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Dot Hybridization Assay for Distinction of Rotavirus Serotypes

JORGE FLORES,[1]* KIM Y. GREEN,[1] DORYS GARCIA,[2] JOHNNA SEARS,[1] IRENE PEREZ-SCHAEL,[2] LUIS F. AVENDAÑO,[3] WILLIAM B. RODRIGUEZ,[4] KOKI TANIGUCHI,[5] SHOZO URASAWA,[5] AND ALBERT Z. KAPIKIAN[1]

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[1]; Instituto de Biomedicina, Ministerio de Sanidad y Asistencia Social, Caracas, Venezuela[2]; Department of Microbiology, University of Chile School of Medicine, Santiago, Chile[3]; Children's Hospital Medical Center and George Washington University Medical School, Washington, D.C. 20010[4]; and Department of Hygiene and Epidemiology, Sapporo Medical College, Sapporo, Japan[5]

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We have developed a hybridization assay that permits distinction of rotavirus serotypes 1, 2, 3, and 4. The serotype of rotaviruses from stool samples or tissue culture was recognized by hybridization of specific probes to (i) blots of viral double-stranded RNAs electrophoresed in agarose gels (Northern blots) or (ii) heat-denatured double-stranded RNAs directly dotted on nylon membranes. The probes consisted of ^{32}P-labeled cDNA synthesized by reverse transcription of in vitro derived rotavirus mRNA from rotavirus serotypes 1 to 4. To prepare these probes, mRNAs were primed with a 17-mer nucleotide common to all four serotypes whose sequence is complementary to bases 375 to 391 of the rotavirus gene encoding the VP7 glycoprotein (gene 8 or 9 depending on the rotavirus strain). The resulting downstream transcripts encompassed areas of major sequence divergence among the four serotypes. Hybridization at high stringency (50°C, 50% formamide, 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) was performed for 16 to 48 h. Autoradiograms of the washed membranes allowed recognition of the rotavirus serotype present in the blotted or dotted specimens since each of them hybridized preferentially to one of the four probes. Twenty-four laboratory specimens and 103 clinical specimens from Washington, D.C., Venezuela, and Chile were "serotyped" with this assay. The results were similar to those obtained with a monoclonal antibody serotyping assay.

Acquisition of further knowledge on the natural history of rotavirus as well as the evaluation of recently developed rotavirus vaccine candidates require methods to detect the serotype specificity of rotaviruses in clinical specimens from field studies. Until recently, serotyping of human rotaviruses was performed by tissue culture isolation followed by neutralization (30, 18) or fluorescent focus inhibition (2) assays that use hyperimmune antibodies to each of the four serotypes. The low efficiency of propagation of human rotaviruses was in some cases circumvented by rescuing the glycoprotein gene (encoding the serotype-specific protein VP7) by reassortment with easily cultivable animal rotavirus strains (15). Several groups have recently developed serotype-specific monoclonal antibodies (5, 16, 27, 29) which have been used successfully in enzyme-linked immunosorbent assays for serotyping field rotavirus strains. We describe here an alternative approach based on our observations of the high degree of sequence conservation in the VP7 gene among rotaviruses belonging to the same serotype and their distinctiveness across different rotavirus serotypes (13, 22). cDNA probes prepared from an area of this gene with 25 to 30% divergence among serotypes were used in blot and dot hybridization assays to recognize and distinguish rotavirus serotypes.

MATERIALS AND METHODS

RNA extraction. Rotavirus double-stranded RNAs (dsRNAs) were obtained from clinical specimens (1 ml of 10% stool suspensions in phosphate-buffered saline) by consecutive extractions with 1,1,2-trichloro-1,2,2,-trifluoroethane, phenol, and chloroform as described previously (7). RNA in the supernatants was precipitated with ethanol by centrifugation at 12,000 × *g* after overnight incubation at −20°C. Rotavirus RNA from infected cultures was obtained in the same manner except for initial concentration of the virus by centrifugation at 35,000 rpm for 2 h in an SW40 rotor (Beckman Instruments, Inc., Palo Alto, Calif.).

