APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1997, p. 3119–3122 Vol. 63, No. 8
0099-2240/97/$04.00+0

Astrovirus Survival in Drinking Water

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Received 21 January 1997/Accepted 5 June 1997

A method based on infection of CaCo-2 cultured cell monolayers (CC) and reverse transcription-PCR (RT-PCR) was developed for the specific detection of infectious astrovirus. The procedure was validated by titrating poliovirus stocks in parallel in CaCo-2 cells by determining the most probable number of cytopathogenic units and by cell culture and subsequent RT-PCR (CC-RT-PCR). CC-RT-PCR was then employed to measure the persistence of astrovirus suspended in dechlorinated tap water. After 60 days, the decay of astrovirus infectivity was 2 log units at 4 ± 1°C and 3.2 log units at 20 ± 1°C, while after 90 days, the titer reduction was 3.3 and 5 log units at 4 ± 1°C and 20 ± 1°C, respectively. Astrovirus decay in the presence of free chlorine (FC) was monitored by CC-RT-PCR. Residual infectivity was found after 2 h in the presence of 1 mg of FC/liter. Under these conditions, astrovirus shows a log titer reduction (LTR) of 4, while 0.5 mg of FC/liter induced an LTR of 2.4. The possibility of acquiring data on the survival of fastidious viruses in the environment opens new perspectives on the epidemiology of some significant infections transmitted by the fecal-oral route.

The genus *Astrovirus* has been classified within the newly established *Astroviridae* family (19). Large outbreaks of astrovirus diarrhea are described with increasing frequency nowadays (20). Recent evidence suggests that astroviruses are a most important cause of acute nonbacterial gastroenteritis in children (17, 25), adults (20), and the elderly (12, 18). Difficulties in the replication of astrovirus in cell cultures have led to the development of diagnostic procedures based on electron microscopy, immunoassays, or molecular techniques, which fail to differentiate between infectious and noninfectious particles (2, 16).

Astroviruses are transmitted by the fecal-oral route and in a previous study (21) were detected in water from an area where a concurrent gastroenteritis outbreak had been reported. One critical environmental issue is whether once in the water environment, significant viruses are able to persist long enough and in high enough concentrations to pose a risk of waterborne infections. No data on the persistence of astrovirus in environmental samples exist at the present time. In this work, we present data on astrovirus survival in drinking water in natural and chlorine disinfection scenarios. Astrovirus infectivity in water samples was monitored by the combined use of CaCo-2 cell infection and detection of specific astrovirus RNA by reverse transcription (RT)-PCR.

MATERIALS AND METHODS

Viruses and cell cultures. Poliovirus serotype 1, strain LSc 2ab, was propagated in BGM cells and titrated in CaCo-2 cells by calculating the most probable number of cytopathogenic units per milliliter (MPNCU/ml) by infecting cell monolayers grown in 96-well microtiter plates (22). Eight wells were infected for each dilution, and 10 μl of inoculum was added to each well. Data were processed with an MPN computer program (13). Astrovirus serotype 4 (kindly provided by W. D. Cubitt) was propagated in CaCo-2 cells after a 30-min preactivation with 10 μg of trypsin (grade IX; Sigma)/ml at 37°C. After adsorption (60 min at 37°C) a serum-free overlay medium with 5 μg of trypsin/ml was added. For astrovirus stock production, infected cells from four T175 flasks were harvested 3 to 5 days postinfection, pelleted by low-speed centrifugation, and resuspended in 1 ml of NT buffer (100 mM NaCl and 10 mM Tris [pH 7.4]). Ten microliters of Nonidet NP-40 (Fluka, Madrid, Spain) was added, and the cells were vortexed for 1 min. After centrifugations at 700 × *g* and 1,300 × *g*, supernatants were collected and stored at −80°C. Astrovirus was assayed by performing an RT-PCR of virus RNA in the infected CaCo-2 cell monolayers.

