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Development of a Method for Detection of Human Rotavirus in Water and Sewage

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The simian rotavirus SA11 was used to develop a simple, reliable, and efficient method to concentrate rotavirus from tap water, treated sewage, and raw sewage by absorption to and elution from Filterite fiberglass-epoxy filters. SA11 adsorbed optimally to Filterite filters from water containing 0.5 mM $AlCl_3$ at pH 3.5. Filter-bound virus was eluted with 0.05 M glycine-NaOH supplemented with 10% tryptose phosphate broth at pH 10. SA11 was quantitated by plaque assay, whereas human rotavirus was detected by immunofluorescence. The method was applied to detect rotavirus in raw and treated sewage at two Houston, Tex., sewage treatment plants. The sewage isolates were identified as rotavirus, probably a human strain, based on several criteria. The sewage isolates were detectable by an immunofluorescence test, using anti-SA11 serum which would detect the simian, human, bovine, and porcine rotaviruses. No reaction was noted by immunofluorescence with the reoviruses or several common enteroviruses. The sewage isolates were neutralized by convalescent sera from a human adult and infant who had been infected by rotavirus as well as by a hyperimmune serum prepared in guinea pigs against purified human rotavirus. Preimmune or preillness sera did not react with the isolates by neutralization or immunofluorescence. The natural isolates were sensitive to pH 11 and other inactivating agents, similar to SA11. The buoyant density of the sewage isolates in CsCl gradients was 1.36 g/cm^3, which is the value usually reported for complete, infectious rotavirus particles. The double-shelled particle diameter was 67.1 ± 2.4 nm. Finally, electron micrographs of cell lysates inoculated with the sewage isolate showed particles displaying characteristic rotavirus morphology.

The major waterborne illness in the United States continues to be gastroenteritis of suspected viral etiology (15). Outbreaks associated with drinking water, bathing water, and shellfish consumption have been suspected to be caused by Norwalk-like agents or rotaviruses (5, 22, 28). This report concerns the rotaviruses, which cause a severe, acute diarrhea that may be fatal to human and animal infants (12, 25). Studies have shown that in some areas greater than 50% of the hospitalized cases of infantile gastroenteritis are associated with rotavirus infections (17). Adults (26, 44) may also be infected with rotavirus either by contact with infected infants (20, 41) or possibly as a "traveler's diarrhea" (3, 34). Rotaviruses appear to be capable of transmission by sewage-contaminated water, as they are excreted in large numbers (10^{10} to 10^{11} particles per g of diarrheic stool) by infected individuals (12), and the virus is able to survive for prolonged periods of time in both fresh and marine waters (16).

Evidence from our laboratory has suggested that infectious rotavirus may not be entirely removed from wastewater by current sewage treatment processes (9). Other preliminary reports have detected rotavirus particles in stools from individuals in outbreaks of waterborne gastroenteritis (22, 29). Unfortunately, the authors had no means to detect the virus in the suspected water to prove that rotavirus was the etiological agent. Farrah et al. (10) and Ramia and Sattar (32) have described methods for concentrating the simian rotavirus SA11 from aqueous solutions, but these procedures cannot detect human rotavirus. A practical method for detecting dilute human rotavirus in either waste or potable water is needed to determine the public health significance of rotavirus in water and the effectiveness of water treatment processes in removing infectious virus.

This report describes the use of SA11 to develop concentration methods for the detection of rotaviruses in tap water and in raw and treated sewage. The concentration methodology was adapted for detection of the human rota-

TABLE 1. Properties of waters used for virus concentration

Water type	Water parameters				
	pH	Conductivity (μmho)	Turbidity (JTU)[a]	A_{254}[b]	Refractive index
Distilled	5.5–6.2 (5.8)[c]	0.5–1.5 (0.9)	0	0	1.3330
Tap	7.5–8.1 (7.8)	65–125 (103)	0.5–1.0 (0.8)	0.01–0.06 (0.03)	1.3330
Secondary sewage effluent	7.6–7.8 (7.7)	135–160 (145)	1.5–3.0 (2.4)	0.20–0.42 (0.29)	1.3330
Raw sewage	7.4–7.5 (7.5)	150–175 (166)	12–50 (34.8)	0.63–1.15 (0.93)	1.3330

[a] Jackson turbidity units.
[b] Absorbance of UV light (254-nm wavelength).
[c] Numbers in parentheses represent mean values.

virus by immunofluorescence (2) and applied in a field study to detect rotaviruses in raw and treated sewage.

MATERIALS AND METHODS

Cell cultures. Two continuous lines of monkey kidney cells were used: MA104 (Microbiological Associates, Bethesda, Md.) and BGM (buffalo green monkey, obtained from Gerald Berg). Both cell lines were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth (TPB), 2% basal Eagle medium vitamins, 0.25% glucose, 0.03% glutamine, 0.075% sodium bicarbonate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 μg of gentamicin per ml. Cells were routinely subcultured with 0.2% trypsin (1:250 tissue culture grade; Difco Laboratories, Detroit, Mich.) in phosphate-buffered saline (PBS) containing 0.025% EDTA.

Viruses and viral assays. SA11 was kindly supplied by H. H. Malherbe (23) and was grown in a continuous line of fetal rhesus monkey kidney cells (MA104) as previously described (36). Human rotavirus was generously donated by R. H. Conklin as a 10% stool suspension. Bovine and porcine rotaviruses were kindly supplied by J. J. Vollet and M. K. Estes, respectively. Plaque-purified poliovirus type 1 (strain LSc) used for this study was grown in the BGM continuous line as previously described (42).

Virus samples were diluted in Tris-buffered saline which contained 20 mM Tris, 140 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 6 mM dextrose, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μg of gentamicin per ml at pH 7.2.

SA11 virus was assayed as previously described by a plaque assay, using MA104 cells and an agar overlay supplemented with pancreatin (0.2 mg/ml [Oxoid Colabs, London, England] or an equivalent dilution of Viokase solution [GIBCO Laboratories, Grand Island, N.Y.]) and DEAE-dextran (100 μg/ml) (36).

Enterovirus plaque assays were performed on BGM cell monolayers in 1-oz (ca. 30-ml) glass prescription bottles. The agar overlay was identical to the method used for SA11 except that 25 mM $MgCl_2$ was added and pancreatin and DEAE-dextran were not. Plaque formation occurred in 2 to 4 days.

Enterovirus natural isolates were quantitated by plaque formation by inoculating 75-cm^2 plastic flasks of BGM cells. The bottles were inoculated with 1.5 ml of sample, virus was adsorbed for 90 min at 37°C with rotational agitation of the inoculum at 15-min intervals, the inoculum was decanted, and an agar overlay was applied as described above for the enteroviruses but supplemented with 2% fetal bovine serum. The samples were observed for 14 days, but plaques generally formed within 4 to 7 days (14).

