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LA THÈSE A ÉTÉ
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SURVIVAL AND DETECTION OF ROTAVIRUSES IN THE WATER ENVIRONMENT

A Thesis submitted to the
School of Graduate Studies,
University of Ottawa

In Partial Fulfillment of
the Requirements for the Degree of
Master of Science,
Department of Microbiology and Immunology,
School of Medicine.

by

Roderick A. Raphael

May, 1984



R.A. Raphael, Ottawa, Canada, 1984.

Dedicated to my Father and Mother
whose love made this possible.

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ABSTRACT

Rotaviruses are among the major causes of acute gastroenteritis in man as well as a variety of animals. Although not much is known about the mechanisms of transmission of these viruses in nature, fecally-contaminated water appears to play a role in this regard. It was with this in mind that the present study was undertaken. The study sought to develop and test suitable methodology for the recovery and detection of naturally-occurring rotaviruses in sewage-polluted surface waters.

1. The first phase of the study involved the determination of the effect of temperature and naturally-occurring particulate matter on the survival of rotaviruses (lab-adapted strains) in raw and treated surface waters collected at different times of the year. The study was divided into short-term and long-term experiments. The short-term experiments were designed to determine the effect of chlorine in drinking water on the infectivity of simian rotavirus SA-11, human rotavirus (strain D) and poliovirus type 1 (Sabin). The water samples were collected as either plant effluent (PE) at a municipal water treatment facility or as tap water (TW) in our laboratory. The viruses, suspended in either distilled water, Earle's balanced salt solution (EBSS) or tryptose phosphate broth (TPB), were added to the water samples and held for 1 hour at 22°C. There was no significant reduction in the plaque titre of the rotaviruses added with EBSS or TPB, but when suspended in distilled water before contamination of PE, there was nearly a 1-log₁₀ loss in the titre of the rotaviruses. There was a greater than 99.9% loss in poliovirus titre in both PE and TW irrespective of the type of suspending medium used.

To study the long-term survival of rotaviruses in the water environment, samples of raw water (RW), TW and filtered raw water (FW) were collected at three different times of the year. The samples were contaminated with the rotavirus (simian, calf or human) under test to give a final concentration of 5.0×10^4 plaque forming units (PFU)/mL. The virus-contaminated water samples were held in the dark at either 4°C or 20°C for a total of 64 days. In TW held at 4°C , there was no significant drop in the virus titre even after 64 days, whereas, at 20°C the titre was reduced by nearly 2-log_{10} over the same period. In RW, a 3-log_{10} drop in PFU occurred in 16 and 32 days at 20°C and 4°C , respectively. In FW there was only a 0.7-log_{10} reduction in virus titre after 64 days, irrespective of the holding temperature.

2. The second phase of the investigation involved the evaluation of the talc-Celite technique and positively charged (30S) Zeta Plus filters for their ability to concentrate rotaviruses from experimentally-contaminated samples of raw water. In the talc-Celite technique, samples had to be preconditioned by the addition of EBSS (1:100) and adjustment of the pH to 6.0. This preconditioning enhanced rotavirus absorption to the talc-Celite layer but did not completely prevent the loss of virus in the filtrate. Using 1X TPB (pH 9.0) as an eluent the mean virus recovery from these layers was 47.3% (± 7.4). With the 30S filter, there was no detectable virus in any of the filtrates. In virus elution, 1% bovine albumin (pH 9.0) and 3% beef extract (pH 9.5) gave mean virus recoveries of 16.7% (± 5.2) and 14.1% (± 4.1), respectively.

1X TPB (pH 9.5) 3% glycine + 3% arginine (pH 9.0) and 6% glycine + 6% arginine (pH 9.0) yielded virus recoveries of 44.1% (± 6.4), 46.1% (± 6.2) + 46.1% (± 3.1), respectively. In an attempt to improve the virus recovery rate, sequential elution was tried. 1X TPB (pH 9.5) and 3% glycine + 3% arginine (pH 9.0), used sequentially, gave a virus recovery of 70.0% (± 2.6) while 1X TPB (pH 9.5) and 6% arginine + 6% glycine (pH 9.0) resulted in the recovery of 70.3% (± 1.5) of the added virus. Large volume experiments with the 30S filter gave a mean virus recovery of 16.3% (± 3.0).

3. The next phase of the study was the application of the immunofluorescence (IF) and immunoperoxidase (IP) techniques and the comparison of their suitability for the detection and quantitation of infectious units of rotaviruses in cell culture. Both methods could detect nearly 3 times the number of infectious units of rotavirus in cell culture as compared with the plaque assay.

4. The final stage was to attempt the detection and quantitation of naturally-occurring rotaviruses in sewage-polluted waters using Zeta Plus 30S filters in conjunction with the immunostaining methods. Infectious rotavirus particles were detected in the concentrates of RW from the Ottawa River. The numbers of rotavirus immunoperoxidase focus units ranged from 19.5 to 40.8/L of RW.

v

LIST OF ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole
Arg	arginine
BA	bovine albumin
BE	beef extract
CFU	colony forming unit(s)
DEAE	diethylaminoethyl
DPD	N,N-diethyl-phenylene diamine
EBSS	Earle's balanced salt solution
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
FCS	fetal calf serum
FFFC	fluorescent focus forming counts
FITC	fluorescein isothiocyanate
FW	filtered raw water
Gly	glycine
g	gram
HA	hemagglutination
IF	immunofluorescence
IP	immunoperoxidase
Km	kilometer(s)
L	litre
MEM	minimal essential medium
mg	milligram
mL	millilitre
PAGE	polyacrylamide gel electrophoresis

PAP	peroxidase-antiperoxidase
PBS	phosphate buffered saline
PE	plant effluent
PEG	polyethylene glycol
PFFC	peroxidase focus forming counts
PFU	plaque forming unit(s)
Post-CE	postchlorinated effluent
Pr. A	protein-A
Pre-CE	prechlorinated effluent
RPM	revolutions per minute
RS	raw sewage
RW	raw water
S.D.	standard deviation
TPB	tryptose phosphate broth
TW	tap water, treated water
ug	microgram
Xg	times gravity
°C	degrees centigrade

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GENERAL INTRODUCTION

DISCOVERY

Light and Hodes (1943, 1949) isolated a filterable agent from the stools of infants with acute gastroenteritis. They were then able to demonstrate that it could produce diarrhea in experimentally-inoculated newborn calves. Almost 30 years later (Hodes, 1977), electron microscopic (EM) analysis of a specimen of calf feces from those early experiments² revealed a morphologically typical rotavirus. After the studies of Light and Hodes in the early forties, other viruses with characteristics of the family Reoviridae were isolated from infant mice (Cheever and Mueller, 1947; Kraft, 1957; Adams and Kraft, 1967; Banfield et al., 1968), monkeys (Malherbe and Strickland-Cholmley, 1967), and calves (Mebus et al., 1969). All of these are now known to be members of the genus Rotavirus.

Bishop et al. (1973), working in Australia, detected a virus in duodenal biopsies from children suffering from acute non-bacterial gastroenteritis. Under the EM this virus resembled the one described by Mebus et al. (1969) from cases of calf scours. In the same year in the United Kingdom, Flewett et al. (1973) using negative-contrast EM, detected similar virus particles in feces of infants with diarrhea. Based on the appearance of these particles, they also suggested the name "Rotavirus".

Rotavirus infections in humans were then reported in Canada (Middleton et al. 1974), the United States (Kapikian et al. 1974) and Australia

(Holmes et al. 1974, Bishop et al. 1974). With these and more reports, rotaviruses were soon recognized as a major cause of acute non-bacterial gastroenteritis in infants and young children as well as many species of warm blooded animals worldwide (Flewett and Woode, 1978; McNulty, 1978; Holmes, 1979; Steinhoff, 1980; Kapikian et al. 1981; Estes et al. 1983).

GENERAL CHARACTERISTICS

Classification

"Rotavirus" was established as a genus of the family Reoviridae by the International Committee on Taxonomy of Viruses at the Fourth International Congress for Virology in 1978 (Matthews, 1979). Members of this genus consist of viruses with a wheel-like (Latin = rota) double-shelled protein coat. The mature and complete virions are approximately 65-70 nm in diameter with a double-stranded RNA genome containing 11 segments (Welch and Thompson, 1973). Rotavirus particles also contain an RNA-dependent RNA polymerase (Cohen 1977) which is consistent with their classification as members of family Reoviridae. As yet, no systematic nomenclature scheme has been formalized for individual members of this genus. They are usually named after the mammalian or avian species from which they have been isolated.

Rotaviruses In Nature

Many of the rotaviruses isolated from different mammalian and avian species can be distinguished by neutralization tests and these viruses

represent different serotypes (Estes et al. 1983). Wyatt et al. (1982) and Gaul et al. (1982) have shown that certain simian and canine rotaviruses are serologically similar to human rotaviruses of subgroup 1. Under experimental conditions, rotaviruses isolated from one animal species have been shown to infect other types of animals. This provides indirect evidence that rotaviruses may be transmitted across species boundaries (Estes et al. 1983).

Morphology

In keeping with other members of the family Reoviridae, rotaviruses have a double-shelled capsid with an icosahedral core (Esparza and Gil, 1978). As has been mentioned earlier, the morphology of intact rotavirus virions is suggestive of a wheel with the core forming a hub, capsomeres radiating from the core as spokes and the marginated outer layer forming the rim (Flewett et al. 1974). All types of rotaviruses examined to date are morphologically indistinguishable from each other. Both double-shelled and single-shelled particles can be seen in most negatively stained EM preparations (Bridger and Woode, 1976; Palmer et al., 1977).

Physicochemical Properties

Double-shelled particles have a bouyant density of 1.36 g/mL in CsCl, and single-shelled particles have a density of 1.38 g/mL in CsCl (Petric et al., 1975). Double-shelled particles have a sedimentation coefficient of 500-530 S (Newman et al., 1975) and single-shelled particles

have a sedimentation coefficient of 380-400 S (Tam et al., 1976). The icosahedral core from bovine rotavirus has a bouyant density of 1.44 g/mL in CsCl and a sedimentation coefficient of 280 S (Bican et al., 1982).

The simian rotavirus SA-11 is stable at low pH (3.5), and is not inactivated by ether, chloroform, freeze-thawing and sonication (Estes et al., 1979). Divalent cation chelators reduce rotavirus infectivity (Cohen et al., 1979) while viral infectivity is enhanced by proteolytic enzymes ((Babiuk et al., 1977).

Ethanol is an effective disinfectant against rotaviruses (Brade et al., 1981; Tan and Schnagl, 1981; Sattar et al., 1983). Snodgrass and Herring (1977) found formalin, lysol and an iodophor preparation to be useful in the disinfection of rotavirus-contaminated material. These viruses have, however, been found to be resistant to inactivation by chlorine (Tan and Schnagl, 1981) even at concentrations of available chlorine several times greater than that used in routine water treatment (Sattar et al., 1983). This suggests that mechanisms such as virus aggregation or virus adsorption to solids might be important for rotavirus survival in sewage treatment plants or in chlorinated tap water (Smith and Gerba, 1982; Keswick et al., 1984).

Serotypes and Hemagglutination

Serotypes of rotaviruses can be identified by serum neutralization tests. Plaque-reduction tests can distinguish between cultivable types

of bovine, canine, feline, human, simian and porcine rotaviruses (Wyatt et al., 1982). Neutralization of fluorescent cell-forming units has been used for the serotyping of those rotaviruses which do not form countable plaques in cell culture (McNulty et al., 1980). There are at least four serotypes of human rotavirus, two serotypes of bovine rotavirus and three serotypes of avian rotavirus (Estes et al., 1983).

In addition to their type-specific antigens, rotaviruses also contain antigens showing group specificity. These common antigens are, however, not shared with viruses of the other genera of the family Reoviridae (McNulty et al., 1979). These group specific antigens are located on the inner capsid of the virus, whereas type-specific antigens form a part of the outer capsid of the virus (Holmes, 1979). The presence of common antigens made it possible to use antisera against cell culture adapted animal rotaviruses in tests for the detection of human rotaviruses in stools and in serological tests for the laboratory diagnosis of rotaviral infections (Schoub et al., 1977; Brade and Schmidt, 1979).

It has been shown that simian rotavirus SA-11 and certain types of bovine, human and avian rotaviruses can hemagglutinate (HA) human "O", guinea pig, and chicken erythrocytes (Hancock et al., 1983). Bastardo and Holmes (1980) showed that treatment of erythrocytes with receptor-destroying enzyme blocks HA by simian rotavirus SA-11. This indicates that the virus interacts with neuraminic acid-containing receptors. Bishai et al. (1978) found that HA activity of bovine rotavirus is inactivated by heating at 45°C, exposure to pH 2.0, freeze-thawing and chloroform treatment without noticeable changes in virus morphology. HA

activity is associated with the double-shelled particles and it may be a type-specific antigen (Spence et al., 1978).

Rotavirus Genome Diversity

Electrophoretic analyses of a number of rotaviruses have shown them to contain eleven segments of double-stranded RNA with a genome molecular weight of $11-12 \times 10^6$ (Welch, 1971; Welch and Thompson, 1973; McNulty, 1978). RNA electropherotypes of rotavirus strains are distinguished on the basis of differences in the migration patterns of the genome segments by polyacrylamide gel electrophoresis (PAGE). The segments can be divided into four size classes based on contour-length measurements of the RNAs by EM and electrophoretic mobility patterns in PAGE (Kalica et al., 1976; Barnett et al., 1978).

In a six-year Australian study, Rodger et al. (1981) detected 19 different human electropherotypes. Lourenco et al. (1981), in a one year study in France, detected 29 different human rotavirus electropherotypes. Rodger et al. (1982) found that in contrast to the diversity of electropherotypes from children and adults, rotavirus specimens from neonates were limited to two electropherotypes and one of these persisted for 4 years.

