

Evaluation of a Probe Hybridisation Serotyping Method for Group A Rotavirus

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ABSTRACT

Serotype-specificity and sensitivity of oligonucleotide probes to serotype human rotaviruses was assessed. Probes could detect as little as 6.3 ng of homologous RNA and none reacted with as much as 100 ng of heterologous RNA. Northern-blot analysis revealed that probes reacted with one of genomic segments 7, 8 or 9 of corresponding serotypes.

Key words : Rotavirus; Oligonucleotide probes; Electrophoresis; Northern-blot; Serotypes.

INTRODUCTION

Group A rotaviruses (RV) are the leading cause of diarrhoeal illness in infants and young children worldwide (4). Current research is aimed at development of an effective vaccine which is safe to administer to babies. Background information on frequencies of different RV strains and their relatedness will aid the formulation and modification of RV vaccines. Enzyme-linked immunosorbent assay (EIA) (3) and, more recently, probe hybridization techniques have been used for conducting serotype analyses (6,11).

We report here a study of specificity and sensitivity of the oligonucleotide probes and characterization of stool RV with regard to electropherotype and serotype. Epidemiological and clinical information on these samples has been published elsewhere (1,2).

MATERIALS AND METHODS

A total of 248 stool specimens, containing RV identified with a commercial kit (DAKOPATTS) used in this study, were collected from patients of all ages that presented for treatment of diarrhoea to the Matlab rural diarrhoea treatment centre of the International Centre for Diarrhoeal Disease Research, Bangladesh between September 1987 and May 1989.

Double-stranded, rotaviral RNA was extracted from 10% (w/v) stool suspension in PBS (pH 7.2)

using sodium acetate, sodium dodecyl sulphate, pH 5, and phenol-chloroform. From RV control cell culture supernatants, precipitation with polyethylene glycol (MW 4000) was performed followed by phenol-chloroform extraction. The amount of dsRNA in the preparation was measured using a spectrophotometer at a wavelength of 260 nm and purity was determined from the ratio of ODs at 260/280 nm (9).

Electropherotypes were determined by performance of PAGE and gels were stained with silver nitrate (5,8).

The dsRNA samples were denatured by treatment with 6.15 M formaldehyde and spotted onto nitrocellulose filters (NC) (Bio-Rad Laboratories, Richmond, CA), air-dried and baked at 80°C for 2 h as performed previously (11).

For Northern-blot analysis, 40 µl of each RNA sample was run in two halves of a gel at 80V for 16 h; one part of the gel was then stained with silver nitrate as described above and the other part of the gel was equilibrated in 0.5 x TAE buffer (1 x TAE = 40 mM Tris, 20 mM Na-acetate and 1 mM EDTA, pH 7.4) for 10 min followed by blotting onto Zeta-probe membrane for 8 h at 40V in a transblot unit (Bio-Rad Laboratories, Richmond, CA) in 0.5 x TAE buffer. RNAs on Zeta-probe membrane were denatured according to

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manufacturer's instructions on filter paper saturated with 0.4 M NaOH for 10 min, washed in 2 x SSC, air-dried and baked at 80°C for 30 min. Rotaviral strains RV4, RV5 and RV3 (provided by R. Bishop, Royal Children's Hospital, Melbourne, Australia; G types 1, 2 and 3 respectively) and ST3 (G type 4) were used as controls.

The blotted or dotted membranes were hybridized with the serotype-specific probes HuG1Ac, HuG2Ac, HuG3Ac, HuG4Ac and HuG8Ac labelled to a specific activity of between 5000 and 7500 Ci/mmol at temperatures as described previously (2,11).

RESULTS

Serially diluted dsRNA purified (as described) from tissue culture supernatants of 5 rotavirus serotypes were spotted onto 5 separate pieces of NC and hybridized with each of the 5 oligonucleotide probes as shown for HuG1Ac in Figure 1. The minimum amount of RV RNA per 15 µl required for a positive signal was 6.3, 12.5, 12.5, 50.0 and 6.3 ng for serotype 1, 2, 3, 4 and 8 probes respectively when probes were used at the concentrations specified by Sethabutr *et al.* (11). The probes did not show crossreactivity with as much as 100 ng of RNA from heterologous serotypes, with the exception that HuG2Ac and HuG3Ac showed some crossreactivity with purified RNAs from other serotypes; however, the signal was significantly weaker than the positive signal (Fig. 1).

Table. Comparison of rotavirus serotypes identified by serotype-specific oligonucleotide probes with RNA patterns from 248 RV ELISA positive stool samples.

Pattern of RNA	Number of specimens of serotype								Total	
	1	2	3	4	1+2	2+4	3+4	8		
Long	32	0	14	28	1	1	1	0	13	90
Short	0	73	0	0	0	0	0	0	10	83
Undetected	7	14	5	6	2	0	0	0	41	75
Total	39	87	19	34	3	1	1	0	64	248

Of the 150 strains to which a serotype could be assigned, 23 electropherotypes were identified. At least one representative of each of the 23 electropherotypes was tested by Northern-blot. Strains were tested against a probe of the serotype to which they reacted in dot blots. The hybridization signal clearly showed that the oligonucleotide probes for serotypes 1-4 hybridized with only one of segments 7, 8 or 9 representing 23 electropherotypes tested, with the exception of pattern L2 which appeared to hybridize with a minor band or degradation product of the major segment (Fig. 2). The intensity of the hybridization signal was consistent with that of corresponding silver

stained RNA segments in the majority of cases; however, some stool RVs that showed strong silver stained bands gave a weak hybridization signal.

