 226	NT		<u>ARG</u>	ACA	ACA	AAC	GUA	GAC	UCA	UUU	GAA	AUG	<u>VAL</u>	GCU	GAG	AAU
HN 254	NT		CAA	ACA	ACA	AAC	GUA	GAC	UCA	UUU	GAA	AUC	<u>VAL</u>	GCU	GAG	AAU
HN 384	NT		<u>ARG</u>	ACA	ACA	AAC	GUA	GAC	UCA	UUU	GAA	AUG	<u>VAL</u>	GCU	GAG	AAU

FIG. 1. Comparison of RNA and deduced amino acid sequences in two regions of VP7 among three of the strains from this study which could not be serotyped with monoclonal antibodies and corresponding sequences of two previously published serotype 1 strains (10). The deduced amino sequence of strain D is presented above its nucleotide sequence. Amino acid differences between the other strains and strain D are enclosed in boxes, and nucleotide differences are underlined. The sequences correspond to hyperdivergent regions B (nucleotides 307 to 351, encoding amino acids 87 to 101) and E (nucleotides 670 to 711, encoding amino acids 208 to 221) previously shown to be conserved within rotavirus strains of the same serotype and divergent among different serotypes (10). NT, Nontypable; \*, previously published sequences (10).

days) than in those infected with serotypes 2 or 4 ( $P < 0.01$  in each case).

Six of the specimens which could not be typed had sufficient double-shelled virus particles to react with the common VP3 antibody. To further investigate these strains, we attempted tissue culture isolation, amplification, and sequencing of regions of the VP7 protein gene involved in neutralization specificity. Only three of the six strains grew in tissue culture. RNA sequences corresponding to two hyperdivergent regions (amino acids 87 to 101 and 208 to 221) are shown in Fig. 1. For comparison, the sequences in the same regions of strains D and M37 (both serotype 1 strains) are also shown. Strain HN384 was identical in both regions to the serotype 1 strain D, except for a single nucleotide substitution at position 702. Strain HN226 had, in addition to the same substitution at nucleotide 702, another substitution at nucleotide 349; neither replacement would result in an amino acid substitution. Strain HN254 was identical in both regions to the serotype 1 M37 strain.

## DISCUSSION

Difficulties in propagation of human rotaviruses have made it impossible to analyze large numbers of field strains to determine the relative frequency and the epidemiological features of infection with each serotype. Serotypic specificities of rotaviruses are determined by neutralization with hyperimmune sera and appear to correspond mostly with epitopes on the outer capsid glycoprotein VP7 (4, 13). Although recent evidence points to VP3 as a separate neutralization locus (12, 18), most efforts have been based on the development of anti-VP7 monoclonal antibodies for use in epidemiological studies to distinguish rotavirus serotypes.

Four human rotavirus serotypes have been described in studies from different regions of the world (9, 25). In

addition, two new serotypes have been proposed but their epidemiological importance is not known (1, 17). Most serotyping ELISAs currently available include only antibodies to serotypes 1, 2, 3, and 4.

We have used the monoclonal antibody test developed by Taniguchi et al. (21) to analyze the serotypic spectrum of rotavirus strains recovered from a group of Venezuelan infants. Most of these strains had been previously subgrouped (24), and some had been studied by RNA hybridization techniques to establish their genetic relatedness with well-characterized laboratory strains (7). Of the stools previously tested in a confirmatory ELISA, 88% could be serotyped. A few (6 of 134) specimens which contained double-shelled virus particles but could not be initially serotyped from the stool were typed after one to three passages in tissue culture.

Serotype 1 rotaviruses were the most commonly detected strains, accounting for 48% of the isolates. Although the number of positive specimens available at each time period was variable, it was clear that serotypes 2, 3, and 4 predominated at the beginning of the study (December 1981 to February 1982) whereas serotype 1 was predominant throughout the rest of the study. Thus, it appears that the relative frequency of a rotavirus serotype is variable over a period of time. Some of the specimens studied had been previously analyzed by RNA electrophoresis and cross-hybridization, and differences in the migration and cross-hybridization patterns could be observed among strains of the same serotype (data not shown), which suggests genetic variation within serotypes. It should be noted that in every case, rotavirus serotype 2 belonged to subgroup 1 and serotypes 1, 3, and 4 belonged to subgroup 2. It is also of interest that a strong correlation was observed between strains serotyped here and their previously reported hybridization patterns with the laboratory prototype strains Wa and DS-1 (7). Thus, while rotavirus serotype 2 presented a high degree of sequence similarity with the DS-1 prototype strain, rotavirus serotypes 1, 3, and 4 hybridized strongly with the Wa virus prototype. These correlations further support our previous observations on the existence of two rotavirus families (5, 7).

No differences were observed in the mean age at which the different serotypes were detected. However, significant differences were observed in the duration of illness. Thus, serotype 1 and 3 strains appear to induce a more prolonged illness than serotypes 2 and 4. It is difficult to assess whether such differences are related to virulence properties among the strains, the presence of preexisting antibodies in the infants, or some other factor.

Universality of any monoclonal antibody-serotyping test is an important requisite for its usefulness. If new rotavirus serotypes are discovered, they could easily be accommodated in a serotyping test by deriving the corresponding monoclonal antibodies. On the other hand, recent studies demonstrate the existence of antigenic variants within a rotavirus serotype which may result in failure to serotype strains of a given serotype with a single monoclonal antibody obtained from a different strain of the same serotype. Such antigenic variants have been described by Heath et al. (11) and Coulson et al. (2) and referred to by the latter group as "monotypes." In the present study, three strains which could not be typed were tentatively identified as serotype 1 strains by their sequence identity in two regions of the VP7 gene with a series of serotype 1 strains that do react with the type 1 monoclonal antibody. Studies in our laboratory have recently demonstrated a strong degree of genetic stability in

the gene encoding VP7 among strains of the same serotype and significant sequence differences with other serotypes (10, 10a). It is possible that the three nontypable strains represent different monotypes or antigenic variants or that their corresponding VP7 epitopes react with the serotyping monoclonal antibodies below the detectable threshold levels of this assay. Recent studies of neutralization escape mutants derived with monoclonal antibody KU-4 (the serotype 1 monoclonal antibody used in this study) indicate that this antibody maps to amino acid 213 (K. Taniguchi, personal observation), which is similar in the three nontypable strains to the corresponding amino acid of other serotype 1 viruses. No amino acid substitutions were detected between strains HN226 and HN384 and the serotype 1 strain D. Amino acid substitutions observed at positions 94, 97, 208, and 218 in one of the strains (HN254) matched the sequence of M37, a serotype 1 laboratory strain which does react with monoclonal antibody KU4. Therefore alternative explanations must be sought to explain the lack of reactivity of the three nontypable strains with the serotype 1-specific monoclonal antibody. Among them, the possibility that changes in a different area of VP7 (which appears to be a highly conformation-dependent antigen) or changes in VP3 (by steric hindrance) may be responsible for the observed phenomena is being explored.

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