A number of electropherotypes may be present in an area and dual infections could result in the appearance of new virus strains by reassortment of genes (Lourenco et al., 1981; Rodger et al., 1981). The high level of genome variation seen in the rotaviruses is analogous to

the heterogeneity of the genome profiles seen with influenza virus and reovirus isolates (Palese, 1977; Hrdy et al., 1979). The link between rotavirus genomic variation and antigenic diversity has not been established due in some part to their fastidious growth in vitro.

Rotavirus Replication Cycle

The replication cycle of cell culture-adapted rotaviruses is approximately 12 hours at 37°C when a high multiplicity of infection is used (Estes et al., 1983). The viral eclipse phase is approximately 2-3 hours (Estes et al., 1983). Viral protein synthesis is maximal at 3-5 hours post infection (Ericson et al., 1982). Viral antigens can be detected by fluorescent antibody technique 4-6 hours post infection (Estes et al., 1979). Progeny virus genome RNA synthesis is first detected during the virus eclipse period at 2-4 hours post infection and continues throughout the replication cycle (McCrae and Faulkner-Valle, 1981).

Morphogenesis

Studies on the early stages of infection suggest that virions enter the cell by endocytosis and are sequestered into the lysosomes (Petrie et al., 1981; Quan and Doane, 1984). Uncoating is thought to occur in the lysosomes producing a 50 nm subviral particle (Chasey, 1977). After the eclipse phase, virus particles 25-35 nm in diameter are seen in large granular cytoplasmic inclusions called viroplasms (Altenburg et al., 1979; Petrie et al., 1982). Within the viroplasmic reticulum, viral RNA

is packaged into 50 nm core particles and viral capsid proteins assemble around the core. Virus particles appear to bud through ribosome-free areas of the rough endoplasmic reticulum into swollen vesicles becoming enveloped in the process (Chasey, 1977). The envelope is lost as the virus particles continue their maturation. Inner capsid proteins of rotaviruses are synthesized throughout the cytoplasm and become concentrated in viroplasmic inclusions, while outer capsid glycoprotein is synthesized only on ribosomes of the rough endoplasmic reticulum (Petrie et al., 1982). The outer capsid layer appears to be acquired during viral budding into cisternae of the endoplasmic reticulum (Petrie et al., 1982).

Cell Culture-Adapted Rotaviruses

Simian rotavirus SA-11 and the "0" agent (Malherbe and Strickland-Cholmley, 1967), bovine rotavirus (Mebus et al., 1971) and porcine rotavirus (Theil et al., 1977) were the first cell culture adapted rotaviruses. Since then other mammalian and avian rotaviruses have been propagated in cell culture (McNulty et al., 1979; Hoshino et al., 1981). In vitro cultivation of human rotavirus has also been achieved (Drozdo et al., 1979; Wyatt et al., 1980; Sato et al., 1981; Urasawa et al., 1981; Birch et al., 1983). The most common cell lines for growing rotaviruses are epithelioid lines derived from monkey kidney (eg. Vero, MA-104, CV-1). A major breakthrough for rotavirus propagation in cell culture was the discovery that pancreatic proteolytic enzymes enhance viral replication (Babiuk et al., 1977; Theil et al., 1978; Ramia and Sattar, 1979a; Graham and Estes, 1980). The role of trypsin

pretreatment of the rotavirus is believed to be due to the cleavage of the major outer capsid polypeptide (VP3) which activates infectivity (Espejo et al., 1981; Clark et al., 1981). The other role of trypsin (by its presence in the agar overlay) is to enhance plaque formation by cell culture-adapted rotaviruses (Matsuno et al., 1977; Ramia and Sattar, 1979a; Estes and Graham, 1980). Diethylaminoethyl (DEAE)-dextran has been found to enhance rotavirus plaque formation in some cases (Smith et al., 1979) when added to the agar overlay.

ROTAVIRUSES AND HUMAN HEALTH

Acute Gastroenteritis

Rotaviruses are the most frequent cause of acute non-bacterial gastroenteritis in infants and children between 6 months and 2 years of age (Kapikian et al., 1976; Tallett et al., 1977). By the age of 5 years most children acquire antibodies to rotaviruses (Steinhoff, 1980; Estes et al., 1983). In general, the incubation period for rotaviral diarrhea is 1-3 days and virus is excreted in feces for 5-7 days (Davidson et al., 1975). The infection can be mild or severe. Rapid dehydration leading to electrolyte imbalance in such cases of diarrhea is known to result in fatalities (Carleson et al., 1978). Rotavirus diarrhea is frequently accompanied by respiratory illness, vomiting, and fever (Lancet, 1979).

Worldwide, 5-6 million children die from acute gastroenteritis of which 30% is attributable to rotavirus infection in combination with malnutri-

tion (Walsh and Warren, 1979; World Health Organization, 1980). The vast majority of these rotavirus-associated deaths occur in the developing world or in those areas within the developed countries where conditions of sanitation and hygiene are poor (Steinhoff, 1980; Kapikian et al., 1981).

Gurwith et al. (1981) reported rotaviruses to be the most common enteropathogen in young children who were outpatients for diarrhea in Canada. Rodriguez et al. (1980) estimated that rotavirus gastroenteritis annually accounts for 220-370 hospitalizations per 100,000 children (United States) less than 2 years of age. With nearly 6.6 million children less than 2 years old in the United States, the annual cost of hospitalization was estimated to be 27 million dollars (US-1980). Nosocomial infections due to rotaviruses also occur frequently; they account for 10%-20% of the infections in control children admitted to hospitals (Ryder et al., 1977; Soenarto et al., 1981).

In a study of enteric pathogens linked with diarrhea at a diarrhea treatment center in rural Bangladesh, rotaviruses and enterotoxigenic Escherichia coli (ETEC) were isolated from approximately 50% and 25% of patients less than two years of age, respectively (Black et al., 1980). ETEC was the most frequently identified enteropathogen for patients of all age groups. Rotavirus and ETEC were found in 31% of diarrhea cases experienced by children less than two years old and in 70% of cases associated with dehydration (Black et al., 1981). Rotavirus and ETEC were detected in stools of 77% of young children with life-threatening dehydration due to diarrhea seen at the treatment center.

Rotavirus vaccine development has been recognized as a top priority in combatting the serious nature of the life-threatening dehydration (Chanock, 1981). Recent developments in immunoprophylaxis have been reported from Finland (Vesikari et al., 1983) and Belgium (Zissis et al., 1983). The immunogenicity and safety of live attenuated oral bovine rotavirus vaccine strain RIT 4237 was tested in adults and young children (Vesikari et al., 1983). In adults the vaccine did not cause clinical symptoms, and booster response in rotavirus serum antibodies was seen. In seronegative young children one oral dose induced seroconversion to homologous virus and it did not produce gastrointestinal or constitutional symptoms. It also did not result in rotavirus excretion in the stools of the vaccinated subjects. In the Belgian study, Zissis et al. (1983) used the same attenuated strain of bovine rotavirus in protection studies in colostrum-deprived piglets. It was found that a two-dose administration either given intramuscularly (twice) or once intramuscularly and once intragastrically was necessary to induce a significant serum antibody response. Prior administration of the live vaccine significantly decreased fecal shedding of the challenge virus when compared with the control animals.

Rotaviruses are believed to spread via the fecal-oral route. They are excreted in large numbers in stools of infected individuals with up to 10 billion virus particles per gram of fecal matter (Flewett and Woode, 1978; McNulty, 1978). The physical particle/infective unit ratio is not known but Gerba et al. (1984) reported an estimated value of 300,000:1. Various vehicles have been implicated in the transmission of rotaviral

infections including air (Foster et al., 1980; Gurwith et al., 1981; Santosham et al., 1983), animate and inanimate surfaces (Halvorsrud and Orstavik, 1980; Murphy et al., 1981; Keswick, 1983; Samadi et al., 1983), food (Hara et al., 1978) and potable and recreational water (Lycke et al., 1978; Linhares et al., 1981; Lam, 1982; Suttmoller et al., 1982; Harris et al., 1983; Murphy et al., 1983; Gerba et al., 1984).

Children

A feature of rotavirus-host interactions is that while neonates are highly susceptible to rotavirus infection, there are many neonatal infections which are asymptomatic (Chrystie et al., 1978). Rotavirus infections occur in premature infants with a mild (Van Renterghem et al., 1980) or mild to severe gastroenteritis (Thomson et al., 1981). It was reported that babies requiring special care are much more likely to develop symptomatic illness after rotavirus infection than are full-term babies (Bishop et al., 1979). Breast-feeding did not always protect infants from diarrhea (Bishop et al., 1979; Crewe and Murphy, 1980). Banatvala and Chrystie (1978) reported a lower incidence of rotavirus infection among breast-fed babies. Rotavirus can be neutralized by immunoglobulins and other factors in milk and colostrum (Otnaess and Orstavik, 1980; Palmer et al., 1980).

Adults

Although primarily a pathogen of early childhood, rotaviruses can cause asymptomatic and symptomatic enteritis in adults as well (von Bonsdorff

et al., 1976; Zissis et al., 1976). Asymptomatic infections occur frequently in adult contacts of ill children (Torres et al., 1978; Blacklow and Cukor, 1981). Outbreaks occur in the elderly (Cubitt and Holzel, 1980; Marrie et al., 1982) and in hospitalized adults (Holzel et al., 1980; Hildreth et al., 1981). In surgical wards outbreaks of rotaviral gastroenteritis have resulted in increased mortality (Yolken et al., 1982). In adult travellers' diarrhea, rotaviruses appear to play a major role (Bolivar et al., 1978, Ryder et al., 1981, Sheridan et al., 1981).

Immunity

By the age of five years, 60-90% of children have detectable serum antibody titres against rotaviruses (Ghose et al., 1978). The protective nature of circulating antibodies is poorly understood because humans and animals can become infected even when they possess detectable levels of serum antibodies (Kapikian et al., 1974; Woode, 1978). However, immuno-suppressed children have been found to be particularly susceptible to attack by rotaviruses (Saulsbury et al., 1980). Local immune factors such as secretory IgA, interferon and general mucosal inhibitors to viral infection may be more important in protection against the more severe deleterious effects of rotavirus infection by being a first line of defence (Riepenhoff-Talty et al., 1981). There appears to be a relationship between the severity of the rotavirus infection and the nature of the systemic and secretory antibody responses (Riepenhoff-Talty et al., 1981).

Rotaviruses in Other Clinical Conditions

In addition to acute gastroenteritis rotaviruses have been found in other clinical conditions in man. They have been detected in cases of intussusception (Konno et al., 1978; Nicolas et al., 1982). Salmi et al. (1978) suggest that rotaviral diarrhea could lead to Reye's syndrome or encephalitis. Recent reports have suggested their role in enterocolitis (Rotbart et al., 1983), sudden infant death syndrome (Yolken and Murphy, 1982), Kawasaki syndrome (Matsuno et al., 1983), SOMPE syndrome (Gordon, 1982) and respiratory illness (Goldwater et al., 1979, Lewis et al., 1979, Gurwith et al., 1981). Santosham et al. (1983) were the first to report the detection of rotavirus antigens (using ELISA) in the nasopharyngeal washings of children with pneumonia.

WATERBORNE OUTBREAKS OF ROTAVIRUS DIARRHEA

Acute non-bacterial gastroenteritis is involved in many waterborne disease ('sewage poisoning') outbreaks (Craun, 1979a). A large proportion of these outbreaks appear to have a viral aetiology (Craun, 1979b). A number of reports published in the past 5 years have implicated rotaviruses as being among the causes of such waterborne outbreaks (Lycke et al., 1978; Zamotin et al., 1981; Sutmoller et al., 1982; Harris et al., 1983; Morens et al., 1983; Murphy et al., 1983; Tulchinsky et al., 1983; Walter, 1983).

There are a number of factors which further reinforce the potential of rotaviruses to spread through the waterborne route. A large number of

virus particles are excreted in the feces of infected individuals (Flewett and Woode, 1978; McNulty, 1978) and this fecally excreted virus has been shown to remain infectious for several months at room temperature (Woode and Bridger, 1975). Rotaviruses have been detected in raw and treated sewage (Smith and Gerba, 1980a; Steinmann, 1981; Hejkal et al., 1984). The resistance of rotaviruses to inactivation by chlorine and other disinfectants is well documented (Snodgrass and Herring, 1977; Tan and Schnagl, 1981) and even at concentrations of available chlorine several times greater than that used in routine water treatment, rotaviruses were not inactivated (Sattar et al., 1983).

2

Samples of drinking water from wells (Keswick et al., 1980) and those from conventional drinking water treatment plants (Smith and Gerba, 1980b; Deetz et al., 1981; Keswick et al., 1984) have been found to contain infectious human rotaviruses. Samples of tap water, meeting U. S. standards for turbidity, coliform and free-chlorine, also had detectable levels of rotaviruses (Deetz et al., 1981, 1984).

PLAN OF STUDY

This study is divided into 3 phases. In phase 1, the emphasis is placed on determining the effect of temperature, naturally-occurring particulate matter and chlorine residuals on the survival of rotaviruses in raw and treated surface waters collected at different times of the year. Phase 2 deals with the comparison of immunofluorescence and immunoperoxidase in the detection and quantitation of infectious units of rotaviruses. Phase 3 of the investigation was to evaluate the talc-Celite technique and positively-charged filters for their ability to concentrate rotaviruses from experimentally-contaminated samples of raw water, and then to attempt the recovery, detection and quantitation of indigenous rotaviruses from sewage-polluted surface waters.

GENERAL MATERIALS AND METHODS

Cells

The MA-104 cell line, derived from embryonic rhesus monkey (Macaca mulatta) kidneys (Microbiological Associates Inc., Bethesda, Md., U.S.A.) was used throughout this study. A seed culture of these cells was originally received by us through the courtesy of Dr. H. Malherbe (Gull Laboratories, Salt Lake City, Utah, U.S.A.). As stock cultures the cells were routinely cultivated as monolayers in 75 cm² plastic culture flasks (Flow Laboratories, Rockville, Md., U.S.A.) using Eagle's minimal essential medium (MEM) in Earle's base (Autopow; Flow Labs.). Each 450 mL of the medium was supplemented with 25 mg. Garamycin (Schering Corporation, Kenilworth, N.J., U.S.A.), 13.5 mL of a 5.6% solution of sodium bicarbonate (J.T. Baker Chemical Company, Phillipsburg, N.J., U.S.A.), 5.0 mL of a 200 mM solution of L-glutamine (Flow Labs.) and 50 mL of virus- and mycoplasma-tested fetal calf serum (FCS; Flow Labs.). Each monolayer was trypsinized using 2.0 mL of a mixture of 0.25% trypsin (Millipore Corporation, Bedford, Mass., U.S.A.) and ethylenediaminetetracetic acid (0.05%) in Ca²⁺ - and Mg²⁺ - free phosphate buffered saline (PBS). A split ratio of 1:4 was generally used for the passage of the cells.

