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Comparison of Enzyme Immunoassay, PCR, and Type-Specific cDNA Probe Techniques for Identification of Group A Rotavirus Gene 4 Types (P types)

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This study was designed to evaluate three techniques most commonly used to identify the VP4 (P) types of human group A fecal rotaviruses. The techniques included PCR with nested primers and hybridization with PCR-generated probes (to determine the P genotypes). The results obtained by these genetic techniques were evaluated against those obtained by an enzyme immunoassay (EIA) incorporating neutralizing monoclonal antibodies (N-MAbs) reacting with three major human P serotypes (serotypes P1A, P1B, and P2A). The P types of the rotaviruses present in 102 fecal specimens were determined under code by each of the three assays. The specificity of each assay was evaluated against a "gold standard" putative P type (P serotype and genotype) deduced from knowledge of the VP7 (G) type and the origin of the fecal specimen. Overall comparison of the results showed respective sensitivities and specificities of 92 and 92% for reverse transcription-PCR, 80 and 99% for hybridization, and 73 and 91% for EIA with N-MAbs. The hybridization assay retained high sensitivity with specimens stored for ≥10 years. Hybridization assays with nonradioactive probes are relatively inexpensive and are suited for use in developing countries. In summary, both genetic assays showed high sensitivities and specificities in assigning a P type to human fecal rotavirus strains. Further evaluation of the EIA with N-MAbs is required, together with incorporation of new N-MAbs for the detection of the additional P types detected in developing countries.

Group A rotaviruses are the single most important cause of severe acute diarrhea in young children worldwide (18). Classification of rotaviruses into serotypes is based on the identification of two outer coat proteins, VP7 and VP4 (coded by separate genes), that initiate the formation of neutralizing antibodies shown to be protective against infection in vivo and in vitro (6, 14). VP7 serotypes (G types) can now be identified by enzyme immunoassay (EIA) incorporating VP7-specific neutralizing monoclonal antibodies (N-MAbs). This assay has permitted extensive worldwide epidemiological studies of G types.

No similar epidemiological information about the diversity and prevalence of VP4 types (P types) is yet available, even though there is evidence that the natural immune response in humans is predominantly directed at VP4 (6, 14). Techniques for determining P types have been difficult to develop. Hyperimmune antisera raised to intact virus particles primarily recognize VP7, so they are unreliable for VP4 typing. The existence of at least four different P types was deduced initially from nucleotide sequence analysis of gene 4 (coding for VP4). Hyperimmune antisera raised to baculovirus-expressed VP4 identified three distinct VP4 serotypes and one subtype identified as P1A (associated with VP7 serotypes G1, G3, G4, and G9, which cause symptomatic illness), P1B (associated with VP7 serotypes G2 and G12), P2 (associated with human rotaviruses causing endemic infections in neonates), and P3 (K8-like). The difficulty in raising specific serological reagents for measuring P-type antigenic differences (serotypes) focused efforts on identifying VP4 types on amino acid sequence comparisons. VP4 relationships established by sequence analysis are termed "genotype" and are denoted by including the genotype number in brackets (6). P serotypes and P genotypes have been shown to be analogous for standard tissue culture-derived strains (10, 11). A predictable correlation between VP4 genotype and serotype is not yet clearly established for fecal rotaviruses (6, 14). The epidemiological field studies that have been performed to date confirm that there are four common rotavirus types (causing severe disease worldwide), classified as P1A[8]G1, P1A[8]G3, P1A[8]G4, and P1B[4]G2. In addition, most strains causing endemic infections in neonatal nurseries have been classified as P2A[6] and are usually associated with G1, G2, G3, G4, or G9 serotypes (8, 12, 15).

Techniques developed to classify the VP4 types of field strains of rotaviruses include RNA-RNA hybridization (24), Northern hybridization with PCR-generated probes (21, 28, 30), PCR typing with nested primers (7), detection of restriction fragment length polymorphisms of the PCR-amplified VP4 genes (17), and reaction with VP4-specific N-MAbs incorporated in an EIA (3, 26). The study described here was undertaken to compare the sensitivities, specificities, and relative costs of three of these P-typing assays. The three techniques were assessed in parallel with a coded collection of human rotaviruses excreted in feces. The resulting evaluation has relevance to the selection of a technique to be used in field studies.