In the persistence experiments, samples were pretreated with trypsin as above, and 10-fold dilutions (200 μl) were inoculated onto CaCo-2 cell monolayers grown in 60-by-15-mm dishes. After adsorption, a serum-free overlay medium (5 ml) containing trypsin was added and the cells were placed at 37°C. Three days postinfection, the cells were harvested, spun at 800 × *g*, and resuspended in phosphate-buffered saline (300 μl). RT-PCR was performed as described below in 3 μl of the cell suspension.

Determination of poliovirus titers by cell culture and RT-PCR (CC-RT-PCR) in CaCo-2 cells was performed as described above for astrovirus.

Poliovirus RT-PCR. Poliovirus RNA was detected in the infected monolayers by a two-step RT-PCR using the pair of primers P1 (5′-CGTTATCCGCTTAT GTACTT-3′) and P2 (5′-CAGCAAACAGATAGATAATG-3′) (5). Three microliters of infected cell suspensions was diluted up to 14 μl with deionized water, heated at 94 to 99°C for 5 min to disrupt the virions, and immediately placed on ice. The other reagents for the RT reaction were added, giving a final volume of 20 μl, and the mixture was incubated for 1 h at 42°C. The RT reaction mixture consisted of 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM spermidine, 1 μM P2 primer, 200 μM (each) deoxynucleoside triphosphates, 2 U of avian myeloblastosis virus reverse transcriptase (Promega, Ingelheim Diagnostica, Barcelona, Spain), and 20 U of an RNase inhibitor (Boehringer Mannheim, Barcelona, Spain). Two microliters of the RT product and 14 μl of salts, nucleotides, primers, and *Taq* polymerase were mixed. Deionized water was added to a final volume of 50 μl. The PCR mix contained 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween 20, 1 μM (each) P2 and P1 primers, 200 μM (each) deoxynucleoside triphosphate, and 2 U of *Taq* polymerase (Progenetics, Barcelona, Spain). The mixture was initially denatured at 94°C for 4 min and then subjected to 30 cycles of amplification, each consisting of 1 min at 94°C, 2 min at 49°C, and 1 min at 72°C. A final extension was carried out at 72°C for 7 min. Ten microliters of each sample was routinely analyzed by electrophoresis on 1.5% agarose gels. The poliovirus amplicon corresponded to a DNA fragment of 445 bp (nucleotides 225 to 670) from the 5′ noncoding region.

Astrovirus RT-PCR. An astrovirus CC-RT-PCR assay was performed on suspensions of CaCo-2 cells infected as described above, with the set of primers A1 (5′-CCTGCCCCGAGAACAACCAAGC-3′) and A2 (5′-GTAAGATTCCCA GATTGGTGC-3′) (24). The A2 primer was used for the RT reaction, while both A1 and A2 were used for the PCR amplification. The procedure used was essentially as described above, but with a different PCR program which consisted of an initial step of 4 min at 94°C followed by 40 cycles of amplification at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 30 s, with a final extension at 72°C for 7 min. The astrovirus strain employed in these studies yielded an amplicon of 192 bp. This fragment belongs to the 3′ terminus of open reading frame 1a of the astrovirus genome.

Water. The test water used throughout these studies was obtained from laboratory faucets. Water was allowed to flow for several minutes and was collected in acid-washed 10-liter high-density polyethylene containers. Dechlorination of

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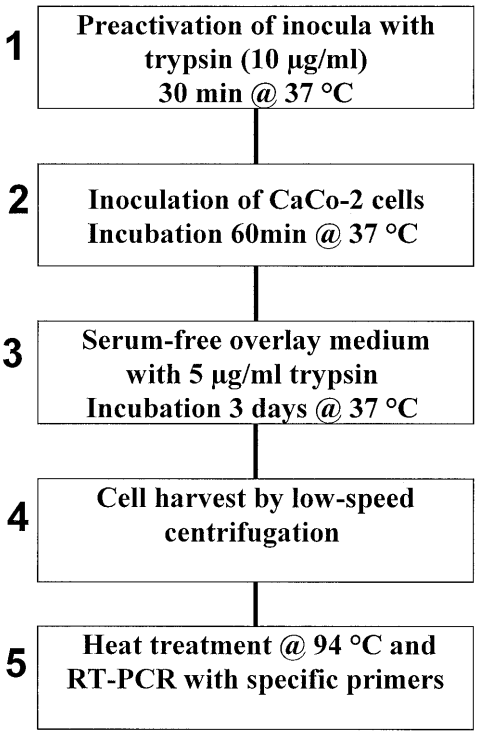