Cultures for plaque assay were put up in 12-well plastic plates (Costar, Cambridge, Mass., U.S.A.). Each well in the plate was seeded with approximately 1.0×10^5 cells in 2.0 mL of growth medium, the plates were then sealed individually in plastic bags (Philips Electronics Ltd.,

Toronto, Canada) before being placed at 37°C in an ordinary walk-in incubator. The monolayers were generally ready for plaque assay within 48 hours of seeding.

Viruses

Three laboratory-adapted strains of rotaviruses were used in this study. Simian rotavirus SA-11 (strain H-96) was supplied to us by Dr. Malherbe. Calf rotavirus (strain C-486) was obtained from Dr. L. Babiuk (University of Saskatchewan, Saskatoon, Sask., Canada). Human rotavirus D strain (subgroup 2, serotype Wa) was sent to us by Dr. R. G. Wyatt (National Institute of Health, Bethesda, Md., U.S.A.). These 3 viruses were first plaque purified in MA-104 cells and the same cell was used for preparing the virus pools.

Since FCS used in cell culture was found to be inhibitory to the rotaviruses, it was necessary to wash the monolayers at least 3 times with Earle's balanced salt solution (EBSS; Flow Labs.) before virus inoculation. After allowing the virus under test to absorb for one hour at 37°C, MEM without FCS and with 5.0 ug/mL of trypsin (1:250; ICN Nutritional Biochemical Company, Cleveland, Ohio, U.S.A.) was introduced into the cultures and they were placed back at 37°C. When nearly 75% of the monolayer was affected by rotavirus cytopathic effects, the cultures were frozen (at -70°C) and thawed 3 times. The cell culture harvest was centrifuged for 15 minutes at 1000 x g in a DPR-6000 centrifuge (Damon, IEC Division, Fisher Scientific Ltd., Pittsburgh, Pa., U.S.A.). The supernatant was extracted with an equal volume of 1,1,2-Trichloro-1,

2,2-trifluoroethane (Freon; J.T. Baker Chemical Co.). The aqueous (top) phase was then removed and centrifuged at 100,000 x g for 90 minutes in a Beckman L-350 centrifuge (Beckman Instruments Inc., Toronto, Canada). The pelleted virus was suspended in distilled water before storage at -70°C.

The Sabin strain of poliovirus type 1 was kindly provided to us by Mr. D.A. McLeod of the Laboratory Center for Disease Control (L.C.D.C.; Ottawa, Canada). The pool of the poliovirus was also prepared in MA-104 cells.

Plaque Assay

The quantitation of virus plaque forming units (PFU) was carried out using the plaque assay technique of Ramia and Sattar (1979a). Cell monolayers in 12-well plates were thoroughly washed with EBSS to remove all traces of FCS present in the culture medium. Each well then received 0.1 mL of the desired inoculum and the cultures were incubated at 37°C for 1 hour on a pitched orbital shaker. Each one of the control wells received 0.1 mL of EBSS. The overlay medium consisted of MEM, 0.6% Agarose Type II (Sigma Chemical Company, St. Louis, Mo., U.S.A.) and 5.0 ug/mL of trypsin (Nutritional Biochemical Co.). The plates were resealed inside the plastic bags and placed in a walk-in incubator at 37°C. After 3 days of incubation at 37°C the cultures were fixed overnight in a 1:10 formalin (J.T. Baker Chemical Co.) - saline mixture. After the removal of the fixative and overlay medium, the monolayers were washed in tap water and then stained with a 1% aqueous solution of crystal violet.

ROTAVIRUS SURVIVAL IN RAW AND TREATED WATER

INTRODUCTION

To permit their waterborne spread, rotaviruses need to retain their infectivity in recreational and potable waters for considerable periods of time. Although these viruses have been detected in various types of waters, very little information exists on the chemical, physical and biological factors which may or may not favor their persistence in such environments (Sattar, 1981; Gerba et al., 1984).

In the case of rotavirus contamination of drinking water in the distribution system, how effective is the residual chlorine in the disinfection of such water? There are conflicting reports in the literature on the efficacy of chlorine in this regard. Grabow et al. (1983) found simian rotavirus SA-11 to be more sensitive to inactivation by free chlorine than hepatitis A virus and poliovirus type 2. Butler and Harakeh (1982) found that, when compared with SA-11, coliphage f2, poliovirus type 1, coxsackievirus B5 and echovirus type 1, human rotavirus was more resistant to inactivation by ozone as well as chlorine dioxide. Rotavirus survival in soil must play an essential role in enabling the virus to pass through to groundwater. Hurst et al. (1980) and Goyal and Gerba (1979) have shown that rotaviruses bind poorly to certain types of soil. SA-11 was also found to survive for about 12 days in a moist loamy and sandy soil (Hurst et al., 1980). This clearly shows the potential for rotavirus contamination of groundwaters.

A limited amount of work has been done to study the long-term survival of rotaviruses in the aquatic environment. Hurst and Gerba (1980) studied the survival of SA-11 in fresh and estuarine waters. The virus was stable for weeks in both types of water. Keswick et al. (1982) observed that in groundwater SA-11 could survive for several days. McDaniel's et al. (1983) examined the survival of calf rotavirus in microorganism-free distilled water and wastewater. The calf rotavirus survived for a month.

Very little has been reported on the capacity of rotaviruses to survive in raw and treated surface waters in our particular climatic and geographic region. This phase of the study was to determine the effect of temperature and naturally-occurring particulate matter on the survival of rotaviruses in raw and treated surface waters collected at different times of the year.

MATERIALS AND METHODS

SHORT-TERM VIRUS SURVIVAL

Water Samples

Samples of conventionally-treated drinking water were collected at 2 different points in a municipal water treatment and distribution system. The first type of samples were those of the effluent (PE) from the Britannia Water Treatment Plant (Ottawa, Ontario); this municipal

facility on the Ottawa River treats about 1.9×10^8 L of water per day and supplies 50% of the drinking water for the greater Ottawa area. Tap water (TW) collected in our laboratory constituted the second type of water samples. This represented a mixture of treated water from the Britannia and Lemieux Island (supplies the remaining 50% of the drinking water for Ottawa area) water treatment plants located 10 km and 6 km, respectively, from our laboratory. The main physicochemical characteristics of raw and treated water from these plants have been described previously (Sattar and Ramia, 1979). Three samples of PE and TW each of 2L were used in this section of the study.

The chlorine measurements on the water samples were carried out using the amperometric titration method and the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method (Standard Methods, 1980).

Test Procedure

The virus under test was suspended in either distilled water, EBSS or tryptose phosphate broth (TPB; Difco Laboratories, Detroit, Mich., U.S.A.) before being added to the water samples. The virus-contaminated sample was then held for one hour at 22°C. To determine the amount of infectious virus remaining in the samples, they were diluted in EBSS and plaque assayed.

LONG-TERM VIRUS SURVIVAL

Bacterial Counts

Determinations of the colony forming units (CFU) of aerobic bacteria in the water samples were carried out using plates (9 cm in diameter) of trypticase soy agar (Difco. Labs.). One mL of the appropriate sample dilution was placed on the agar surface and the inoculum was evenly spread with the help of a glass rod. The plates were incubated at 37°C for 24 hours prior to counting of the bacterial colonies.

pH Measurements

All pH measurements on the water samples were carried out using a Fisher Scientific pH meter (Accumet, model no. 220) with a combination electrode.

Water Samples

One sample each of raw water (RW) was collected from the Britannia Water Treatment Plant during the fall (1982), winter and spring (1983). TW samples were collected concomitantly from taps in our laboratory. The third type of water samples were obtained by filtering RW through a membrane filter (47 mm diameter) with a pore size of 0.22 um (Millipore Corp.).

Test Procedure

For performing long-term virus survival tests, water samples were contaminated with the test virus which was suspended in distilled water after ultracentrifugation. It was diluted using portions of the water under test as a diluent and added to the sample to give a final concentration of 5.0×10^4 PFU/mL. Immediately after addition of the virus, a 9.0 mL volume of the water sample was removed to determine the starting titre of the virus. Clean and sterile glass flasks (500 mL capacity) were used as containers for incubating the contaminated water samples at either 4°C or 20°C in the dark. Aliquots of the sample under test were removed aseptically after 1, 2, 4, 8, 16, 32 and 64 days of incubation. To determine the amount of infectious virus present, 1.0 mL of sterile 10X TPB (pH 9.0) was added to 9.0 mL of the water sample. It was then passed through a 0.22 µm membrane filter and stored at -70°C until titrated. The addition of TPB to the water samples was necessary to avoid loss of virus by absorption to the membrane filters (Ramia and Sattar, 1980).

For bacterial counts and pH measurements aliquots were withdrawn from a separate flask containing the water under test but without rotavirus contamination.

RESULTS

SHORT-TERM SURVIVAL

The data for chlorine measurements are presented in Table 1. The samples of PE were found to contain 0.75 mg/L of free and 0.89 mg/L of total chlorine. There was a 15- to 18- fold reduction in these amounts by the time the treated water was collected as TW in our laboratory. Addition of TPB alone to PE to a final concentration of 1:1000 reduced the amounts of free and total chlorine by approximately 66%. When EBSS was used instead, the reduction in chlorine values in PE amounted to about 20%.

As can be seen from the data summarized in Tables 2 and 3, there was no significant decrease in the plaque titre of SA-11 and human rotavirus when they were suspended in TPB, EBSS, or distilled water before being added to the samples of TW. Similarly, the virus titre remained almost the same, in samples of PE when TPB or EBSS was used for virus suspension. However, there was a greater than 1- \log_{10} loss in the titre of SA-11 (Table 2) and almost 2- \log_{10} reduction in the titre of human rotavirus (Table 3) in samples of PE when the viruses were suspended in distilled water prior to sample contamination. There was a greater than 3- \log_{10} (99.9%) reduction in the plaque titre of the poliovirus in the samples of both PE and TW irrespective of the type of suspending medium used (Table 3).

TABLE 1.

AMOUNTS OF FREE AND TOTAL CHLORINE PRESENT
IN THE SAMPLES OF TAP WATER AND PLANT EFFLUENT

Sample type	Chlorine mg/L	
	free	total
Tap water	< 0.05	0.05 (.00)
Plant effluent (PE)	0.75 (.05)	0.89 (.05)
PE with 1 X tryptose phosphate broth (TPB)	0.26 (.04)	0.30 (.04)
PE with Earle's balanced salt solution (EBSS)	0.58 (.06)	0.72 (.07)

TPB or EBSS was added to the PE samples to a final concentration of 1:1000. The chlorine estimations were made using the amperometric titration method (Standard Methods, 1980). The number in the brackets represents the standard deviation.

TABLE 2.

SHORT-TERM SURVIVAL OF ROTAVIRUS SA-11 IN
SAMPLES OF PLANT EFFLUENT AND TAP WATER

Virus suspended in	Input PFU/mL	PFU/mL recovered	
		plant effluent	tap water
1 X tryptose phosphate broth	5.7×10^3	5.2×10^3	4.8×10^3
Earle's balanced salt solution	5.7×10^3	4.9×10^3	5.1×10^3
Distilled water	5.7×10^3	5.0×10^2	4.7×10^3

The virus was added to the sample under test and after a contact time of 1 h at 22°C it was diluted and plaque assayed in MA-104 cells.

TABLE 3.
SHORT-TERM
SURVIVAL OF HUMAN ROTAVIRUS (STRAIN D) AND POLIOVIRUS (TYPE 1, SABIN)
IN SAMPLES OF PLANT EFFLUENT AND TAP WATER

Virus and suspending medium	Input PFU/mL	Recovered PFU/mL	
		Plant effluent	Tap water
Human rotavirus			
distilled water	7.0×10^3	4.0×10^2	5.0×10^3
tryptose phosphate broth	2.0×10^2	2.0×10^2	2.1×10^2
Poliovirus			
distilled water	1.5×10^4	<10	<10
tryptose phosphate broth	1.2×10^4	<10	<10

The virus under test was suspended in either distilled water or tryptose phosphate broth before its addition to the water sample. The virus-contaminated sample of water was held at 22°C for 1 h. It was then diluted and plaque assayed in MA-104 cells.

TABLE 4.
CHARACTERISTICS OF RAW WATER SAMPLES USED IN THIS STUDY

Characteristics	Sampling Dates		
	Nov. 15, 82	March 23, 83	May 25, 83
Temperature (°C)	4.5	0.2	12
pH	7.0	7.2	7.2
Color (Hazen units)	50	95	38
Turbidity (N.T.U.)	2.6	14	3.3
Spec. Cond. (Microhms/cm)	70	81	68
Total Alkalinity (CaCO ₃)	18	24	20
Standard Plate Count/mL	630	2210	1210
Fecal Coliforms/100 mL	560	155	60

The information listed here has been taken from the records kept at the Britannia Water Treatment Plant.

LONG-TERM SURVIVAL

Characteristics of the Waters Used

Three samples of RW and TW were tested in this study. Their characteristics are summarized in Table 4. The temperatures of RW collected in November, 1982, March, 1983 and May, 1983 were 4.5°C, 0.2°C and 12°C, respectively. The fall sample showed a fecal coliform count of 560/100 mL as opposed to 155/100 mL and 60/100 mL for the early and late spring samples. There was no significant difference in the pH, color, and specific conductivity values of the three RW samples. The increase in turbidity value for March resulted from increased volume and turbulence during the spring runoff.

