A comparison of two sets of primers for the RT-PCR detection of astroviruses in environmental samples

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Abstract

Human astroviruses (HAstV) are associated with sporadic cases and outbreaks of diarrhoea. The faecal-oral route is the predominant mode of transmission and contaminated drinking water and shellfish have been implicated as vehicles of transmission. Conventional diagnostic techniques have limited sensitivity and in this study two primer pairs, designated *Jon* and *Mon*, were compared for the detection of HAstV in environmental specimens by the reverse transcriptase-polymerase chain reaction (RT-PCR). Both primer pairs yielded positive RT-PCR products for the cell culture adapted HAstV-1 positive control. The *Jon* primers, however, also yielded positive results for other viruses as well as for a number of water samples. These data suggest that the regions amplified by the *Jon* primers are not unique to HAstV. The *Mon* primer pair yielded positive RT-PCR results only for HAstV serotypes 1 to 4 and some environmental samples. The results obtained using the *Mon* primer pair could be confirmed by either hybridisation with an oligonucleotide probe specific for HAstV or a HAstV specific enzyme immunoassay (EIA). RT-PCR, using the *Mon* primers, proved more sensitive than electron microscopy (EM), immune electron microscopy (IEM) and EIA for the direct detection of HAstV in river water. Cell culture amplification using the PLC/PRF/5 human primary liver carcinoma cell line improved the sensitivity of HAstV detection by EIA and RT-PCR, but not EM and IEM. The sensitivity of the RT-PCR assay system was enhanced by prior viral recovery by a glass wool adsorption-elution technique.

Introduction

Human astroviruses (HAstV) are associated with both sporadic episodes and outbreaks of human gastroenteritis. Although HAstV infections mainly occur in young children and the elderly, people of all age groups may be affected (Oishi et al., 1994). Eight serotypes of HAstV have been associated with human infections, with HAstV serotype 1 (HAstV-1) being the most prevalent (Jonassen et al., 1995). HAstV are typically transmitted by the faecal-oral route, and outbreaks have been associated with the consumption of contaminated water and food, particularly oysters (Kurtz and Lee, 1987). Routine diagnostic methods rely on electron microscopy (EM) (Appleton and Higgins, 1975), immune electron microscopy (IEM) (Lee and Kurtz, 1994) and enzyme immunoassays (EIA) (Herrmann et al., 1990; Moe et al., 1991). The detection limit of EM is about 10^6 viral particles/mℓ of test suspension, and the reverse transcriptase-polymerase chain reaction (RT-PCR) has been proven to be more sensitive than EIA for the detection of HAstV in clinical specimens (Mitchell et al., 1995). RT-PCR would therefore be more suitable for research on the low titres of HAstV which may occur in faecally polluted water environments. RT-PCR has successfully been used to detect HAstV in stool specimens (Jonassen et al., 1993; Mitchell et al., 1995), sea water (Myint et al., 1994) and environmental water samples (Marx et al., 1995).

In a previous study, using a set of nested primers reportedly highly specific for HAstV-1, we found that approximately 70% of the environmental samples tested yielded positive RT-PCR results (Marx et al., 1995). This 70% incidence of HAstV was higher than expected for environmental samples, consequently the evaluation of another set of primers was indicated to confirm or negate these findings. We report on a comparison of the sensitivity and specificity of these two sets of primers for the detection of HAstV in environmental samples. The efficacy of a glass wool adsorption-elution viral recovery procedure and the effect of cell culture amplification on the detectability of HAstV by RT-PCR were also examined.

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Received 16 July 1996; accepted in revised form 24 December 1996.

Experimental

Samples

Samples (500 mℓ) were collected from water environments in the Pretoria area over a period of four months. These samples included sludge from a sewage plant, hospital effluent collected from the HF Verwoerd hospital sewage system, water from a river that flows through a suburban area (River II) and source water for a water purification system (River I). Viruses were recovered from 20 to 40 ℓ of river water (River I) using a glass wool adsorption-elution procedure (Grabow and Taylor, 1993).

