

Detection and Distribution of Rotavirus in Raw Sewage and Creeks in São Paulo, Brazil

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Rotaviruses were concentrated from 8-liter samples of raw domestic sewage and sewage-polluted creek water by adsorption to and elution from positively charged microporous filters (Zeta Plus 60S), followed by ultracentrifugation of the filter eluates. Indirect immunofluorescence and direct immunoperoxidase methods allowed detection and enumeration of rotavirus in 6 (20.6%) of 29 sewage samples and in 19 (34.5%) of 55 creek water samples. Levels of rotaviruses ranged from <3 to 63 focus-forming units (FFU)/liter, and the geometric means were 2.2 FFU/liter in sewage, 2.9 FFU/liter at creek Tremembé, and 2.6 FFU/liter at creek Pirajussara. Wastewater samples examined during autumn and winter months showed a higher rate of positivity for rotavirus than those collected in spring and summer, corresponding to the seasonal variation of rotaviral diarrhea in the city of São Paulo.

Viral gastroenteritis is a common disease characterized by diarrhea and vomiting (7). Rotavirus infection has been shown to be an important cause of hospitalization and mortality of infants and children in developing countries (14). In Brazil, occurrence of diarrheal disease is widespread (5, 11, 17, 22), especially in areas where the water supply and sewage disposal systems are usually in precarious condition (21, 22). Under such conditions, the presence of human enteric pathogenic viruses in water assumes great importance in terms of public health. Transmission of rotaviruses through consumption of sewage-polluted water and food has to be considered, since many outbreaks of waterborne diseases with suspected rotavirus etiology have been reported (10, 12, 21). Many types of enteric viruses have been detected in sewage and water supplies in Brazil (6, 13, 19), but there is a lack of information about the possible presence of rotaviruses in such waters. In this report, we describe the detection, quantitation, and distribution of rotaviruses in domestic sewage and sewage-polluted creeks in the city of São Paulo.

MATERIALS AND METHODS

Water samples. Eight-liter samples of raw sewage and polluted surface waters (creeks) were collected at three different sites of the city of São Paulo: the sewage pumping station (SPS) of Edu Chaves and creeks Tremembé and Pirajussara. These creeks receive sewage mainly from the low-income segment of the population of São Paulo. All samples were collected between 8 and 9 a.m. on weekdays.

Concentration of rotavirus from wastewater. All water samples were concentrated by a two-step concentration procedure (15). In brief, rotaviruses were concentrated by filtration of the water samples through a Zeta Plus 60S microporous positively charged filter (AMF Cuno Div., Meriden, Conn.) and ultracentrifugation at $180,000 \times g$ using a Beckman 70.1 Ti rotor for 2 h at 4°C. The sediment was suspended in 1.0 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.4, effecting an 8,000-fold concentration. Samples were subsequently stored at -20°C until use.

Cell cultures. The MA104 continuous line of monkey kidney cells was originally obtained from T. H. Flewett (Regional Virus Laboratory, Birmingham, United Kingdom). The cells were grown in Eagle minimum essential medium supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 100 U of penicillin G per ml, and 100 µg of streptomycin per ml. Cells were routinely subcultured with 0.25% trypsin (1:250; tissue culture grade; Difco Laboratories, Detroit, Mich.) in PBS (without EDTA), pH 7.4.

Indirect immunofluorescence (IIF) method. IIF was performed as described by Bryden et al. (4), with modifications (8). Infected cells were detected with goat anti-rotavirus serum (1:40) and fluorescein-conjugated guinea pig anti-goat immunoglobulin antibody (1:8; Kallestad, Austin, Tex.) and then examined under code with a Reichert-Jung microscope (wavelength, 450 to 490 nm). Results of these assays were expressed as focus-forming units (FFU) per liter. Simian rotavirus (SA-11) was used as a positive control, and a fecal sample (10% suspension) was used as a negative control.