The TW samples collected in November, March and May gave initial temperature readings of 8°C, 4°C and 12°C, respectively. No total or fecal coliforms were detected in the TW samples. The November and March samples gave a standard plate count of 2 CFU/mL while the one collected in May had 3 CFU/mL. Other characteristics of the treated waters from the Ottawa River have been described in detail elsewhere (Sattar and Ramia, 1979a).

Virus Survival

Figures 1 through 12 show the survival of the three types of rotaviruses in RW, TW and FW collected in November (1982), March (1983) and May (1983) and held at 4°C and 20°C. Figure 1 shows the survival of calf rotavirus in RW, FW and TW collected in November 1982 and held at 4°C. The drop in the virus did not exceed 0.7- \log_{10} even after 64 days in TW. In RW, 2.7- \log_{10} of infectious virus were lost after 64 days of

incubation. As indicated in Fig. 2, TW incubated at 20° showed a less than 2.0- \log_{10} reduction in the virus titre over 64 days. At this higher temperature, the virus suspended in RW underwent a 3- \log_{10} drop in its titre after 16 days. In Figures 1 and 2, the drop in calf rotavirus titre did not exceed 0.7- \log_{10} even after 64 days in FW irrespective of the holding temperature. Fig. 3 summarizes the data for the RW, FW and TW collected in May, 1983 and held at 4°C. The extent of virus loss in the TW sample was almost the same as was seen in TW collected in the fall of 1982. The drop in the infectivity titre of the calf rotavirus in the RW sample amounted to approximately 3- \log_{10} over a period of 64 days. Fig. 4 represents the data for the RW, FW and TW samples collected in May, 1983 and incubated at 20°C. In TW, the reduction in the plaque titre of the virus was found to be about 2- \log_{10} . There was a greater than 3- \log_{10} loss in the virus titre after 64 days when the RW sample was held at 20°C. Again in Fig. 3 and 4, the drop in infectivity titre of calf rotavirus in FW did not exceed 0.7- \log_{10} even after 64 days irrespective of temperature of incubation. The pattern seen in Figs. 1,2,3, and 4 for calf rotavirus survival in RW, FW and TW can be seen for SA-11 (Figs. 5-8) and human rotavirus (Figs. 9-12) at both incubation temperatures (4°C and 20°C). The times required in days for a 99% (T-99) and 99.9% (T-99.9) drop in the plaque titre of the rotaviruses in the water samples incubated at the two different temperatures are presented in Table 5.

TABLE 5.

TIMES IN DAYS FOR 99.0% (T-99) AND 99.9% (T-99.9)
LOSS OF INFECTIOUS ROTAVIRUS IN THE WATER SAMPLES

Water Samples	T-99			T-99.9		
	4°C	20°C	4°C	4°C	20°C	20°C
Tap Water	> 64	64	> 64	> 64	> 64	> 64
Raw Water	32	10	64	64	16	16
Filtered Water	> 64	> 64	> 64	> 64	> 64	> 64

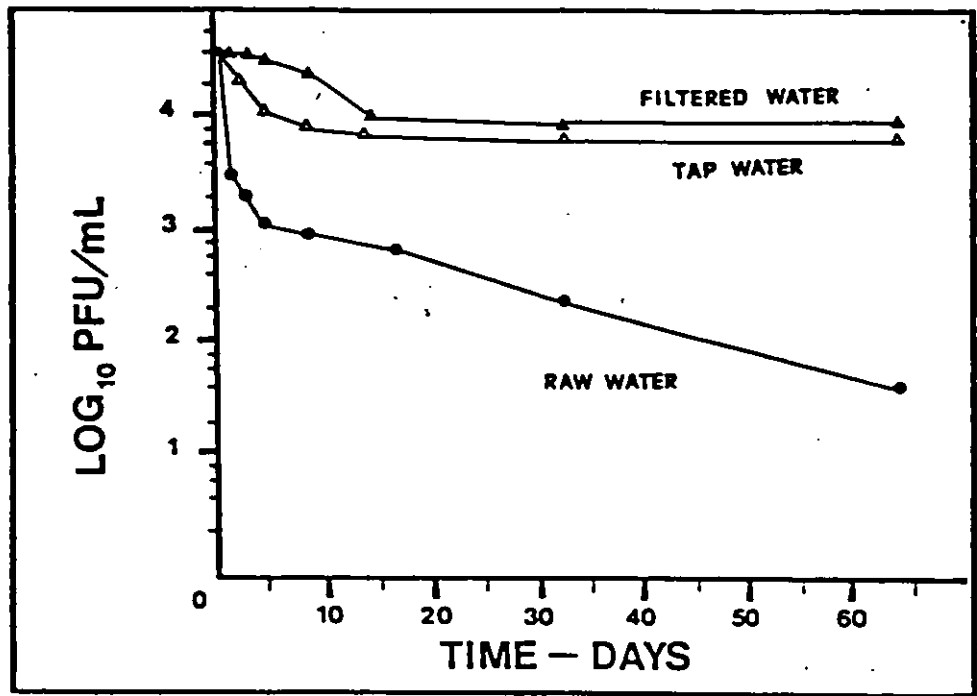


Fig 1. Survival of Calf Rotavirus at 4°C in the Water Samples Collected in November of 1982.

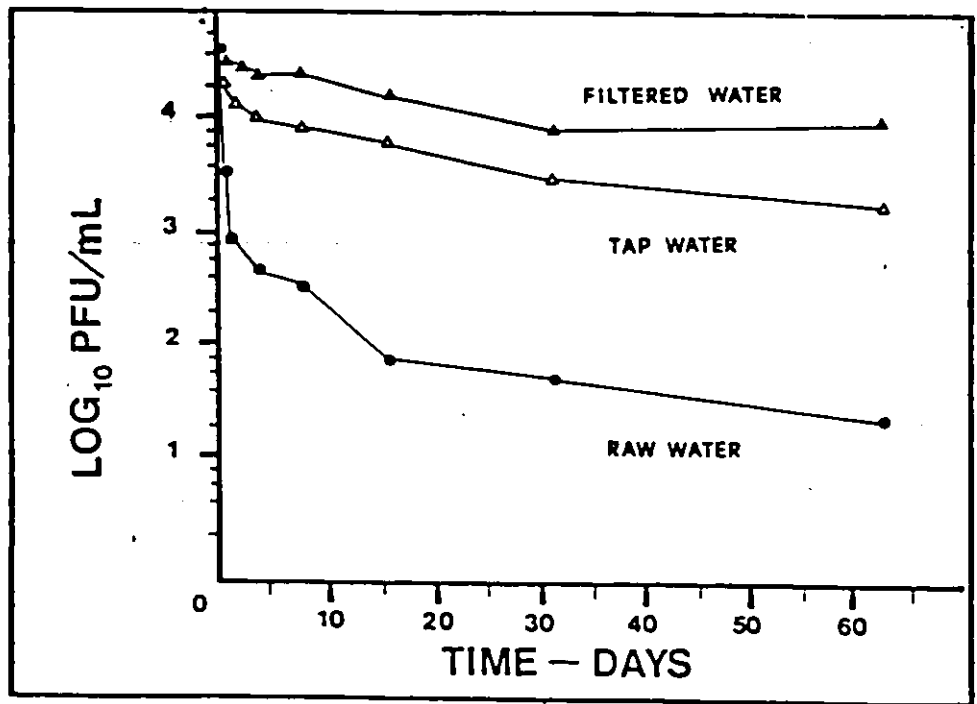


Fig 2. Survival of Calf Rotavirus at 20°C in the Water Samples Collected in November of 1982.

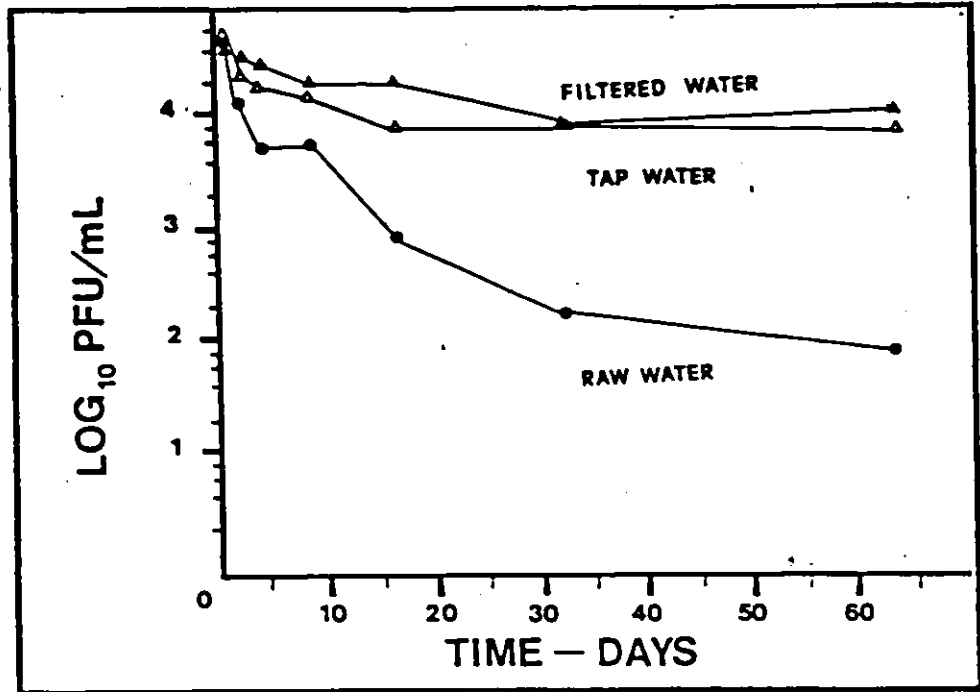


Fig 3. Survival of Calf Rotavirus at 4°C in the Water Samples Collected in May of 1983

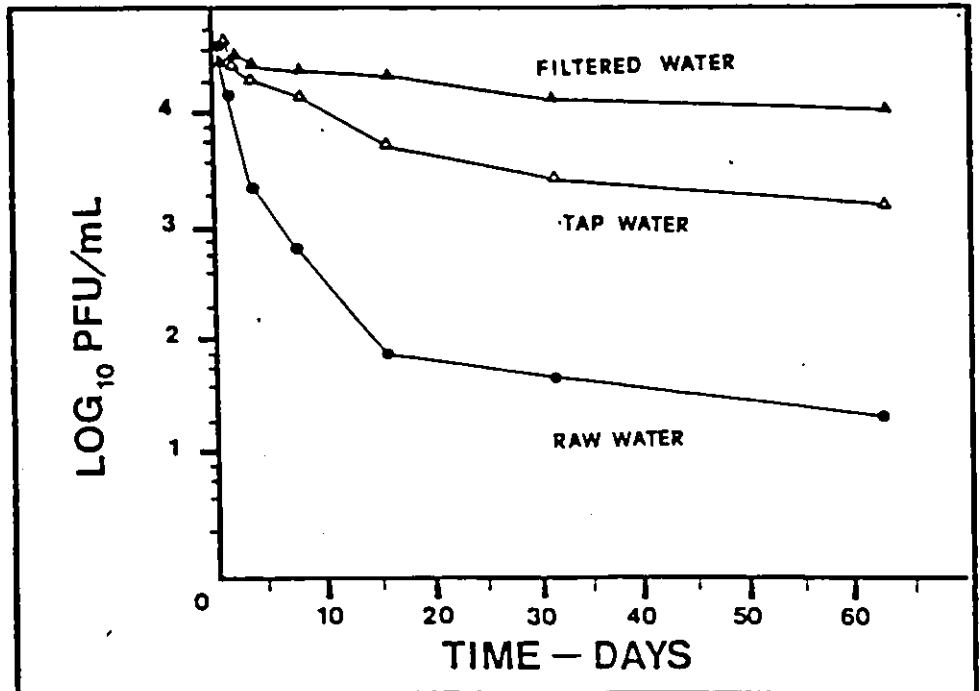


Fig 4. Survival of Calf Rotavirus at 20°C in the Water Samples Collected in May of 1983

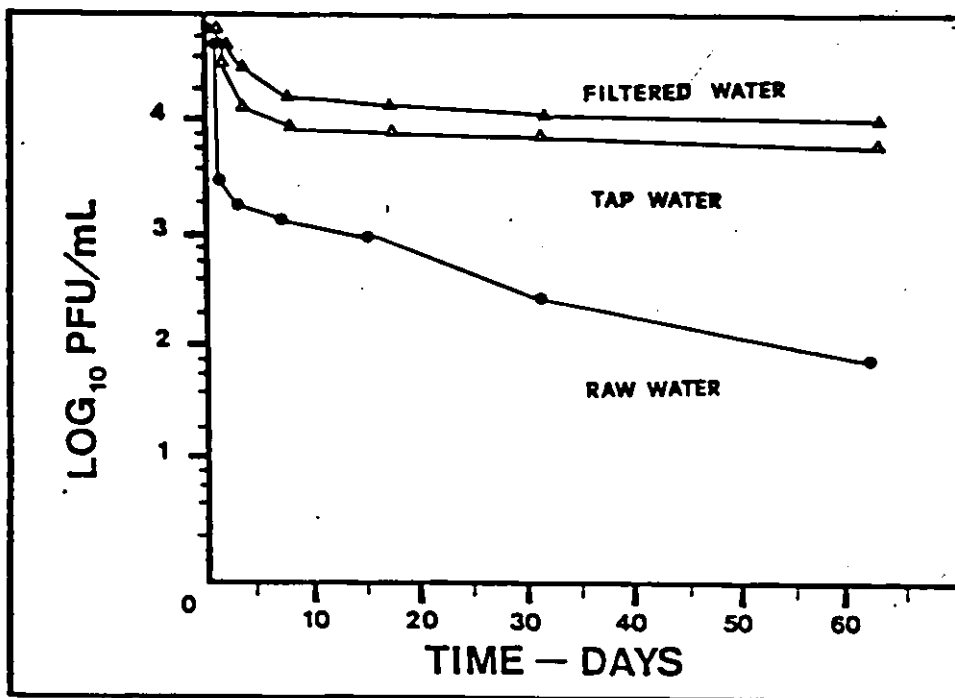


Fig 5. Survival of Rotavirus SA-11 at 4°C in the Water Samples Collected in November of 1982.

