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A dot-blot hybridization procedure for the detection of astrovirus in stool samples

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SUMMARY

We have developed a nucleic acid dot–blot hybridization test for the detection of astroviruses in stool samples. The test was not as sensitive as electron microscopy for the detection of low numbers of well preserved astrovirus particles, but was able to identify astroviruses in stools containing particles of indistinct morphology. In total, this procedure identified astroviruses in more samples than did electron microscopy, and the data indicate that the incidence of astroviruses may be substantially underestimated.

INTRODUCTION

Electron microscopic examination of stool samples has led to the discovery of several viral causes of gastroenteritis. Many of these viruses (rotavirus, enteric adenovirus, calicivirus, and astrovirus) possess a distinctive morphology easily seen when well-preserved particles are examined in the electron microscope [1]. The morphology of the other major groups of viruses implicated in viral gastroenteritis, the small round viruses (SRV) and the small round structured viruses (SRSV) is less distinctive. SRVs are seen as small (20–30 nm) spherical particles with no visible surface structure [2], SRSV or Norwalk-like agents are generally slightly larger (30–35 nm in diameter) with an ill-defined surface structure and an irregular surface margin [3].

Only rotaviruses and enteric adenoviruses can be detected by commercially available enzyme immunoassays, but an immunoassay for astroviruses has recently been developed [4]. Consequently, electron microscopy remains the only widely applicable diagnostic method, and success depends on good preservation of virus morphology in the sample, as well as on the skill and experience of the microscopist.

In the case of astroviruses, only some 10% of particles show the characteristic five- or six-pointed star on their surface [5], and the use of morphology alone as a diagnostic criterion has resulted in underestimation of the incidence of astrovirus and the misclassification of these agents [2]. Identification of viruses

can be improved by various forms of immune-electron microscopy but these can be difficult techniques and are not widely used. For instance, the Harlow agent of gastroenteritis did not show any typical astrovirus surface morphology, but was identified as astrovirus by immune electron microscopy and by immunofluorescence [3]. Similarly the Marin County agent was originally classified by morphology as a Norwalk or SRS virus [6]. Monoclonal antibodies were later used to reclassify the virus as an astrovirus [7]. A recent retrospective study examined samples previously classified as SRV by electron microscopy. Immune-electron microscopy and buoyant-density determination revealed that approximately 25% of the samples contained astrovirus as the major virus present [2].

Because of these difficulties in positive identification, the prevalence of astrovirus in faecal samples must be underestimated. There is, therefore, a need for a simple objective test which is suitable for widespread application to determine their true prevalence. We have developed a simple nucleic acid hybridization dot–blot test which we describe here.

MATERIALS AND METHODS

Probe preparation

Astrovirus-specific cDNA for use as a probe was prepared from cloned RNA obtained from CaCo-2 cells infected with astrovirus serotype 1. Growth of astroviruses in these cells has already been reported [8], and cloning of virus-specific sequences will be described elsewhere (Willcocks and colleagues in preparation). The cDNAs obtained represent a sequence of approximately 1000 bp from the 3′ end and a sequence of approximately 900 bp from an internal region of the astrovirus genome. These two sequences do not cross hybridize. Each was excised from the vector and purified by gel extraction. Each cDNA was labelled separately with [^{32}P]dCTP by the method of Feinberg and Vogelstein [9], this method achieved specific activities in excess of 10^9 cpm/μg. The mixed probe used in this study was produced by combining equal quantities of the two radiolabelled cDNAs.

Blotting procedure

Nucleic acid was extracted from stool samples of virus-infected cells and applied to nitrocellulose in a dot–blot apparatus (Schleicher and Schuell). Each sample (20 μl) was denatured at 100 °C for 60 s, 60 μl of 20× concentrated SSC (saline sodium citrate; 3 M NaCl, 0·3 M sodium citrate) was added and mixed before application to the nitrocellulose. Blots were air-dried and nucleic acid was fixed by baking for 2 h in a vacuum oven at 80 °C. Filters were prehybridized overnight at 65 °C in 5× concentrated Denhardt's solution (0·1% polyvinylpyrrolidone, bovine serum albumen, and Ficoll 40000), 6× concentrated SSC, 100 μg/ml, salmon sperm DNA, and 0·1% SDS (sodium dodecyl sulphate), and hybridized overnight at 65 °C in the same solution containing 10^6 cpm/ml ^{32}P-labelled astrovirus-specific cDNA probe. After hybridization the blot was washed at 65 °C for 45 min each in three changes of 2× concentrated SSC containing 0·1% SDS and exposed to X-ray film for 1–2 days with an intensifier screen at −70 °C.

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Sample preparation

Samples used in this work were obtained from virus-infected cells or from diarrhoeal faeces.