Viruses and bacteriophages

The following laboratory strains of viruses were used to determine the specificity of the primer sets: HAstV-1 to -4 (TW Lee, Public Health Laboratory, John Radcliffe Hospital, Oxford, UK); poliovirus 1 (polio 1)(vaccine strain LSc 2ab); coxsackievirus B1 to B6 (cox B1-6), coxsackievirus A9 (cox A9), and echovirus 1 (echo 1)(National Institute of Virology, Johannesburg); cytopathogenic hepatitis A virus strain pHM-175 (HAV) (Cromeans et al., 1989); reovirus (reo); simian rotavirus SA-11 (rota SA-11)(ATCC VR-899); human rotavirus HRV-3 (rota HRV3); adenovirus type 40 (Ad40) strain Hovi-X (Grabow et al., 1992) and type 41 (Ad41) strain 23341-77 (Grabow et al., 1992); somatic coliphage V1 (Grabow et al., 1984); and male specific coliphage MS2 (ATCC 15597-B). Unless otherwise specified, the origin of these viruses has been documented previously by Grabow et al. (1995).

Electron microscopy

For direct EM a drop of specimen, concentrated by centrifugation, was mixed with an equal volume of 2% phosphotungstic acid (PTA; pH 6.8) and examined as described by Taylor et al. (1993). IEM studies were carried out using an agar-diffusion method (Cubitt et al., 1979).

Enzyme immunoassay

A commercial EIA (IDEIA™ Astrovirus, DAKO Diagnostics Ltd, Cambs) was used for the detection HAstV in the samples and cell culture extracts.

Cell culture amplification

Confluent monolayers of the human primary liver carcinoma cell line, PLC/PRF/5 (ATCC CRL 8024), in 25 mℓ flasks, were inoculated with 300 µℓ of the same freon-treated environmental samples as used for RNA extraction (Jiang et al., 1992). After adsorption, Eagle's minimal essential medium (MEM) supplemented with 10 µg/mℓ trypsin was added and the infected cells were incubated at 37°C. Serum-free MEM was used as negative control and HAstV-1 as positive control. Aliquots (500 µℓ) of cell culture suspensions were withdrawn after 24, 48 and 72 h incubation for EM, IEM and RT-PCR.

RNA extraction

Total RNA was extracted from 300 µℓ of concentrated and unconcentrated environmental samples using a polyethylene glycolcetyltrimethylammonium bromide (PEG/CTAB) method described by Jiang et al. (1992) as modified by Marx et al. (1995). The same procedure was used for the RNA extraction from cell culture extracts.

Oligonucleotide primers

Two published sets of primers were used. The nucleotide (nt) positions are numbered according to the published sequence of the HAstV genome (Willcocks and Carter, 1992; Genebank Accession nr. Z11682/S38068). The first set of primer pairs (*Jon*), designed from a region at the 3'-end of the HAstV-1 genome, was used in a nested RT-PCR reaction and reportedly selectively detected HAstV-1 (Jonassen et al., 1993). The primer pair used in the first reaction of the RT-PCR amplify a region from nt 6176 to nt 6586, resulting in a 410 bp amplicon, while in the second or nested reaction a 193 base pair (bp) region from nt 6254 to nt 6447 is amplified. The second primer pair (*Mon*), designed from conserved sequences at the 3'-end of the HAstV-1, -2 and -4 genomes, amplify a 89 bp region from nt 6727 to nt 6813 and are used in a single reaction (Mitchell et al., 1995). All primers were synthesised by the Midland Certified Reagent Company, Midland, TX.