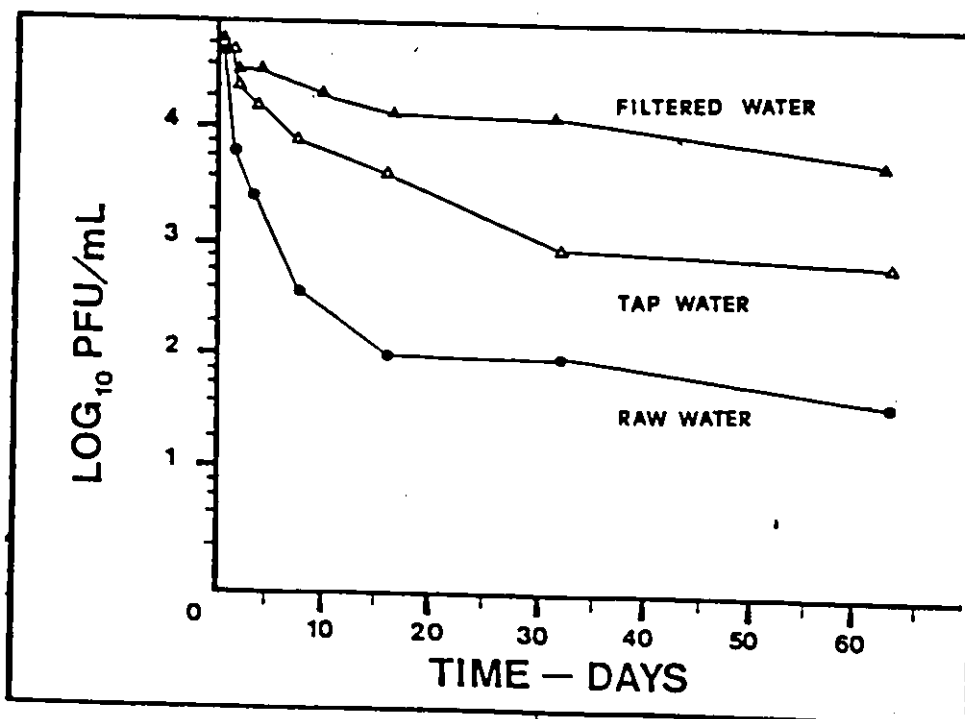


Fig 6. Survival of Rotavirus SA-11 at 20°C in the Water Samples Collected in November of 1982.

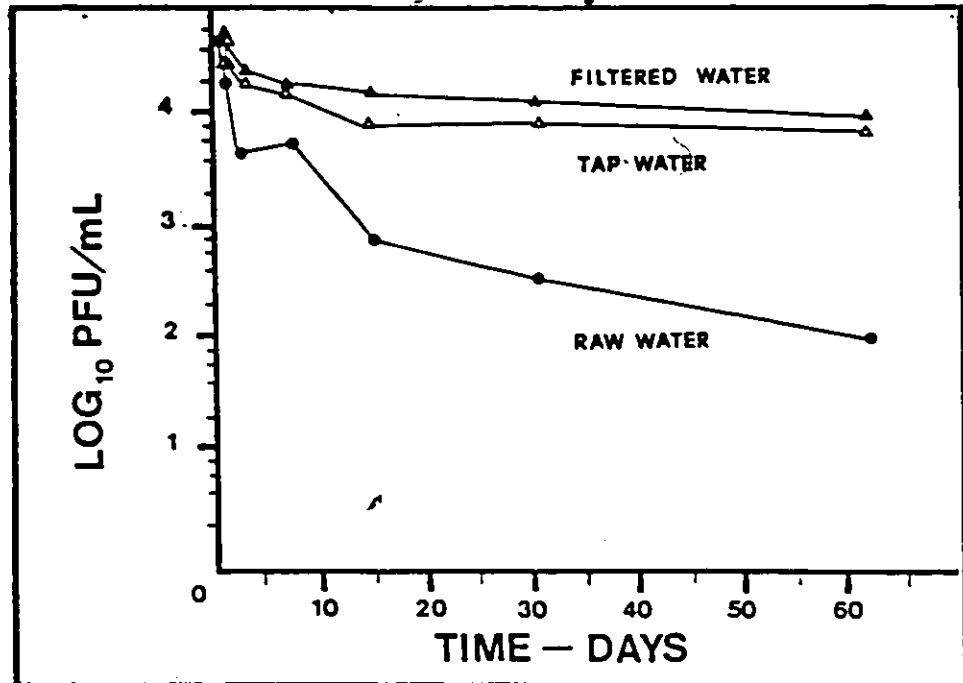


Fig 7. Survival of Rotavirus SA-11 at 4°C in the Water Samples Collected in March of 1983.

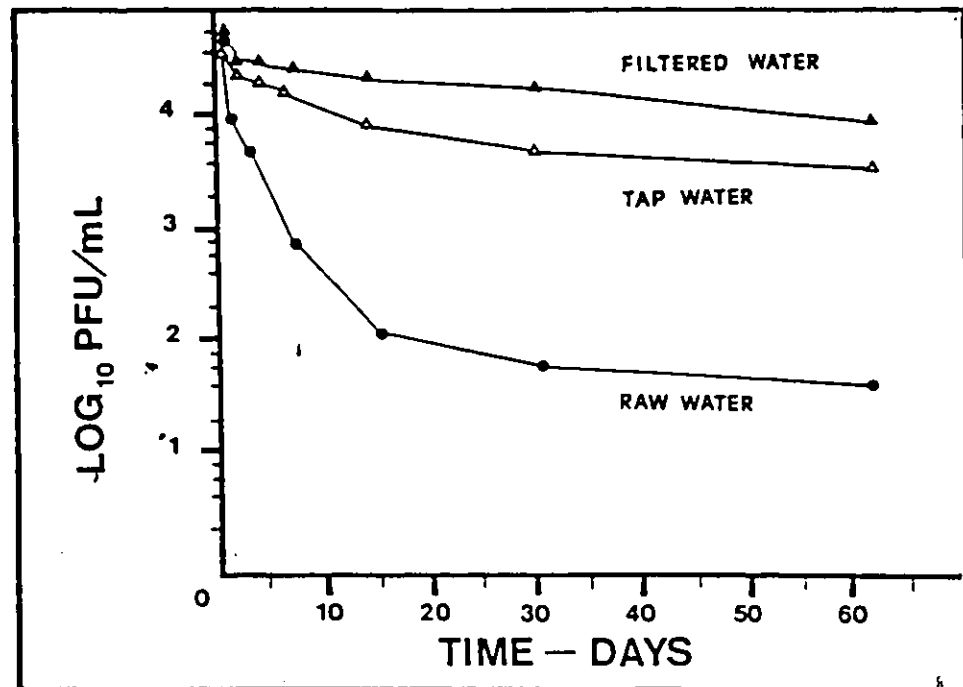


Fig 8. Survival of Rotavirus SA-11 at 20°C in the Water Samples Collected in March of 1983

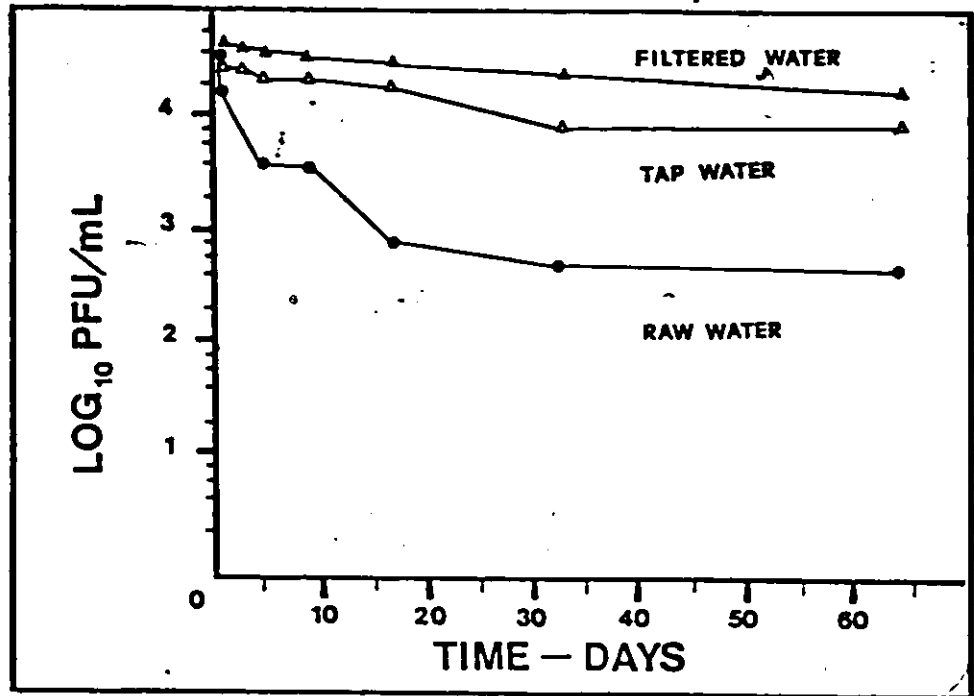


Fig 9. Survival of Human Rotavirus at 4°C in the Water Samples Collected in March of 1983.

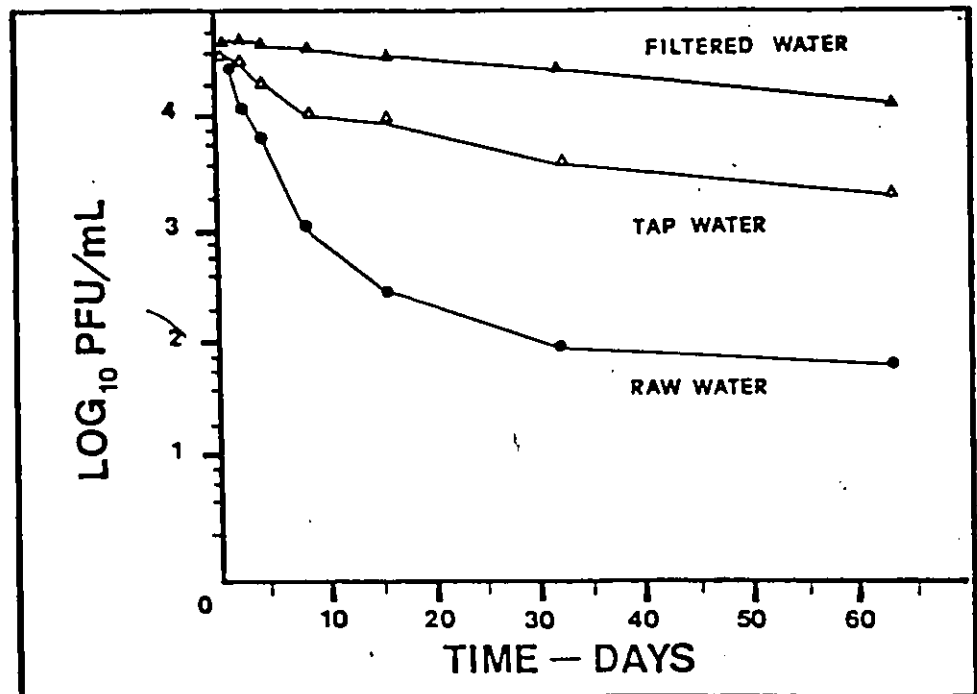


Fig 10. Survival of Human Rotavirus at 20°C in the Water Samples Collected in March of 1983.

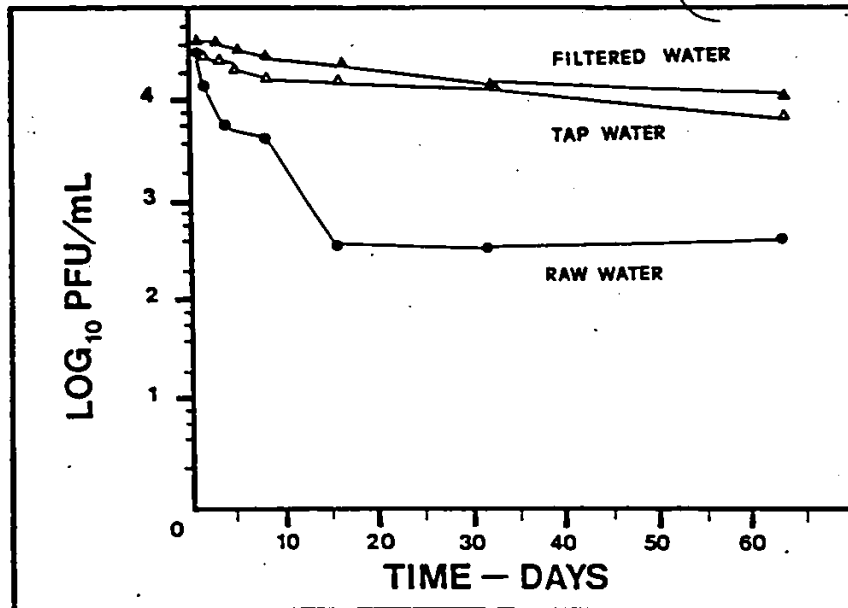


Fig 11. Survival of Human Rotavirus at 4°C in the Water Samples Collected in May of 1983.