Rotavirus strains. Several human and animal tissue culture-adapted rotavirus strains of known serotype (as previously determined by plaque neutralization assays) were tested. They included the following: D, Wa, M37, and Mont (serotype 1), DS1, HN126, and 1076 (serotype 2), P, M, McN13, RRV, SA11, and CU1 (serotype 3), and ST3, VA70, Hosokawa, and Gottfried (serotype 4) (30, 18, 15). Clinical specimens tested were obtained from previous studies in Caracas, Venezuela, which included samples isolated from infants hospitalized with diarrhea (10) and from asymptomatic newborns (25). Also, specimens from Washington, D.C. (14), and Chile (1) were tested.

Serotype-specific probes were prepared from single-stranded RNA (ssRNA) transcripts derived from single-gene substitution reassortants described previously (23, 24). Type 1 probe was synthesized from the D × RRV reassortant ssRNA; type 2 probe, from the DS1 × RRV reassortant; type 3 probe, from the P × UK reassortant; and type 4 probe, from the ST3 × RRV reassortant. These reassortants are easier to cultivate and transcribe than their corresponding human rotavirus parents. The genotype of these reassortants consists of 10 genes of the animal strain (either the RRV [rhesus rotavirus vaccine] or the UK bovine strain) and 1 gene (the eighth or ninth gene which encodes the serotype-specific glycoprotein VP7) from the corresponding human strain (D, DS1, P, or ST3).

Preparation of probes. ssRNAs were prepared by in vitro transcription of rotavirus-purified cores as described previ-

* Corresponding author.

ously (9). These ssRNAs served as the template for serotype-specific cDNA probe synthesis; 2 to 4 μg of total ssRNA was mixed with 100 ng of the oligodeoxynucleotide 5'GATCCTGTTGGCCATCC3', which is complementary to the region of the VP7 mRNA between nucleotides 375 and 391 (13). The RNA-primer mixture was boiled for 1 min, chilled on ice, and incubated for 30 min at 43°C in a mixture of the following ingredients at the given concentrations: dATP, 100 μM; dGTP, 100 μM; TTP, 100 μM; dCTP, 20 μM; avian myeloblastosis virus reverse transcriptase, 300 U/ml; KCl, 50 mM, $MgCl_2$, 10 mM; Tris hydrochloride, 50 mM; pH 8.3. In addition, the mixture contained 50 μCi of [^{32}P]dCTP.

Northern (RNA) blots. Northern blot hybridizations were carried out with some modifications from our previously described method (8). Rotavirus ds-RNAs were electrophoresed in agarose gels (1.2 to 1.4%), blotted after staining with ethidium bromide, and photographed under UV light. RNAs were denatured and partially digested by incubating the gel in 0.2 M NaOH for 10 min. The NaOH was neutralized by successive washes in 1 M, 100 mM, 50mM, and, finally, 20mM Tris hydrochloride, pH 7.0. Two pieces of nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) cut to the gel size, previously rinsed in 20 mM Tris hydrochloride, pH 7.0, were placed over and under the gel to obtain duplicate blots from it. The gel with the nylon membranes was sandwiched in stacks of blot paper. After overnight blotting, the membranes were lightly rinsed in water and baked at 80°C for 2 h. The membranes were kept at room temperature until time of hybridization.

Dot blots. dsRNAs or ssRNAS (prepared as described above) from rotavirus-infected tissue culture or from clinical specimens were denatured by boiling for 3 min. After 1 to 3 min on ice, the RNAs were dotted (0.8 to 1 μl) on Nytran membranes marked with grids which had been previously rinsed in 20 mM Tris, pH 7.0, and allowed to dry. Several identical membranes were prepared from the same set of specimens. After all the samples had been dotted on the gridded membranes, membranes were baked for 2 h at 80°C and kept at room temperature until hybridization was carried out. In each case, several controls of known serotype were dotted on the membranes.