FIG. 1. General outline of the methodology employed for the detection of infectious astrovirus.

this water was accomplished by the addition of sodium thiosulfate to a final concentration of 30 mg/liter. Water was then autoclaved and kept at 4°C. Physicochemical analyses were performed according to procedures adapted from Standard Methods for the Examination of Water and Wastewater (3) and provided the following mean values: pH, 8.38; conductivity, 500 μS; A_{254}, 0.0310; A_{650}, 0.0065; A_{720}, 0.063; turbidity, 0.51 NTU; salinity, 0.00‰.

Studies of astrovirus persistence in drinking water. Astrovirus persistence was assayed in tap water at 4 ± 1°C and 20 ± 1°C. Semipurified astrovirus suspensions (1 ml) were added to 10-ml aliquots of water, which were then placed at the designated temperature. At 0, 1, 3, 7, 15, 30, 45, 60, and 90 days, the sample aliquots were removed and kept at −80°C until assayed. All experiments were performed in triplicate, and all samples from a given experiment were assayed concurrently. Each sample was titrated in duplicate.

Astrovirus decay was figured by determining the $\log_{10}(\text{RT-PCRu}_t/\text{RT-PCRu}_0)$, where RT-PCRu_0 is the reciprocal end point dilution detectable by CC-RT-PCR at time zero, and RT-PCRu_t is the reciprocal end point dilution detectable by CC-RT-PCR at various time intervals. The analysis of variance test (23) was performed with log-transformed data to determine significant differences.

Chlorination experiments. Glassware used for systems involving free chlorine (FC) was kept for over 15 h in a solution of 0.8 mg of FC/liter to satisfy the chlorine demand. FC solutions of 0.5 and 1.0 mg/liter were prepared from a stock solution of sodium hypochlorite (5%). FC concentrations were determined by the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) method (3) by using a test kit (Aquamerck 11735; Merck, Darmstadt, Germany). As before, each test system consisted of 10 ml of autoclaved tap water with the appropriate FC concentration. For each disinfection system, autoclaved water without FC was used as the control. Experiments were performed in triplicate at 20 ± 1°C and pH 7.5 ± 0.2. Astrovirus was added to the various disinfection systems, and at predetermined time intervals (0, 2, 5, 15, 30, 60, and 120 min) samples were taken and neutralized with 14.6% sodium thiosulfate (3). Astrovirus survival in the presence of FC was evaluated by determining the $\log_{10}(\text{RT-PCRu}_t/\text{RT-PCRu}_0)$, as described above.

RESULTS

Validation of the CC-RT-PCR method for infectious virus quantitation. A procedure based on CC-RT-PCR in CaCo-2 cells has been developed for the specific detection of infectious astrovirus. To assess the validity of this CC-RT-PCR methodology (Fig. 1) in studies of virus persistence, dilutions of a stock of poliovirus, a cytocidal virus, were titrated by determining both the MPNCU/ml in CaCo-2 cells and the reciprocal end point dilution detectable by CC-RT-PCR. Figure 2 shows the regression analysis between the decimal logarithms of the MPNCU and reciprocal end point dilutions detectable by RT-PCR, both determined in CaCo-2 cells. Poliovirus titers figured by these techniques showed a strong correlation ($P < 0.05$).