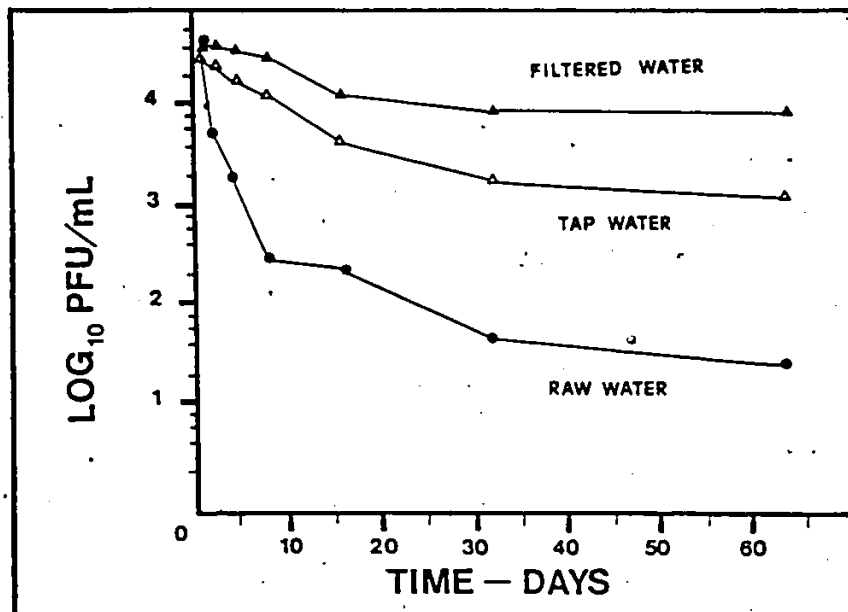


Fig 12. Survival of Human Rotavirus at 20°C in the Water Samples Collected in May of 1983.

Changes in pH

The initial pH of the samples of TW used in this study was 8.4. There was a gradual but slight reduction in the pH (final value 7.8) of these samples over the 64 day period when they were held at 20°C. At 4°C the final pH value for the fall sample was 8.2 and that of the spring samples were recorded as 7.7. The starting pH values for RW samples collected in November, March and May were 7.4, 7.2 and 7.2, respectively. In the fall sample the pH remained essentially unchanged at 4°C throughout the test period. However, there was a gradual increase to a final pH value of 8.2 when this sample was held at 20°C for a period of 64 days. The pH of both spring samples increased to 7.8 and 7.9 over the test period at 4°C and 20°C, respectively. In the fall sample of FW the starting pH was 6.8 and it increased over a 64 day period to 7.2 and 7.5 at 4°C and 20°C, respectively. In the spring samples the initial pH values were 7.6. In these two samples there was a slight increase in pH to 7.8 at 4°C and 20°C.

Bacteriological Tests

The starting number of aerobic bacteria in the samples of TW were found to be 2, 3 and 2 CFU/mL for November, March and May, respectively. In the November sample the counts after 64 days of incubation were recorded as 21 (4°C) and 100 (20°C). In the March sample of RW there were 1100, 1500 and 1950 CFU/mL, respectively. At 4°C the count in the fall sample gradually reached 1.4×10^4 CFU/mL after 64 days, whereas, at 20°C there was a more pronounced increase in the number of bacteria and the final

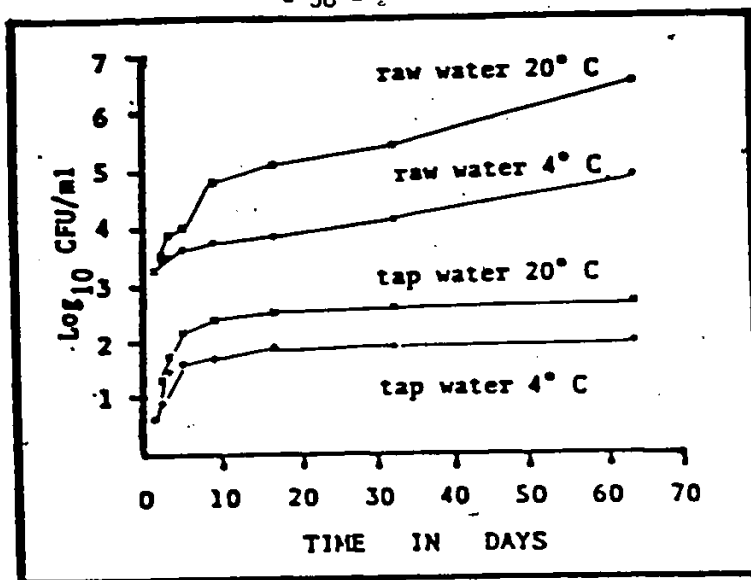


Fig 13. Increase in the number of colony forming units of aerobic bacteria in the water samples collected in March of 1983.

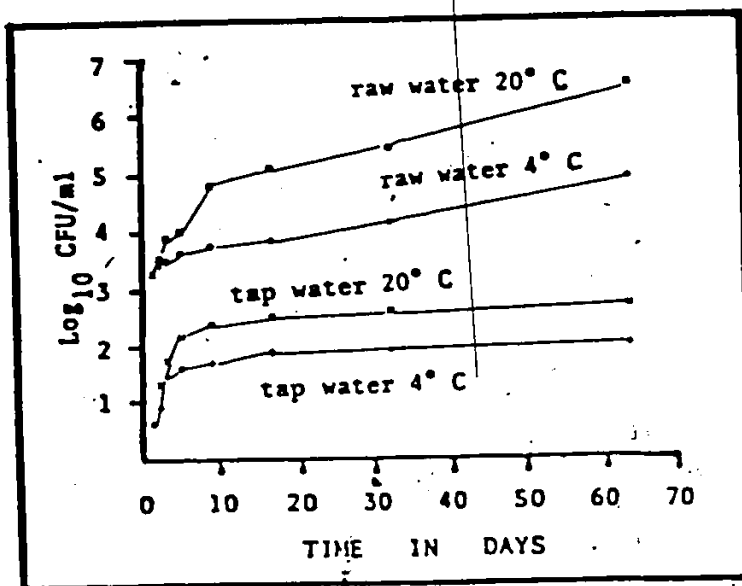


Fig 14. Increase in the number of colony forming units of aerobic bacteria in the water samples collected in May of 1983.

count was 2.0×10^6 CFU/mL. In the March sample of RW the final readings for the bacterial numbers were 6.8×10^4 (4°C) and 3.5×10^6 CFU/mL (20°C). In the May sample of RW the final bacterial counts were 5.0×10^4 (4°C) and 3.0×10^6 CFU/mL (20°C). The initial numbers of aerobic bacteria in the three samples of FW were 0 CFU/mL. In the spring samples there were no detectable bacteria after 64 days of incubation at either 4°C or 20°C . In the fall sample there were detectable bacteria after 64 days incubation; the final counts were 10 (4°C) and 12 CFU/mL (20°C). Figure 13 represents the bacteriological data for TW and RW samples collected in March and incubated at 4°C and 20°C . Likewise, Figure 14 contains the bacteriological data for TW and RW samples collected in May.

DISCUSSION

SHORT-TERM SURVIVAL

The rotaviruses and poliovirus used in these experiments were subjected to ultracentrifugation to remove extraneous organic and inorganic matter from the virus pools. Grabow et al. (1983) and Barrio and O'Brien (1984) reported the use of density gradient-purified rotavirus in their experiments. Moreover, these investigators used chlorine demand free buffers to minimize the interference extraneous organic and inorganic matter could cause with the activity of the chlorine. Both Grabow et al. (1983) and Barrio and O'Brien (1984) made up their chlorine containing solutions in chlorine demand free buffers which in no way resembles the make-up of tap water in a natural setting. In nature, contamination of treated water with sewage introduces into it not only microorganisms but also a variety of organic (particularly proteins and lipids) and inorganic impurities. In order to simulate this, TPB was used as a source of such substances. TPB was chosen because, unlike many other organic loads (e.g. fecal extracts, sera and skim milk), it has been shown to be non-inhibitory to rotaviruses (Ramia and Sattar, 1980).

The results of the short-term rotavirus survival experiments (Tables 1, 2, 3) show that the amounts of chlorine present in TW were unable to inactivate the virus to any significant degree even in the absence of added organic or inorganic matter. The experiments of Butler and Harakeh (1982), Grabow et al. (1983) and Barrio and O'Brien (1984) did not investigate the capacity of TW to inactivate rotaviruses. Their

experiments were done in an artificial environment using purified virus and chlorine demand-free buffers. This study was designed to evaluate the effect of the chlorine residuals in TW and PE on rotaviruses. The levels of chlorine in PE affected the rotavirus titre in only those preparation which were suspended in distilled water prior to sample contamination; the reduction in virus titre was approximately 1-log_{10} . Earlier studies on virus disinfection have shown rotaviruses to be able to withstand levels of chlorine many times higher than those used in terminal chlorination of drinking water (Snodgrass and Herring, 1977; Tan and Schnagl, 1981; Sattar et al., 1983).

The marked difference in the behaviour of human rotavirus and poliovirus in samples of PE and TW is another example of the different characteristics of rotaviruses and enteroviruses. Farrah et al. (1978) have shown that rotaviruses adsorb poorly to activated sludge flocs in comparison with enteroviruses and this leads to their inefficient removal during sewage treatment. Deetz et al. (1984) and Keswick et al. (1984) detected rotaviruses in finished water from municipal drinking water treatment plants. The rotaviruses were able to escape the alum and silica flocculation and survive terminal chlorination. This suggests that upon its contamination with rotaviruses, conventionally-treated drinking water could act as a vehicle for their spread.

Examples now exist where sewage-contamination of conventionally-treated drinking water has led to outbreaks of rotavirus diarrhea in people consuming such water (Lycke et al., 1978; Suttmoller et al., 1982; Harris et al., 1983; Walter, 1983).

LONG-TERM SURVIVAL

A number of factors are known to influence the survival of viruses in the water environment (Sattar, 1981). Among the many factors, temperature and naturally-occurring particulate matter are of particular interest. The two incubation temperatures selected for these experiments (4°C and 20°C) represent the temperature extremes encountered in water treatment, storage and distribution systems in temperate regions. Tests with holding temperatures higher than 20°C would be required to assess rotavirus survival in the water environment in tropical settings. McDaniels et al. (1983) have shown that rotavirus can survive at least 30 days in water samples held at 26°C . The water samples in our study were incubated in the dark again to simulate conditions found in underground water reservoirs as well as in water treatment, storage and distribution systems.

The findings of this phase of the study demonstrate that the survival capacity of rotaviruses in the raw water environment is dependent on the temperature of the water. When the virus-contaminated samples of RW were held at 4°C , the rotaviruses remained viable for a much longer period than at 20°C . The influence of temperature may be indirect in the sense that the higher temperature was more favorable for the growth of bacteria and other types of micro-organisms in the water. That such microbial activity in certain types of water may be deleterious to the survival of viruses has been demonstrated by the studies of Magnusson et al. (1966, 1967), Shuval et al. (1971), O'Brien and Newman (1977),

Loh et al. (1979) and Toranzo et al. (1983). In most instances this antiviral activity was directly or indirectly associated with the growth of marine microorganisms. In contrast to the work of Toranzo et al. (1983) on poliovirus, we passed raw water through bacteria retaining filters (FW) then seeded it with rotavirus and found that rotavirus survival was greatly enhanced in the absence of microbial activity even at 20°C. This suggests that the virucidal activity in fresh water may be different from the system operating in the marine environment.

In comparison with RW, and irrespective of the holding temperature, FW was found to be the most favorable for the survival of rotaviruses. The maximum drop in the virus titre in FW was 0.7- \log_{10} after 64 days (at either 4°C or 20°C).

The samples of TW had been subjected to full conventional treatment, including terminal disinfection by chlorine, at the municipal water treatment facilities in Ottawa. The total and free chlorine values in the TW samples were approximately 0.05 and <0.05 mg/L, respectively. No attempt was made to neutralize the chlorine in TW samples before the addition of the virus. The absence of an immediate and noticeable reduction in the plaque titre of the input virus indicated that the chlorine levels in the TW samples did not inactivate the virus. The maximum drop in virus titre recorded in this study for TW was about 2- \log_{10} after 64 days at 20°C. This indicates that if sewage contamination occurs in the water distribution system, rotaviruses could survive long enough to make such contaminated potable waters a health hazard. Sewage-polluted groundwaters have been implicated in outbreaks of rotavirus gastroenteritis (Murphy et al., 1983; Tulchinsky et al., 1983).

Hurst and Gerba (1980) studied the stability of simian rotavirus SA-11 in sewage-polluted and non-polluted samples of fresh and estuarine waters. The virus was added to these samples without the prior removal of indigenous microorganisms and they were held under indoor lighting conditions. McDaniels et al. (1983) investigated rotavirus and reovirus stability in microorganism-free distilled and wastewater samples held in the dark. In the wastewater samples the microorganism-free state was achieved by using a combination of pasteurization (62°C, 20 min.), freezing, thawing and addition of penicillin (500 units/mL), streptomycin (500 ug/mL) and amphotericin B (5 ug/mL). The elimination of indigenous microorganisms from such wastewater samples made the experimental setting highly artificial. In our experiments we sought to use RW and TW samples in their native state. The FW samples were included in our study as controls to test the influence of the raw water microflora on rotavirus survival.

IMMUNOCYTOCHEMICAL STAINING METHODS FOR WATERBORNE ROTAVIRUSES

INTRODUCTION

Rotaviruses and/or their antigens are present in fecal samples of infected individuals. More recently, antigens of these viruses have also been detected in tracheal aspirates from cases of viral pneumonia (Santosham et al., 1983). The following immunocytochemical techniques are used for the in vitro detection and identification of infectious rotavirus particles (Banatvala et al., 1975; Moosaf et al., 1978; Graham and Estes, 1979; Kapikian et al., 1979; Chasey, 1980; Graubelle et al., 1981; Hopley and Doane, 1983; Cevenini et al., 1984): immunofluorescence (IF), immunoperoxidase (IP) and peroxidase-antiperoxidase (PAP).

Whereas, a number of techniques are available for the routine laboratory diagnosis of rotaviral infections, detection and identification of naturally-occurring rotaviruses found in the water environment pose some problems. Conventional EM examination of concentrates of sewage and water is usually not feasible because of the low numbers of physically-intact virus particles present. However, Murphy et al. (1983) were successful in detecting rotaviruses by direct EM of concentrates of drinking water heavily contaminated with sewage, but these virus particles did not produce cytopathological effects in cell culture. Steinmann (1981) used direct EM as well as ELISA to detect rotaviruses in concentrates of domestic sewage; this study made no attempts to determine whether the detected viruses were infectious or

not. Therefore, until and unless the virus particles detected in such samples are shown to be infectious, their potential hazard to health remains undetermined.

