Epidemiol. Infect. (1992), **108**, 175–184
Printed in Great Britain

Rotavirus detection by dot blot hybridization assay using a non-radioactive synthetic oligodeoxynucleotide probe

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(*Accepted 31 July 1991*)

SUMMARY

A synthetic oligodeoxynucleotide of 40 nucleotides corresponding to nucleotides 33–72 of the gene coding for the viral protein VP7 of rotavirus, was used as a nucleic acid probe to develop a non-radioactive hybridization method for rotavirus detection. The probe was labelled at the 3′ end with biotin-7-dATP. The sensitivity and specificity of the dot blot hybridization assay for rotavirus detection was evaluated with 303 stool specimens. The results indicate that the hybridization assay has a higher sensitivity than both PAGE and EIA. Among the rotavirus strains tested 37 different electropherotypes were found. The results suggest that rotavirus diagnosis by dot hybridization using a non-radioactive probe may become routine laboratory procedure because it is simple, highly specific and very sensitive.

INTRODUCTION

Rotavirus infections are one of the major causes of acute diarrhoeal disease in infants and young children in developing countries [1–3]. The detection of rotavirus in hospitalized patients with acute gastroenteritis is important in ensuring use of adequate therapy and also in preventing nosocomial infections [4–7].

Rotavirus diagnosis has been helped by the extremely large number of viral particles excreted in stools, making possible virus detection by electron microscopy [8]. Infection with rotavirus can also be diagnosed by detecting viral antigens using an enzyme-linked immunosorbent assay [9, 10]. Immunological detection of rotavirus antigens provides a fast and convenient way of screening the large number of samples necessary for epidemiological studies [11, 12]. Unfortunately most of the immunological systems currently in use which do not include a blocking stage, have been reported to give false positive results.

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Another method used to detect rotavirus makes use of the fact that this virus has a unique genome of 11 double-stranded RNA segments which can be easily detected by polyacrylamide gel electrophoresis [4, 13, 14]. This latter method may be particularly useful since it has been suggested that the electrophoretic pattern can be used to trace nosocomial infections using the viral electropherotype as a marker to define a particular viral strain [6, 14, 15].

The need for methods to detect viruses that lack the group A antigens found in the original isolates, has led to the development of nucleic acid probes as an alternative to detect the presence of rotavirus [16–20]. Several detection methods have been developed based on nucleic acid hybridization. They mostly use ^{125}I- or ^{32}P-labelled cDNA or RNA probes to detect the presence of rotavirus in either dot blot of Northern-blot assays and these can allow even viral subgroups and serotypes to be distinguished [21–24]. Although these probes have shown a high level of specificity and sensitivity, the use of radioactive isotopes limits their routine use. Because radiolabelled probes have a short half-life are expensive and require sophisticated equipment for their use, their use becomes impractical for many routine laboratories.

Recently a method using biotinylated single-stranded RNA transcripts as probes has been developed [25]. The procedure employs a streptoavidin-alkaline phosphatase conjugate to detect the biotin-labelled probe, thus avoiding the problems of using radioactive probes [26–28]. Nevertheless this system, as well as all the others described to date, are still difficult to use in routine diagnosis, or in large epidemiological studies. This is due to size of the probe and the difficulties involved in either obtaining large amounts of pure transcripts or storing them for long periods without degradation. For reliable assays the probes must be small, readily available in large quantities and free from contamination with non-specific DNA. It is still not easy to prepare such pure, highly concentrated probes by cloning in recombinant plasmid.

In this paper we report the use of a small synthetic biotinylated oligodeoxynucleotide for the detection of the genomic RNA of rotavirus. The oligonucleotide is derived from a highly conserved sequence located in the 5′ end region of the rotavirus gene encoding the structural glycoprotein VP7 of the NCDV strain [29, 30]. This system provides the basis for a fast, sensitive and simple assay for rotavirus diagnosis.