Hybridization. The blotted or dotted membranes were prehybridized for 1 to 2 h at 50°C in a mixture containing 50% formamide, 4× SSC (1× SCC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll (Pharmacia, Inc., Piscataway, N.J.), and 50 mM phosphate buffer, pH 6.5. A second prehybridization period was applied in fresh prehybridization mixture containing, in addition, 50 μg of salmon sperm DNA per ml. Hybridization was carried out in a similar solution, which in addition to the salmon sperm DNA contained 10% dextran sulfate and 3×10^6 to 5×10^6 cpm of probe; hybridization took place at 50°C for 16 to 48 h and was terminated by washing the membranes in 2.5× SSC–0.1% sodium dodecyl sulfate at room temperature (four times), followed by two 15-min washes at 50°C in 1.25X SSC–0.05% sodium dodecyl sulfate. The membranes were allowed to dry at 37°C and exposed for 2 to 24 h to X-ray film with intensifying screens.

RESULTS

Northern blots of electrophoresed RNAS. Initially, to determine optimal hybridization conditions and the potential hybridization of the probes to other than the gene 9 RNA segment, tissue culture rotavirus specimens of known serotype were tested after electrophoretic separation and blotting as described above. Figure 1 shows an example of the results obtained when duplicate gels were blotted (in both directions) to obtain four identical blots; each of the blots was hybridized to a different serotype probe (1, 2, 3, or 4). Similar experiments were carried out on 24 specimens derived from Venezuelan children with diarrhea (Fig. 2). In each case, one of the probes hybridized with greater intensity than the other three to the blotted RNA in the area corresponding to segments 7, 8, and 9, which cannot be separated in these gels.

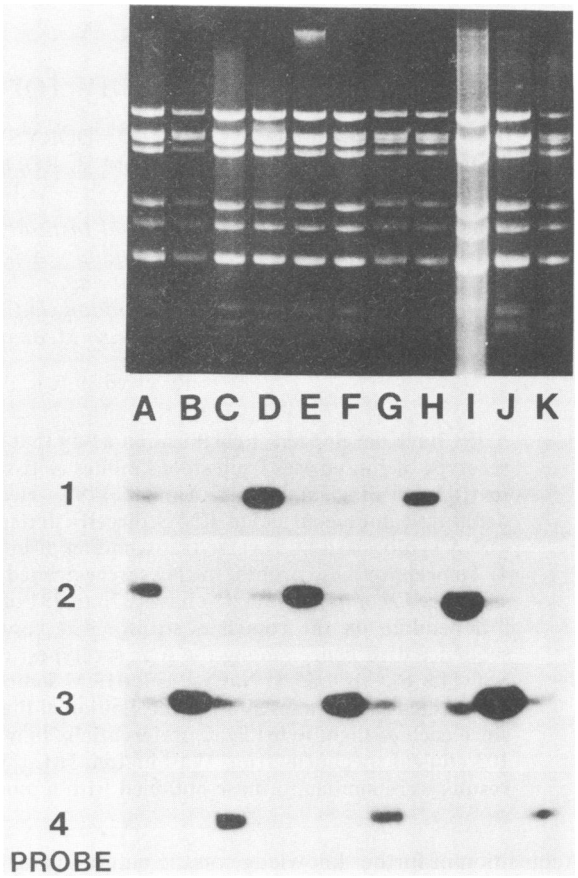

FIG. 1. Northern blot hybridization of several rotavirus dsRNA preparations. Eleven samples were electrophoresed in two 1.2% agarose duplicate gels, blotted as described in the text to obtain four blots, and hybridized to probes derived from D × RRV (probe 1), DS1 × RRV (probe 2), P × UK (probe 3), and ST3 × RRV (probe 4). (Top) UV-light photograph of one of the gels after ethidium bromide staining. (Bottom) Autoradiograms of the areas corresponding to the 7-8-9 gene complexes after hybridization to the probes. The following dsRNA preparations were run: A, DS1 (type 2); B, P × UK reassortant (type 3); C, ST3 × RRV reassortant (type 4); D, Wa (type 1); E, DS1 × RRV reassortant (type 2); F, P (type 3); G, ST3 (type 4); H, D (type 1); I, DS1 (type 2); J, P × UK reassortant (type 3); K, VA70 (type 4).