Cells. RNA was extracted from CaCo-2 cells infected with one of each of the astrovirus serotypes 1–5 [10], by the method of Barrett and co-workers [11]. RNA was also prepared from replicate cultures of uninfected, or echovirus 25-infected CaCo-2 cells for use as controls for specificity of hybridization. The RNA was applied to nitrocellulose and hybridized with the astrovirus cDNA probe.

Stool samples. Each stool sample was suspended at 10% in Hanks balanced salt solution (HBSS). tRNA (5 μg) was added to 0·5 ml of suspension to act as a carrier. This sample was clarified at low speed (3000 g) and the supernatant removed to a fresh tube. This was then adjusted to 10 mM-Tris-HCl (pH 7·8); 5 mM-EDTA and 0·5% SDS (final concentrations) by addition of a 10× concentrate and digested with proteinase K (50 μg/ml) at 37 °C for 30 min. Any remaining protein was removed by phenol extraction, and the nucleic acid was precipitated from 0·1 M sodium acetate with ethanol. Precipitates were collected by centrifugation and resuspended in 20 μl of distilled water prior to dot-blotting as described above.

RESULTS

Specificity of the probe

The probe was prepared from serotype 1 astrovirus and it was therefore necessary to assess its usefulness in the detection of other astrovirus serotypes. The mixed probe recognized RNA derived from all five astrovirus serotypes, but did not react with RNA from uninfected- or echovirus 25-infected CaCo-2 cells (Fig. 1). The two probes do not cross-hybridize and must therefore recognize different regions of the virus genome. Individually the probes show varying levels of cross-reactivity with the astrovirus serotypes 2–5 (Willcocks and colleagues, in preparation) and contributions from each are additive. Consequently, their use in combination maximized cross-serotype reactivity and achieved greater sensitivity.

Examination of diarrhoeal stools

A panel of 77 stool samples from cases of diarrhoea submitted for routine electron microscopic examination was also tested by dot–blot hybridization. Figure 2 shows the reactivity of the mixed probe with a selection of these stool samples.

The probe did not react with either of the controls for the stool nucleic acid extraction procedure (HBSS alone or HBSS plus tRNA carrier; both processed identically to the stool samples). It also showed no hybridization with any of the stool samples negative for virus by electron microscopy, nor with any containing rotavirus, adenovirus, echovirus or calicivirus. The probe did however react strongly with 6 and weakly with a further 5 of the 15 stools known to contain astrovirus included on this blot. Five of the 7 samples characterized by electron microscopy as SRV and 1 of the 10 as SRSV included on this blot also showed reactivity with the astrovirus probe.

The original classification made by electron microscopy and the result obtained

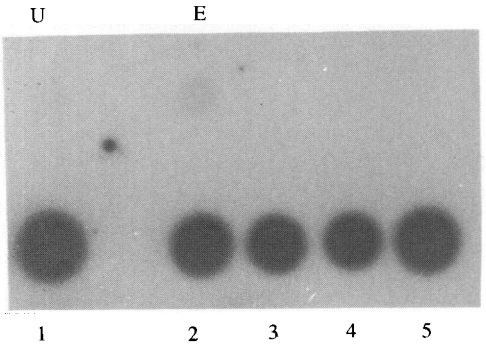

Fig. 1. Detection of astrovirus serotype 1–5 RNA with serotype 1 cDNA probe. RNA was extracted from uninfected CaCo-2 cells (U); and replicate cultures infected with human astrovirus serotypes 1–5 (1–5) or human echovirus 25 (E). RNA was dot-blotted and hybridized with the astrovirus-specific probe prepared from serotype 1, as described in the text.