MATERIALS AND METHODS

Stool samples

The samples tested consisted of stools collected between 1986 and 1988. Stool samples were obtained from 303 infants under 2 years old admitted to Roberto del Rio Children's Hospital of Santiago, Chile. Stool samples from children hospitalized with diarrhoea were shown to contain rotavirus by RNA gel electrophoresis using a 10% polyacrylamide gel (PAGE) and the electropherotype for each isolate was determined [6, 14]. Rotavirus infection was also diagnosed by detecting viral antigens using the enzyme-linked immunoassay (EIA) described previously [31]. The virus isolates used as controls (adenovirus, pararotavirus

(strain NOCIRAM), reovirus and respiratory syncytial virus) were made from patients and identified by current diagnostic procedures.

Synthesis of the deoxyoligonucleotide probe

A 40-base deoxyoligonucleotide was synthesized with an automated DNA synthesizer (Applied Biosystems, USA), using phosphoramidite chemical methods [23]. After synthesis the probe was purified using Applied Biosystems' Oligonucleotide purification cartridges following the manufacturer's instructions and stored at −20 °C until they were labelled. The sequence of the synthesized 40mer was the following:

5′-CGGTTAGCTCCTTTTAATGTATGGTATTGAATATACCACA-3′.

This sequence corresponds to nucleotides 33–72 of the VP7 gene of the NCDV strain of rotavirus. This is a highly conserved region in all rotavirus strains sequenced up till now, such as UK, Wa, HU-5 and SA-11 [29, 30]. In this particular region only one base change has been observed in strain Hu-5 (in position 11 of the probe where a C residue is changed to a T, and in position 2 where a G to C change is observed in the SA-11 strain).

Labelling of deoxyoligonucleotide probes with biotin

The probes were labelled with biotin-7-dATP (Bethesda Research Lab., USA) at the 3′ end using terminal deoxynucleotidyl transferase (TdT). The labelling reactions were performed in a volume of 50 μl containing 1 μg of the 40mer probe, 1x tailing buffer (100 mM potassium cacodylate pH 7·2, 2 mM-$CoCl_2$ and 0·2 mM dithiothreitol), containing 30 μM-dATP, 100 μM biotin-7-dATP and 15 units of TdT. The mixture was incubated at 37 °C for 4 h and the reaction was terminated by adding 5 μl of 150 mM-EDTA pH 8·0. The labelled probes were stored at −20 °C until required.

Processing of stool samples for dot blot hybridization assays

Viral RNA was extracted from stool samples by two consecutive extraction cycles with phenol and chloroform and precipitated with ethanol as previously described [6, 14]. To denaturate the RNA, the samples were resuspended in distilled water, boiled for 5 min and quenched on ice for 2 min. The denatured samples were then applied onto nitrocellulose membranes (Schleicher and Schuell) which had been presoaked with 10 × SSC buffer (1 × SSC: 0·15 M-NaCl and 0·015 M sodium citrate), using a 96–well BRL filtration manifold. After air drying, the filters were baked in a vacuum oven for 2 h at 80 °C. Viral RNA concentration was determined by UV absorption at 260/280 nm.

Dot blot hybridization conditions

The filters with the immobilized nucleic acids were prehybridized for 12 h at 50 °C in a solution containing: 50% formamide, 2.5 × SSC buffer, 5 × Denhardt's solution (0·1% each of bovine serum albumin, polyvinylpyrrolidone, and ficoll), 30 mM phosphate buffer pH 6·5, 0·2% SDS, and 100 μl/ml of salmon sperm DNA.

https://doi.org/10.1017/S0950268800049621 Published online by Cambridge University Press

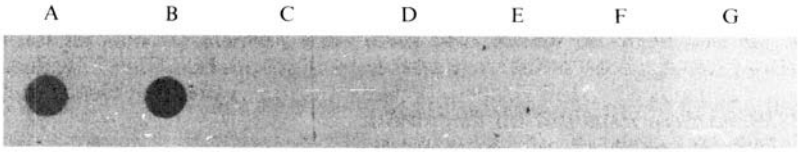

Fig. 1. Specificity of the synthetic oligonucleotide probe on dot blot hybridization. Rotavirus RNA or nucleic acids (200 ng), derived from other viruses, were denatured and spotted onto nitrocellulose membranes. As indicated hybridization of the probe was evaluated with: rotavirus RNA from a clinical isolate, long electropherotype, serotype 1 (A), rotavirus RNA from a clinical isolate, short electropherotype, serotype 2 (B), pararotavirus genomic RNA (C), reovirus genomic RNA (D), respiratory syncytial virus genomic RNA (E), yeast tRNA (F), and adenovirus genomic DNA (G) are shown.

