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Rotavirus (RV) in stools of children with diarrhea in Thailand were serotyped by monoclonal enzyme immunoassay (MEIA), and RNA extracted from these specimens were tested for hybridization with oligonucleotides constructed

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from the nucleotide sequences of VP7 of human serotypes 1, 2, 3, and 4. Of 178 specimens that contained RV as identified with a monoclonal antibody to group A RV, 84% (149/178) hybridized with serotype-specific oligonucleotides, and 42% (74/178) were serotyped by MEIA (P .001). Of the 74 specimens that were serotyped by MEIA, 92% (35/38) of type 1, 97% (34/35) of type 2, and the one type 4 RV hybridized with the HuG1Ac, HuG2Ac, and HuG4Ac oligonucleotides, respectively. RV strains identified in children in Thailand in 1987 and 1988 to which a serotype could be assigned by either method were either type 1, type 2, or, less often, type 4. Testing RV for hybridization with oligonucleotides for genes encoding VP7 is an alternate method of determining RV serotypes.

Serotyping of Human Group A Rotavirus with Oligonucleotide Probes

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Rotaviruses (RV) in stools of children with diarrhea in Thailand were serotyped by monoclonal enzyme immunoassay (MEIA), and RNA extracted from these specimens were tested for hybridization with oligonucleotides constructed from the nucleotide sequences of VP7 of human serotypes 1, 2, 3, and 4. Of 178 specimens that contained RV as identified with a monoclonal antibody to group A RV, 84% (149/178) hybridized with serotype-specific oligonucleotides, and 42% (74/178) were serotyped by MEIA ($P < .001$). Of the 74 specimens that were serotyped by MEIA, 92% (35/38) of type 1, 97% (34/35) of type 2, and the one type 4 RV hybridized with the HuG1Ac, HuG2Ac, and HuG4Ac oligonucleotides, respectively. RV strains identified in children in Thailand in 1987 and 1988 to which a serotype could be assigned by either method were either type 1, type 2, or, less often, type 4. Testing RV for hybridization with oligonucleotides for genes encoding VP7 is an alternate method of determining RV serotypes.

Rotavirus (RV) is an important cause of childhood gastroenteritis worldwide [1]. The World Health Organization considers the development of an effective RV vaccine a high priority to reduce the morbidity and mortality of diarrheal disease in young children. It has been estimated that an effective RV vaccine might reduce diarrheal deaths by 30% in children 6–24 months old [2]. A heterologous bovine strain RIT 4237 was used to immunize children in Finland [3], Peru [4], and the Gambia [5], and was partially effective in preventing RV infections. It appears, however, from recent vaccine trials [6, 7] that an effective vaccine must contain strains antigenically similar to those circulating in the community [8]. The evaluation of future RV vaccines in clinical trials will require a simple, inexpensive, and sensitive RV serotyping test. This test could also be used to determine the epidemiology and compare the natural histories of infections with different serotypes of RV.

RVs of human and animal origin have a core containing double-stranded RNA (dsRNA) that can be separated into 11 discrete bands by gel electrophoresis [9]. RV group and subgroup antigens are coded by gene 6 and are in the major inner coat polypeptide (VP6) of relative molecular weight 42,000–46,000. Antibodies against VP6 polypeptides neutralize infectivity slightly [9, 10]. The RV outer shell comprises two proteins that are known to evoke a neutralizing antibody response. These are the glycosylated product of gene 7, 8, or 9, molecular weight 34,000 (VP7) [9], and the product of gene 3 or 4, molecular weight 86,000–87,000 (VP4) [11].

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Although human RV strains are now cultivable in cells in research laboratories [12], serotyping either by immunofluorescent focus neutralization with hyperimmune sera [13] or by plaque reduction assay [14] is still limited and suitable only for cultivable virus strains. Using hyperimmune antisera, numerous cross-reactions between serotypes have been observed that are predominantly related to the sharing of VP4 among different serotypes [15]. Monoclonal antibodies (MAbs) to serotype-specific antigens have been described and used to define each of these proteins separately. Serotypes as currently defined have been shown to be consistent with identification of VP7 [16, 17]. Eventually it may be necessary to develop a binary system of classification based upon identification of VP4 and VP7. Two sets of MAbs specific for VP7 of each of the four major human serotypes have been used in a simple and specific enzyme immunoassay (EIA) to serotype human RV [18–20]. VP7 MAbs have been shown to identify ~70% of field isolates [21]. The inability to assign a serotype may be partly due to inherent insensitivity of the assay and partly due to degradation of the VP7 antigen during storage. Alternative methods for characterizing RV serotypes are to sequence the genes encoding serotype-specific VP7 [17] or to compare RV by RNA segmental oligonucleotide mapping [22].