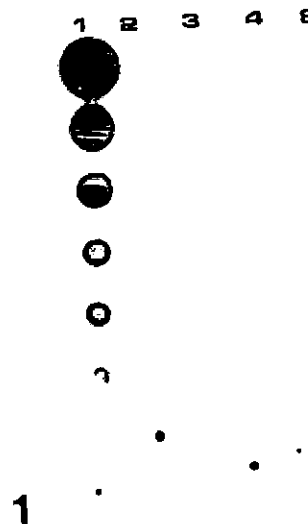


Fig. 1. Dot hybridization assay of five reference rotavirus dsRNAs; Wa (serotype 1), RV5 (serotype 2), RV3 (serotype 3), ST3 (serotype 4) and B37 (serotype 8). Two-fold dilutions of dsRNA were spotted onto nitrocellulose filters and hybridized with serotype 1 probe. Spots closest to the top of the figure contained 100 ng of dsRNA. Serotypes of strains are indicated at the top of the figure.

The results of serotyping and correlation with electropherotype are shown in Table. Five specimens (2%) hybridized with two probes and 64 (25.8%) failed to hybridize with any of the 5 probes, of which 23 (35.9%) showed an identifiable electropherotype. Of these 23 strains, 13 were long patterns, and 10 were short patterns. Of the 5 strains that hybridized with two probes, 3 had long patterns (of which one had an extra band and 2 had unique patterns), and 2 failed to show a detectable pattern. Of the 184 (74.2%) samples which could be typed by the probes, 34 (18.4%) failed to show any RNA pattern. Of the 34 RV positive specimens that were typed but no RNA pattern was found, 7 were serotype 1, 14 were serotype 2, 5 were serotype 3, 6 were serotype 4, and 2 were serotypes 1+2.

DISCUSSION

Probes were found to be specific and sensitive down to a detection limit of 6.3 ng of dsRNA. Northern-blotting experiments showed that probes hybridized with bands 7, 8 or 9 and results suggest that hybridization probably occurred with only one band. Some samples showed a weak but clear hybridization signal on Zeta-probe membrane, despite displaying strong silver stained bands in polyacrylamide gels. Insufficient transfer is unlikely