Hybridization was carried out at 50 °C for 12 h, in the same solution used for prehybridization, with 150 ng/ml of the biotinylated probe. After hybridization, the filters were washed four times with 2 × SSC containing 0·1 % SDS, twice with 0·2 % SSC with 0·1 % SDS and finally twice with 0·01 % SSC containing 0·1 % SDS, the latter was performed at 65 °C. Detection of the hybrids was accomplished with the use of BlueGENE (Bethesda Research Laboratories, Maryland, USA), following the manufacturer's instructions.

RESULTS

The 40-nucleotide synthetic oligodeoxynucleotide was used as a probe to develop a nonradioactive hybridization method for rotavirus detection. The detection of rotavirus RNA by dot blot hybridization was evaluated by hybridizing the synthetic oligodeoxynucleotide labelled at the 3′ end with biotin-7-dATP, to 200 ng genomic RNA of rotavirus isolates which had been previously denatured and blotted on nitrocellulose membranes as described in Methods. As controls we tested tRNA, adenovirus genomic DNA, pararotavirus (strain NOCIRAM), reovirus genomic RNA, and respiratory syncytial virus genomic RNA, As seen in Fig. 1, the synthetic probe recognized RNA from non-group A rotavirus strains tested while all the controls tested were negative in the assay. Optimal conditions for hybridization of the oligonucleotide probe were at 50 °C with 50 % formamide in 2·5 × SSC for 12 h.

The sensitivity of the assay was evaluated by spotting decreasing amounts (200 ng–200 pg) of denatured dsRNA of rotavirus on nitrocellulose membranes. These results were compared with those obtained with samples of rotavirus containing similar amounts of dsRNA analysed by polyacrylamide gel electrophoresis. As shown in Fig. 2, the electrophoresis method used allowed detection down to 20 ng of genomic rotavirus RNA. On the other hand, hybridization assays using the non radioactive oligonucleotide probe, permit the detection down to approximately 200 pg of rotavirus RNA, as seen in Fig. 3.

To compare the efficiency of the dot blot hybridization assay with polyacrylamide gel electrophoresis as well as with another conventional method such as an enzyme immunoassay, 303 stool specimens obtained from children under the age of 2 years with acute diarrhoea were used, as shown in Table 1. Analysis of the

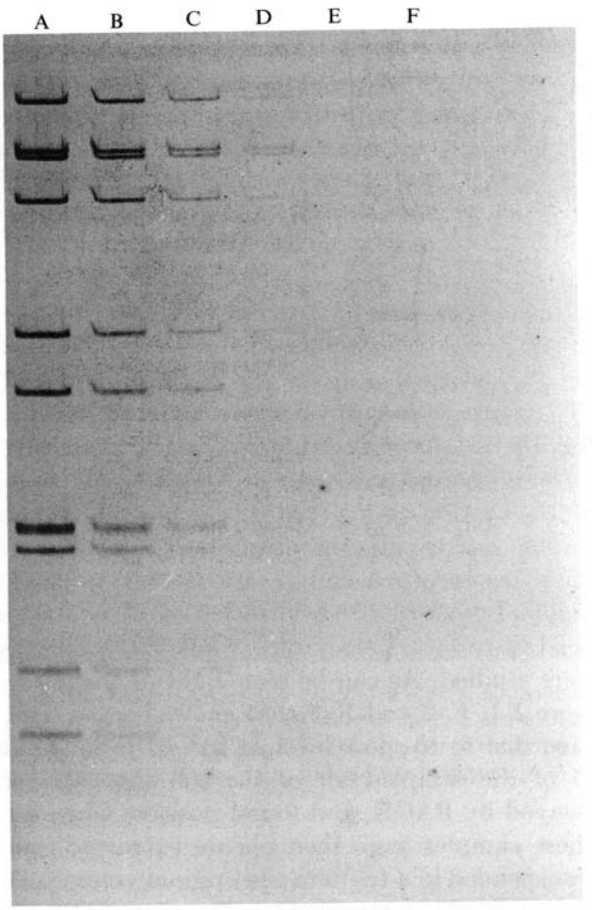

Fig. 2. Sensitivity of detection of rotavirus genomic RNA by polyacrylamide gel electrophoresis. Amounts added to the gel varied between 200 ng and 200 pg of rotavirus genomic RNA. Approximate amounts corresponding to: 200 ng (A), 100 ng (B), 50 ng (C), 20 ng (D), 1 ng (E), 200 pg (F) are shown. RNA was detected by silver nitrate staining. The arrows indicate the position of each of the eleven segments of rotavirus RNA.