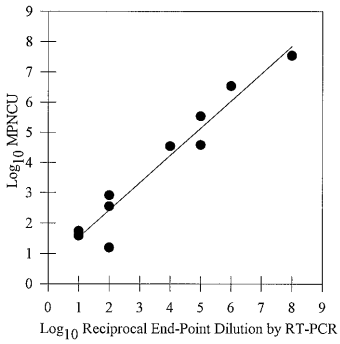

FIG. 2. Titration of a stock of poliovirus type 1 in CaCo-2 cells by cytopathogenicity and RT-PCR.

Astrovirus persistence in drinking water. Astrovirus residual infectivity in dechlorinated drinking water samples was estimated through the reduction in the titers determined by CC-RT-PCR, by following the procedure depicted in Fig. 1. Astrovirus displayed remarkable stability at 4°C, with only a 1.2 log titer reduction (LTR) after 45 days (Fig. 3). After 60 and 90 days at 4°C, the losses of infectivity in tap water were 2.0 and 3.3 log units, respectively. As expected, astrovirus persisted less in tap water at 20°C than in tap water at 4°C ($P < 0.05$). At 20°C, astrovirus showed an LTR greater than 2 after 30 days, while the decay at 60 and 90 days was 3.2 and 5 log units, respectively (Fig. 3).

Astrovirus disinfection by chlorine. Figure 4 depicts the virucidal action of FC on astrovirus in drinking water. In the presence of 0.5 mg of FC/liter, astrovirus displayed an LTR of 2.5 after a 1-h contact time. After 2 h, astrovirus inactivation was lower than 3 log units. When the FC concentration was increased to 1 mg/liter, the loss of astrovirus titer was around

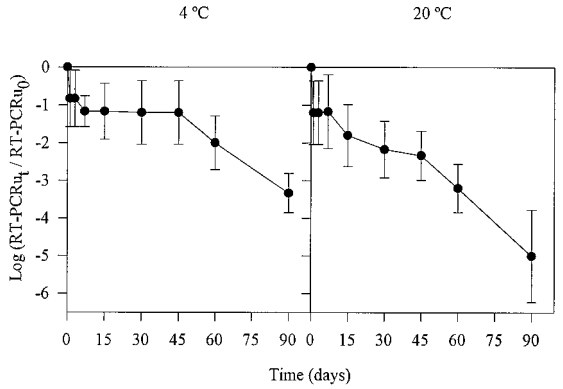

FIG. 3. Loss of astrovirus infectivity in drinking water at 4 ± 1°C and 20 ± 1°C. Values shown are means of three independent trials, with vertical error bars corresponding to 1 standard deviation.

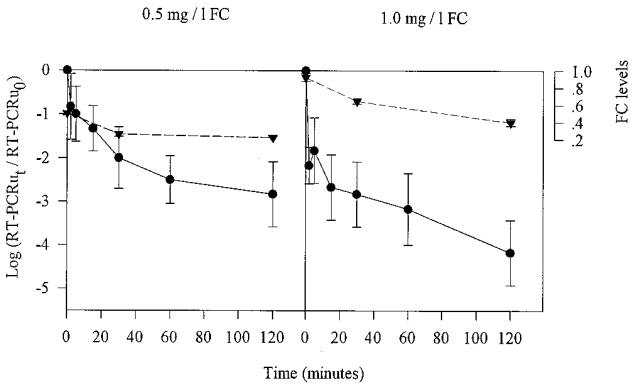

FIG. 4. Astrovirus disinfection in drinking water by 0.5 and 1.0 mg of FC/liter. Circles, virus persistence; triangles, FC levels. Values shown are means of three independent trials, with vertical error bars corresponding to 1 standard deviation.

3 log units. Residual infectivity was found after 2 h in the presence of 1 mg of FC/liter, showing an LTR of 4.17.

The evolution of FC levels was monitored throughout these experiments. The concentration of FC decreased during the disinfection experiments (Fig. 4). Initial FC levels in the disinfection systems averaged 0.50 and 0.92 mg/liter; after 30 min, only 52 and 70% of these levels, respectively, were detected in these systems, while after 2 h, residual FC represented 44 and 43% of the original FC input, respectively.