MATERIALS AND METHODS

Rotavirus standard strains. The tissue culture-adapted rotaviruses used in this study included RV4 (P1A[8]G1), P (P1A[8]G3), F45 (P1A[8]G9), RV5 (P1B[4]G2), RV3 (P2A[6]G3), ST3 (P2A[6]G4), and SA11 (P[2]G3). All were grown in MA104 cells in the presence of trypsin, extracted with fluorocarbon, and concentrated 100-fold by ultracentrifugation. These preparations were used to eval-

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TABLE 1. Characteristics of VP4 N-Mabs used to determine P types of human rotaviruses

N-Mab	P-type specificity	VP4-reactive epitope (amino acid no.)	Rotavirus strains bound in EIA	Reference
F45:4	P1A[8]	392	Wa, KU, RV-4, YO, P, VA70, Hoso, F45	3, 19
IA10	P1A[8]	458	Wa, P, WI61, VA70, KU	25, 26
RV5:2	P1B[4]	148	DS-1, S2, RV-5, RV-6, L26	3, 19
ST3:3	P2A[6]	397	M37, RV-3, ST-3	3, 19
HS6	P2A[6]	72	M37, McN13, ST-3, Gottfried	25, 26
HS11	P2A[6]	217	M37, McN13, ST-3	25, 26

uate working dilutions of N-MAbs for P typing by EIA and were also incorporated as control antigens in P-typing assays.

Rotavirus field strains. Rotavirus field strains were assembled as 102 coded specimens and included 73 rotavirus-positive fecal specimens (containing 19 different rotavirus electropherotypes) collected as part of an Australia-wide epidemiological survey of rotaviruses infecting young children admitted to hospital for treatment of severe acute diarrhea from 1993 to 1995. The specimens had been frozen at −70°C for various lengths of time in the laboratory of origin and were transferred in a frozen state to our laboratories for G typing with VP7 type-specific N-MAbs in a serotyping EIA (4). The specimens were then stored as unextracted feces or as 10% extracts in phosphate-buffered saline (PBS) at −70°C. Further specimens included 29 rotavirus-positive specimens that had been stored in our laboratory at −20°C (21 specimens) for 10 to 15 years or −70°C (8 specimens) for 20 years. They were stored as unextracted feces (15 specimens), electron microscopy pellets (2 specimens), or 10% fecal homogenates in PBS (12 specimens). In preparation for P typing, all specimens were thawed, prepared as 10% PBS extracts after homogenization and clarification by low-speed centrifugation (where appropriate), and immediately assayed for the rotavirus P type by EIA.

In addition, a subset of 17 specimens collected during 1995 and later identified as G1P1A[8] (10 strains), G2P1B[4] (5 strains), and G4P1A[8] (2 strains) were subjected to seven rounds of thawing and refreezing at −70°C and were retested by EIA after zero, three, five, and seven freeze-thaw cycles.

RNA from aliquots of all specimens was prepared by phenol-chloroform extraction, followed by purification by adsorption to hydroxyapatite. The RNA was eluted in 200 mM potassium phosphate buffer and was stored at −20°C until assayed.

MAbs. The N-MAbs used for P typing were chosen from hybridoma cell lines raised and characterized in our laboratories (3, 19) and from others listed in published papers (14, 20, 25, 26, 32, 33). The VP4 N-MAbs that were selected had been raised to human rotaviruses and appeared to be P type specific by neutralization assay and to show P type-specific binding in EIAs when they were tested against a wide range of standard human and animal rotavirus strains of different P types (Table 1).

Ascitic fluids containing N-MAbs were prepared from all cell lines in our laboratories by standard techniques (3). Ascitic fluids from BALB/c mice were purified by protein A or protein G affinity chromatography. The working dilution of each N-MAb incorporated into the P-typing assay was determined by checkerboard titration against tissue culture-adapted P1A[8] viruses (RV4, P, and F45), P1B[4] virus (RV5), and P2A[6] viruses (RV3 and ST3). The control specimens included in each assay were rotavirus-positive specimens representing P1A[8], P1B[4], and P2A[6]; mock-infected MA104 cells; and a rotavirus negative-specimen comprising a pool of fecal extracts negative for rotavirus by the EIA with N-MAbs. In addition, cell culture-grown simian rotavirus strains SA11 and RRV (representing nonhuman P types) were included to check the specificity of the assay.

EIA for P types. A double-sandwich EIA was used to establish the optimal dilutions of N-MAbs to be used and to assay all fecal specimens (3). The type of rabbit antirotavirus hyperimmune serum used as capture antibody was matched to the G type of the fecal virus (previously determined by the VP7 serotyping EIA). The six N-MAbs being tested were added at the optimal dilutions assessed previously by checkerboard titration. A fecal sample was considered to be positive for a particular P type if the optical density at 450 nm (OD_{450}) with the N-MAb was at least twice the OD_{450} of the negative fecal extract.