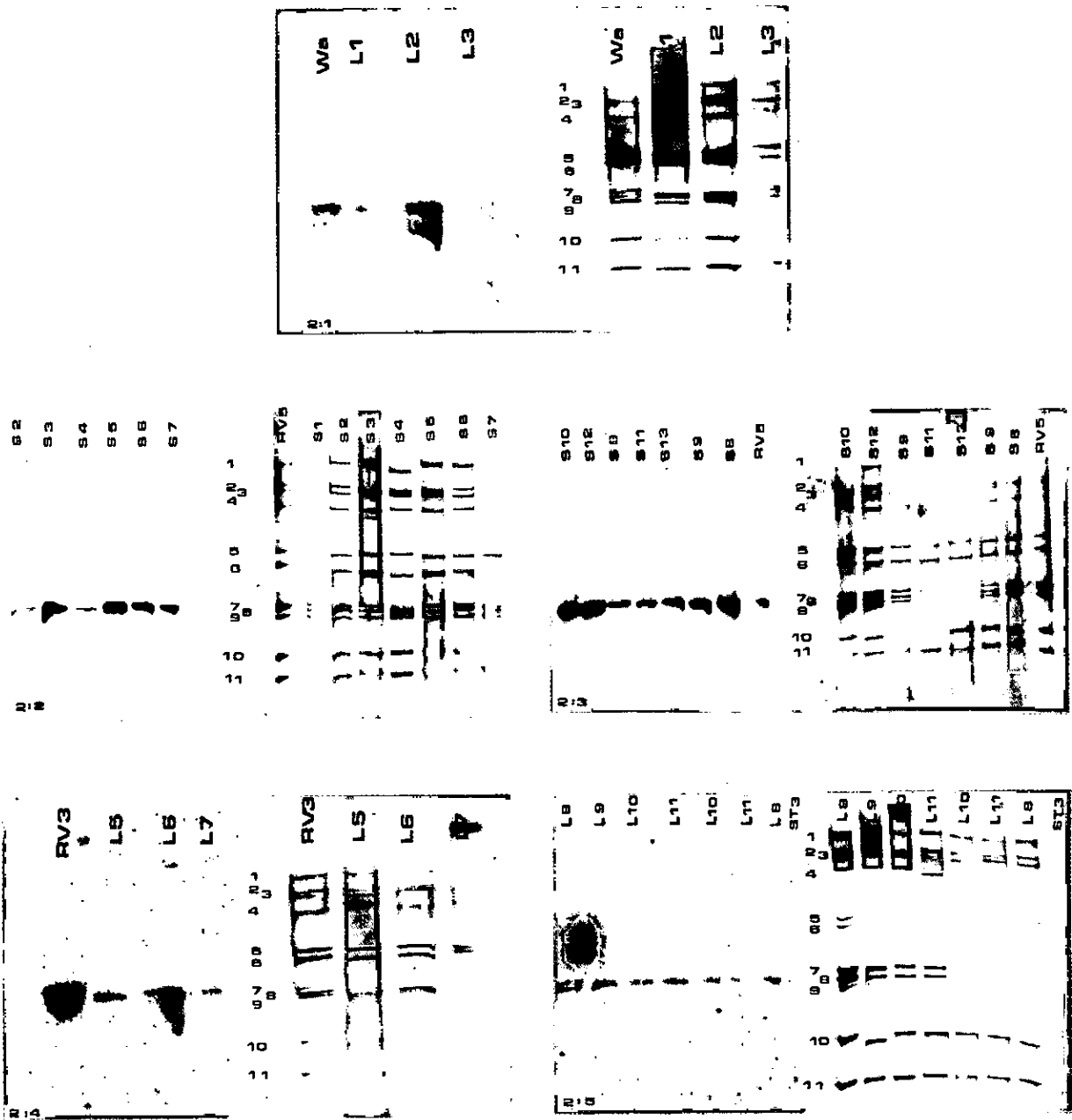


Fig. 2. Northern-blot hybridization: 40 µl of dsRNA was electrophoresed in two halves of a gel for 16 h at 80V, the right side was stained with silver nitrate and the left side was blotted onto zeta probe at 40V for 8 h followed by denaturation with NaOH. Filters were reacted with ³²P-labelled oligonucleotide probes of the corresponding serotype and washed and autoradiographed. RNA segments are indicated in the middle of each panel. RNA electropherotypes (L and S) and reference strains are indicated on the top. Patterns L1-L3, LS-L11 (long pattern) and S1-S13 (short pattern) are shown.

NOTE: 2.2 - no signal was found in this particular Northern-blot for RV5 and S1 and these lanes are, therefore, not shown. RV5 was found to react on other blots, e.g. 2.3. 2.5 - no signal was found in this particular Northern-blot for ST3. As can be seen in the silver-stained portion, only very little viral RNA was present.

to be the cause of such a phenomenon as gels stained after transfer showed no retained RNA. These strains probably show a lower degree of homology with the oligonucleotide probes, yielding weaker signals or the denaturation method using NaOH (according to manufacturer's instructions) may have been suboptimal for some strains.

A comparison of RNA pattern and serotype revealed expected correlations between long and short patterns and serotypes 1-4 (Table). Of the serotyped strains, 34 (19.6%) did not display a detectable RNA pattern. Using a tissue culture RV strain, it was found that 10 ng of RNA was required for all 11 bands of dsRNA to be detected (data not shown), compared with hybridization where a positive signal was found with 6.3 ng of dsRNA indicating only a very small difference in sensitivity. Therefore, the strains displaying no PAGE pattern have undergone degradation by stool enzymes, for example, and smaller RNA fragments may have possessed intact target sequences capable of giving a positive signal in dot hybridization. Among these strains, the majority were serotype 2 (14/34), indicating that the serotype 2 probe may be extremely sensitive and/or serotype 2 strains may be more sensitive to degradation, as has been suggested previously (7).

Strains found that were not typable but had a detectable RNA pattern (13 were patterns that were found among serotype 1 strains and 10 were short pattern), suggest that HuG1Ac and HuG2Ac probes may differ sufficiently in sequence homology not to allow hybridization to occur. Sequence specificity of the oligonucleotide probes may limit their use to detect RV serotypes where probed sequences differ by more than one base and/or different hybridization conditions may be necessary. There are similar limitations with monoclonal enzyme immunoassays. Some serotype specific monoclonal antibodies have difficulty in detecting all strains of a serotype compared with other monoclonal antibodies (10). The strains described may be serotype variants (or monotypes). The polymerase chain reaction technique, which has been performed for RV serotyping, may be appropriate for such strains when sequence diversity is a limitation of the serotyping technique.

ACKNOWLEDGEMENTS

This study was funded by the United States Agency for International Development under grant No. DPE-5986-A-00-1009-00 and by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The ICDDR,B is supported by countries and agencies which share its

concern for the health problems of developing countries. Current donors include: the aid agencies of the Governments of Australia, Bangladesh, Belgium, Canada, Denmark, France, Japan, the Netherlands, Norway, Saudi Arabia, Sweden, Switzerland, the United Kingdom and the United States; international organizations including the United Nations Children's Fund, the United Nations Development Programme, the United Nations Population Fund and the World Health Organization; and private foundations, including the Ford Foundation and the Sasakawa Foundation.

We thank P. Echeverria for providing the oligonucleotides, Mr. J. Gomes, Mr. Shahjahan Mian, Ms. Nurun Nahar Banu for their assistance in rotavirus testing and Mr. M.S.I. Molla and Mr. Manzurul Haque for secretarial services.

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