Water. Table 1 shows the different types and physical characteristics of water used for these experiments. Distilled water was prepared by glass distillation of deionized tap water. Conductivity was measured in microohms with a conductivity meter (Markson Scientific, Del Mar, Calif.). Turbidity was measured in Jackson turbidity units with a turbidimeter (Hach Chemical Co., Loveland, Colo.). UV absorbance was measured at 254 nm in a spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.) as a measure of relative indicator of inorganic compounds. The refractive index of each water type was measured with the aid of an AO T/C refractometer. In all cases, distilled water was used to zero the instruments.

Tap water was obtained from laboratory faucets that had been allowed to flow for several minutes to clear standing water from the lines. This water was then dechlorinated by the addition of sodium thiosulfate to a final concentration of 0.05 mg/liter. Residual chlorine was determined by adding 2 ml of a 0.1% *o*-toluidine solution to 5 ml of water. The presence of a yellow color was indicative of residual chlorine (1).

Adsorption and elution. Distilled water, tap water, and raw sewage were seeded with SA11 virus at 10^4 to 10^5 PFU/ml. After virus addition, the pH was lowered as required with 1 N HCl and the addition of 1 M $AlCl_3$ to the specified final concentration. In large-volume experiments (20 liters), 100 ml of the water was seeded with virus and assayed for infectious virus. The 100 ml was then mixed with the rest of the water for concentration. This procedure prevented dilution beyond the limits of the plaque assay.

Water, seeded with virus, was passed through membrane filter units (either nylon or stainless steel; Millipore Corp., Bedford, Mass.) of diameters 13, 25, 47, 90, or 142 mm, depending on the experiment. Water samples of 1 liter or less were processed at flow rates of 50 to 100 ml/min or less. The filters used for virus concentration were fiberglass-epoxy, melamine-treat-

ed paper disks (Duo-Fine series; Filterite Corp., Timonium, Md.) of the appropriate diameter for the filter holders. Most of the experiments utilized 3.0- and 0.45-μm nominal pore diameter filters in series in one holder. Millipore nitrocellulose microporous membranes and Zeta-plus (AMF CUNO, Meriden, Conn.) positively charged depth filters were also used (37). Pleated cartridge filters (10 inch [ca. 25 cm]; Filterite) with a nominal porosity of 0.45 μm were used to clarify sewage before virus concentration. During clarification, the native pH and salt concentration of the sewage were unaltered to prevent virus adsorption to the filter or suspended solids.

Eluents evaluated for desorbing viruses from the filters included TPB (Difco), beef extract (BBL Microbiology Systems, Cockeysville, Md.), and glycine-HCl buffer. TPB and beef extract eluents were prepared in 0.05 M glycine-NaOH buffer as a weight/volume percentage. Necessary pH adjustments of eluents were made with concentrated HCl or NaOH, whereas any virus-containing solutions were adjusted with 1 N HCl or 1 N NaOH.

All samples were neutralized before assay or storage of samples either by dilution in Tris-buffered saline or by titration with 1 M glycine buffer, pH 2.

Concentration of natural isolates. Twenty-liter samples of raw sewage and secondary treated sewage effluent were collected from a trickling filter (West University) or activated sludge (Bellaire) sewage treatment plant. All samples were collected between 9 and 11 a.m. on weekdays. Initially, the sewage was clarified by passage through an 0.45-μm nominal porosity fiberglass-epoxy cartridge filter (25.5 cm long; Duo-Fine series, Filterite). Next, the pH was lowered to 3.5 with 1 N HCl, and 10 ml of 1 M $AlCl_3$ was added while mixing thoroughly to a final concentration of 0.5 mM. This mixture was passed through a 142-mm-diameter filter series (3.0- and 0.45-μm nominal pore size; Duo-Fine series, Filterite). The flow rate was maintained at 1 liter/min. Fifty milliliters of eluent (0.05 M glycine buffer–10% TPB, pH 10) was used to elute filter-bound virus. The eluent was neutralized with 1 M glycine buffer at pH 2, divided into three equal portions, and stored at −80°C until assayed.

IIF test. The indirect immunofluorescence (IIF) test described by Porter et al. (31) was used with minor modifications. MA104 cells were grown to confluence (≈5 × 10^4 cells/chamber) in eight-chamber tissue culture slides (Lab-Tek Products, Naperville, Ill.). Fifty microliters of virus or sewage sample was inoculated per chamber, the slides were incubated at 37°C for 15 min, and 0.2 ml of maintenance medium was placed on the monolayer. Next, the inoculated slides were taped to a test tube carrier for an IEC 269 rotor (Damon/IEC, Needham Heights, Mass.) and centrifuged at 25°C for 90 min at 1,200 × *g* (2). The inoculated slides were then incubated for 24 h at 37°C in a 5% CO_2 incubator.

At the end of the infection period, the medium was decanted, and the monolayers were washed three times with 0.85% saline and then air dried. Next, the cells were fixed in methanol at −4°C for 5 min and air dried. Fixed slides were rehydrated for 5 min in PBS before staining. Each chamber or well was treated with 25 μl of preimmune or hyperimmune guinea pig serum specific for SA11 (7) and diluted 1:60 with PBS. The slides were washed and stained with goat anti-guinea pig immunoglobulin G (1:20) conjugated to fluorescein isothiocyanate. The fluorescenisothiocyanate-stained cells were then washed and dried, and the cover slips were mounted in a solution of 8% Elvanol (grade 51-05; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), 0.15 M NaCl, 0.01 M KH_2PO_4–$Na_2HPO_4 \cdot 12H_2O$, and 16% (vol/vol) glycerol.

Slides were examined under code with a Zeiss microscope fitted for epifluorescence, using a filter and beam splitter combination for blue-green excitation (wavelength, 450 to 490 nm; filter pack no. 487709).

Immunofluorescent neutralization test. Specific antiserum was used in a neutralization test based on the method of Thouless et al. (40) for rotavirus identification. Equal volumes of virus or unknown sample (diluted in PBS) were mixed with dilutions of antiserum and incubated for 1 h at 37°C. MA104 cells grown on glass chamber slides were inoculated with 50 μl of virus and antiserum, incubated, and stained by the IIF method described above. Ideally, the diluted virus sample contained 50 to 100 fluorescent foci, and neutralization was considered to be positive when a greater than 50% reduction in foci occurred. SA11 plus PBS containing no antiserum or a preimmune serum was incubated for 1 h at 37°C and inoculated into MA104 cells as a control.