In this study RNA extracted from stools of children with RV diarrhea in Thailand were tested for hybridization with oligonucleotides constructed from sequences coding for VP7 of four human group A RV reference strains. Stools were examined with a MAb RV serotype-specific EIA, and results of both methods were compared.

Materials and Methods

Source of specimens. Stools containing RV were initially identified with a monoclonal EIA (Pathfinder; Kallestad Laboratories,

Table 1. *Sequences of oligonucleotides constructed from the nucleotide sequences of human group A rotavirus.*

VP7 serotype	Oligonucleotide	Sequence	°C for hybridization
1	HuG1Ac	5′ GTC ACC ATC ATT GAT TTG AGT ACT T 3′	42
2	HuG2Ac	5′ TTC ATC ATC TGA AAT CTC ATT TTT A 3′	38
3	HuG3Ac	5′ TGA ATT ATC ATT TAT TTC TGT TGC T 3′	38
4	HuG4Ac	5′ TTC AGT GTC ACT AAT TTG AGT TGG A 3′	42

Austin, TX). Stools were collected from children <6 months old with diarrhea seen at the outpatient department of Children's Hospital in Bangkok in January and February 1988 and from children <5 years with diarrhea seen at the Ubon Hospital in northeastern Thailand in January through April 1987 [23]. Stools that were positive in the Kallestad EIA were stored at −70°C until examined.

Monoclonal enzyme serotyping immunoassay. Stools containing RV, as identified in the Kallestad EIA, were examined with the MAb serotyping EIA (MEIA) [20] to identify type 1, 2, 3, and 4 RV. At the time RV was serotyped in Australia a high affinity MAb, RVA, directed to the inner capsid protein, VP6, of RV and reactive by EIA to all serotypes of group A RV tested was included as a positive control for the presence of rotaviral antigens. Specimens shown to contain group A RV were compared with the results of hybridization with serotype-specific oligonucleotides.

Hyperimmune sera produced in rabbits to human group A RV types 1, 2, 3, and 4 were diluted in phosphate-buffered saline (PBS) and 100 μl of the predetermined optimal dilution was added to the wells of polystyrene Immuno-1 microtiter plates (A/S NUNC, Copenhagen) and incubated at 4°C for 18 h [20]. After the plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T), 75 μl of PBS-T containing 2.5% skimmed milk powder (PBS-T-SMP) was added, followed by 25 μl of a 10% dilution of stool in PBS, RV EIA, or buffer controls. Plates were incubated at 37°C for 2 h and washed three times with PBS-T, and 100 μl of MAbs was added that was reactive with one of the four virus serotype antigens, matching the coating antisera diluted 1:2000 to 1:60,000 in PBS-T-SMP. After incubation at 37°C for 2.5 h and washing, 100 μl of a 1:800 dilution of goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (Silenus Laboratories, Melbourne) in PBS-T-SMP was added. The conjugate was incubated at 37°C for 1.5 h and then washed; 100 μl of 1.01 *M* sodium-citrate-acetate buffer (pH 6.0) containing 0.1 mg of 3,3',5,5'tetramethyl benzidine per milliliter and 1.3 mmol H_2O_2 was added. The reaction was stopped after 10 min with 50 μl of 2 *N* H_2SO_4, and the absorbance was read at 450 nm. A fecal specimen was considered positive for a particular serotype if the optical density (OD) at 450 nm (OD 450) of the sample against antibodies produced to a given serotype was at least twice the mean of the OD 450 of the sample against antibodies to the other nonreacting serotypes. MAbs used in the assay have been shown to be VP7 specific and included RV4:2 (type 1), RV5:3 (type 2), RV3:1 (type 3), ST 3:1 (type 4), and RVA (group A) [20].

Construction of oligonucleotides. The nucleotide sequences of the four oligonucleotide probes used to identify RV serotypes were selected from the sequences complementary to nucleotides 315–339 of known sequences of VP7 in human group A RV serotype 1 [24], serotype 2 [25], serotype 3 [17], and serotype 4 [17]. The sequences of these oligonucleotides, shown in table 1, were selected because they encoded part of the major antigen A region for serotype-specific neutralization [26] and covered sequences with maximum interserotype variation.

The four oligonucleotides were constructed by phosphoramidite chemistry on a 380 B DNA synthesizer (Applied Biosystems, Foster City, CA) [27]. The deprotected, detritylated DNA was desalted by passage through Sep Pak C 18 cartridges (Waters, Bedford, MA). Oligonucleotide DNA (8 pmol) was labeled with γ-^{32}P by 5′ end labeling (New England Nuclear, Boston) [28]. The labeled oligonucleotides were purified by Sephadex G 25 chromatography (5 Prime 3 Prime, West Chester, PA).

