

## Detection of Rotavirus in Sewage and Creek Water: Efficiency of the Concentration Method

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*Simian rotavirus SA-11, experimentally seeded, was recovered from raw domestic sewage by a two-step concentration procedure, using filtration through a positively charged microporous filter (Zeta Plus 60 S) followed by ultracentrifugation, effecting an 8,000-fold concentration. By this method, a mean recovery of 81% ± 7.5 of the SA-11 virus, was achieved.*

Key words: rotavirus - sewage - virus concentration - direct immunoperoxidase

Rotaviruses have been recognized as a major cause of severe infantile gastroenteritis, both in temperate (Kapikian & Chanock 1990) and in tropical and sub-tropical countries, including Brazil (Linhares et al. 1981, Candeias et al. 1989, Pereira et al. 1993, Stewien et al. 1993). In tropical countries, acute gastroenteritis has accounted for very high morbidity and mortality rates, particularly in poor and malnourished populations living in precarious conditions of water supply and sewage disposal systems (Suttmoller et al. 1982, Trabulsi et al. 1985, Linhares et al. 1986). Several outbreaks of waterborne gastroenteritis with suspected rotavirus etiology have been reported in our country and in other parts of the world (Suttmoller et al. 1982, Hopkins et al. 1984, Linhares et al. 1988).

The presence of rotavirus in the suspected waters have not been determined because of the lack of a suitable method for detecting them at low concentrations (Hejkal et al. 1984). Most methods proposed for the recovery of viruses from sewage and other polluted waters have been developed to detect polio- and other enteroviruses, but they showed low efficiency for rotaviruses (Smith & Gerba 1982, Hejkal et al. 1984, Rao et al. 1986).

Previously, we presented results of the first longitudinal study conducted in Brazil, which determined the presence and levels of rotaviruses in raw domestic sewage and in sewage-polluted creeks in

the city of São Paulo (Mehnert & Stewien 1993). In the present paper we describe in details the two-step concentration procedure used in the study and the efficiency evaluation of this method.

### MATERIALS AND METHODS

*Cell cultures* - The MA104 continuous line of monkey kidney cells, maintained as previously described (Mehnert & Stewien 1993), was used for DIP assay.

*Simian rotavirus* - Simian rotavirus (SA-11), originally obtained from Dr TH Flewett, was grown in MA104 cells maintained with Eagle's MEM, without FBS and supplemented with 10 µg/ml trypsin (1:250 tissue culture grade, Difco Lab., Detroit, MC) as described by Hársi and Candeias (1991).

The SA-11 virus titers were determined by the DIP. In brief, ten-fold dilution of the virus sample was prepared in Eagle's MEM without FBS, supplemented with trypsin (10 µg/ml). Fifty µl volumes of each dilution were added to MA104 cell monolayers, cultured in a 96 well plate in quintuple and incubated for 18 hr at 37°C. Then, the cells were fixed and rotavirus assayed by DIP (Mehnert & Stewien 1993). Results are reported as focus forming units (FFU)/50 ml. SA-11 virus was used as a positive control in each assay.

*Two-step procedure for concentration of natural occurring rotaviruses in wastewater* - Eight-liter samples of raw sewage and sewage polluted creek water were collected at three sites in São Paulo (Mehnert & Stewien 1993). Initially, the pH was measured and adjusted to 6.5 with 1N HCl, if necessary. Then each water sample was passed sequentially through a 140 mm-diameter AP<sub>20</sub> filter membrane (porosity of 0.45 µm, Millipore Corp. - Bedford, MA) placed on a Zeta Plus 60 S microporous positively charged filter (AMF CUNO

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Div., Meridien, CO) in a pressure filter holder (FABBE, São Paulo, Brazil). The flow rate was maintained at 50 ml/min. The filter-bound viruses were eluted with 100 ml of a 3% Beef-Extract - 0.05M Glycine pH 9.0 solution (BE-GLY). After an exposure of 10 min, the BE-GLY solution was filtered twice and then immediately neutralized with 1N HCl. The viruses were re-concentrated by ultracentrifugation at 180,000 x g using a Beckman 70.1 Ti rotor for 2 hr at 4°C. The sediment was resuspended in 1.0 ml of 0.15 M phosphate buffered saline pH 7.4 (PBS) effecting an 8,000 fold concentration.