Field strains of rotaviruses are slow growing and generally non-cytopathogenic in cell culture (Sato et al., 1981; Urasawa et al., 1981; Estes et al., 1983). At the same time, most concentrates of sewage and water are known to contain a variety of other enteric viruses which can grow faster and produce extensive cytopathology in vitro (Sattar, 1979; Hejkal et al, 1984). In view of this, IF staining has been applied in the detection as well as quantitation of infectious rotavirus particles in concentrates of samples from the water environment (Smith and Gerba, 1980a, 1980b, 1982; Deetz et al., 1984; Hejkal et al., 1984). However, no published data have been available on the suitability of other types of immunocytochemical methods in this regard. The purpose of this part of the study was, therefore, to compare the efficiency of IF and IP for the rapid detection and quantitation of infectious units of waterborne rotaviruses.

MATERIALS AND METHODS

Anti-rotavirus Antisera

Mouse ascitic fluid (clone 6B1/A3/M4) containing rotavirus group specific monoclonal antibodies was kindly supplied to us by Dr. T. H. Flewett (East Birmingham Hospital, Birmingham, Great Britain). This material was diluted 1:1000 in PBS for immunofluorescence and

immunoperoxidase studies. Rabbit immunoglobulins against human rotavirus prepared by DAKO-Immunoglobulins were purchased from Cedarlane Laboratories Limited (Hornby, Ontario, Canada). Rabbit antiserum to the Norden strain of calf rotavirus was a gift from Dr. R. S. Roy (Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Quebec, Canada). Both of these rabbit antisera were tested at a 1:40 dilution. Guinea pig serum against simian rotavirus SA-11 was kindly supplied to us by Dr. R. K. Chaudhury (LCDC, Ottawa, Canada) and was used at a 1:20 dilution.

The relevant information on the materials and reagents used in this part of the study is summarized in Tables 6 and 7. The methods for the fixation, staining and counting of rotavirus-infected cultures are given in detail in Appendix 1.

Cell Culture for Immunocytochemical Detection of Rotaviruses

MA-104 cells were propagated in Lab-Tek cell culture slide chambers (Miles Laboratories, Westmount, Ill., U.S.A.). Confluent monolayers were washed with EBSS and inoculated with 0.1 mL of the appropriate inoculum. The inoculated cell cultures in slide chambers were placed in holders (Damon, IEC Division, Fisher Scientific) and were centrifugal at 1000 RPM in a GLC-1 centrifuge (Sorval, Newton, Conn., U.S.A.) for 10 min. at room temperature. The chambers were processed in three different ways: (1) 8-10 hr incubation with MEM (no FCS) and trypsin (5 ug/mL), (2) 24 hr incubation with MEM (no FCS) and trypsin (5 ug/mL), (3) 24-72 hr incubation with the semi-solid overlay used in the plaque assay.

TABLE 6.

MATERIALS USED IN STAINING

Item	Manufacturer
Acetone	Fisher Scientific Co.
3-amino-9-ethylcarbazole	Sigma Chemical Co.
Dimethylformamide	Fisher Scientific Co.
Hydrogen peroxidase	BDH Chemicals, Toronto, Canada
Methanol	Fisher Scientific Co.
Merthiolate	Sigma Chemical Co.
Sodium acetate	Fisher Scientific Co.
Succinic acid	Fisher Scientific Co.
Cell culture chamber/slides	Miles Laboratories Inc., Westmont, Illinois, U.S.A.

TABLE 7.

INFORMATION ON REAGENTS USED IN STAINING

Item	Lot No.	Manufacturer	Dilution used
<u>Fluorescence (FITC)</u> Rabbit antimouse	022	Dako-Immunoglobulins*	1:20 in PBS
Protein A	20817	Zymed Laboratories*	1:10 in PBS
Goat antirabbit	20602	Zymed Laboratories	1:20 in PBS
Rabbit antiguinea pig	20902	Zymed Laboratories	1:20 in PBS
Rabbit antiguinea pig	011A	Dako-Immunoglobulins	1:20 in PBS
<u>Peroxidase</u> Rabbit antimouse	031A	Dako-Immunoglobulins	1:10 in PBS
Rabbit antimouse	11106	Zymed Laboratories	1:10 in PBS
Rabbit antiguinea pig	030	Dako-Immunoglobulins	1:20 in PBS
Rabbit antiguinea pig	104192	Zymed Laboratories	1:20 in PBS
Protein A	20618	Zymed Laboratories	1:10 in PBS
Rabbit antihuman rotavirus	031A	Dako-Immunoglobulins	1:20 in PBS

*Cedarlane Laboratories is the Canadian distributor.

Detection of Rotaviruses in Wastewater

In order to test the immunocytochemical methods in the detection of naturally-occurring rotaviruses, it was decided to experiment with samples of wastewater. Such samples were obtained from the Green Creek Pollution Control Centre of the Regional Municipality of Ottawa-Carleton. It is the largest wastewater treatment facility in this area, treating nearly 400 million litres of sewage per day. It subjects the sewage to primary sedimentation, phosphate removal and terminal disinfection by chlorine. The treated effluent is then discharged into the Ottawa River. Samples of raw sewage (RS), prechlorination (Pre-CE) and postchlorination (Post-CE) effluents were collected in February, 1984. Three samples of each of RS, Pre-CE and Post-CE were obtained from the pollution control centre. All samples were found to be positive for rotavirus by indirect IP and IF of infected cell cultures.

To 180 mL of the wastewater under test 20 mL of 10X TPB was added and the mixture was passed through a 47 mm diameter membrane filter with a pore size of 0.22 μ m. The filtrate was collected and inoculated onto MA-104 cell cultures on slides, incubated at 37°C for 24 hr then fixed and stained by IF and IP.

RESULTS

Although all the polyclonal antisera against rotaviruses could be used in the detection of the 3 types of rotaviruses in slide culture IF and IP studies, for the comparison and quantitation experiments, the mouse ascitic fluid containing monoclonal antibodies to human rotavirus was employed. The plaque assay was compared with the fluorescent cell forming assay and the peroxidase cell forming assay. The slide chamber cultures of MA-104 cells were inoculated with the virus; a liquid overlay with trypsin was placed on them and they were kept at 37°C for 8-10 hours. After fixation and staining, the slides were examined under the microscope and cells showing IF or IP staining were counted. Table 8 summarizes the data for the quantitative comparison of the staining methods. The IF assay and the IP assay both gave counts of infectious rotavirus which were at least 2-fold higher when compared with the plaque assay. Figure 15 shows the bright cytoplasmic fluorescence of an MA-104 cell infected with human rotavirus (strain D) and stained with mouse monoclonal antibody to human rotavirus and FITC-antimouse IgG. Figure 16 shows profuse cytoplasmic red chromogen in inclusion bodies in MA-104 cells infected with human rotavirus (strain D) and stained with mouse monoclonal antibody and peroxidase-labeled antimouse IgG with 3-amino-9-ethylcarbazole as the reaction substrate.

As can be seen from Figures 17 (IF) and 18 (IP), naturally-occurring infectious rotavirus particles could be demonstrated in RS. The Pre-CE and Post-CE samples tested in this study also revealed the presence of similar infectious particles of rotaviruses.

TABLE 8.
COMPARISON OF IMMUNOFLOUORESCENCE, IMMUNOPEROXIDASE
METHODS WITH PLAQUE ASSAY FOR THE IN VITRO
DETECTION AND QUANTITATION OF ROTAVIRUSES

EXPT. NO.	PLAQUE ASSAY PFU/mL (X10 ⁶)	METHODS WITH PLAQUE ASSAY FOR THE IN VITRO			PCFU/mL ^b (X10 ⁶)
		FCFU/mL ^a (X10 ⁶)	PROTEIN A ANTI MOUSE IgG	PROTEIN A ANTI MOUSE IgG	
1	2.0	3.7	4.6	5.2	4.1
2	3.9	4.8	6.1	6.5	5.0
3	3.0	NDC	ND	10.2	7.1

The virus used was human rotavirus strain D and the primary antiserum was the mouse monoclonal (ascites fluid). Slide chamber cultures of MA-104 cells were fixed 9 hrs post inoculation and stained as described in Appendix I.

- a. FCFU/mL: Fluorescent cell forming units per mL
- b. PCFU/mL: peroxidase cell forming units per mL
- c. Not done because of microscope failure.

Fig 15. Cytoplasmic Fluorescence of MA-104 Cells Infected with Human Rotavirus and Stained with Mouse Monoclonal Antibody to Human Rotavirus and FITC-Antimouse IgG. (X 882)

Fig 16. Immunoperoxidase Staining of MA-104 Cells Infected with Human Rotavirus. Mouse Monoclonal Antibody to Human Rotavirus and Peroxidase-Antimouse IgG. (X 220)

15



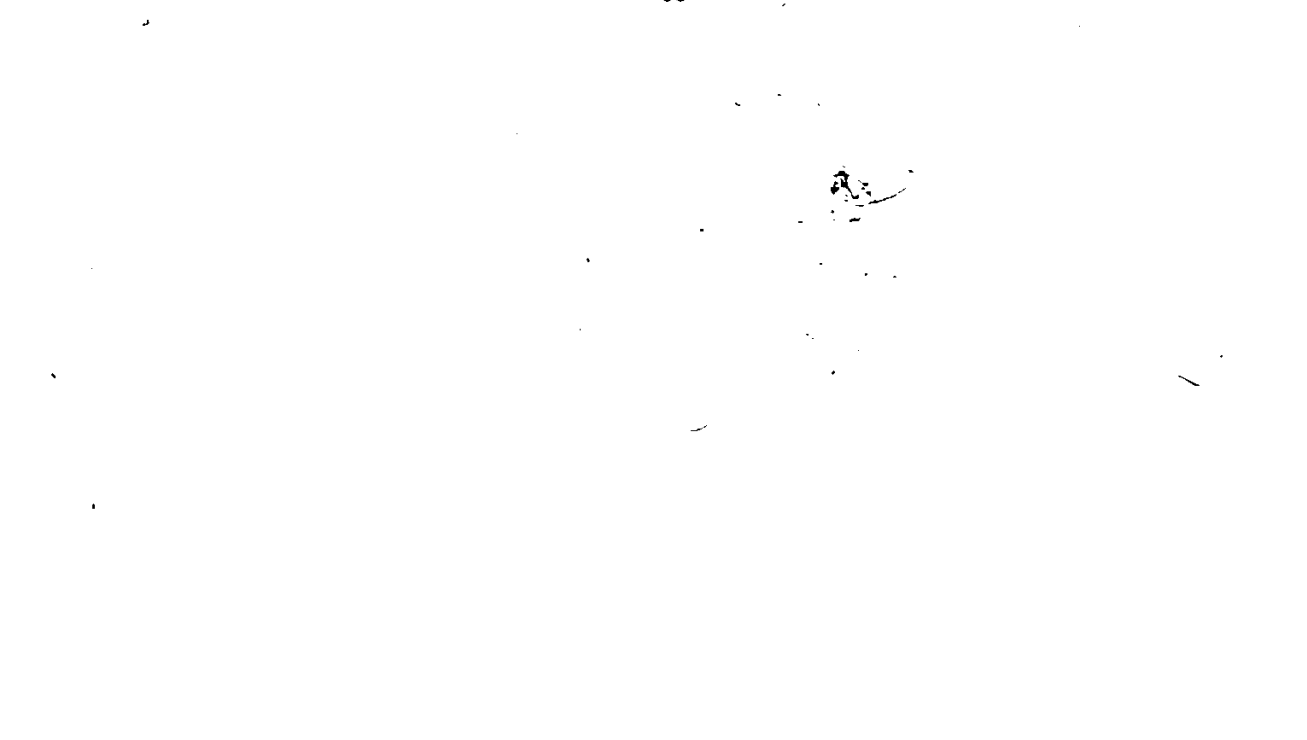


Fig 17. Immunofluorescence Staining of an MA-104 Cell Infected with Rotavirus from Raw Sewage. (X 882)

Fig 18. Immunoperoxidase Staining of MA-104 Cells Infected with Rotavirus from Raw Sewage. (X 220)

17



18

DISCUSSION

IMMUNOCYTOCHEMICAL QUANTITATION OF ROTAVIRUSES

In the indirect IF technique, a single preparation of fluorochrome-labeled secondary antibodies can be used for the detection of primary antibodies of different specificities from a single animal species (Sternberger, 1979). IP techniques are similar to those for IF but with an enzyme reaction used to localize an antigen instead of ultraviolet-induced excitation of a fluorochrome. Antibodies are covalently linked with peroxidase without impairing the enzymatic activity of the peroxidase (Farr and Nakane, 1981; Falini and Taylor, 1983).

The protein-A component of the cell wall of Staphylococcus aureus has been shown to bind specifically to the Fc portion of most mammalian IgG subclasses (Forsgren and Sjoquist, 1966; Goding, 1978). Coupling of protein-A to other substances such as fluorescein or peroxidase does not affect the biological properties of the native protein (Falini and Taylor, 1983). Protein-A methods in IF and IP techniques offer certain advantages such as economy, versatility, rapidity and decreased background staining (Sternberger, 1975; Falini, 1983). In this study protein-A, labeled with either peroxidase or fluorescein, was used for the detection and quantitation of rotaviruses in infected cells. Such preparations were always free from non-specific background staining and the specific stain was as bright as that seen with the IF and IP methods.

Due to the difficulties encountered in working with coverslip cultures, Lab-Tek slide cultures were employed in this study. Such chambers have also been used in earlier immunocytochemical studies involving rotaviruses (Graham and Estes, 1979; Smith and Gerba, 1982). In our experiments we centrifuged the inoculum onto monolayers of MA-104 cells in slide chambers. This technique has been shown to enhance the process of virus absorption (Banatvala et al., 1975) and at the same time increase the sensitivity of the fluorescent focus assay for rotaviruses in comparison with the plaque assay (Estes et al., 1983).