VP4 typing assay by nested PCR. Nested PCR was carried out as described by Gentsch et al. (7) with gene 4-specific primers con2 and con3 in first-round reverse transcription-PCR (RT-PCR). The second-round typing PCR incorporated con3 and the P-type specific primers 1T-1 (P[8] specific), 2T-1 (P[4] specific), and 3T-1 (P[6] specific).

VP4 typing with cDNA probes. Northern hybridization of viral double-stranded RNA was performed with PCR-derived digoxigenin-labelled cDNA probes made from gene segment 4 of standard P1A[8] (RV4), P1B[4] (RV5), and P2A[6] (ST3) strains as described previously (9). Alkaline rather than heat denaturation of viral double-stranded RNA was used. Briefly, 16 μl of each sample or 3.5 μl of each control (RV4, RV5, and ST3) was diluted to 80 μl in distilled H_2O in a sterile polystyrene, 96-well microtiter tray (Nunc). An equal volume of 100 mM NaOH was added to each well, and the plate was left at room temperature for 5 min. Samples and controls (50 μl) were then loaded onto triplicate nylon membranes by using a Bio-Dot Microfiltration Apparatus (Bio-Rad) and were washed through with 100 μl of 2.5 M NaCl–0.5 M Tris (pH 8.0). Hybridization and chemiluminescence detection were carried out as described previously (9).

RESULTS

The sensitivity and specificity of each assay were calculated by reference to a "gold standard" whereby the correct P type was considered to be that inferred from the known G type and from the clinical sources of the specimens. Thus, strains excreted by children admitted to hospital with acute rotavirus diarrhea were considered to represent P1A[8] if they were G1, G3, or G4 and P1B[4] if they were G2. Strains excreted by neonates during the first 10 days of life while housed in obstetric hospital nurseries where rotavirus infections were endemic were considered to represent P2A[6] strains. The rationale for these decisions is based on tabulated data (6, 8, 10, 12, 14, 15).

Comparison of P-typing MAbs. The N-MAbs representing alternative EIA typing systems were compared by reaction with rotavirus-positive specimens of known G types. The P1A N-MAbs F45:4 and IA 10 assigned a P type to 38 of 44 (86%) and 9 of 44 (20%) putative P1A[8] strains, respectively. The P2A N-MAbs ST3:3, HS6, and HS11 assigned a P type to 8 of 12 (67%), 1 of 12 (8%), and 2 of 12 (17%) putative P2A[6] types, respectively. All N-MAbs exhibited some cross-reactions between P types. Overall, a P type inconsistent with the putative P type was assigned by N-MAb F45:4 to 3% (2 of 77) of the specimens, by N-MAb IA10 to 1% (1 of 77) of the specimens, by N-MAb RV5:2 to 4% (3 of 77) of the specimens, by N-MAb ST3:3 to 8% (6 of 77) of the specimens, by N-MAb HS6 to 6% (5 of 77) of the specimens, and by N-MAb HS11 to 5% (4 of 77) of the specimens.

Comparison of P-typing techniques. (i) Sensitivity. All results obtained by RT-PCR and hybridization were obtained with coded specimens. The need to match hyperimmune capture antibody to the G type of rotavirus strains in the EIA partly decoded this assay. The tabulated results apply only to N-MAbs F45:4, RV5:2, and ST3:3. The results obtained with N-MAbs IA10, HS6, and HS11 have been excluded due to low sensitivity (described above). The results obtained independently from each assay were decoded and compared after the completion of all assays. All standard rotavirus strains and negative and positive control specimens examined under code reacted as expected. The sensitivities of the P-typing results obtained by the EIA with N-MAbs, RT-PCR, and cDNA hybridization are presented in relation to the VP7 types of fecal viruses for specimens stored for 1 to 3 years (Table 2) or for 10 to 20 years (Table 3).

Overall, a P type could be assigned to 73, 92, and 80% of 1- to 3-year-old specimens assayed by the EIA with N-MAbs, RT-PCR, or hybridization, respectively. The ability of EIA and RT-PCR to assign a P type appeared to be affected by the duration of storage of specimens (Table 3). These results may also have been influenced by the nature of the specimen stored (electron microscopy pellet, unextracted feces, or fecal homogenate), but the numbers are too small to allow further analysis. The sensitivity of each assay varied in relation to the G type. The low ability of EIA to assign a P type to G2 strains was not influenced by electropherotype, since each particular electropherotype was equally likely to be typed or to remain untyped in this assay (data not shown).