The samples were detoxified by mixing it with an equal volume of trichlorotrifluoroethane (Freon TF, DuPont) and decontaminated by treatment with antibiotics (1,000 IU Penicillin G and 1,000 µg Streptomycin per ml). All concentrates were stored in aliquots at -20°C, until use.

*Efficiency of the two-step concentration procedure* - Four 8-l samples of raw domestic sewage were collected at sewage pumping station Edu Chaves at the same day and period to test the efficiency of rotavirus recovery of the concentration method. The wastewater samples were initially autoclaved at 121°C for 1 hr and cooled to reach room temperature. Then about  $3.5 \times 10^4$  FFU of SA-11/50 µl (Table) were inoculated in each water sample and incubated for 30 min at room temperature with constant magnetic stirring. The rotaviruses were concentrated as described above. Concentrates were then treated with Freon TF and antibiotics and assayed by DIP.

*DIP method* - DIP was performed as previously described and results expressed as FFU/l (Mehnert & Stewien 1993).

*Fecal coliforms counts* - The numbers of fecal coliforms in raw sewage were determined by the membrane filtration technique and the results expressed as colony forming units CFU/l (APHA 1985).

## RESULTS

The ability of the two-step concentration procedure to recover SA-11 from raw domestic sewage was determined in four experiments. Four 8-l samples of raw domestic sewage were collected uniformly at the same site, day and period. The samples presented a pH of 6.8 and the mean number of fecal coliforms was  $6.4 \times 10^7$  CFU/l.

A SA-11 virus stock suspension with a mean titer of  $9.8 \times 10^6$  FFU/50 ml was diluted to contain about  $3.5 \times 10^4$  FFU/50 ml and aliquots of 50 ml of these dilutions were seeded into four sewage samples and concentrated by the two-step procedure (Fig. 1). Rotavirus recovery averaged  $81\% \pm 7.5$  of the input virus, with a range of 68 % to 88 %

in individual trials, as shown in Table.

Rotavirus infected cells are easily recognized by DIP technique at low or medium magnification. The infected cells show dark brown granula around the nuclei as observed on a Carl Zeiss Jena inverted microscope (Fig. 2). Cytotoxic effects were not observed in cell cultures.

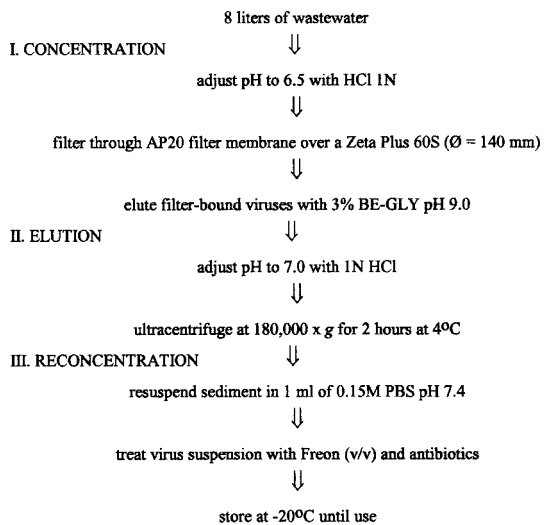


Fig. 1: scheme for concentration of rotavirus from sewage and sewage-polluted creek waters.

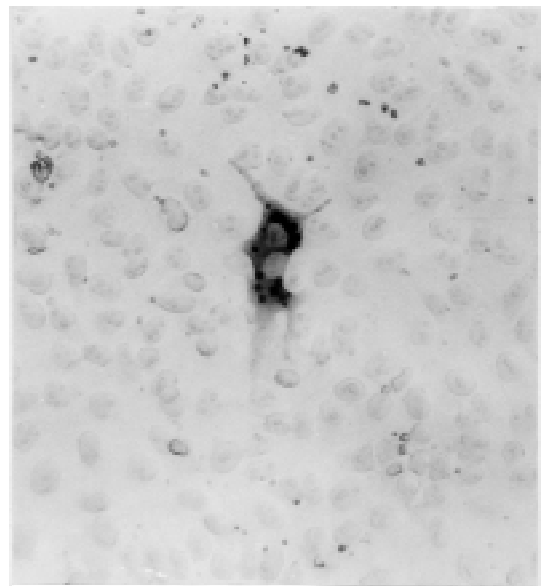


Fig. 2: MA104 cells inoculated with a concentrated water sample and stained by DIP method, showing one focus-forming unit of rotavirus. Magnification 400 X.