Reverse transcriptase-polymerase chain reaction

In all RT-PCR reactions a positive control (cell-cultured HAstV-1) and negative control (distilled water) were included. To avoid the possibility of false positive RT-PCR results, all procedures were carried out according to standard recommendations (Kwok and Higuchi, 1989). Unless otherwise specified, all reagents used for PCR were from Boehringer Mannheim GmbH, Mannheim The details and conditions for the nested RT-PCR using the *Jon* primers have been described previously (Marx et al., 1995). For the *Mon* primer pair, 5 µℓ of the sample RNA was used in a 30 µℓ RT-mixture (final concentration): 1 x AMV buffer; 0.5 mM dNTPs; 0.5 µM reverse primer; 0.3 mM $MgCl_2$ (Promega Corp., Madison, Wi); 40U RNase inhibitor and 10U AMV reverse transcriptase. The reaction mixture was incubated at 42°C for 1 h. The 80 µℓ RT-PCR reaction mixture contained (final concentration): 1 x RT-PCR buffer; 0.45 µM forward primer; 0.5 mM dNTPs; 0.3 mM $MgCl_2$ (Promega Corp., Madison, WI); 2.5U Taq DNA polymerase and 30 µℓ RT reaction product. The reaction conditions were: denaturation at 92°C for 4 min followed by 30 cycles of denaturation at 90°C for 1 min, annealing at 40°C for 2 min, elongation at 72°C for 1 min and final extension at 72°C for 10 min.

Polyacrylamide gel electrophoresis and hybridisation analysis

Ten microlitres of the RT-PCR products were analysed by polyacrylamide gel electrophoresis and visualised by ethidium bromide staining and UV illumination. The cDNA amplicons which co-migrated with that of the HAstV-1 control were considered to be presumptively positive for HAstV. After denaturation presumptively positive RT-PCR products, as detected by the *Mon* primer pair, were transferred to a nylon membrane (Hybond⁺; Amersham, Little Chalfont, Bucks) using a vacuum blotter (TE 80 Transvac™ vacuum blotter, Hoeffer Scientific Instruments, San Francisco, CA). Nucleic acid was fixed to the membrane by UV cross-linking for 5 min followed by baking at 80°C for 15 min in a vacuum oven, and pre-hybridised for 2 h at 55°C in 50 µℓ hybridisation solution/cm² membrane (hybridisation solution: 6 X saline sodium citrate (SSC) (Sambrook et al., 1989), 5 X Denhardt's reagent (Sambrook et al., 1989), 16 mM Tris-HCl (pH 8.3); 10 µg/mℓ denatured salmon sperm DNA and 0.1% sodium dodecyl sulphate (SDS).

A positive sense synthetic oligonucleotide probe (5' ATC ACC ATT TAA AAT TgA TTT AAT CAg AAg 3'), designed from a conserved sequence at the 3'-end of the HAstV-1, HAstV-2 and HAstV-4 genomes as well as being homologous to a region within the cDNA amplicon of the *Mon* primer, was non-radioactively labelled with digoxigenin (DIG)-dUTP using the DIG oligonucleotide 3'-end labelling kit (Boehringer Mannheim GmbH, Mannheim). Hybridisation was carried out overnight at 55°C in the same pre-hybridisation solution with the addition of 30 pMol DIG labelled probe. Hybridised membranes were washed twice with 6 X SSC and 0.01% SDS for 15 min each at 65°C. RT-PCR amplicon-oligonucleotide probe hybrids were detected by immunological and colourimetric detection using the DIG nucleic acid detection kit (Boehringer Mannheim GmbH, Mannheim) and DIG wash and block buffer set (Boehringer Mannheim GmbH, Mannheim) according to the manufacturer's instructions.

Results

Primer specificity

In tests on RNA extracted from laboratory strains of a variety of DNA and RNA viruses, differences in the specificities of the two sets of primers were observed. Both sets of primers produced RT-PCR products of the expected size for the HAstV-1 control. In addition to HAstV-1, the *Jon* primer pair yielded positive RT-PCR products of the expected size for HAstV-2 and HAstV-3, as

well as for polio 1, cox B1-6, cox A9, echo 1, HAV, rota SA-11, rota HRV3, Ad40, and Ad41, but none for reo and the somatic and male specific coliphages. Primer pair *Mon*, however, only yielded RT-PCR products of the expected size for HAstV-1 to -4. Bands were observed for some of the other viruses, but these were lower in intensity and of an incorrect size. Hybridisation analysis showed that the *Mon* primer RT-PCR products from HAstV-1 to -4 hybridised with the probe, while no hybridisation could be demonstrated with the other viruses tested (results not shown).