Extraction of rotavirus dsRNA. Ten percent suspensions of fecal specimens in 0.1 *M* sodium acetate, 1% sodium dodecyl sulfate (SDS), pH 5 (Sigma, St. Louis), were extracted with phenol chloroform and precipitated with ethanol at −70°C.

Detection of dsRNA by polyacrylamide gel electrophoresis. RV dsRNA was examined by polyacrylamide gel electrophoresis (PAGE) after silver staining as described by Herring et al. [29].

Hybridization. The dsRNA samples were mixed with an equal volume of 6.15 *M* formaldehyde, 10× SSC (standard saline citrate; 1× SSC = 150 m*M* NaCl and 15 m*M* sodium citrate), and incubated at 65°C for 15 min; 20 μl of the sample was spotted on a 0.45-μm-pore nitrocellulose filter (BA-85; Schleicher and Schuell, Keene, NH) using a Bio-Dot apparatus (Bio-Rad, Richmond, CA), rinsed with 10× SSC, air-dried, and baked at 80°C for 2 h.

Hybridization with 10^6 cpm/ml of each oligonucleotide probe was performed at the temperature shown in table 1 in 3× SSC, 0.5% polyvinyl pyrrolidone, 5% bovine serum albumin, and 1% SDS for 24 h. Filters were washed for 10 min twice at the same temperature used for hybridization in 3× SSC and then in 2× SSC at 25°C for 1 h. Filters were exposed to X-Omat AR film (Kodak, Rochester, NY) with a Cronex intensification screen (Du Pont, Wilmington, DE) overnight at −70°C and developed according to the manufacturer's instruction (Kodak).

Results

A total of 211 stool samples containing RV, as identified with the Kallestad EIA, were collected from children with diarrhea in Thailand and examined; 167 samples from children in Ubon were initially tested for RV in May 1987 and 44 from children in Bangkok were examined in March 1988. Stools underwent four freeze-thaw cycles before being examined by PAGE and for hybridization with oligonucleotides in August and September 1988 in Thailand and six freeze-thaw cycles before being examined by MEIA, November–January 1988, in Australia.

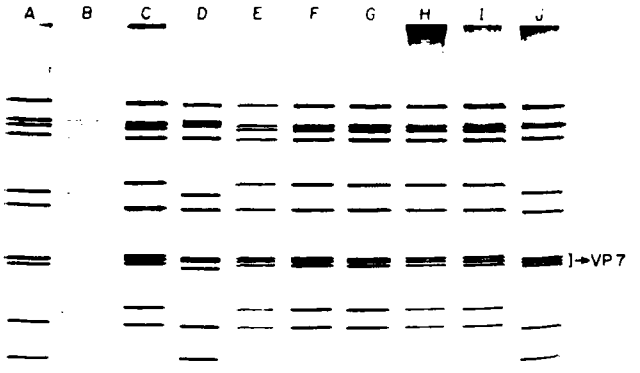

Figure 1. Double-stranded RNA electropherotypes of group A rotavirus. Lane, strain, pattern by monoclonal enzyme immunoassay, and oligo probe that was hybridized: A, W-5, long (serotype 4), HuG4Ac; B, W-59, long (serotype 1), HuG1Ac; C, UH-21, short (serotype 2), HuG2Ac; D, UH-55, long (not typable), HuG1Ac. E–I all hybridized with HuG2Ac oligo probe. Strains and patterns were: E, UH-108, short (not typable); F, UH-151, short (serotype 2); G, ND-78, short (not typable); H, U-35, short (serotype 2); I, U-85, short (serotype 2); J, U-274, long (not typable), and did not hybridize with any oligo probe. Bracket and arrow denote dsRNA segments 7, 8, or 9 that encode for VP7.

A dsRNA RV electropherotype was found in 76% of the 211 stools examined. An example is shown in figure 1. Of the 160 specimens with clearly discernible dsRNA RV patterns, long electropherotypes alone were found in 42%, short patterns alone were found in 57%, and both long and short patterns were found in 1% of the specimens.

Of the 211 specimens examined for hybridization with oligonucleotides, RNA samples from 27% (57/211) hybridized with the HuG1Ac (type 1) alone, 43% (90/211) with the HuG2Ac (type 2) alone, 1% (3/211) with both the HuG1Ac and HuG2Ac, <1% (2/211) with the HuG4Ac (type 4) alone, and 28% (59/211) could not be typed. Of the 59 untypable specimens dsRNA patterns were found in 25% (15/59), 40% (6/15) of which hybridized with three or more oligonucleotides, and 60% (9/15) hybridized with none of the four oligonucleotides. Of the 44 untypable specimens in which an RNA electrophoresis was not found, 68% (30/44) hybridized with three or more and 32% (14/44) with none of the oligonucleotides.