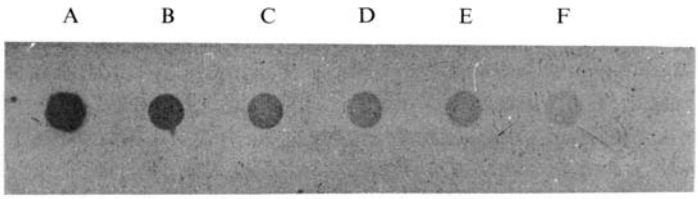

Fig. 3. Sensitivity of detection of rotavirus genomic RNA by dot blot hybridization. Rotavirus RNA amounts varying between 200 ng and 200 pg, were denatured and spotted on nitrocellulose membranes and hybridized with an oligonucleotide probe, which was labelled with biotin-7-dATP at the 3′ end with the use of terminal deoxynucleotydyl transferase. Approximate amounts, corresponding to: 200 ng (A), 100 ng (B), 50 ng (C), 20 ng (D), 1 ng (E), 200 pg (F), are shown.

Table 1. *Comparison of rotavirus detection by different methods*

Detection methods			303 clinical samples
Dot blot	Page	Eia	
+	+	+	218
+	+	−	12
−	−	+	7
−	−	−	66

303 isolates by electrophoresis indicated the presence of rotavirus in 230 stool samples while the remaining 73 samples were negative. A study of electrophoretic patterns was also carried out using an electrophoretic mobility diagram. By PAGE analysis 37 different electropherotypes were present in the 303 samples analysed. Six were different short electropherotypes and 31 were long ones. All the electropherotypes found had patterns already detected by us [6, 14]. The analysis of the same samples by dot blot hybridization assays demonstrated that the oligonucleotide probe was capable of detecting all samples found positive by PAGE, regardless of the serotype and/or electrophoretic pattern of the sample analysed. The analysis by dot blot hybridization of some of these samples (144) is shown in Fig. 4. In rows I to L 48 samples initially scored as negative by gel electrophoresis were studied. As can be seen eight of them were positive by dot blot and three more I-1, K-2 and K-3 that showed some level of reaction were considered negative due to the low level of hybridization. Similar results were obtained with 24 of the samples out of the 303 that were initially scored as negative when assayed by PAGE, and found positive when assayed by dot blot hybridization. These samples were then phenol extracted, ethanol precipitated once more, and resuspended in a tenth of the original volume. They were analysed again by polyacrylamide gel electrophoresis. In all samples tested this time the presence of genomic rotavirus RNA was demonstrated, showing that the initial inability to detect viral RNA was due to the low concentration of RNA in the samples.

When the same 303 samples were analysed by EIA, 225 samples were positive while 78 were negative. The samples scoring positive for the presence of rotavirus by EIA included seven samples that were negative when analysed by electrophoresis as well as by the dot blot hybridization system. On the other hand the EIA system used failed to detect the presence of rotavirus in 12 samples in which the presence of viral rotavirus RNA was detected by both RNA gel electrophoresis and dot blot hybridization, indicating the possibility of false negative results. Thus, our findings confirm that the EIA system developed by WHO against both rotavirus antigenic subgroups can produce false positive as well as false negative results [32]. There was complete coincidence in the results obtained with the three detection assays in 218 samples. From the 303 samples studied 230 were positive by dot-blot and RNA PAGE assays and only 95% of them were detected by EIA, suggesting that the latter is a less reliable diagnostic procedure.