Hyperimmune animal and human convalescent sera were used for the neutralization tests. Preillness and convalescent human sera were obtained from an adult who was exposed to human rotavirus by a laboratory incident and subsequently developed diarrhea. A human infant convalescent serum was kindly supplied by John J. Vollet. The infant had diarrheic stools in which rotavirus particles were visible by electron microscopy; the infant's serum had an enzyme-linked immunosorbent assay blocking titer of 1:128. Mary K. Estes generously provided specific antirotavirus sera: a guinea pig antibovine rotavirus serum (courtesy of G. Woode), a gnotobiotic porcine antiporcine rotavirus serum (courtesy of E. Bohl), and a guinea pig hyperimmune anti-human rotavirus serum (prepared by M. Petric and obtained from Leslie Spence) with a complement fixation titer of 1:8,000 (11).

Inactivation of natural isolates. Sewage concentrates were treated by various inactivating procedures to reduce the number of fluorescent foci as an indication of biological activity. One-milliliter samples of a sewage concentrate positive for rotavirus by immunofluorescence were: (i) autoclaved for 20 min; (ii) boiled for 20 min; (iii) treated with chlorine (10 mg/liter) for 30 min, and the chlorine was neutralized with sodium thiosulfate; (iv) adjusted to pH 11 for 30 min; or (v) treated with a 1:2,000 dilution of Formalin for 4 days at 37°C followed by dialysis against 1 liter of PBS to remove the Formalin. Samples of a sewage concentrate negative for rotavirus were seeded with SA11 and treated identically. Rotavirus was detected by the IIF test.

Buoyant density of natural isolates. One hundred-milliliter samples of three secondary treated sewage concentrates were pooled and sonically treated for 60 s, and large particulate matter was removed by centrifugation at 1,000 × *g* for 10 min. Virus in the supernatant fluid was pelleted at 100,000 × *g* for 60 min at 4°C in a type 30 rotor (Beckman Instruments). The pellet

was suspended in 10 ml of CsCl (ρ = 1.37 g/cm^3). This mixture was divided into two tubes and ultracentrifuged in an SW50.1 rotor (Beckman Instruments) at 114,000 × *g* for 20 h at 4°C. Fractions (0.4 ml) were collected by bottom puncture, and the density of the CsCl was determined from the refractive index. Each fraction was assayed for rotavirus by the IIF procedure.

Electron microscopy of natural isolates. The raw sewage concentrate samples were pooled (300 ml) and purified by centrifugation in a CsCl buoyant density gradient as described above. MA104 cells were inoculated with the virus found in the fraction with a density of 1.36 g/cm^3. After a 48-h incubation, the medium was replaced with 0.2 ml of distilled water, and the monolayer was scraped off the glass and sonically treated for 60 s. The resulting lysate was prepared for negative-stain electron microscopy by the pseudoreplica method of McCombs et al. (24). Duplicate samples of this fraction were tested for infectivity and identified by the fluorescent foci neutralization test, using human adult and infant convalescent and preillness sera as well as hyperimmune anti-SA11 sera.

RESULTS

Rotavirus adsorption to filters. Optimization of adsorption-elution conditions was a compromise in conditions for the greatest adsorption and flow rates versus minimal virus inactivation and filter clogging. The initial adsorption experiments were performed to determine the necessary ionic conditions enabling rotavirus to adsorb to filters. Fiberglass-epoxy, melamine-treated paper filters (3.0- and 0.45-μm nominal porosity; Duo-Fine series, Filterite) were used for these experiments since they resist clogging and are effective for concentrating enteroviruses (8). The pH of virus-containing tap and distilled water was made acidic, the water was passed through the filter, and the percentage of adsorption was determined by assaying the input water and the filtrate for SA11. Optimal virus adsorption was obtained at pH values below 4 (Fig. 1).

Increased flow rates, large volumes, and organic material present in raw sewage or other natural waters often make it necessary to add cations to neutralize repulsive charges (27). $AlCl_3$ at a concentration of 0.5 to 1.5 mM enhanced SA11 adsorption to filters from distilled and tap water as well as from raw sewage (Fig. 2). It is interesting to note that SA11 virus in tap water at pH 3.5 adsorbed very efficiently to the fiberglass-epoxy filters, but this was not true with distilled water or sewage. At $AlCl_3$ concentrations greater than 1.5 mM, virus adsorption was decreased, and flocs formed which clogged the filter.

Constant developments in filter technology are providing alternative filter materials. Several filter types were tested for the ability to adsorb SA11 virus. A resin- and diatomaceous earth-containing cellulose filter (Zeta-plus) was compared with the standard virus-adsorbing filters, fiberglass-epoxy and cellulose ester (Table 2). The resin and diatomaceous earth filter adsorbed SA11 effectively at pH 7, but all three filter types adsorbed 100% of the virus under the appropriate conditions. The fiberglass-epoxy filters (Filterite) were much more resistant to clogging, were capable of higher flow rates than the other two, and therefore were the more practical for processing raw sewage. Various combinations of fiberglass-epoxy filters with nominal pore diameters of 3.0, 0.45, and 0.25 μm were tested for optimal adsorption of SA11 from dechlorinated tap water. A combination of all three pore size filters was the most effective for

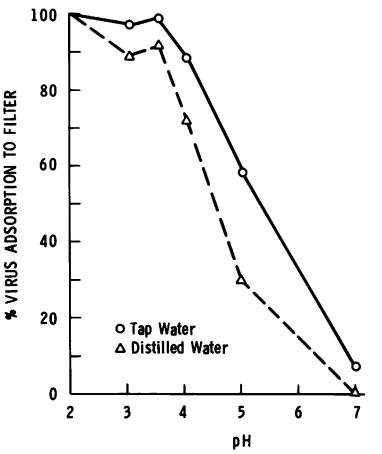

FIG. 1. Comparison of SA11 adsorption to Filterite filters at different pH values from tap and distilled water. Tap or distilled water (100 ml) was seeded with 10^5 PFU of SA11 per ml, and the pH was adjusted as indicated. The water was passed through a 25-mm-diameter Filterite filter series (3.0- and 0.45-μm nominal pore diameter), and the filtrate was collected, mixed, and assayed for nonadsorbed virus.

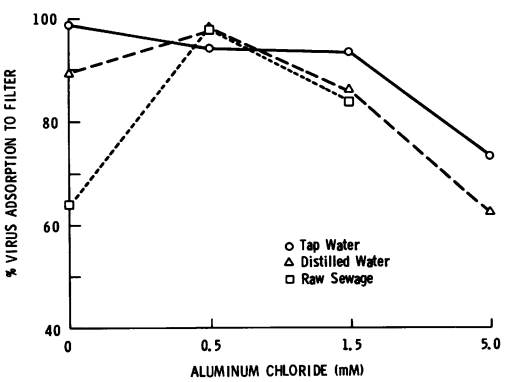

FIG. 2. Enhancement of rotavirus adsorption to Filterite filters by $AlCl_3$. Conditions were identical to those described for Fig. 1, except the pH was 3.5 and the concentration of $AlCl_3$ was the variable.