TABLE 2. Sensitivities of P-typing assays in assigning a P serotype or genotype to rotavirus-positive fecal specimens (1993 to 1995) from hospitalized children with acute diarrhea

Rotavirus VP7 type	No. of specimens	No. (%) of specimens positive in each reaction system		
		EIA[a]	PCR	Hybridization
G1	26	22 (84.6)	25 (96.2)	22 (84.6)
G2	21	11 (52.3)	18 (85.7)	17 (81)
G3	6	4 (66.7)	5 (83.3)	4 (66.7)
G4	12	11 (91.7)	12 (100)	9 (75)
Not known	8	5 (62.5)	7 (87.5)	6 (75)
Total	73	53 (72.6)	67 (91.8)	58 (79.5)

[a] Based on results with N-MAbs F45:4 (P1A), RV5:2 (P1B), and ST3:3 (2PA).

TABLE 4. Specificities of P-typing assays in assigning inferred P type to rotavirus-positive fecal specimens

Infection and rotavirus VP7 types	Inferred P type	% positive for inferred P type in reaction system		
		EIA[a]	PCR	Hybridization
Community infection				
G1, G3, G4	1A[8]	98 (40/41)[b]	98 (45/46)	100 (40/40)
G2	1B[4]	93 (14/15)	94 (29/31)	97 (35/36)
Neonatal	2A[6]	64 (7/11)	73 (11/15)	100 (12/12)

[a] Based on results with N-MAbs F45:4 (P1A), RV5:2 (P1B), and ST3:3 (2PA).
[b] Data in parentheses represent number of specimens whose type was in agreement with inferred P type/total number of specimens identified to have that P type.

(ii) Specificity. The specificity of each assay is listed in Table 4. By using the criteria designated above for determination of "putative" P type, the specificity of each assay was found to vary and appeared to be influenced by P type. Overall, cDNA hybridization gave highly specific results for the three P types. EIA and PCR gave highly specific results for symptomatic strains but were less specific for neonatal (putative P2A[6]) strains.

Effect of storage of specimens on determination of P type by EIA. Seventeen fecal specimens that initially showed high OD results by the P-typing EIA were stored at −70°C as 10% fecal extracts or unextracted feces and were then thawed and refrozen three, five, or seven times prior to repeat assay. Fourteen specimens remained positive for their respective P types (P1A[8] and P1B[4]) after seven freeze-thaw cycles. Decreased absorbance values were recorded for 5 of the 14 specimens. Three specimens were non-P typeable after thawing on three occasions. Cross-reactivity with other P-typing N-MAbs appeared for 8 of 17 specimens after freezing-thawing, such that P1A strains reacted with P1A and P2A N-MAbs and P1B strains reacted with P1A N-MAbs.

DISCUSSION

RT-PCR proved to be the most sensitive assay for assignment of a P type to human fecal rotaviruses stored for ≤3 years prior to assay. These sensitivities compared well with the results of published surveys that separately assessed one of the three assays and showed sensitivities of 65 to 95% (median, 75%) for RT-PCR (5, 16, 27, 29), 67 to 69% for hybridization (20, 28), and 83% for EIA (2, 3). The decreased sensitivity of EIA in this survey (compared with those in published surveys) may reflect the diversity of the strains examined (representing 19 different electropherotypes) compared with those in previous surveys. In particular, the P1B N-MAb (RV5:2) detected only 52% of G2 strains tested. Improvement in the sensitivity of the EIA is likely to require development of an additional hybridoma cell secreting VP4-specific N-MAbs, perhaps by using a variety of G2 strains as inocula prior to splenic fusion.

TABLE 3. Sensitivities of P-typing assays in assigning a P serotype or genotype to rotavirus-positive fecal specimens stored for 10 to 20 years

Infection and rotavirus VP7 types	No. of specimens	No. (%) of specimens positive in each reaction system		
		EIA[a]	PCR	Hybridization
Symptomatic				
G1	2	0	1	2
G2	12	0	6	12
G4	3	3	2	3
Neonatal	12	6	10	11
Total	29	9 (31)	19 (65.5)	28 (96.6)

[a] Based on results with N-MAbs F45:4 (P1A), RV5:2 (P1B), and ST3:3 (2PA).