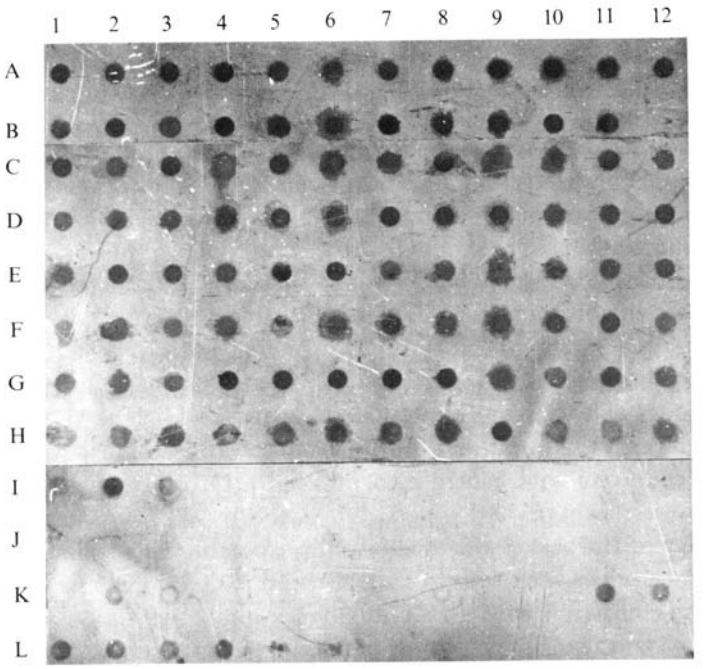

Fig. 4. Detection of rotavirus RNA in 144 diarrhoea stools. Rotavirus RNA genomic obtained from patients with acute diarrhoea were dotted on nitrocellulose membranes and hybridized to a biotinylated oligonucleotide probe as described. Including 103 positive and 41 negative to both dot blot and gel electrophoresis.

DISCUSSION

A synthetic non-radioactive oligodeoxynucleotide probe with a sequence selected from the 5′ end of gene coding for the polypeptide VP7, was tested in rotavirus diagnosis. The sequence corresponding to nucleotide 33-72 was selected because it is almost completely conserved in all known sequences of the VP7 gene coding for different viral serotypes [29, 30]. The data presented in this paper indicate that the use of this probe represents a simple and sensitive assay for the detection of rotavirus in stools samples from patients with acute diarrhoea or from asymptomatic ones.

Although several probes have been obtained for the detection of rotavirus, they are all based on the use of cDNA or RNA transcripts made *in vitro*. These cDNA probes are difficult to obtain free of contamination with non-specific nucleic acids and at the high concentrations required for this type of assay. In the case of RNA probes, as well as the problems described above they are also very unstable for use for diagnostic purposes. In contrast, the sequence we selected can be easily obtained in large quantities and in pure form by using an automated oligonucleotide synthesizer, a simple and inexpensive procedure. A further advantage is that a small oligonucleotide probe, like the one described here, can be hybridized completely in a much shorter time than that required for larger probes [26, 27]. Another aspect to be considered is that a single-stranded probe will hybridize in a more efficient way than a double-stranded probe. Self-annealing

of double stranded probes lowers the effective concentration of the probe available for hybridization with the samples.

Another very important aspect is that the dot hybridization assay described in this work employs a non-radioactive probe. Most probes utilized up to now consist of ^{125}I or ^{32}P-labelled cDNA or RNA which, although being highly specific and sensitive, have several disadvantages. They are expensive to prepare, have a short half-life and require trained personnel for the use and disposal of radioisotopes. Furthermore radioisotopes are not readily available in most laboratories in developing countries where this assay would be most valuable.

The probe used in this work was labelled with biotin-7-dATP by tailing at the 3′ end with terminal deoxynucleotydyl transferase [33]. We have selected this system because it allows the incorporation of an adequate number of biotin residues. It has been shown that oligonucleotide probes with unhybridized 'tails' of biotin-dNTP residues provide a non-radioactive detection substrate that is more sensitive than one with biotin incorporated into internucleotide linkage by chemical synthetic methods [33].

The sensitivity of the assay was evaluated by spotting rotavirus genomic RNA directly on nitrocellulose membranes and the limit was found to be of the order of 200 pg. This was compared with a PAGE/silver staining detection assay that was found to be less sensitive in detecting genomic rotavirus RNA with a limit of 20 ng.