TABLE 2. Adsorption of SA11 to different filter material[a]

Membrane	Membrane composition	pH of adsorption	Aluminum chloride (mM)	Virus titer (PFU)		% Adsorption
				Input	Filtrate	
Filterite	Fiberglass-epoxy	3.5	1.5	3.5×10^5	0	100
Millipore	Mixed cellulose esters	3.5	1.5	4.0×10^5	0	100
Zeta-plus 60S[b]	Cellulose-diatomaceous earth-"charge modified" resin	7.0	0	1.4×10^6	0	100

[a] A 100-ml sample of dechlorinated tap water seeded SA11 was passed through a 3.0- and 0.45-μm nominal pore size filter series under the above conditions. The entire filtrate as collected, mixed, and assayed by the plaque method.

[b] With a nominal porosity of 0.45 μm.

virus adsorption and had the greatest resistance to clogging (data not shown).

Elution of SA11 from filters. The ability of protein-containing compounds to interfere with SA11 adsorption to filters makes these components useful for eluting adsorbed virus. High-pH glycine buffer, which is very effective in eluting adsorbed enteroviruses, was tested along with TPB (Table 3) for effectiveness in eluting SA11 virus. An eluent at pH 10 and containing 10% TPB was the most effective.

The results of the adsorption-elution studies suggested that the optimal method of concentrating rotaviruses from water should consist of adsorbing the virus to Filterite filters (3.0- and 0.45-μm nominal porosity) at pH 3.5 with 0.5 mM $AlCl_3$ and eluting the virus with 0.05 M glycine containing 10% TPB at pH 10. To use this as a quantitative method, the efficiency had to be determined for the viruses, volumes, and types of water expected to be concentrated. The efficiency of concentrating SA11 was highest with distilled water (80%) and decreased to 29% with raw sewage (Table 4). The effectiveness of concentrating poliovirus 1 was also tested for these same waters. The method was much more efficient at concentrating poliovirus, probably due to its greater stability at pH 10 (7). The initial step of clarifying the raw sewage with a 0.45-μm clarifying filter did not remove any detectable amount of seeded virus. Because of the relatively low efficiency with raw sewage, several modifications were evaluated to improve effectiveness. Efficiency could not be increased with larger volumes of eluate, whereas smaller volumes reduced efficiency. It was possible to increase the elution efficiency by passing the eluate through the filter twice, but no increase was found with a third passage. Smaller volumes (10 liters) with relatively smaller filters (90-mm diameter) were no more efficient than 20-liter samples and 142-mm-diameter filters.

TABLE 3. Effect of different eluents[a]

Molarity of glycine buffer	TPB (%)	pH	Elution (%)
0.05	0	9.0	0.33 ± 0.4
	0	10.0	37.8 ± 5.2
	0	10.5	20.4 ± 1.0
	2.5	9.0	10.0 ± 0
	1	10.0	48.0 ± 12.2
	10	10.0	96.0 ± 1.2
	1	10.5	13.0 ± 2.8
	10	10.5	9.0 ± 0
	10	11.0	0 ± 0
0.25	0	10.0	17.4 ± 2.3
	0	10.5	8.2 ± 0.7

[a] A 100-ml sample of tap water containing approximately 10^5 PFU of SA11 was concentrated on 13-mm-diameter Filterite filters (3.0- and 0.45-μm nominal pore size). The virus was eluted by passing 3 ml of eluent through the filter. Infectivity was measured by plaque assay. The results are the mean of three experiments ± standard deviation.

Detection of rotavirus by immunofluorescence. The guinea pig anti-SA11 antiserum (7) was tested for specificity in neutralization and immunofluorescence tests with several common enteroviruses. Only SA11 virus was neutralized by the antiserum, and only SA11-infected monolayers stained positive (Table 5). Staining of rotavirus-infected cells was abolished by absorbing the anti-SA11 serum (1 ml of a 1:60 dilution) with SA11-infected MA104 cells (10^7). Individual fluorescing foci of rotavirus-infected cells were easy to distinguish, correlated directly with the number of PFU added, and were not inhibited by contaminating enteroviruses at concentrations less than 2×10^4 PFU/ml (data not shown).

Detection of rotavirus in Houston sewage. Raw sewage and secondarily treated sewage effluents from a Houston, Tex., trickling filter sewage treatment plant (West University) were concentrated as described, and the concentrates were examined for the presence of rotavirus and enterovirus, using the immunofluorescence and plaque assays, respectively. Both rotavirus and enterovirus were found in the raw sewage as

TABLE 4. Concentration efficiencies of SA11 and poliovirus from large volumes of water[a]

Type of water	Expt no.	SA11		Poliovirus	
		PFU of virus added to sample (20 liters)	PFU recovered (% ± SD)	PFU of virus added to sample (20 liters)	PFU recovered (% ± SD)
Distilled	1	7.0×10^5	87	4.6×10^6	104
	2	6.8×10^5	72	4.6×10^6	102
			Mean = 80 ± 10.6		Mean = 103 ± 1.4
Tap	1	4.1×10^6	54.8		—
	2	4.4×10^6	55.7		—
	3	1.6×10^6	60.0	5.0×10^6	100
	4	1.2×10^6	48.2	4.0×10^6	100
			Mean = 54.6 ± 4.8		Mean = 100 ± 0
Secondarily treated sewage	1	1.0×10^7	51.0	4.6×10^7	132
	2	6.0×10^6	40.0	3.4×10^7	94
	3	6.4×10^6	56.2	3.3×10^7	106
	4	6.4×10^6	43.8	3.7×10^7	92
			Mean = 47.8 ± 7.2		Mean = 106 ± 18.4
Raw sewage	1	5.2×10^6	27.0	4.5×10^7	84
	2	2.7×10^6	43.9	4.4×10^7	68
	3	6.7×10^5	30.3	4.9×10^7	72
	4	5.1×10^6	13.5	3.8×10^7	92
			Mean = 28.6 ± 12.5		Mean = 79 ± 11.0

[a] Twenty liters of water for each experiment was seeded with SA11 or poliovirus and concentrated by adsorption to 3.0- and 0.45-μm nominal pore size filter series (142-mm diameter; Duo-Fine series, Filterite). Viruses were eluded with 50 ml of 0.05 M glycine containing 10% TPB at pH 10.

well as in secondarily treated effluent samples (Table 6). The level of rotavirus in the treated sewage was almost equal to the input in the raw sewage. In contrast, enterovirus levels were reduced by the treatment process. Rotavirus was also found in both raw sewage and treated effluent from a more modern activated sludge treatment plant (Bellaire). Some of the raw sewage isolates were toxic to the cell monolayers, so the IIF procedure was modified by washing the inoculum off after the 15-min incubation period before the centrifugation step. No loss of efficiency was apparent, and the toxicity problem usually was eliminated.