Comparison of the sensitivity of the HAstV detection methods

In a parallel comparison of the two sets of primer pairs using RNA extracted from selected environmental samples, 15/22 (68%) samples tested yielded RT-PCR products of comparable size to that of the HAstV-1 control with the *Jon* primers, compared to 7/22 (31%) observed with the *Mon* primers (Table 1). All seven cDNA amplicons resulting from amplification using the *Mon* primers hybridised with the probe (results not shown). The seven samples found to be positive for HAstV using the *Mon* primer pair were amongst those found to be positive when using the *Jon* primer pair.

In a comprehensive comparison of different techniques, namely EM, IEM, EIA and RT-PCR, for the detection of HAstV in environmental samples, HAstV were demonstrated by EM, IEM, EIA and RT-PCR in the positive control, i.e. cell culture

TABLE 1
RESULTS OBTAINED USING TWO SETS OF PRIMERS FOR THE RT-PCR* DETECTION OF HUMAN ASTROVIRUSES IN ENVIRONMENTAL SAMPLES

	Number of positive samples/ number of samples tested (%)	
	Primer set	
Samples	*Jon*	*Mon*
Sewage sludge	4/6 (67%)	3/6 (50%)
Hospital effluent	4/5 (80%)	2/5 (40%)
River I	7/7 (100%)	2/7 (29%)
River II	0/4 (0%)	0/4 (0%)
Total	15/22 (68%)	7/22 (31%)

*RT-PCR = reverse transcriptase-polymerase chain reaction

TABLE 2
COMPARISON OF METHODS FOR THE DETECTION OF HUMAN ASTROVIRUSES IN ENVIRONMENTAL SPECIMENS

	Sample			
	HAstV-1 (control)	River I	River I (concentrated*)	River II
Direct				
EM	+	-	-	-
IEM	+	-	-	-
EIA	+	-	-	-
RT-PCR: *Jon* primers	++	±	+	-
RT-PCR: *Mon* primers	++	-	+	-
Cell culture amplification (24 h)				
EM	+	-	-	-
IEM	+	-	-	-
EIA	+	-	+	-
RT-PCR: *Jon* primers	++	+	+	+
RT-PCR: *Mon* primers	++	-	+	-
Cell culture amplifcation (48 h)				
EM	+	-	-	-
IEM	+	-	-	-
EIA	+	-	+	-
RT-PCR: *Jon* primers	++	+	++	+
RT-PCR: *Mon* primers	++	+	++	-

concentrated* = Virus recovery by a glass wool adsorption-elution method
- = negative; ± = weak positive; + = positive; ++ = strong positive
EM = electron microscopy; IEM = immune electron microscopy; EIA = enzyme immunoassay; RT-PCR = reverse transcriptase-polymerase chain reaction