A study using the 40mer probe to detect rotavirus RNA was carried out with 303 stool specimens from children with acute diarrhoea, and showed than our hybridization assay has higher specificity and sensitivity that either PAGE/silver staining or an EIA system. Twenty-four samples scored as negative when tested initially by PAGE, were found positive in the hybridization assay. All these samples where shown to be positive when retested using samples with concentrated RNA, showing that the PAGE system is highly specific but less sensitive that our dot hybridization assay. Another aspect to be taken into account is the specificity of the probe. As shown in Fig. 1, the probe did not cross-hybridize with adenovirus, respiratory syncytial virus or with other more closely related viral RNAs such as reo or pararotavirus.

Among the rotavirus strains detected by our dot blot hybridization assay were both long and short electropherotypes (serotypes 1 and 2 respectively), and including 37 different electropherotypes. On the other hand the EIA system failed to detect 12 of the 230 positive samples and gave false positive results with 7 samples, showing that it was less specific and sensitive than the probe.

In addition we have also used the probe with samples from asymptomatic children in which rotavirus was also identified, this represents also an advantage since immunological methods usually give false results with this type of sample [32].

The results suggest that rotavirus diagnosis by dot blot hybridization using a synthetic deoxyoligonucleotide probe may become a useful tool as a simple routine laboratory procedure which ensures high sensitivity and specificity even if other enteropathogens are present in the stool sample.

ACKNOWLEDGEMENTS

We wish to thank Mr P. Ortiz and J. P. Sanchez for their excellent technical assistance. This work was supported in part by FONDECYT Grant No. 398-88, 1017-89, UNDP Programan RLA/83/009.

REFERENCES

1. Estes Mk, Palmer EL, Obijeski JF. Rotaviruses: a review. Curr Top Microbiol Immunol 1983; **105**: 123–84.
2. Kapikian AZ, Chanock RM. Rotaviruses, In Fields BN, Knipe DM, Chanock RM, Melnick JL, Roizman B, Shope RE. eds. Virology. New York: Raven Press 1985; 863–906.
3. Avendaño LF, Spencer E, Calderon I, Martinez A. Infección por rotavirus en lactantes con diarrea aguda. Aspectos clínicos y epidemiológicos. Rev Med Chile 1983; **111**: 240–6.
4. Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR, Menzies JD. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver -strained polyacrilamide gels. J Clin Microbiol 1982; **16**: 473–7.
5. Avendaño LF, Barraza P, Calderon A, Matamala I, Duarte E, Spencer E. Infección intrahospitalaria por rotavirus en lactantes. Revista Chilena de Infectología 1986; **3**: 89–98.
6. Fernández J, Sandino AM, Pizarro J, Pizarro JM, Avendaño LF, Spencer E. Characterization of rotavirus electropherotypes excreted by symptomatic and asymptomatic infants. Epidemiol Infect 1991; **106**: 189–98.
7. Flores J, Sears J, Green KY, et al. Genetic stability of rotaviruses recovered from asymptomatic neonatal infections. J Virol 1988; **62**: 4778–81.
8. Brandt CD, Kim HW, Rodriguez WJ, et al. Comparison of direct electron microscopy, immune electron microscopy, and rotavirus enzyme-linked immunosorbent assay for detection of gastroenteritis viruses in children. J Clin Microbiol 1981; **13**: 976–81.
9. Yolken RH, Barbour B, Wyatt RG, Kalica AR, Kapikian AZ, Chanock RM. Enzyme-linked immunosorbent assay for identification of rotavirus from different animal species. Science 1978; **201**: 259–61.
10. Beards GM, Campbell AD, Cottrell NR, et al. Enzyme-linked immunosorbent assays based on polyclonal and monoclonal antibodies for rotavirus detection. J Clin Microbiol 1984; **19**: 248–54.
11. Taniguchi K, Urasawa T, Morita Y, Greenberg HB, Urasawa S. Direct serotyping of human rotavirus in stool by an enzyme-linked immunosorbent assay using serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies to VP7. J Infect Dis 1987; **155**: 1159–66.
12. Cromien JL, Himmelreich CA, Glass GI, Storch GA. Evaluation of new commercial enzyme immunoassay for rotavirus detection. J Clin Microbiol 1987; **25**: 2359–62.
13. Chanock SJ, Wenske EA, Fields B. Human rotavirus and RNA genome. J Infect Dis 1983; **148**: 49–5.
14. Spencer E, Avendaño LF, Araya M. Characteristic and analysis of electropherotypes of human rotavirus isolated in Chile. J Infect Dis 1983; **148**: 41–8.
15. Lourenco MH, Nicolas JC, Cohen J, Scherrer R, Bricout F. Study of human rotavirus genome by electrophoresis: attempt of classification among strains isolated in France. Annales de Virologie 1981; **132**: 161–73.
16. Pedley S, Bridger JC, Chasey D, McCrae MA. Definition of two new groups of atypical rotaviruses. J Gen Virol 1986; **67**: 131–7.
17. Lowe JB. Clinical applications of gene probes in human genetic disease, malignancy, and infectious disease. Clinica Chimica Acta 1986; **157**: 1–32.
18. Viscidi RP, Yolken RH. Molecular diagnosis of infectious diseases by nucleic acid hybridization. Molec Cell Probes 1987; **98**: 253–65.
19. Dimitrov DH, Graham DY, Estes MK. Detection of rotavirus by nucleic acid hybridization with clones DNA of simian rotavirus SA11 genes. J Infect Dis 1985; **152**: 293–300.
20. Flores J, Purcell RH, Perez I, et al. A dot hybridization assay for detection of rotavirus. Lancet 1983; ii: 555–8.
21. Lin M, Imai M, Ikegami N, et al. cDNA probes of individual genes of human rotavirus distinguish viral subgroups and serotypes. J Virol Methods 1987; **15**: 285–9.