TABLE 5. Specificity of anti-SA11 guinea pig antiserum

Virus	Neutralization[a]	Immunofluorescence[b] (% positive cells)
SA11[c]	1:30,000	100
Reovirus 1	<1:10	0
Reovirus 2	<1:10	0
Reovirus 3	1:10	0
Poliovirus 1	<1:10	0
Echovirus 1	Not done	0
Coxsackievirus B4	Not done	0
Coxsackievirus B6	Not done	0

[a] Dilution of guinea pig antisera at which 100 PFU of the virus was reduced by greater than 80%.

[b] MA104 cells were infected with the indicated viruses and stained by an IIF test, using the anti-SA11 guinea pig serum (1:60) and fluorescein isothiocyanate-conjugated goat anti-guinea pig immunoglobulin G (1:20).

[c] Preimmune guinea pig serum had a neutralization titer of <1:10 with SA11.

Identification of natural isolates. Treatment of sewage concentrates by heat, chlorine, pH 11, or Formalin destroyed the ability of the isolates to form fluorescent foci in the immunofluorescence test (Table 7). SA11 fluorescent focus formation was similarly inhibited. Experiments using guinea pig preimmune serum or the hyperimmune anti-SA11 serum which had been adsorbed with SA11-infected MA104 cell extracts found no specific fluorescence with SA11-, human rotavirus-, or sewage isolate-infected cells (data not shown).

Since the IIF test detects the rotavirus group antigen, a neutralization test involving the reduction of fluorescent foci was used to identify the natural isolates. The domestic sewage isolates were neutralized by a human convalescent serum and not significantly by the preillness serum (Table 8). A checkerboard neutralization test was used to characterize the specificity of several hyperimmune antirotavirus sera and the human convalescent serum and to support the identification of the natural isolates as human rotavirus. The hyperimmune sera neutralized the homologous virus. A cross-reaction was found between the hyperimmune guinea pig anti-human rotavirus serum and the SA11 virus. The natural isolates were not neutralized by the animal rotavirus antisera.

TABLE 6. Detection of rotavirus and enteroviruses in Houston domestic raw and treated sewage

Sewage type	Date	Location[a]	Enteroviruses		Rotavirus	
			No. of plaques counted/6-ml eluate	PFU in 20-liter sample	No. of foci counted/0.4-ml eluate	No. of foci in 20-liter sample
Raw	12/5/78	WU	NT[b]	NT	13	1,914
	1/30/79	WU	40	395	13	1,914
	2/10/79	WU	1	10	0	ND[c]
	3/6/79	WU	300	2,962	11	1,329
	3/27/79	WU	80	790	28	3,480
	4/6/79	Bellaire	140	1,382	13	1,595
	4/9/79	WU	80	790	2	290
Secondary treated	3/9/79	WU	280	2,800	50	7,488
	3/23/79	WU	0	ND	9	1,296
	3/26/79	WU	0	ND	8	1,152
	4/2/79	WU	0	ND	5	823
	4/6/79	Bellaire	0	ND	1	150
	4/9/79	WU	20	200	4	600
	4/17/79	WU	140	1,400	2	300

[a] West University (WU) and Bellaire sewage treatment plants, Houston, Tex.
[b] NT, Not tested.
[c] ND, No virus detected.

Buoyant density of natural isolates. Pooled sewage concentrates positive by the IIF test were clarified, pelleted at 100,000 × *g*, and then ultracentrifuged in a CsCl buoyant density gradient as described in Materials and Methods. No bands were visible or detectable by UV absorbance scanning of gradient fractions. Assay of the gradient fractions for rotavirus by immunofluorescence showed the greatest number of foci in the fraction with a density of 1.36 g/cm^3 (Fig. 3). Gradient fractions exhibiting fluorescent foci were neutralized by human adult and infant convalescent sera but not by an antiserum to SA11 (Table 8). Immunoelectron microscopy of the positive fractions was unable to detect any rotavirus particles.

Morphology of the sewage isolate virus. Cell lysates from MA104 cells inoculated with the sewage isolate were found to contain virus particles which had the distinct "wheel-like" morphology characteristic of the rotaviruses (Fig. 4A). These cells had been inoculated with material from a CsCl gradient fraction of density 1.36 g/cm^3. No typical rotavirus cytopathic effects were evident 48 h postinoculation, but immunofluorescence with anti-SA11 serum of duplicate samples showed 5% of the cells to be fluorescing positive for rotavirus. The virus particles in the lysates were observed singly or occasionally as aggregates containing membranous material and primarily single-shelled rotavirus particles (Fig. 4B). These aggregate structures resembled the areas of endoplasmic reticulum distended cisternae observed in thin sections of rotavirus-infected cells (25). The diameter of complete particles possessing the outer rim or capsid layer ranged from 63 to 91 nm, with a mean diameter of 83 particles being 67.1 ± 2.4 nm. No rotavirus particles were observed in lysates from cells inoculated with a sewage isolate that had been neutralized with a human infant convalescent serum. Immunofluorescence tests on this sewage isolate found the virus to be neutralized by human adult and infant convalescent sera but not by preillness adult or hyperimmune anti-SA11 sera (Table 8). Thus, the rotavirus particles from this sample appeared to be a human strain and certainly not an SA11 laboratory contaminant.

TABLE 7. Inactivation of fluorescent foci[a]

Treatment	Virus titer (foci/0.05 ml)	
	SA11	Sewage isolate
None	107	18
Autoclave (20 min)	0	0
Boil (20 min)	0	0
pH 11 (30 min)	0	0
Chlorine (10 mg/liter for 30 min)	0	0
Formalin (1:2,000 for 4 days)	10	0

[a] Sewage concentrates seeded with SA11 or positive for rotavirus by immunofluorescence were treated as indicated and assayed for fluorescent foci.

DISCUSSION

Previous concentration methods for rotavirus have been effective with SA11 in small volumes of cell culture medium (10) or tap water (32), but no practical methods have been reported capable of detecting human rotavirus in large volumes of tap water and sewage. In this study, SA11 was concentrated efficiently from 20-liter

TABLE 8. Serum neutralization of rotaviruses

Rotavirus	Normal guinea pig serum (1:200)[b]	Neutralization with given antiserum[a]						
		Guinea pig antisimian rotavirus (1:10,000)	Guinea pig antibovine rotavirus (1:1,000)	Porcine antiporcine rotavirus (1:10,000)	Guinea pig anti-human rotavirus (1:10,000)	Human adult		Human infant, convalescent (1:500)
						Preillness (1:200)	Convalescent (1:200)	
SA11	106	0	90	100	100	78	92	102
Bovine	116	102	0	NT[c]	100	120	112	NT
Porcine	132	119	NT	0	140	117	126	NT
Human	30	38	30	24	0	28	0	1
Sewage isolate A	24	26	28	20	0	16	0	0
Sewage isolate B	18	16	NT	NT	NT	21	0	NT
Gradient purified sewage isolate	56	48	NT	NT	NT	62	0	0