Dot hybridization. Over 20 cultivable rotaviruses, or rotavirus reassortants, whose serotype had been previously determined by neutralization assay were repeatedly dotted and hybridized to the serotyping probes. These included rotavirus strains of serotypes other than 1 to 4, such as OSU (serotype 5), NCDV and UK (serotype 6), and the newly described Wi61 (4) and 69 M (21); specific hybridization to any of the probes was not observed with these strains. An

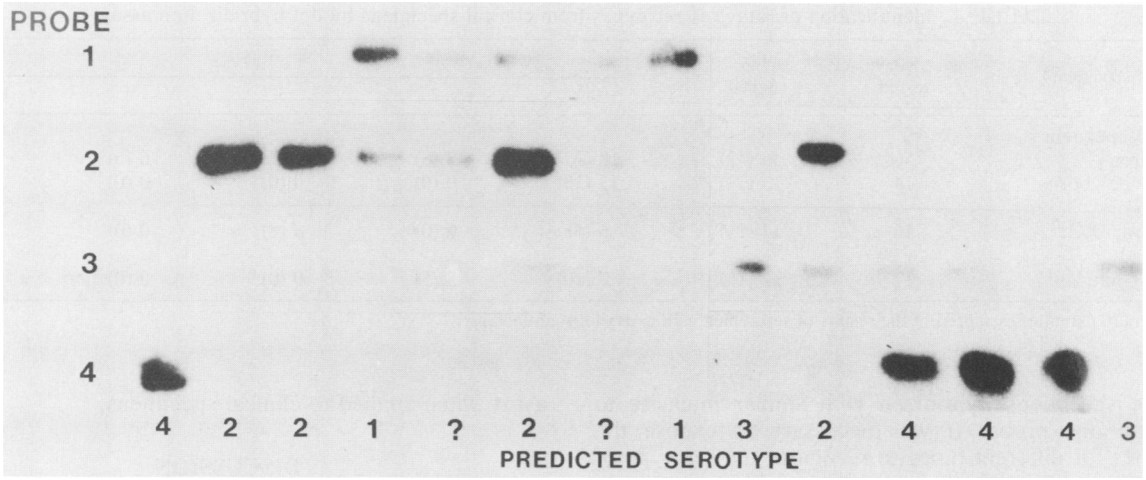

FIG. 2. Northern blot hybridization of dsRNAs obtained from 14 stool specimens from Venezuelan infants with diarrhea. The RNAs were run in duplicate gels and blotted in both directions to obtain four similar blots, which were hybridized to serotype 1, 2, 3, and 4 probes. Autoradiograms from the gene 7-8-9 region have been lined up such that each vertical set of lanes corresponds to the same RNAs. The predicted serotype corresponding to each specimen is given at the bottom.

example is presented in Fig. 3 in which 10-fold dilutions of denatured dsRNAs from four such strains (Wa, DS1, P, and ST3) were blotted, hybridized, and exposed to X-ray film for various times. Table 1 presents the results of dot "serotyping" 103 stool specimens from Caracas, Venezuela; Washington, D.C.; and Santiago, Chile. The serotype of 18 specimens could not be identified, in most cases because of failure to hybridize with any of the four probes; in three

