Detection of Infectious Astroviruses in Water

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A method based on the infection of CaCo-2 cells and molecular hybridization with a specific cDNA probe has been developed for the detection of infectious astroviruses in environmental samples. By this procedure wild-type astroviruses have been detected in water from an area where a concurrent gastroenteritis outbreak was reported.

Astroviruses were originally described in 1975 in association with outbreaks of gastroenteritis in newborns (1) and were named for the unique stellate configuration that appears on their surface. Astrovirus infections occur worldwide and are most frequent in young children; by the age of 10 years, more than 80% of the population shows evidence of past infection (8). Illness rates attributable to astrovirus infection among the elderly are also high (6, 9). The actual relative contribution of astroviruses to the total incidence of virus-associated diarrhea is not known, mainly because of the difficulties in the diagnosis of astrovirus infections. However, some recent reports indicate that astrovirus infections may have been underestimated and are likely to be the second most common cause of viral gastroenteritis (16). Astroviruses are transmitted by the fecal-oral route, and outbreaks have been associated with the consumption of sewage-polluted shellfish (8) and ingestion of water from contaminated streams (4). To date, no reports on the occurrence of astroviruses in the environment exist. In this study we describe the occurrence of infectious astroviruses in water.

The continuous cell line CaCo-2 has been described as an *in vivo* amplification system for the detection of a wide range of fastidious human enteric viruses in clinical samples (13). CaCo-2 cells support the growth of rotaviruses, reoviruses, enteroviruses, enteric adenoviruses, and, particularly, astroviruses. Fecal samples, obtained from patients at the Hospital for Sick Children, London, United Kingdom, and diagnosed to contain astrovirus serotype 1 by immunoelectron microscopy (5), were used to develop the method described herein for the detection of wild-type astroviruses in water samples. Figure 1 shows the results of a dot blot hybridization assay of astrovirus 1 from a stool sample before and after infection of CaCo-2 cells. Confluent monolayers of CaCo-2 cells (passage level, 80 to 100) in T25 flasks were inoculated with 0.1 ml of 10-fold dilutions of trypsin-treated (30 min at 37°C with 10 µg of trypsin per ml; 1:250 tissue culture grade; Difco, Detroit, Mich.) (13) fecal suspensions, containing 10^8 to 10^6 physical particles as determined by electron microscopy. After adsorption for 45 min at 37°C, 10 ml of serum-free maintenance medium with 5 µg of trypsin per ml was added (13). At 48 h postinfection, RNA was extracted from infected cell lysates by heating at 60°C for 30 min in the presence of 100 µg of proteinase K per ml followed by phenol extraction (water-equilibrated phenol [pH 5.2]) and ethanol precipitation. A dot

blot hybridization assay was performed with the digoxigenin-11-dUTP-labelled probe 21T43 (16), following standard procedures. Prehybridization and hybridization (50 ng of probe per ml of solution) were carried out at 42°C in the presence of 50% formamide. Positive signals were detected by the use of a photochemical signal-generating system (Boehringer Mannheim, Barcelona, Spain). After infection of CaCo-2 cells, the sensitivity was significantly enhanced, and 10^6 physical particles could be easily detected (Fig. 1).

A cell culture-adapted strain of astrovirus serotype 4 (kindly provided by W. D. Cubitt) was used to determine the detection limit of the method after three passages in CaCo-2 cells. One milliliter of water obtained directly from the Sant Pere Martir well located in Esplugues de Llobregat, Barcelona, Spain, was seeded with 10^{10} astrovirus particles (titrated by electron microscopy). This water suspension was treated with trypsin and 10-fold diluted. An aliquot (0.1 ml) of the diluted suspension containing 10^9 to 10^2 physical particles per ml was inoculated into T25 flasks of confluent cells. After 48 h of incubation, 0.1 ml of the supernatant was removed, trypsin treated, and reinoculated onto CaCo-2 cells. The RNA of the infected cells was extracted as described above. One more passage was performed, in order to obtain and to determine the amplification after three cell passages. The viral RNA was detected by hybridization as described above. The detection limits were established as 10 virus particles after three passages, 10^3 virus particles after two passages, and more than 10^7 virus particles after a single passage (Fig. 2). The different detection limits found for the cell-adapted strain (10^8 physical particles without cell amplification and more than 10^7 after one cycle of cell infection) and the wild-type strain (10^9 physical particles without amplification and 10^6 after a single cell amplification [13]) may be due to strain and/or serotype differences or to variations in the infectious and noninfectious particle ratio for each virus.

The same method was used to detect wild-type astroviruses in concentrated water samples from sites with sewage pollution. Viruses were concentrated on location from 500 liters of river water by filtration through positively charged Zeta Plus

FIG. 1. Molecular hybridization of wild-type astrovirus from a stool sample after infection of CaCo-2 cells. Lanes: +, probe (positive control); -, RNA extracted from mock-infected cells; A, RNA extracted from a stool sample, corresponding to 10^9 particles; B to D, RNA extracted from CaCo-2 cells infected with 10^8 , 10^7 , and 10^6 particles, respectively.