Direct immunoperoxidase (DIP) method. MA104 cell monolayers, cultured in 96-well plates, were inoculated with 50-µl aliquots of the concentrated water samples as described above. After incubation for 18 h at 37°C, the cells were fixed by the method described by Pauli et al. (16). Briefly, the inoculated cells were fixed with a 3% formaldehyde solution containing 0.006% H₂O₂ for 10 min at room temperature. This solution was removed, and 100 µl of a 1% Triton X-100 solution was added before incubation for 30 min. The monolayers were washed once with 0.01 M PBS (pH 7.2) and air dried. For the DIP method, rabbit anti-human rotavirus serum conjugated to peroxidase (Dakopatts, Copenhagen, Denmark) that had previously been titrated and diluted to 1:100 in PBS-T-BSA (0.15 M PBS [pH 7.4], 0.05% Tween 20, 1% bovine serum albumin) was used to detect infected cells. A solution of 0.1% 3,3'-diaminobenzidine tetrahydrochloride (D-56637; Sigma Chemical Co., St. Louis, Mo.) containing 0.006% H₂O₂ was used as the substrate. The reaction was stopped after 30 min at room temperature by washing the monolayers with distilled water. Infected cells stained dark brown, and the results of this assay technique were expressed in FFU per liter of water

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TABLE 1. Rates of rotavirus positivity in sewage and creek water samples collected from different sites in the city of São Paulo, Brazil, from October 1987 to July 1988

Sampling site	No. of water samples examined	No. (%) of samples positive by:			
		IIF	DIP	IIF and DIP	IIF and/or DIP
SPS Edu Chaves	29	3 (10.3)	5 (17.3)	2 (6.9)	6 (20.6)
Creek Tremembé	31	3 (9.7)	8 (25.8)	0	11 (35.5)
Creek Pirajussara	24	4 (16.7)	8 (33.4)	4 (16.7)	8 (33.4)
Total	84	10 (11.9)	21 (25.0)	6 (7.1)	25 (29.8)

sample. Simian rotavirus was included as a positive control, and a 10% fecal sample was included as a negative control.

Fecal coliforms counts. The numbers of fecal coliforms in sewage and creek water samples were determined by the membrane filtration technique (1), and the results were expressed as CFU per liter.

RESULTS

During a period of 10 months, concentrates prepared from 84 samples of domestic sewage and highly polluted surface water (creeks) were examined by IIF and DIP. Rates of positivity for rotavirus were 20.6% at SPS Edu Chaves, 35.5% at creek Tremembé, and 33.4% at creek Pirajussara. The total percentage of rotavirus-positive sewage and creek water samples was 29.8% (Table 1). Ten (11.9%) of the 84 water samples were positive by IIF, 21 (25.0%) were positive by DIP, and 6 (7.1%) were positive by both methods.

The rotavirus levels measured in samples of sewage and creek water by DIP and IIF ranged from <3 to 63 FFU/liter (Tables 2 and 3). Rotavirus levels ranged from <3 to 63 FFU/liter in sewage, <3 to 45 FFU/liter in creek Tremembé, and <3 to 30 FFU/liter in creek Pirajussara. The corresponding geometric means were 2.2, 2.9, and 2.6 FFU/liter (Table 3). Levels of <3 FFU/liter were included in the calculation of geometric means by assigning them a value of one-half of the detection limit (9).

Geometric means of fecal coliform levels in the water samples examined ranged from 24.8×10^6 to 68.9×10^6

(mean, 49.2×10^6) CFU/liter in sewage, from 0.9×10^6 to 11.7×10^6 (mean, 4.8×10^6) CFU/liter in creek Tremembé, and from 0.9×10^6 to 10.1×10^6 (mean, 3.8×10^6) CFU/liter in creek Pirajussara (Tables 2 and 3).

Rotavirus levels in sewage and creek water were expressed as mean values of FFU/liter obtained for each sample by IIF and DIP and are presented monthly for the period investigated in Fig. 1. It is shown that rotaviruses were detected during all months of the survey. During the period from March to July 1988, 32.1% (17 of 53) of the samples examined were positive, compared with 25.8% of the samples obtained from October 1987 to February 1988.

DISCUSSION

This is the first study conducted in Brazil which determined the presence and levels of rotaviruses in raw domestic sewage and in sewage-polluted creeks over a long period. From October 1987 to July 1988, 84 wastewater samples from the city of São Paulo were examined and rotaviruses were detected in 6 (20.6%) of 29 sewage samples and in 19 (34.5%) of 55 creek water samples examined by IIF and/or DIP (Table 1).