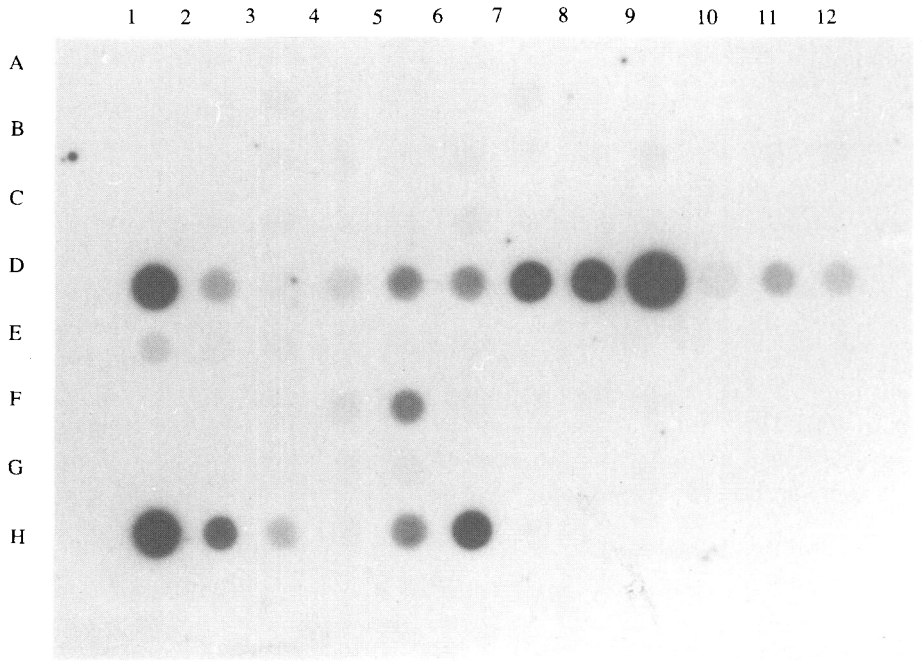

Fig. 2. Detection of astrovirus by dot–blot analysis of stool samples. Nucleic acid was extracted from stool samples, dot-blotted and hybridized as described. Exposure to X-ray film was for 48 h with an intensifier screen at −70 °C. Controls: A1, HBSS; A3, HBSS plus tRNA carrier. Stool samples (EM or culture designation): A5, A7, echovirus; B1–B6, stools negative by EM; B7–B12, rotavirus; C1–C6, adenovirus; D1–D12 and E1–E3, astrovirus; F1–F10, SRSV; G1–G6, calicivirus; H1–H7, SRV. Seventeen positive hybridization reactions are illustrated on this blot, these comprise D1, 2; D4–9, D11, 12; E1, F5, H1–3; H5, 6. Some dots are less clear in reproduction.

by hybridization of nucleic acid extracted from each stool sample with the mixed probe is shown in Table 1. Astroviruses were detected in 26/77 (34%) samples by hybridization and in 22/77 (29%) by electron microscopy. Of the 22 samples positive by electron microscopy 18 (82%) were also positive by hybridization.

There was an approximate correlation between the number of virus particles

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Table 1. *Astrovirus dot–blot hybridization of 77 stool samples previously classified by electron microscopy*

Sample classification by EM	Number of samples	Number positive by hybridization
Astroviruses detected by EM	22	
Astroviruses detected by hybridization		26
Rotavirus	6	0
Adenovirus	7	0
Echovirus	2	0
Calicivirus	10	0
Astrovirus	22	18
SRSV/Norwalk-like	17	3
SRV	7	5
Negative	6	0
Total number of samples	77	77

seen in the electron microscope and the intensity of reaction obtained in the hybridization, for instance samples D7, D8 and D9 shown in Fig. 2 had each been estimated as 3+ in the electron microscope (indicating that approximately 25–50 astrovirus particles were seen per grid square). Those astrovirus samples negative by the dot–blot test were predominantly those in which only an occasional particle had been observed in the electron microscope (for instance samples D3 and E2, shown in Fig. 2), though samples D10 and E3, also shown, had both been estimated as 1+ in the electron microscope.

The astrovirus-specific probe also reacted with 5/7 (71%) of the stool samples which had been classified as containing SRV particles, and 3/17 (18%) of those which contained SRSV. Taking this into account, the dot–blot test identified more astroviruses in total from this panel than electron microscopy. When the identifications by both techniques were combined astroviruses were identified in 30/77 (39%) samples, as opposed to 22/77 (29%) by electron microscopy alone and 26/77 (34%) by hybridization. These data indicate that the use of electron microscopy as the sole diagnostic method results in the underestimation of the prevalence of astroviruses in diarrhoeal faeces.

DISCUSSION

Astroviruses do not always show the typical appearance which enables this virus to be recognized in the electron microscope. We have previously reported that astrovirus virions released after the initial and subsequent two or three passages in CaCo-2 cells appeared larger and with less distinct margins than virions from later passages. However, both types of released virions contained identical polypeptides, and both were shown to be astrovirus serotype 1 by serology [8]. Underestimation of the incidence of astrovirus infection has been suggested previously. Oliver and Phillips [2] re-examined stool viruses classified as SRV by electron microscopy, and reported that 14/53 samples contained astroviruses as the major virus present. We have found that 5/7 stools originally thought, on electron microscopic evidence, to contain SRV were shown by hybridization to contain astrovirus. In some laboratories, SRV are identified as the second most

common virus in faeces after rotaviruses [12]. Consequently any such underestimate could be substantial.

The capital expenditure required to set up the hybridization technique is small, and less skill and experience is required than in finding characteristic particles by electron microscopy, but the technique is slower to perform than electron microscopy. The method would however be suitable for batch processing and becomes logistically more efficient as sample number rises.

The samples examined here were deliberately selected from those known to contain different types of virus, and also included samples in which no virus had been detected by electron microscopy in order to test the specificity of the dot–blot test. Consequently these numbers do not reflect the incidence of astroviruses in the population at large, and further work is needed before the true incidence of astroviruses in diarrhoeal faeces can be estimated. We are currently conducting a wider survey to address this question.

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