22. Flores J, Green KY, Garcia D, et al. Dot hybridization assay for distinction of rotavirus serotypes. J Clin Microbiol 1989; **7**: 29–34.
23. Sethabutr O, Unicomb LE, Holmes IH, Taylor DN, Bishop RF, Echeverria P. Serotyping of human group A rotavirus with oligonucleotide probes. J Infect Dis 1990; **162**: 368–72.
24. Flores J, Midthun K, Hoshino Y, et al. Conservation of the fourth gene among rotaviruses recovered from asymptomatic newborn infants and its possible role in attenuation. J Virol 1986; **60**: 972–9.
25. Bellinzoni R, Jiang X, Tanaka TN, Scodeller E, Estes MK. Rotavirus gene detection with biotinylated single-stranded RNA probes. Molec Cell Probes 1989; **3**: 233–44.
26. Olive MD, Sethi SK. Detection of human rotavirus by using an alkaline phosphatase–conjugated synthetic DNA probe in comparison with enzyme-linked immunoassay and polyacrilamide gel analysis. J Clin Microbiol 1989; **27**: 53–7.
27. Arens M, Swierkosz EM. Detection of rotavirus by hybridization with a nonradioactive synthetic DNA probe and comparison with commercial enzyme immunoassays and silver-stained polyacrylamide gels. J Clin Microbiol 1989; **27**: 1277–9.
28. Jablonski E, Moomaw EW, Tullis RH, Ruth JK. Preparation of oligodeoxynucleotide alkaline phosphatase conjugates and their use as hybridization probes. Nucleic Acids Res 1986; **14**: 6115–28.
29. Glass RI, Keith J, Nakagomi O, et al. Nucleotide sequence of the structural glycoprotein VP7 gene of Nebraska Calf Diarrhea virus rotavirus: Comparison with homologous genes from four strains of human and animal rotavirus. Virology 1985; **141**: 292–8.
30. Huang J, Nagesha HS, Dyall-Smith ML, Holmes IH. Comparative sequence analysis of VP7 genes from five Australian porcine rotavirus. Arch Virol 1989; **109**: 173–83.
31. Greenberg HB, Flores J, Kalica AR, Wyatt RG, Jones R. Gene coding assignments for growth restriction neutralization and subgroup specificities of the Wa and DS-1 strains of human rotavirus. J Gen Virol 1983; **64**: 313–20.
32. Araya M, Spencer E, Brunser O, Espinoza J, Sandino AM. Estudio Comparativo de dos metodos en el diagnóstico de rotavirus en lactantes con diarrea aguda y asintomáticos. Rev Chil Pediatr 1985; **56**: 441–4.
33. Cook AF, Vuocolo E, Brakel CL. Synthesis and hybridization of a series of biotinylated oligonucleotide. Nucl Acids Res 1988; **16**: 4077–96.