As shown in Tables 1 and 2, DIP was twice as sensitive as IIF for detection of rotaviruses in concentrated environmental water samples. Furthermore, infected cells assayed by DIP showed dark brown granula around the nuclei, which are very easy to recognize under an inverted microscope at low or medium magnification. More details will be presented

TABLE 2. Levels of rotaviruses and fecal coliforms in sewage and sewage-polluted creeks from the city of São Paulo, Brazil, during the period from October 1987 to July 1988^a

Mo	SPS Edu Chaves			Creek Tremembé			Creek Pirajussara		
	No. of positive samples/total	Rotavirus level (FFU/liter)	Geometric mean no. of CFU of fecal coliforms (10^6 /liter)	No. of positive samples/total	Rotavirus level (FFU/liter)	Geometric mean no. of CFU of fecal coliforms (10^6 /liter)	No. of positive samples/total	Rotavirus level (FFU/liter)	Geometric mean no. of CFU of fecal coliforms (10^6 /liter)
October	ND ^b	ND	ND	1/1	35.0	0.9	ND	ND	ND
November	1/3	3.0	24.8	0/3	40.0	6.2	0/1	<3.0	0.9
December	1/3	5.0	58.6	0/3	<3.0	11.7	1/3	10.0	8.9
January	0/3	<3.0	61.9	1/3	5.0	2.9	0/1	<3.0	2.4
February	1/3	63.0 ^c	40.4	1/3	8.0	2.7	0/1	<3.0	10.1
March	1/3	25.0 ^d	55.4	2/3	3.0, 3.0 ^d	1.4	2/4	3.0, ^c 3.0 ^c	2.4
April	1/3	3.0	60.9	1/4	5.0	3.4	1/3	30.0 ^d	4.9
May	0/4	<3.0	48.2	1/3	3.0 ^d	6.9	0/3	<3.0	4.3
June	0/4	<3.0	68.9	1/5	5.0	8.1	3/5	17.0, ^c 5.0, 5.0	2.8
July	1/3	8.0 ^d	33.2	2/3	45.0, 10.0 ^d	9.5	1/3	10.0 ^c	4.4
Total	6/29			11/31			8/24		

^a Unless otherwise noted, all levels were obtained by DIP only.

^b ND, not done.

^c Mean level obtained by IIF and DIP.

^d Level obtained by IIF only.

TABLE 3. Results of bacteriological and virological analyses and pH values of sewage and creek water samples from the city of São Paulo, Brazil, for 1987 to 1988

Sampling site	No. of water samples	pH range (mean)	Range of geometric mean no. of CFU of fecal coliforms (10^6 /liter) (mean)	Range of rotavirus levels, FFU/liter (mean) ^a
SPS Edu Chaves	29	6.0–8.0 (7.0)	24.8–68.9 (49.2)	<3–63.0 (2.2)
Creek Tremembé	31	6.5–8.0 (7.3)	0.9–11.7 (4.8)	<3–45.0 (2.9)
Creek Pirajussara	24	5.5–7.0 (6.3)	0.9–10.1 (3.8)	<3–30.0 (2.6)

^a Values of one-half of the detection limit (3 FFU/liter) were included in calculations of geometric means.

in another study, showing that DIP is a very reliable and cost-effective method for rapid detection of rotaviruses in environmental samples (15).

Rotavirus levels ranged from <3 to 63 FFU/liter in sewage, with a geometric mean of 2.2 FFU/liter (Table 2). Geometric means of sewage-polluted creeks were almost the same (2.9 and 2.6 FFU/liter). Similarity between these levels can be attributed to the high level of discharge of domestic sewage into these creeks by a low-income and crowded population living along this surface water, where enteropathogens easily circulate all year round.

Lower fecal coliform levels detected in creeks than in sewage may be explained by the finding that these bacteria are not able to survive as long as enteric viruses in such polluted surface waters (2).