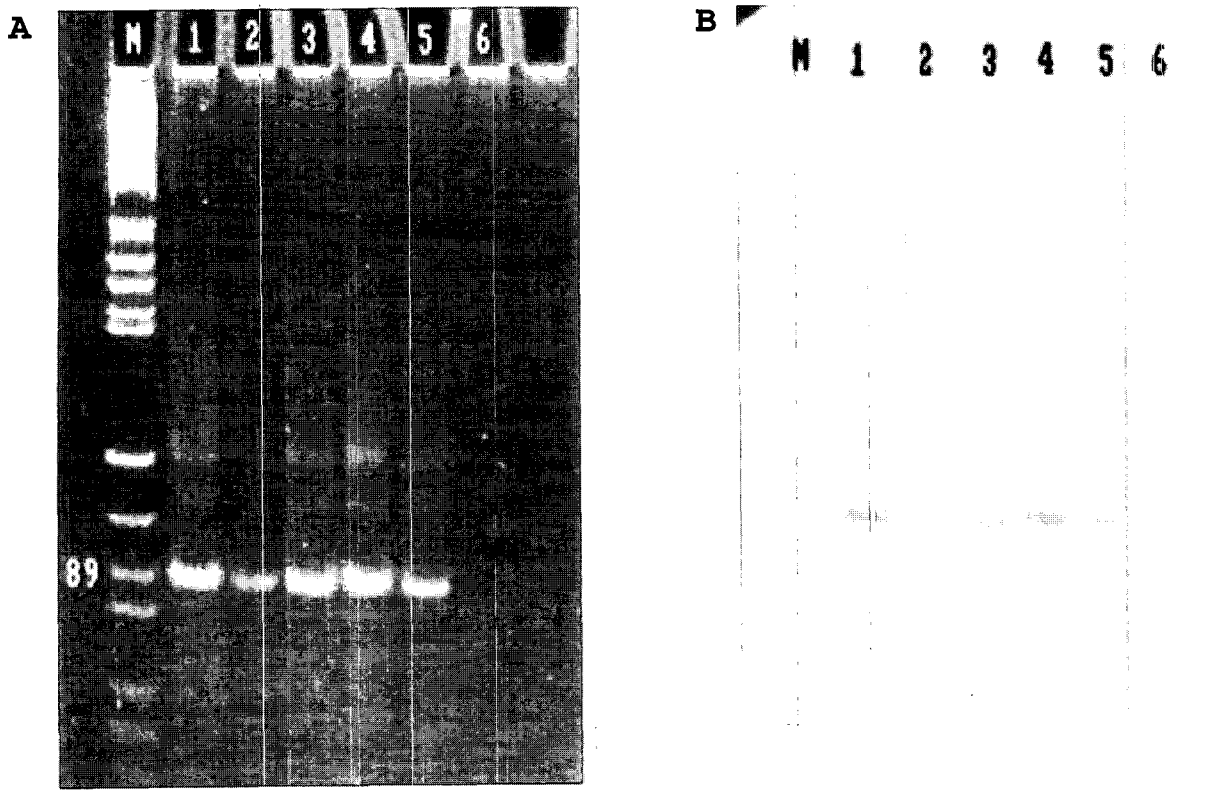

Figure 1

Ethidium bromide visualisation and hybridisation analysis of human astrovirus (HAstV) cDNA amplicons after amplification by the reverse transcriptase polymerase chain reaction (RT-PCR)using the Mon primers.
***A**: Polyacrylamide gel migration of ethidium bromide-stained products.*
***B**: Southern blot of RT-PCR products hybridised with digoxigenin-labelled HAstV specific probe.*
Lanes: (M) Molecular Marker V (Boehringer Mannheim); (1) HAstV-1 (positive control); (2) River I concentrated [prior to cell culture amplification]; (3) River I concentrated [after cell culture amplification for 24 h); (4) River I concentrated [after cell culture amplification for 48 h); (5) River I (direct) [after cell culture amplification for 48 h]; (6) Uninfected PLC/PRF/5 cell cultures (Negative control). [concentrated = viral recovery by a glass wool adsorption-elution method]

amplified HAstV-1 (Table 2). No viruses could be detected, by EM or IEM, in any of the environmental samples or their cell culture extracts. The EIA detected HAstV in the River I samples, but only after recovery by glass wool adsorption-elution and subsequent cell culture amplification for 24 h and 48 h, and not in any of the other environmental samples or their cell culture extracts. RT-PCR, however, detected HAstV directly in the River I water sample, both before and after virus recovery using the glass wool adsorption-elution method, as well as in the cell culture extracts of the water samples after cell culture amplification (Table 1 and Figure 1). However, no difference was observed in the detectability of HAstV by RT-PCR after 48 h incubation in the cell cultures.