[a] Expressed as number of fluorescent foci per 0.1 ml after treatment with the indicated antiserum. Equal volumes of virus and serum were incubated for 1 h at 37°C and then assayed by IIF in duplicate.
[b] Serum dilution.
[c] NT, Not tested against that particular virus.

volumes of tap water, treated sewage, or raw sewage by use of a filter adsorption-elution technique with only minor modifications of the standard procedure for concentration of enteroviruses (1, 13, 43). Since the virus is labile at pH values greater than 11 (7), bound rotavirus was eluted from the filters by competition with a proteinaceous compound rather than with pH 11.5 buffer which is commonly used to elute enteroviruses (1, 13, 43). The differences in the concentration efficiencies of SA11 and poliovirus (Table 4) can be accounted for by the greater stability of poliovirus at pH 10 (7). The filter adsorption-elution concentration procedure is capable of processing up to 1,800 liters of tap water for enteroviruses (8) and should work as well with rotaviruses. Concentrates of raw sewage were sometimes toxic to cell monolayers, but this effect may be eliminated by filtration of the sample through Zeta-plus 50S filters (15a).

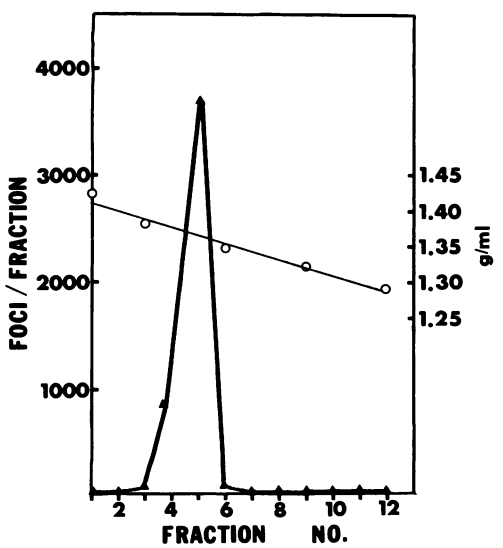

FIG. 3. Buoyant density of sewage isolate. Three 20-liter secondary sewage samples were concentrated to 180 ml by filter adsorption-elution; the virus was pelleted at 100,000 × *g* and centrifuged at 114,000 × *g* for 20 h in a CsCl buoyant density gradient. Fractions (0.4 ml) were collected by bottom puncture, dialyzed against TBS, and assayed for rotavirus by the immunofluorescence test.

An immunological procedure was used to detect the human rotavirus in water samples because the virus replicates poorly in cell culture, and the formation of cytopathic effect is inconsistent if present at all (47). The immunofluorescence test (2) is a highly specific procedure that detects incomplete human rotavirus replication in cell culture (35), can easily identify viral strains (46), and, with the proper antisera, distinguishes between viral serotypes (39). Antisera to SA11 will detect all rotaviruses by immunofluorescence because of a shared group antigen that cross-reacts between all rotavirus strains (Table 8) (46). Therefore, it is easy to make specific antirotavirus antisera capable of detecting the human virus by inoculating animals with purified bovine or simian rotavirus which can be grown to high titer in cell culture (4, 6).

The main advantage of the immunofluorescence test is that it is the only practical, specific method for measuring human rotavirus infectivity (12, 25). It is important to measure infectivity, since the presence of noninfectious rotavirus particles in water is of little public health significance. A second feature of the immunofluorescence test is its sensitivity, being theoretically capable of detecting one infectious unit per

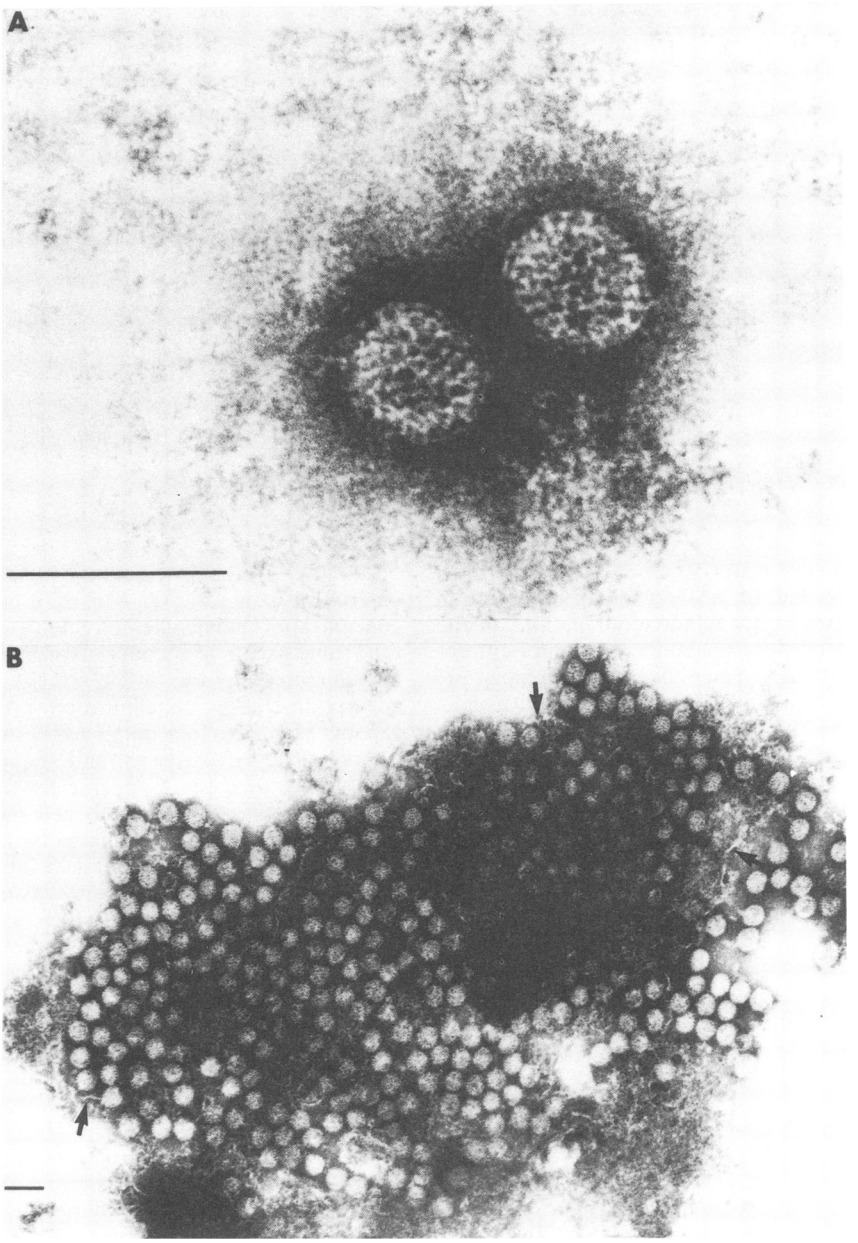

FIG. 4. Electron microscopy of negative-stained preparations of a sewage isolate. MA104 cells were inoculated with a sewage isolate banding at a buoyant density of 1.36 g/cm^3 in CsCl. After 48 h of incubation, the monolayers were lysed with water, sonically treated, and then stained with 1% phosphotungstic acid (pH 6.8) by the pseudoreplica technique. (A) Complete double-shelled rotavirus particles from cells inoculated with a sewage isolate. (B) An aggregate of primarily single-shelled particles from cells inoculated with a sewage isolate. Structure resembles the distended cisternae of endoplasmic reticulum seen in thin sections of rotavirus-infected cells (arrow). Bar = 100 nm.