Previously, levels of rotaviruses were determined only in some investigations. Hejkal et al. (9) reported levels of 1 to 321 FFU/liter in raw domestic sewage of the city of Houston, Tex., with a geometric mean of 9.8 FFU/liter. Bosch et al. (3) detected 36 to 653 FFU/liter (mean, 153 FFU/liter) during November 1985 and 25 to 492 FFU/liter (mean, 158 FFU/liter) in July 1986 in the municipal sewage of Barcelona, Spain. Rao et al. (18) found levels of 119 to 1,000 FFU/378 liters (0.3 to 2.6 FFU/liter) in sewage-polluted estuarine water samples from Galveston Bay, Gulf of Mexico. Although an exact comparison between the results of these investigations cannot be made, in view of methodological differences, the reported levels of rotavirus in sewage were about four times higher in Houston and about 70 times higher

in Barcelona than in São Paulo. Reasons for these marked differences are unknown, but the differences may be explained by different dilution factors of raw sewage in the cities investigated.

On the basis of the finding that levels of rotaviruses were lower in São Paulo sewage than previously reported for other cities (3, 9), enhancement of the sensitivity of our method will be necessary to obtain higher detection rates of these viruses. This can be achieved by inoculating larger volumes of the concentrated samples into cell cultures. For example, by inoculation of 500- μ l volumes instead of the 50 μ l used in the present study, we can increase the sensitivity of our method 10-fold.

Seasonal variation in rotavirus levels was observed for the first time by Hejkal et al. (9) in Houston sewage. As shown in Fig. 1, rotaviruses were detected during the whole period of our study. Although a marked seasonal variation in rotavirus levels was not observed, samples examined from March to July of 1988 showed more positive results than did water samples collected from October 1987 to February 1988 (32.1 versus 25.8%) (Table 2 and Fig. 1). This variation in rotavirus positivity agreed well with the incidence of rotaviral diarrhea in the city of São Paulo, which increases during the period between March and September (autumn and winter) (5, 20).

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REFERENCES

1. American Public Health Association. 1985. Membrane filter technique for members of the coliform group, p. 886–902. In A. E. Greenberg, R. R. Trussell, and L. S. Clesceri (ed.), Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. Berg, G., and T. G. Metcalf. 1978. Indicators of viruses in waters, p. 267–293. In G. Berg (ed.), Indicators of viruses in water and food. Ann Arbor Science Publishers Inc., Ann Arbor, Mich.
3. Bosch, A., R. M. Pinto, and J. Jofre. 1988. Non-seasonal distribution of rotavirus in Barcelona raw sewage. Zentralbl. Bakteriell. Abt. 1 Orig. B Hyg. Krankenhaushyg. Betriebshyg. Praev. Med. 186:273–277.
4. Bryden, A. S., H. A. Davies, M. E. Thouless, and T. H. Flewett. 1977. Diagnosis of rotavirus infection by cell culture. J. Med. Microbiol. 10:121–125.
5. Candeias, J. A. N., M. L. Racz, L. R. Trabulsi, and J. Murahowsky. 1989. Relative prevalence of rotavirus diarrhoea in children attending outpatient departments of hospitals and general practitioners in S. Paulo, Brazil. J. Diarrhoeal Dis. Res. 7:24–27.
6. Christovão, D. A., J. A. N. Candeias, and S. T. Iaria. 1967. Condições sanitárias das águas de irrigação de hortas do município de S. Paulo. II. Isolamento de vírus entéricos. Rev. Saude Publ. 1:12–17.
7. Flewett, T. H., and G. N. Woode. 1978. The rotaviruses. Arch. Virol. 57:1–23.
8. Hãrsi, C. M., and J. A. N. Candeias. 1991. A reação de imunofluorescência indireta no diagnóstico de rotavírus. Rev. Microbiol. 22:282–287.
9. Hejkal, T. W., E. M. Smith, and C. P. Gerba. 1984. Seasonal occurrence of rotavirus in sewage. Appl. Environ. Microbiol.