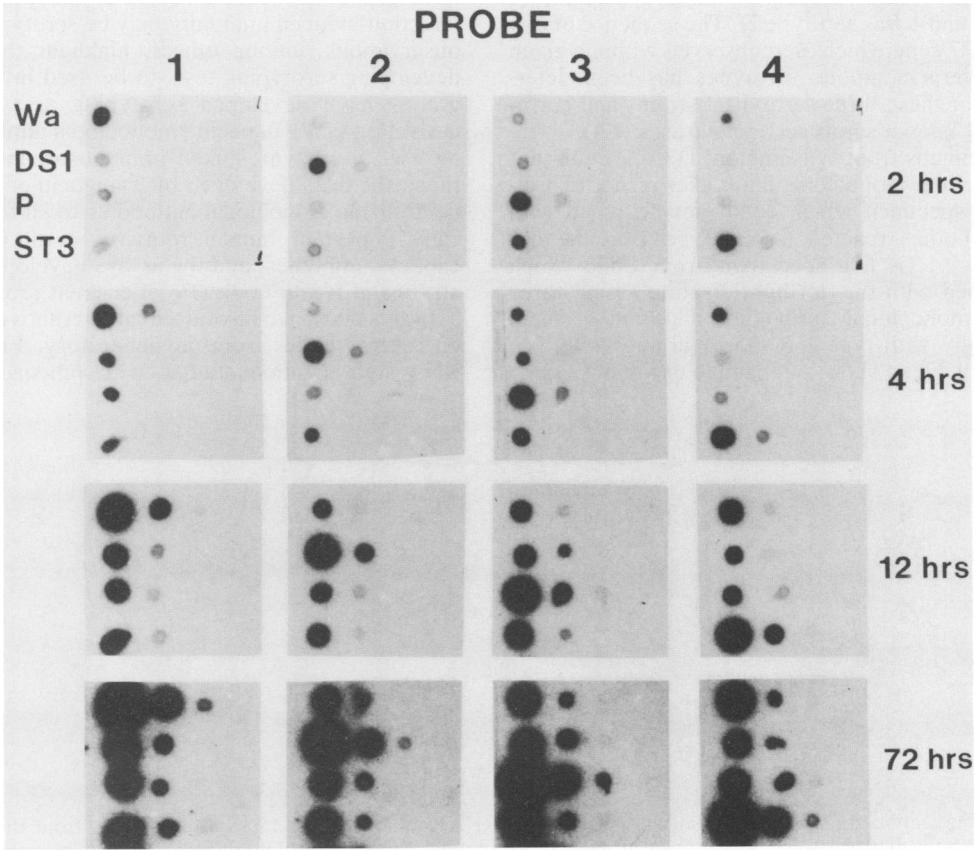

FIG. 3. Dot hybridization assay of four control RNAs. dsRNAs from Wa (serotype 1), DS1 (serotype 2), P (serotype 3), and ST3 (serotype 4) were dotted in 10-fold dilutions (starting with 10 ng on the left) in quadruplicate membranes and hybridized to probes prepared from D × RRV (serotype 1), DS1 × RRV (serotype 2), P × UK (serotype 3), and ST3 × RRV (serotype 4). The membranes were then exposed for different time periods (as indicated on the right) to X-ray film with intensifying screens.

TABLE 1. Identification of rotavirus serotypes from clinical specimens by dot hybridization assay[a]

Origin of specimens	No. tested	No. typed	No. of type:				
			1	2	3	4	Unidentified
Caracas, Venezuela							
Ill children	56	46 (52)	18 (23)	9 (8)	13 (15)	6 (6)	10 (4)
Healthy newborns	14	13 (13)	13 (13)	0 (0)	0 (0)	0 (0)	1 (1)
Washington, D.C.	16	14 (13)	6 (5)	0 (0)	8 (8)	0 (0)	2 (3)
Santiago, Chile	17	12 (15)	7 (10)	5 (5)	0 (0)	0 (0)	5 (2)

[a] Numbers in parentheses represent the results of serotyping with monoclonal antibodies.

cases the specimens hybridized with similar intensity to more than one probe. It was necessary to expose the membranes for different times to recognize the serotype of specimens giving signals that were too weak or too strong; when this was done, analysis of serotype for a given specimen was carried out from membranes exposed to the four probes for the same length of time.

All but 10 of the 103 specimens were typed with monoclonal antibodies. Monoclonal antibody typing had not yet been done before analysis of the dot assay results on the Venezuelan specimens. Of 56 specimens from the Venezuelan ill children, 10 which could not be identified with the dot hybridization were typed with monoclonal antibodies (8 were serotype 1 and 2 were serotype 2). On the other hand, 4 of the 56 specimens could not be identified with monoclonal antibodies but were serotyped by dot hybridization (3 were serotype 1, and 1 was serotype 2). The sequence of two regions of the VP7 gene which are conserved within a given serotype but divergent among serotypes has been determined recently for these three serotype 1 strains and corresponds to that of known serotype 1 rotaviruses (12).

Of the 16 specimens from Washington, D.C., 2 could not be identified by either monoclonal antibodies or dot hybridization. A third specimen which could not be typed with monoclonal antibodies reacted as serotype 1 in the dot hybridization assay. Of five specimens from Chile which could not be typed with the dot hybridization assay, three were typed by monoclonal antibodies; all three of them reacted specifically with type 1 monoclonal antibody. The autoradiograms of Fig. 4 show an example of the dot typing assay when applied to clinical specimens.