sample, providing the entire sample can be assayed (18). If low numbers of infectious virus particles are inoculated with a short incubation period, individual fluorescent foci form (similar and directly related to plaque formation) that can be quantitated as infectious units. Our results and those of other investigators (18, 19) have shown that as few as 10 infectious units per liter can be reliably detected by immunofluorescence. The short incubation period for the virus

assay (less than 24 h) has the added advantage of limiting bacterial growth that can destroy cell cultures (18).

Because this is the first reported isolation of rotavirus from sewage, the sewage isolates were identified by several criteria involving physical and immunological techniqes as well as direct observation. The physical evidence showed that the agent was biologically active and sensitive to common inactivation treatments. Immunological evidence showed that the virus-infected cells reacted specifically with an antirotavirus antiserum and that the infectivity of the isolates could be neutralized only by antisera against human strains of rotavirus. Direct observation of virus-infected cell lysates showed virus particles possessing the characteristic rotavirus morphology and size. Taken together, these data show the sewage isolates were rotavirus and suggest that they were a human strain.

As a result of sampling Houston sewage for rotavirus it appears that, at least in certain treatment plants, infectious virus can survive sewage treatment processes as previously suggested by Farrah et al. (9). Future studies that use sequential samples of sewage through the treatment processes are necessary to accurately determine the overall efficiency of the processes to remove or inactivate rotavirus.

Detection of rotavirus in water has several applications for future studies. The relative prevalence of enteric virus infection in a community and the serological types present should be reflected by the concentration and type of viruses present in a community's sewage (21, 30, 33, 38, 45). Rotavirus infections are thought to be seasonal in areas with a temperature climate (12, 25), and such changes may also be reflected in a community's sewage (45). Finally, the ability to detect rotaviruses in water provides a means to identify suspected sources for rotavirus contamination in outbreaks of waterborne gastroenteritis.

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LITERATURE CITED

1. **American Public Health Association.** 1978. Standard methods for the examination of water and wastewater, 15th ed. American Public Health Association, Washington, D.C.
2. **Banatvala, J. E., B. Totterdell, I. L. Chrystie, and G. N. Woode.** 1975. In-vitro detection of human rotavirus. Lancet **ii**:821.
3. **Bolivar, R., R. H. Conklin, J. J. Vollet, L. R. Pickering, H. L. DuPont, D. L. Walters, and S. Kohl.** 1978. Rotavirus in travelers' diarrhea: study of an adult student population in Mexico. J. Infect. Dis. **137**:324–327.
4. **Clark, S. M., B. B. Barnett, and R. S. Spendlove.** 1979. Production of high-titer bovine rotavirus with trypsin. J. Clin. Microbiol. **9**:413–417.
5. **Craun, G. F.** 1979. Waterborne disease outbreaks in the United States. J. Environ. Health **41**:259–265.
6. **Estes, M. K., D. Y. Graham, C. P. Gerba, and E. M. Smith.** 1979. Simian rotavirus SA11 replication in cell cultures. J. Virol. **31**:810–815.
7. **Estes, M. K., D. Y. Graham, E. M. Smith, and C. P. Gerba.** 1979. Rotavirus stability and inactivation. J. Gen. Virol. **43**:403–409.
8. **Farrah, S. R., C. P. Gerba, C. Wallis, and J. L. Melnick.** 1976. Concentration of viruses from large volumes of tapwater using pleated membrane filters. Appl. Environ. Microbiol. **31**:221–226.
9. **Farrah, S. R., S. M. Goyal, C. P. Gerba, R. H. Conklin, and E. M. Smith.** 1978. Comparison between adsorption of poliovirus and rotavirus by aluminum hydroxide and activated sludge flocs. Appl. Environ. Microbiol. **35**:360–363.
10. **Farrah, S. R., S. M. Goyal, C. P. Gerba, R. H. Conklin, C. Wallis, J. L. Melnick, and H. L. DuPont.** 1978. A simple method for concentration of enteroviruses and rotaviruses from cell culture harvests using membrane filters. Intervirology **9**:56–59.
11. **Fauvel, M., L. Spence, L. A. Babiuk, R. Petro, and S. Bloch.** 1978. Hemagglutination and hemagglutination-inhibition studies with a strain of Nebraska calf diarrhea virus (bovine rotavirus). Intervirology **9**:95–105.
12. **Flewett, T. H., and G. N. Woode.** 1978. The rotaviruses. Arch. Virol. **57**:1–23.
13. **Gerba, C. P., S. R. Farrah, S. M. Goyal, C. Wallis, and J. L. Melnick.** 1978. Concentration of enteroviruses from large volumes of tap water, treated sewage, and seawater. Appl. Environ. Microbiol. **35**:540–548.
14. **Goyal, S. M., C. P. Gerba, and J. L. Melnick.** 1978. Prevalence of human enteric viruses in coastal canal communities. J. Water Pollut. Control Fed. **50**:2247–2256.
15. **Haley, C. E., R. A. Gunn, J. M. Hughes, E. C. Lippy, and G. F. Craun.** 1980. Outbreaks of waterborne disease in the United States, 1978. J. Infect. Dis. **141**:794–797.

15a. **Hejkal, T. W., C. P. Gerba, and V. C. Rao.** 1982. Reduction of cytotoxicity in virus concentrates from environmental samples. Appl. Environ. Microbiol. **43**:731–733.