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FIG. 2. Molecular hybridization of cell-adapted astrovirus from seeded water samples before (row A) or after one (row B), two (row C), and three (row D) amplification passages in CaCo-2 cells. Lanes: 1, 10^8 (row A) and O (rows B to D) physical astrovirus particles; 2 to 8, serial 10-fold dilutions from 10^7 to 10^1 , respectively; - and +, negative and positive controls, respectively, as in Fig. 1.

MK II filters (AMF Cuno, Sefiltra, Alcobendas, Spain), elution with 500 ml of 3% beef extract-0.05 M glycine buffer, and organic flocculation to a final volume of 50 ml, following the procedures described elsewhere (3). Confluent monolayers of CaCo-2 cells in T25 flasks were inoculated with 0.2 ml of a 1:1 dilution of trypsin-treated concentrated water samples. Specific astrovirus nucleic acids were not detected in any of six different concentrated water samples (Fig. 3, row A). None of the samples was positive after one passage in CaCo-2 cells (Fig. 3, row B). Because of the low sensitivity of the astrovirus probe 21T43, the samples were blind passaged twice in CaCo-2 cells to amplify the target nucleic acid and thus increase the chances of detection as described above. A positive hybridization signal was obtained with one sample after three passages in CaCo-2 cells (Fig. 3, column 2, row D). This positive sample was confirmed by reverse transcription-PCR following the procedure described by Willcocks and Carter (15). Three microliters of the supernatant from the third blind passage was diluted up to 14 μ l and heated to 99°C for 5 min before reverse transcription-PCR to extract the viral RNA. After the amplification step, a band of 190 nucleotides, as expected for astrovirus, could be observed (Fig. 4). This astrovirus DNA segment contains several sites for the *Dde*I restriction endonuclease, the number of sites being different for each serotype. Serotype 1 contains one site (10), serotype 2 contains three sites (7), and serotype 4 contains at least two sites (Fig. 4), since at least three segments, the longest of which is approximately 72 bp, are generated after digestion. *Dde*I digestion of the amplicon from the water isolate gave a pattern similar to those of serotypes 2 and 4. No data on the restriction patterns of astrovirus serotypes 5, 6, and 7 exist thus far.

To our knowledge, this is the first description of astrovirus detection in environmental samples. After three passages in CaCo-2 cells, the detection limit for the cell-adapted strain of

FIG. 3. Detection of wild-type astrovirus in water samples by dot blot hybridization after successive passages in CaCo-2 cells. Lanes: +, probe (positive control); -, RNA extracted from mock-infected cells; 1 to 6, six different water samples. Rows: A, samples assayed with cell infection; B to D, sample after one, two, and three passages, respectively, in CaCo-2 cells.

FIG. 4. Reverse transcription-PCR of astrovirus samples. Lanes labeled: 2, supernatant of the third passage of the water sample positive for astrovirus by hybridization (shown in Fig. 3); +, astrovirus serotype 4; *Dde*I, the same samples as those used for the first two lanes but digested with endonuclease; M, *Hae*III-digested ϕ X174 DNA size marker (with sizes [in base pairs] indicated on the right).

astrovirus serotype 4 was 10^2 physical particles per ml. Assuming the same replication pattern for the wild-type astrovirus strain isolated from water, the water concentrate and unconcentrated sample could be estimated to contain 10^2 and 10 particles per liter, respectively. However, since the average efficiency of virus recovery after filtration of the water samples through positively charged MK filters is around 50% (11), the positive hybridization signal suggests that the unconcentrated sample actually contained a minimum of 20 astroviruses per liter.

Mixed infections involving astrovirus and other potential gut pathogens are, unfortunately, not uncommon, as one route of spread of these agents is through sewage-contaminated water or shellfish (2, 8). In fact, the water sample containing astrovirus was also positive for infectious enterovirus, rotavirus, and adenovirus 40 after one passage in CaCo-2 cells. The water was sampled from a dam receiving sewage effluents from an area where a concurrent gastroenteritis outbreak was reported. Stool samples from some affected individuals had been diagnosed to contain abundant astrovirus particles by immunoelectron microscopy (data not shown).

Large gastroenteritis outbreaks associated with astroviruses are being reported with increasing frequency (12), suggesting that astrovirus may be an important agent of epidemic acute nonbacterial gastroenteritis in children and adults.

In certain situations, it appears of primary importance to differentiate infectious and noninfectious particles in environmental samples. The procedure described herein for infectivity assays for astroviruses broadens the spectrum of fastidious enteropathogenic viruses that can be monitored in the environment.

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