DISCUSSION

Studies on the epidemiology of rotavirus have been limited by the lack of information about the serotype specificity of the strains identified in the field. Whereas some aspects of the molecular epidemiology of this agent have been widely studied because of the relative ease of obtaining viral RNA for electrophoretic and hybridization analysis, studies on the prevalence, distribution, virulence, and natural protection induced by infection with specific rotavirus serotypes have not been carried out because of the difficulties encountered in cultivation of rotavirus for serotyping.

Recent experience with rotavirus vaccines (11, 19) as well as studies of nosocomial rotavirus infection (3) indicate that infection-induced immunity may be serotype specific. These observations, among others, highlight the importance of developing serotyping tests to be used in the field. Several groups have developed serotyping assays based on the derivation of VP7-specific monoclonal antibodies which can be used in enzyme-linked immunosorbent assays. Among them, the tests developed by Taniguchi et al. (29), in which neutralizing monoclonal antibodies to all four epidemiologically important human rotavirus serotypes are used as capture antibodies, and the assays developed by Coulson et al. (5) and Heath et al. (16) offer great promise.

In this study we have used an alternative approach based on recent studies from our laboratory. First, liquid RNA-RNA hybridization studies with full-size ssRNA probes

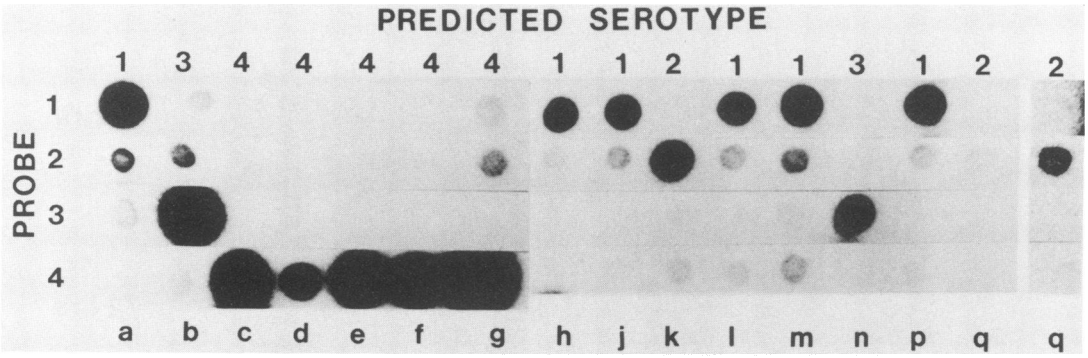

FIG. 4. Dot hybridization for serotyping clinical specimens. dsRNAs obtained from 15 stool specimens from the same number of Venezuelan infants with diarrhea were dotted in quadruplicate membranes, which were hybridized to probes prepared from D × RRV (serotype 1), DS1 × RRV (serotype 2), P × UK (serotype 3), and ST3 × RRV (serotype 4). The autoradiograms were lined up such that each set of vertical dots represents dotted RNAs from the same specimen. The predicted serotype for each specimen is indicated on the top. Prolonged exposures of the same membranes to X-ray film resulted in sharper distinction of serotype. An example of this can be seen in the last two sets of dots (specimen q, serotype 2), which were photographed after short and long exposure times.

TABLE 2. Comparison of nucleotide sequence divergence in the region of the glycoprotein gene (gene 8 or 9) comprising nucleotides 1 to 391 of four human retrovirus serotypes

Serotype	No. of bases different between intersecting serotypes		
	Serotype 1 (D)	Serotype 2 (DS1)	Serotype 3 (P)
1			
2	107 (27.4)[a]		
3	97 (24.9)	108 (27.7)	
4	101 (25.9)	116 (29.7)	111 (28.9)

[a] Number in parentheses represents percent divergence.