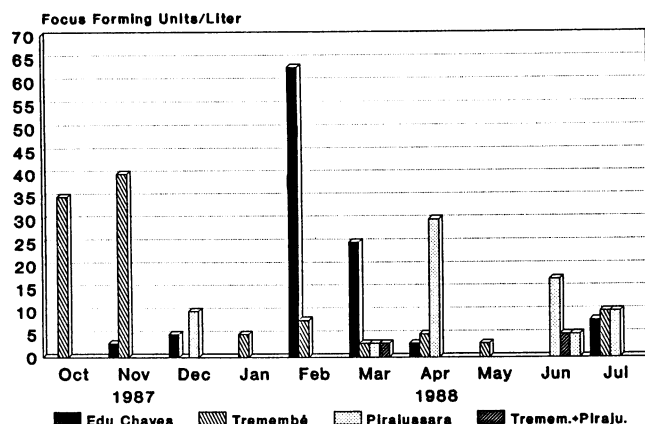


FIG. 1. Distribution of rotaviruses in domestic sewage and creeks of the city of São Paulo, Brazil, from October 1987 to July 1988. The data shown are mean values obtained by IIF and DIP.

- 47:588-590.
10. Hopkins, R. S., G. B. Gaspard, F. P. Williams, R. J. Karlin, G. Cukor, and N. R. Blacklow. 1984. A community waterborne gastroenteritis outbreak: evidence for rotavirus as the agent. *Am. J. Public Health* 74:263-265.
 11. Linhares, A. C., Y. B. Gabbay, J. D. P. Mascarenhas, R. B. Freitas, T. H. Flewett, and G. M. Beards. 1988. Epidemiology of rotavirus subgroups and serotypes in Belém, Brazil. *Ann. Inst. Pasteur Virol.* 139:89-99.
 12. Linhares, A. C., F. P. Pinheiro, R. B. Freitas, Y. B. Gabbay, J. A. Shirley, and G. M. Beards. 1981. An outbreak of rotavirus diarrhea among a nonimmune isolated South American Indian community. *Am. J. Epidemiol.* 113:703-710.
 13. Martins, M. T., P. S. Sanches, E. Marques, C. K. Monteiro, and A. G. Molina. 1986. A ten year survey of salmonella and enterovirus in raw and treated water in the great S. Paulo, Brazil. *Water Sci. Technol.* 18:53-60.
 14. McNulty, M. S. 1978. Rotaviruses. *J. Gen. Virol.* 40:1-18.
 15. Mehnert, D. U., and K. E. Stewien. Unpublished data.
 16. Pauli, G., J. P. Gregersen, and H. Ludwig. 1984. Plaque focus immunoassay: a simple method for detecting antiviral monoclonal or others antibodies and viral antigens in cells. *J. Immunol. Methods* 74:337-344.
 17. Pereira, H. G., R. S. Azeredo, J. P. G. Leite, J. A. N. Candeias, M. L. Racz, A. C. Linhares, Y. B. Gabbay, and L. R. Trabulsi. 1983. Electrophoretic study of the genome of human rotavirus from Rio de Janeiro, São Paulo and Pará, Brazil. *J. Hyg.* 90:117-125.
 18. Rao, V. C., T. G. Metcalf, and J. L. Melnick. 1986. Development of a method for concentration of rotavirus and its application to recovery of rotaviruses from estuarine waters. *Appl. Environ. Microbiol.* 52:484-488.
 19. Stewien, K. E. 1979. Comparative study of two methods for concentrating enterovirus in sewage and determination of coliform/virus ratios in environmental samples. Ph.D. thesis. University of São Paulo, São Paulo, Brazil.
 20. Stewien, K. E., et al. Viral, bacterial and parasitic pathogens associated with severe diarrhoea in the city of São Paulo, Brazil. Submitted for publication.
 21. Sutmoller, F., R. S. Azeredo, M. D. Lacerda, O. M. Barth, H. G. Pereira, E. Hoffer, and H. G. Schatzmayr. 1982. An outbreak of gastroenteritis caused by both rotavirus and *Shigella sonnei* in a private school in Rio de Janeiro. *J. Hyg.* 88:285-293.
 22. Trabulsi, L. R., M. R. F. Toledo, B. S. O. Ceballos, and J. A. N. Candeias. 1985. Epidemiology of diarrheal diseases in South America, p. 121. *In* T. Tzipori et al. (ed.), *Infectious diarrhea in the young*. Elsevier Biomedical Press, Amsterdam.