TABLE  
Efficiency of recovery of SA-11 virus from raw sewage water by the two-step concentration procedure

Trial	Sample volume (liter)	Virus input (FFU/81)	Virus recovered (FFU/81)	% of virus recovery
1	8	$3.6 \times 10^4$	$3.0 \times 10^4$	83
2	8	$5.0 \times 10^4$	$3.4 \times 10^4$	68
3	8	$2.5 \times 10^4$	$2.1 \times 10^4$	84
4	8	$3.2 \times 10^4$	$2.8 \times 10^4$	88

FFU: focus forming units, as assayed by direct immunoperoxidase.

The mean  $\pm$  standard deviation percent efficiency was  $81 \pm 7.5$ .

## DISCUSSION

Reliable, sensitive and practical methods for detecting small quantities of rotaviruses in sewage and polluted surface waters are needed to determine the public health significance of these pathogens in the environment. In Brazil, about 30 millions people have no access to treated drinking water and only a limited segment of populations in the large cities has an urban sewage system. Otherwise, sewage is discharged into creeks and rivers, specially in the periphery of the cities.

Previous concentration methods for rotaviruses have been effective with SA-11 seeded in tap and estuarine waters (Ramia & Sattar 1980, Guttman-Bass & Armon 1983, Rao et al. 1986), but they were of limited efficiency for the recovery of these viruses from raw sewage (Smith & Gerba 1982, Guttman-Bass et al. 1987). In the present study, SA-11 was concentrated efficiently from 8-l samples of raw domestic sewage by use of a two-step concentration procedure, with a mean recovery of  $81\% \pm 7.5$  (Table).

Adsorption of virus particles on positively charged filter Zeta Plus occurred at neutral pH range obviating the need for acidification of water samples to pH 3.0-3.5 when negatively charged filters are used (Sobsey & Jones 1979, Sobsey & Glass 1980, Rose et al. 1984). Ultracentrifugation was chosen as a second step after filter elution of virus with BE-GLY at pH 9.0, avoiding other pH variations, which may result in loss of rotavirus infectivity (Estes et al. 1979).

Since rotavirus levels in sewage and sewage-polluted water seem to be quite different in some countries (Smith & Gerba 1982, Bosch et al. 1988), the successful detection of these viruses depends on the concentration factor of the procedure. In a longitudinal survey conducted in Jerusalem, Israel, by Guttman-Bass et al. (1987), rotaviruses were not detected, even during the winter months, prob-

ably due to the low mean concentration of 287-fold attained by the method. Smith and Gerba (1982) detected rotaviruses in raw and secondary treated sewage in Houston after a 400-fold concentration. With the present two-step concentration procedure, achieving an 8,000-fold concentration, Mehnert and Stewien (1993) detect rotaviruses in 29.8% of the sewage and creek water samples, assaying only volumes of 50  $\mu$ l. Higher rates of positivity are to be expected by assaying greater volumes of the wastewater concentrates.

DIP is a simple, cost-effective and reliable technique for detecting rotaviruses in wastewater, because the infected cells are easily recognized with an inverted microscope. Dark brown granula are seen in the cytoplasm around the nucleus, exhibiting a very characteristic picture (Fig. 2). These granula were also observed in infected cell cultures during ultrastructural studies of rotavirus replication (Altenburg et al. 1980, Petrie et al. 1982). A very slight background staining was observed, without interfering in the recognition of the infected cells.

Cytotoxicity of the environmental samples was totally removed in associating the use of Zeta Plus filter to Freon treatment (Hejkal et al. 1982).

Recently, molecular techniques have improved the virus detection in sewage samples, but these methods do not permit the detection of infectious virus particles, which are of great significance in Public Health.

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