Discussion

In this study two sets of primers for the RT-PCR detection of HAstV in environmental samples were compared. The observed differences in results obtained when using the two primer sets could possibly be ascribed to differences in specificity and/or sensitivity of the primers. The primers amplify different regions at the 3'-end of the HAstV genome which may have some affect on either or both their sensitivity and specificity. The *Jon* primer set yielded RT-PCR positive results for several different viruses, which implies that the *Jon* primers are not specific for HAstV or that there was a contamination problem. The assays with the laboratory strains of the different viruses were repeated with freshly extracted RNA, and the results were found to be the same as those found previously. As strict control measures were taken to avoid contamination during all procedures, the possibility of contamination is negligible, which indicates that the *Jon* primers

are possibly not as specific as the author's claim. The *Mon* primer pair, however, yielded positive results only for HAstV-1 to -4 and have been previously been shown, in the clinical setting, to be highly specific for all serotypes of HAstV (Mitchell et al., 1995). In this study RT-PCR using the *Mon* primers was found to be highly specific for the detection of HAstV in environmental samples as shown by the detection of HAstV in cell culture extracts using a HAstV specific EIA, and by the hybridisation of the cDNA amplicon with a HAstV specific probe. This suggests that the RT-PCR results obtained using the *Mon* primers are more specific and therefore more reliable than those obtained with the *Jon* primers. As the presence of HAstV in cell culture extracts after inoculation with River II could not be confirmed by either EIA or RT-PCR with the *Mon* primers, the RT-PCR results obtained using the *Jon* primers could possibly be false positive and would therefore require further confirmation before they could be accepted as positive.

In patient stool specimens, EIA has been found to be more sensitive than EM for the detection of HAstV (Moe et al., 1991; Herrmann et al., 1991), while RT-PCR using the *Mon* primers was more sensitive than EIA (Mitchell et al., 1995). Similar findings for environmental samples were shown in this study (Table 2). Using the *Mon* primers, HAstV could be detected directly in an environmental sample (River I) after viral recovery by glass wool adsorption-elution by RT-PCR, but not by EM, IEM or EIA. After cell culture amplification of the same sample HAstV could be detected in cell culture extracts, by EIA and RT-PCR, but not by EM or IEM. These results indicate that the PLC/PRF/5 cell line supports not only nucleic acid amplification but the production of viral protein as well, as demonstrated by the detection of virus/antigen by EIA. HAstV could only be detected by RT-PCR, and then only after cell culture amplification of the virus, in the unconcentrated River I sample. These results suggest that RT-PCR is currently the most sensitive method available for the detection of HAstV in environmental samples.

HAstV have previously been detected in environmental samples using oligonucleotide probe hybridisation in conjunction with cell culture amplification (Pintó et al., 1996). This study, however, describes an RT-PCR/oligonucleotide probe combination assay, used in conjuction with cell culture amplification in the PLC/PRF/5 cell line, which has been shown to be more sensitive than EM, IEM and EIA for the detection of HAstV in environmental samples. The sensitivity of the assay system is further enhanced by prior viral recovery by a glass wool adsorption-elution method. The more intense HAstV RT-PCR-positive band observed on the ethidium bromide agarose gels for the samples after virus recovery by the glass wool adsorption-elution recovery method show that HAstV could be successfully recovered by this technique, although no indication of the efficiency of recovery could be determined from the intensity of RT-PCR bands.

From this study it is evident that RT-PCR detection of HAstV in environmental water samples is feasible and the most sensitive method available at present. The primers, however, have to be carefully selected for both sensitivity and specificity, with a well defined confirmatory technique. This study also clearly shows that virus recovery by glass wool adsorption-elution is more effective than cell culture amplification for the rapid detection of the HAstV in environmental specimens by RT-PCR, and that a combination of these two methods is more efficient than either alone, and would be recommended method for environmental studies.

Acknowledgements

This work was supported by the Water Research Commission and The Poliomyelitis Research Foundation. We thank Dr DK Mitchell and Prof X Jiang, Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, USA, for the kind gift of the *Mon* primers.

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