indicated that a high degree of conservation in the VP7 gene exists among rotaviruses from the same serotype and that this gene is distinct from its alleles present in other serotypes. In these experiments, ssRNA probes were obtained from four RRV or UK rotavirus reassortants in which the VP7 had been replaced by the corresponding gene from one of the four commonly detected human rotavirus serotypes. In later experiments, the VP7 sequences of a number of human rotavirus strains were derived by primer extension (13). Direct comparison of the complete sequences (except for the 30 to 40 bases corresponding to the 3′ end of the mRNA) from 13 human strains confirmed that within a given serotype a high degree of sequence conservation occurs (>96%), whereas sequence divergence of over 20% occurs between different serotypes (13). The divergent bases appear to be grouped in regions of the gene which encode neutralization-associated epitopes. Further sequence information from two highly divergent regions in 25 additional strains confirmed these observations (14). Both liquid hybridization and direct sequencing of divergent areas can be used to identify rotavirus serotypes. In our current more practical approach, we prepared cDNA probes specific to nucleotides 1 to 391 of the glycoprotein gene from the four rotavirus serotypes by priming their synthesis with an oligonucleotide complementary to bases 375 to 391 (Table 2). These probes were subsequently hybridized to quadruplicate membranes in which RNAs had been blotted after electrophoretic separation or directly dotted. After initial trials using different stringencies, optimal conditions were established that allowed distinction of the serotypes of the strains blotted or dotted. In theory, these conditions would allow the distinction of RNAs of up to 15% divergence (8, 28). In practice, hybridization could be detected with all four probes by prolonged exposures to X-ray film; however, the intensity of the hybridization signal was clearly greater with one of the probes than with the other three. The background seen with the nonhomotypic probes may be due to highly conserved areas of the gene (for instance, sequence similarity of >95% occurs in nucleotides 1 to 70). In some instances, hybridization was observed with two of the four probes. In two of these cases (not included in Table 1), two different viruses were present in the same specimen as judged by the presence of mixed RNA patterns and their reactivity to two monoclonal antibodies. In three other instances, the hybridization signal was similar for two or more probes.

Comparison of the results with those obtained with the monoclonal antibody assay indicates that, at this time, the dot assay is less efficient. However, it should be noted that four specimens which failed to be recognized by the monoclonal antibodies reacted clearly with either serotype 1 (three specimens) or serotype 2 (one specimen) probes. Sequence information from two of the specimens reacting with serotype 1 probe revealed that they share the sequence of the probed area with serotype 1 rotaviruses (data not shown). Thus, it is possible that in some cases the dot assay can indirectly detect serotype specificities not recognizable with monoclonal antibodies. Coulson et al. found that some of the specimens they studied did not react with their specific monoclonal antibody to serotype 1 used for typing but did react with other monoclonal antibodies to the same serotype (5) and refer to these antigenic variants as "monotypes." This suggests that different monotypes existing within a given serotype may complicate the widespread use of currently available monoclonal antibodies, and eventually a battery of hybridomas for each serotype may be needed. It is likely, however, that the small differences detected by the monoclonal antibodies may correspond to one or a few base changes in the gene. If this were the case, the probes developed here could possibly identify the serotypes of different monotypes as being the same. Three of the strains from Venezuela which hybridized as serotype 1 in this assay but could not be typed by monoclonal antibodies have been recently studied by sequence analysis of two regions of the VP7 gene (12). Although these strains have not been serotyped by plaque neutralization assays, it is likely from previous comparisons among the VP7 genes of rotavirus serotypes 1 to 6 that they do belong to serotype 1 (13, 14).

Improvements on the present dot serotyping assay currently ongoing in our laboratory include the use of cloned DNA probes in which the highly conserved 70 bases at the 5′ end have been removed, the use of nonradioactive labeling, and the inclusion of probes to the newly described human rotavirus serotypes.

The same approach used here could be used to detect differences in the fourth gene which encodes VP4 (formerly referred to as VP3), the other outer capsid protein involved in neutralization (17) and perhaps in virulence (8). Once the areas of the fourth gene which determine these two properties are defined, it would be feasible to recognize corresponding specificities in strains from the field.

Others have used cDNA probes from clones of the VP7 gene to distinguish serotypes 1 and 3 (6), or 3 (20); however, testing of field strains was not performed. In our experience, use of cDNA probes representing all or most of the gene have resulted in unacceptably higher levels of background when compared with the probes described here.

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