The specificities of the assays in assigning a P type were determined by comparing the results obtained by each assay with the putative P type inferred from knowledge of the VP7 (G) type and the source of each specimen. These putative P types were originally identified from deduced amino acid sequence homologies for VP4 (10) and were later confirmed by serological reactions (11, 15) and reinforced by further reviews (6, 8, 12, 14). The specificities were uniformly high when assigned by hybridization assay, but reduced specificities were encountered for neonatal strains (inferred P2A[6] types) analyzed by RT-PCR and EIA. The high specificity achieved by the hybridization assay suggests that the mechanism for assigning a putative P type was valid and that the current EIA and RT-PCR assays may be inherently less specific than the hybridization assay. Cross-reactive results have been encountered previously in EIAs with VP4-specific N-MAbs (3, 26). The majority of cross-reactions examined in this survey involved reaction of putative P2A strains with P1A-specific N-MAbs. This may have been partly attributable to cross-reactions that occurred after prolonged storage, since most of the P2A strains had been stored for >10 years.

The N-MAbs selected for this comparative study recognized epitopes present on the VP8* or VP5* subunits of VP4. It has been suggested that the major antigenic sites responsible for P-type-specific neutralization occur on the VP8* subunit, whereas those occurring on VP5* are predominantly cross-reactive. The three N-MAbs reacting most effectively in this study (MAbs F45:4, RV5:2, and ST3:3) have been mapped to amino acids 392 (on VP5*), 148 (on VP8*), and 397 (on VP5*), respectively. All represent regions containing neutralization epitopes (6, 14). In particular, N-MAb F45:4 (directed at amino acid 382 on VP5*) gave highly specific results in the identification of P1A[8] types. This position is in a region that has been shown to be a critical neutralization site on VP4 of human and animal rotaviruses (20, 22, 25, 32, 33). The development of new N-MAbs that react with this region of VP5* may improve the overall specificity of the EIA.

The sensitivities of the assays appeared to be influenced by the ages and the conditions of storage of the specimens. There was experimental evidence of deterioration of antigenic deter-

minants on VP4 for 82% of the specimens after three freeze-thaw cycles and for 47% of the specimens after seven freeze-thaw cycles. It seems likely that specimens with low initial positive absorbance values might become untypeable after several freeze-thaw cycles. Definition of optimal methods and duration of storage requires further study. However, it seems wise to recommend that EIA P typing of fecal specimens be performed within 3 years (at least) of the time of specimen collection and that specimens be maintained in a frozen state (preferably at −70°C) prior to initial P typing. Hybridization assays appear to be particularly well suited to epidemiological studies of P types in historical, stored collections of human rotaviruses.

The assays used in this study were not designed to identify P types other than the major human P types (P1A[8], P1B[4], and P2A[6]) described in surveys to date (1, 8, 16). Recent surveys have identified additional P types including P3[9], P3B[13], P4[10], P5[3], and P8[11] as causes of acute diarrhea in young children (5, 13, 23, 27, 31). RT-PCR and hybridization can be adapted to the detection of newer P types more readily than EIA, since the latter is dependent on the derivation and characterization of additional N-MAbs. The major advantage of using EIA to identify P types is that it is inexpensive ($A0.50/sample) and EIA technology is readily available in most laboratories worldwide. The hybridization assay used in this survey (9) shares some of these advantages since it is also inexpensive ($A0.40/sample) and requires relatively inexpensive equipment. Probes can readily be made and will retain activity during reuse and storage for longer than 12 months. By comparison, RT-PCR analysis is expensive ($A5.00/sample) but is suitable for use with small numbers of samples.

Overall, this comparison identifies RT-PCR and hybridization with cDNA probes as sensitive and specific techniques for determination of the P types of human fecal rotaviruses. Both genotyping methods are also easily adaptable to accommodating emerging P types. Hybridization appears to be particularly well suited to use with specimens stored for long periods (≥10 years). In addition, it is a relatively inexpensive assay. Use of nonradioactive probes makes it suitable for use in developing countries. EIA with the currently available N-MAbs could be useful as a screening assay for the detection of field strains for further detailed study. The assay also requires the development of further N-MAbs to improve the sensitivity of EIA and to identify newer human P types. It is important to continue to improve this serological assay since epitope diversity within a given P serotype may not be revealed by genetic methods. Large-scale surveys of fecal strains of human rotaviruses should eventually lead to a clearer understanding of the epidemiology of P types, which may be crucial to vaccine development.

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