After six freeze-thaw cycles, RV specimens were serotyped by MEIA; 18% (38/211) of these strains were identified as type 1, 17% (35/211) as type 2, <1% (1/211) as type 4, and 65% (137/211) were untypable.

The results of the MEIA and hybridization with VP7-specific oligonucleotides with 178 specimens that contained group A RV, as identified with MAb RVA in Australia, are shown in table 2. Of the 74 specimens that were serotypable by MEIA, 92% (35/38) of type 1, 97% (34/35) of type 2, and the 1 type 4 RV hybridized with the HuG1Ac, HuG2Ac, or HuG4Ac oligonucleotides, respectively. Three samples that contained both the long and short electropherotypes hybridized with both the HuG1Ac and the HuG2Ac oligonucleotides. One of these was serotyped as 1; the other two were not typable by MEIA. Of the 178 identified as group A RV in Australia 42% (74/178) of RV could be serotyped by MEIA compared with 84% (149/178) that hybridized with the HuG1Ac, HuG2Ac, or HuG4Ac oligo probes ($P < .001$).

Discussion

RV strains identified in children in Bangkok in January and February 1988 and Ubon in January through April 1987 that were serotypable by either method were either type 1, type 2, or less often type 4. Unicomb et al. [21] serotyped 71% of group A RV strains in Australia and Nakagomi et al. [30] serotyped 73% of RV strains in Japan with MAbs; however, only 27% of RV strains in India were serotypable [31]. In Australia the MEIA was most successful in serotyping specimens stored <3 years and specimens containing 10^4 or more particles per milliliter [21]. The comparatively low rate of serotyping achieved with the Thai specimens (42%) may be due to subjecting the specimens to six freeze-thaw cycles before examining them by MEIA.

It is imperative for future RV vaccine trials in Thailand and

Table 2. Comparison of 178 rotavirus specimens examined with the monoclonal enzyme immunoassay and for hybridization with rotavirus serotype-specific oligonucleotides.

		Hybridization with					
Serotype	No. of specimens	HuG1Ac	HuG2Ac	HuG4Ac	HuG1Ac + HuG2Ac	Multiple*	None
1	38	34	0	0	1	2	1
2	35	0	34	0	0	1	0
3	0	0	0	0	0	0	0
4	1	0	0	1	0	0	0
Untypable	104†	23	53	1	2†	10	15

* Hybridized with three or more oligonucleotides.
† Two specimens with long and short electropherotypes that were untypable with serotype-specific monoclonal antibodies hybridized with the HuG1Ac and HuG2Ac oligo probes.

elsewhere that an accurate RV serotyping system be established. MEIA has been developed as a simple, accurate method of determining RV serotypes. Although a serotype can be determined in >70% of specimens tested [21, 30], only 27%–42% of RV from developing countries sent to reference laboratories are serotypable (this study, [31]). RV serotyping MAbs are now commercially available, are relatively inexpensive, and are easy to use. If specimens can be tested promptly by MEIA, this method may be preferable. Alternatively, specimens could be tested for hybridization with RV serotype-specific oligonucleotides. With a DNA synthesizer it is possible to make milligrams quantities of four different oligonucleotides in a few days. Since only 8 pmol of each oligonucleotide is used to examine 500 strains and oligonucleotides can be dried and shipped without refrigeration, they could be disseminated worldwide.

The one obvious disadvantage of oligonucleotides is the reliance on frequent shipments of radioisotopes and the hazard to untrained technicians of using radioactive material. It is unlikely that most laboratories, especially in developing countries, will have access to either a DNA synthesizer or radioisotopes. Alkaline phosphatase–conjugated oligonucleotide probes, however, have been constructed that can be shipped lyophilized at room temperature.

When additional RV serotypes are found their VP7 genes can be sequenced and oligonucleotides constructed to identify these additional serotypes. We recently constructed two other oligonucleotides, HuG8Ac and HuG9Ac. HuG8Ac (5′ CGA ACT ATC TGC TAT CTC TGT CTC T 3′) was based on the sequence of RV B37 isolated by Albert et al. [32] and sequenced by Hum et al. [33]. HuG9Ac (5′ TTC TGT ATC TCC AAT TTG AGT TGA T 3′) was constructed based on the sequence of RV F45, described by Hum et al.; the sample was obtained from Ikegami in Japan (Hum, personal communication). RV F45 is similar to RV WI61 isolated in Philadelphia and subsequently characterized by Green et al. [34].

Our experience with RV serotype-specific oligonucleotides suggests they are a practical alternative to MEIA in determining RV serotypes. Assigning a serotype to RV strains with an oligonucleotide for nucleotides coding for VP7 appears to be more sensitive than serotyping strains by MEIA. Both methods need to be compared with recently collected specimens in the same laboratory.

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