16. **Hurst, C. J., and C. P. Gerba.** 1980. Stability of simian rotavirus in fresh and estaurine water. Appl. Environ. Microbiol. **39**:1–5.
17. **Kapikian, A. Z., H. W. Kim, R. G. Wyatt, W. L. Cline, J. L. Arrobio, G. D. Brandt, W. J. Rodriquez, D. A. Sack, R. M. Chanock, and R. H. Parrott.** 1976. Human reovirus-like agent associated with "winter" gastroenteritis. N. Engl. J. Med. **294**:965–972.
18. **Katzenelson, E.** 1976. A rapid method for quantitative assay of poliovirus from water with the aid of the fluorescent antibody technique. Arch. Virol. **50**:197–206.
19. **Kedmi, S., and E. Katzenelson.** 1978. A rapid quantitative fluorescent antibody assay of poliovirus using tragacanth gum. Arch. Virol. **56**:337–340.
20. **Kim, H. W., C. D. Brandt, A. Z. Kapikian, R. G. Wyatt, J. O. Arrobio, W. J. Rodriquez, R. M. Chanock, and R. H. Parrott.** 1977. Human reovirus-like agent infection. Occurrence in adult contacts of pediatric patients with gastroenteritis. J. Am. Med. Assoc. **238**:404–407.
21. **Kuwert, E., P. G. Hoher, and C. A. Primavesi.** 1970. Intratypic differentiation of poliovirus strains isolated from sewage: consequences for the practice of oral vaccination. Zentralb. Bakteriol. Parastenkd. Infektionskr. Hyg. Abt. 1 Orig. **215**:16–25.
22. **Lycke, E., J. Blomberg, G. Berg, A. Eriksson, and L. Madsen.** 1978. Epidemic acute diarrhea in adults associated with infantile gastroenteritis virus. Lancet **ii**:1056–1057.
23. **Malherbe, H. H., and M. Strickland-Cholmley.** 1967. Simian virus SA11 and the related O agent. Arch. Gesamtl Virusforsch. **22**:235–245.
24. **McCombs, R. M., M. Benyesh-Melnick, and J. P. Brunschwig.** 1968. The use of Millipore filters in ultrastructural studies of cell cultures and viruses. J. Cell Biol.

36:231–243.
25. **McNulty, M. S.** 1978. Rotaviruses. J. Gen. Virol. **40**:1–18.
26. **Meurman, O. H., and M. J. Laine.** 1977. Rotavirus epidemic in adults. N. Engl. J. Med. **296**:1298–1299.
27. **Mix, T. W.** 1974. The physical chemistry of membrane-virus interaction. Devl. Ind. Microbiol. **15**:136–142.
28. **Morbidity and Mortality Weekly Report.** 1978. Viral gastroenteritis—Pennsylvania. Morbid. Mortal. Weekly Rep. **27**:403–404.
29. **Morens, D. M., R. M. Zweighaft, T. M. Vernon, G. W. Gary, J. J. Eslien, B. T. Wood, R. C. Holman, and R. Dolin.** 1979. A waterborne outbreak of gastroenteritis with secondary person-to-person spread. Association with a viral agent. Lancet **i**:964–966.
30. **Nelson, D. B., R. Circo, and A. S. Evans.** 1967. Strategic viral surveillance of sewage during and following an oral poliovirus vaccine campaign. Am. J. Epidemiol. **86**:641–652.
31. **Porter, D. D., I. Wimberly, and M. Benyesh-Melnick.** 1969. Prevalence of antibodies to EB virus and other herpesviruses. J. Am. Med. Assoc. **208**:1675–1679.
32. **Ramia, S., and S. A. Sattar.** 1980. Concentration of seeded simian rotavirus SA-11 from potable waters by using talc-celite layers and hydroextraction. Appl. Environ. Microbiol. **39**:493–499.
33. **Riordan, J. T.** 1962. IX. Isolation of enteroviruses from sewage before and after vaccine administration. Yale J. Biol. Med. **34**:512–521.
34. **Sack, R. B., H. L. DuPont, B. Rowe, S. L. Gorbach, N. R. Blacklow, S. B. Formal, R. L. Guerrant, A. Z. Kapikian, D. A. Sack, and L. R. Trabulsi.** 1978. International conference on the diarrhea of travelers—new directions in research: a summary; etiology—Summary of session. J. Infect. Dis. **137**:358–360.
35. **Schoub, B. D., A. R. Kalica, H. B. Greenberg, D. M. Bertram, M. M. Sereno, R. G. Wyatt, R. M. Chanock, and A. Z. Kapikian.** 1979. Enhancement of antigen incorporation and infectivity of cell cultures by human rotavirus. J. Clin. Microbiol. **9**:488–492.
36. **Smith, E. M., M. K. Estes, D. Y. Graham, and C. P. Gerba.** 1979. A plaque assay for the simian rotavirus SA11. J. Gen. Virol. **43**:513–519.
37. **Sobsey, M. D., and J. S. Glass.** 1980. Poliovirus concentration from tap water with electropositive adsorbent filters. Appl. Environ. Microbiol. **40**:201–210.
38. **Tarabcak, M., I. Kratochvil, and A. Milosovicova.** 1971. Study of the effect of vaccination with live poliomyelitis vaccine on the circulation of enteroviruses in the population. J. Hyg. Epidemiol. Microbiol. Immunol. **15**:258–266.
39. **Thouless, M. E., A. S. Bryden, and T. H. Flewett.** 1978. Serotypes of human rotavirus. Lancet **i**:39.
40. **Thouless, M. E., A. S. Bryden, T. H. Flewett, G. N. Woode, J. C. Bridger, D. R. Snodgrass, and J. A. Herring.** 1977. Serological relationships between rotaviruses from different species as studied by complement fixation and neutralization. Arch. Virol. **53**:287–294.
41. **von Bonsdorff, C. H., T. Hovi, P. Mäkelä, and A. Mörttinen.** 1978. Rotavirus infections in adults in association with acute gastroenteritis. J. Med. Virol. **2**:21–28.
42. **Wallis, C., M. Henderson, and J. L. Melnick.** 1972. Enterovirus concentration on cellulose membranes. Appl. Microbiol. **23**:476–480.
43. **Wallis, C., J. L. Melnick, and C. P. Gerba.** 1979. Concentration of viruses from water by membrane chromatography. Annu. Rev. Microbiol. **33**:413–437.
44. **Wenman, W. M., D. Hinde, S. Feltham, and M. Gurwith.** 1979. Rotavirus infection in adults. Results of a prospective family study. N. Engl. J. Med. **301**:303–306.
45. **Wilterdink, J. B., H. T. Weiland, and J. D. Verlinde.** 1970. A longitudinal study on the significance of examination of sewage for the presence of poliovirus in the population. Arch. Gesamtl Virusforsch. **32**:82–90.
46. **Woode, G. N., J. C. Bridger, J. M. Jones, T. H. Flewett, A. S. Bryden, H. A. Davies, and G. B. B. White.** 1976. Morphological and antigenic relationships between viruses (rotaviruses) from acute gastroenteritis of children, calves, piglets, mice and foals. Infect. Immun. **14**:804–810.
47. **Wyatt, R. G., V. W. Gill, M. M. Sereno, A. R. Kalica, D. H. Van Kirk, R. M. Chanock, and A. Z. Kapikian.** 1976. Probable *in vitro* cultivation of human reovirus-like agent of infantile diarrhoea. Lancet **i**:98–99.