DISCUSSION

The advent of molecular biology has prompted the development of procedures for the detection of fastidious enteric viruses (5, 8, 11, 14). However, molecular techniques fail to distinguish between infectious and noninfectious particles, which may be of critical relevance in environmental virology. For instance, it has been pointed out (2, 16) that molecular procedures are not adequate to monitor the presence of infectious viruses after disinfection. In previous experiments, viruses inactivated by low levels of FC were still detectable by molecular techniques (2). Chemical alteration of the nucleocapsids by chlorine produces noninfectious virions which, however, encapsidate a detectable RNA.

CaCo-2 cells have been described as supporting the propagation of a wide variety of enteric viruses, including astrovirus (21, 22). The combined use of CaCo-2 cell infection and subsequent RT-PCR enabled the development of a semiquantitative procedure (CC-RT-PCR) for the detection of infectious astrovirus. The described methodology may be adapted for other fastidious infectious agents replicating on CaCo-2 cells simply by using appropriate primers. Infected and noninfected CaCo-2 cells usually become detached after 24 to 48 h due to the action of trypsin. However, they remain viable in suspension and able to support virus growth. The viral RNA in infected cells is made readily available for RT-PCR by heating the sample for 2 or more minutes at 94°C (15). Titration of poliovirus stocks by CC-RT-PCR and determination of MPNCU confirmed the validity of the procedure for measurement of virus decay in studies of virus persistence. In order to reproduce the actual conditions of the viruses in the environment, virus stocks used in these studies were not subjected to equilibrium or rate zonal centrifugation. Viruses tend to occur in the environment as aggregates rather than as monodispersed particles. However, it is reasonable to assume that environmental factors and the compositional makeup of water samples may differ from one geographical location to another and differentially affect virus survival. Salts and organic materials were introduced into the test water samples concomitantly with virus inocula and could affect virus survival. However, in situations of actual virus contamination, salts and organic matter contaminate drinking water too.

The survival of astrovirus in dechlorinated tap water is comparable to the data found in similar studies performed with human rotavirus serotype 3 and enteric adenovirus types 40 and 41 (1), which are all important agents of waterborne gastroenteritis (4, 7, 10). In those studies (1), enteric adenoviruses and human rotaviruses show an LTR of 3.2 after 60 days at 20°C. A temperature effect is observed; the astrovirus decay is more pronounced at high temperatures.

Chlorine is widely used for the routine inactivation of pathogenic microorganisms in water, particularly in drinking water. According to previous studies (1, 2), the behavior of astrovirus in the presence of FC is not far from that of adenovirus, which showed titer reductions of 2.5 and 3 log units in the presence of 0.5 and 1 mg of FC/liter, respectively, after 120 min. FC disinfection was much less efficient at inactivating astrovirus than poliovirus. However, the persistences reported in the same studies for human rotavirus or hepatitis A virus in the presence of FC are higher than that observed in the present work for astrovirus. The reduction observed in FC levels in the course of the disinfection experiments, in these or other similar studies (2), may be due to various environmental factors, such as temperature, light, or halogen demand of the sample (6, 9), and could account for the bimodal kinetics observed in the virus inactivation curves.

The transmission of gastroenteritis-causing enteric viruses, such as astrovirus, via contaminated drinking water is an important public and environmental health concern. However, nothing is known about the survival of astrovirus in such a vehicle. The possibility of studying the persistence of this and other fastidious enteric viruses in the water environment will shed new light on the health significance of their occurrence in sewage-polluted waters.

ACKNOWLEDGMENTS

We thank W. D. Cubitt, Hospital for Sick Children, London, United Kingdom, for kindly providing astrovirus samples and for helpful discussion. We are also grateful to M. J. Carter and M. M. Willcocks, School of Biological Sciences, University of Surrey, Guilford, United Kingdom, for kindly providing the 21T43 probe and for helpful comments.

F. X. Abad was a recipient of an F.I. fellowship from the Generalitat de Catalunya.

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