A liquid medium supplemented with trypsin (no FCS) was used as an overlay and it proved to be more advantageous than the semi-solid overlay. Problems arose during the removal of the semi-solid overlay prior to the fixation step in the staining process. Moreover, use of the semi-solid overlay often resulted in crystalline deposits on the cells as well as the detachment of the monolayer.

In the initial experiments incubation periods of 24, 48 and 72 hrs were tried but were found to be too long because they resulted in the generation of secondary foci of virus infection. Further work showed an incubation period of 8-10 hrs to be suitable in the immunocytochemical quantitation of the lab-adapted strains of rotaviruses.

Acetone, methanol and glutaraldehyde have been used as fixatives in IF and IP assays for rotaviruses (Banatvala et al., 1975; Moosai et al., 1978; Graham and Estes, 1979; Chasey, 1980; Graubelle et al., 1981; Smith and Gerba, 1982; Cevenini et al., 1984). In the IF assay we

employed acetone as the fixative but in the IP assay a combination of acetone, methanol and hydrogen peroxide were used instead. Methanol and hydrogen peroxide were used in order to eliminate endogenous peroxidase activity of the cells thereby reducing non-specific background staining (Sternberger, 1979). Other methods for eliminating background staining include methanol and nitroferricyanide (Straus, 1971), acid alcohol (Benjamin, 1977), sodium azide and hydrogen peroxide (Desmond et al., 1979), phenylhydrazine (Straus, 1979) and egg albumin and hydrogen peroxide (Zehr, 1978).

Both the IF and IP assays were found to be more sensitive than the plaque assays because not all infectious virus particles can form countable plaques. The average time required to complete the immunocytochemical assays was 2 hrs for IF and 2.5 hrs for IP following the 8-10 hours of incubation of inoculated cultures. The plaque assay, on the other hand, took 3 days for its completion. Moreover, the analysis of concentrates of water using plaque assay for rotaviruses is not feasible because field strains of rotaviruses do not form countable plaques. For these reasons, immunocytochemical methods are considered more appropriate for the detection and quantitation of field strains of rotaviruses. The reagents used in the IF and IP assays of these viruses are readily available from commercial sources.

There are two major difficulties associated with the IF technique. The first is the expense involved in the purchase and maintenance of a fluorescence microscope. The second difficulty is the fading of fluorescence during the process of specimen examination. Methods now

available to retard fading include the addition of p-phenylenediamine to buffered glycerol (Johnson and de Nogueira-Araujo, 1981) or the use of commercially available mountants such as Fluormount (Gurr, BDH Chemicals) and DPX mountant (BDH Chemicals).

L
IP assays, besides being more time consuming, also have some associated problems. Some of the peroxidase substrates are carcinogenic (Voogd et al., 1980). In particular, benzidine (which produces a blue reaction product) and diaminobenzidine (which produces a brown reaction product) are known to be carcinogenic. Other less harmful substrates which may be employed in this technique are 3-amino-9-ethylcarbazole (AEC), 4-chloro-1-naphthol, p-phenylenediamine plus pyrocatechol (OPD), 5-amino-salicylic acid (5-AS) and o-dianisidine (DIA) (Marcollet et al., 1980; Voogd et al., 1980; Farmilo, 1983). In tissue-antigen localization by light microscopy, a color change with a stable and insoluble reaction product is desired (Farmilo, 1983). The AEC reaction product is insoluble in aqueous solvents. Therefore, using an aqueous mounting medium and no organic solvents makes AEC a good chromogenic substrate for the IP detection of rotaviruses, as shown by this study. To reduce crystalline build-up in the reaction product, the substrate solution was prepared fresh and filtered just prior to use.

Two advantages of the IP over the IF technique are its lower cost as well as its capability of producing preparations which do not fade during examination and storage.

Some preliminary experiments have been done with PAP staining of MA-104 cells infected with cell culture-adapted rotaviruses. These preliminary findings show it to be very sensitive. It has increased sensitivity over conventional IP because of the high overall binding affinity of peroxidase for antiperoxidase in the cyclic PAP molecule (Petralli et al., 1974). A drawback with PAP techniques is the number of steps involved in the procedure and therefore more time is required to complete this technique. Protein-A methods may be employed in the PAP scheme but choice of reagents is important because Notani et al. (1979) showed that protein-A has a high affinity for rabbit and guinea pig PAP and a lower affinity for rat and goat PAP.

Recently the use of avidin-biotin in immunocytochemical studies has been shown to increase the sensitivity of the basic immunocytochemical techniques for the detection of rotaviral antigens (Hopley and Doane, 1983; Yolken, 1983).

DETECTION OF ROTAVIRUSES IN WASTEWATER USING IF AND IP

Figures 17 and 18 show the immunocytochemical demonstration of rotaviruses detected in wastewaters from the Ottawa area. The sewage treatment process apparently did not eliminate the rotaviruses. In our experiments, we did not encounter cytotoxicity of the concentrates. Hejkal et al. (1984) encountered cytotoxicity of the concentrates of sewage for MA-104 cells and used filtration through positively-charged filters to reduce it. Hejkal et al. (1984) reported a seasonal occurrence of rotaviruses in sewage with a peak from December through March. There is evidence that rotavirus gastroenteritis peaks in the winter.

months in temperate climates (Kapikian, et. al., 1981). This may correlate with the seasonal pattern of the appearance of rotaviruses in sewage as seen by Hejkal et al. (1984).

RECOVERY OF ROTAVIRUSES FROM SEWAGE-POLLUTED SURFACE WATER

INTRODUCTION

In order to properly evaluate the potential risk to human health due to rotaviruses in the aquatic environment, suitable methods are required for recovering these agents from water. In general, such recovery methods are based on the physicochemical properties of viruses with the adsorption/ elution techniques appearing to be the most useful.

The 2 types of adsorbents which are used for concentrating waterborne enteric viruses are microporous filters and particulates (Primrose et al., 1981). The particulates used include insoluble polyelectrolytes (Wallis et al., 1970), aluminum hydroxide flocs, aluminum phosphate flocs and calcium phosphate (Wallis and Melnick, 1967; Primrose and Day, 1977), iron oxides (Rao et al., 1968; Bitton and Mitchell, 1974), talc-Celite (Sattar and Westwood, 1976; Sattar and Ramia, 1979a, 1979b; Ramia and Sattar, 1980) and glass powder (Sarrette et al., 1977). The 2 classes of microporous filters are the negatively-charged (Farrah et al, 1976) and the positively-charged (Sobsey and Jones, 1979) filters.

The water sample (experimentally- or naturally-contaminated) is passed through the adsorbent layer and then an appropriate eluent (usually protein in nature) is used to release the virus from the layer. Further concentration of the eluted material may be achieved by ultracentrifugation, ultrafiltration, acidic flocculation or hydroextraction (Primrose et al., 1981).

Ramfa and Sattar (1980) efficiently concentrated simian rotavirus SA-11 from experimentally-contaminated tap water using the talc-Celite technique. Pretreatment of the water was necessary and this involved the adjustment of sample pH to 6.0 and the addition of divalent cations. Elution was performed with proteinaceous solutions at alkaline pH. This study, however, did not investigate the suitability of the talc-Celite method for rotavirus recovery from samples of raw surface waters.

Microporous filters have also been used in concentrating rotaviruses from the water environment (Smith and Gerba, 1980b; Steinmann, 1981; Goyal and Gerba, 1983; Guttman-Bass and Armon, 1983; Deetz et al., 1984; Hejkal et al., 1984; Keswick et al., 1984). Smith and Gerba (1980b) used a fiberglass-epoxy filter system (Viradel, a negatively-charged filter) to concentrate these viruses from experimentally-contaminated samples of tap water and sewage. Pretreatment of the sample was needed and it involved pH reduction and the addition of aluminum chloride. Virus elution was carried out with amino acids in combination with tryptose phosphate broth. It was noted that the optimum conditions for rotavirus adsorption and elution were different than those for enteroviruses. This confirmed the earlier observations of Farrah et al. (1978). The Viradel method has been applied to the detection of naturally-occurring rotaviruses in sewage (Smith and Gerba, 1982; Hejkal et al., 1984), and raw and treated waters (Keswick et al., 1980; Smith and Gerba, 1980b; Deetz et al., 1981; Deetz et al., 1984; Keswick et al., 1984).

Steinmann (1981) used the positively-charged Seitz filters in combination with ultracentrifugation and precipitation with aluminum sulphate to concentrate rotaviruses from sewage. Guttman-Bass and Armon (1983) reported the use of positively-charged Zeta Plus filters to concentrate simian rotavirus SA-11 from experimentally-contaminated samples of tap water. Toranzos and Gerba (1983) showed that the positively-charged 1-MDS filters were capable of concentrating rotaviruses from water and sewage. Keswick (1983) demonstrated that rotaviruses (as well as enteroviruses) could retain their infectivity for several weeks after adsorption to the 1-MDS filters. This meant that immediate elution of the viruses from these filters was not necessary, thus making them suitable for use in field studies. This type of filters have in fact been used to detect human rotaviruses in sewage (Hejkal et al., 1982) and conventionally-treated tap water (Gerba et al., 1983).

Since this study was aimed at the recovery of naturally-occurring rotaviruses from fresh surface waters, it was considered necessary to determine which of the available methods of rotavirus concentration from water would be suitable for this purpose. Therefore, based on the information summarized above, it was decided to study the comparative rotavirus concentrating efficiency of the talc-Celite layers and positively-charged Zeta-Plus filters.

MATERIALS AND METHODS

TECHNIQUES FOR CONCENTRATION OF ROTAVIRUSES FROM SEEDED SAMPLES OF
SEWAGE-POLLUTED SURFACE WATERS

Water Samples

RW samples from the Ottawa River were collected at the Britannia Water Treatment Plant during 1982 and 1983. TW samples were collected in our laboratory during the same time period.

Processing of the Water Samples

In experiments with talc-Celite layers Sattar and Ramia (1979a) found that preconditioning of the water was necessary to maximize rotavirus absorption to the layers. Before experimental contamination of the water sample with the virus under study, the preconditioning steps, therefore, involved dechlorination, addition of EBSS and acidification. Dechlorination was carried out by adding 0.4% solution of sodium thiosulphate (Fisher Scientific Co.) to the sample to a final concentration of 1:100. EBSS, which acted as a source of cations, was added to give a final concentration of 1:100. The sample pH was adjusted to 6.0 with 0.05N HCl solution (Fisher Scientific Co.).

Virus Adsorbents

Talc-Celite Layers

Talcum powder (magnesium silicate; J. T. Baker Chemical Co.) and Celite 503 (Flux-calcinated diatomite; J. T. Baker Chemical Co.) were purchased from Canadian Laboratory Supplies (Toronto, Canada). Before use, both of these powders were washed 2 times in distilled water. After washing, they were left in the incubator at 37°C for drying. A mixture of 10 grams of talc and 3.3 grams of Celite was added to one litre of distilled water.

Layers prepared for the processing of small volumes (up to one litre) were held in 47 mm diameter glass filter holders (Millipore Corp.). A Whatman No. 114 filter paper of 47 mm diameter was moistened in sterile distilled water and placed in the holder. The talc-Celite suspension (30 mL) was poured into the holder and suction was applied to remove the water. The talc-Celite mixture was deposited as a uniform layer in the filter paper. The layer was carefully covered with a second moistened filter paper. An AP-25 prefilter disc (Millipore Corp.) was placed on the sandwiched layer, which was then autoclave sterilized.

Positively-Charged Filters

The positively-charged microporous filters used were Zeta Plus (30S, 10S, 5S) cellulose-diatomaceous earth- "charge modified" resin filters (AMF, Cuno Division, Meriden, Conn., U.S.A.). The 30S, 10S, and 5S filters have pore sizes of 2 µm, 4 µm, and 10 µm, respectively. The 47 mm glass filter holders were used without any prefilters. For larger volumes (20L RW), the 142 mm diameter stainless steel membrane filter

holder (Sartorius, GmbH., 34 Gottingen, Federal Republic of Germany) was employed.

Eluents

Substances tested for their rotavirus-eluting efficiency included the following: powdered beef extract (BE), tryptose phosphate broth (TPB), bovine albumin (BA), L-arginine (Arg) and glycine (Gly). Further information on these eluents is summarized in Table 9.

Experimental

Virus under test was added to the water sample and a 9.0 mL portion of it was immediately removed to determine the starting titre of the virus in the sample. The contaminated sample was then passed through the adsorbent under vacuum at room temperature.

The starting titre of virus in the water sample was determined by adding 1.0 mL of 10X TPB (pH 9.0) to 9.0 mL of the sample and then passing it through a 0.22 um membrane filter before plaque assay. To determine the amount of virus lost in the filtrate, a 9.0 mL volume of it was treated in the same manner as the control sample. The eluent under study was then passed through the adsorbent in order to recover the virus retained. The eluate was also membrane filtered and plaque assayed to calculate the efficiency of virus recovery.

For large volume (20L RW) contamination experiments only the 30S Zeta Plus filter was used to recover the seeded virus. Elution was carried out with 100 mL of the appropriate eluent. A second-step concentration of the eluent was required and this involved hydroextraction with polyethyleneglycol (PEG; J. T. Baker Chemical Co.). The virus-contaminated eluate was poured into a dialysis sac (2.7 cm diameter, 4.8 nm pore size; Fisher Scientific Co.) and the sac was sealed and placed in a styrofoam container. Enough PEG powder was poured into the container to completely surround the sac and it was placed at 4°C. Hydroextraction was allowed to proceed overnight. The material remaining inside the sac was resuspended in 10 mL of EBSS and plaque assayed after membrane filtration.

RECOVERY OF NATURALLY-OCCURRING ROTAVIRUS FROM SEWAGE-POLLUTED SURFACE
WATERS

Water Samples

RW samples (20L-40L) were collected in stainless steel pressure vessels at three sites along the Ottawa River (Britannia and Lemieux Is. Water Treatment Plants and Canadian International Paper Company, Gatineau, Quebec) in the winter of 1983.

Experimental

The water samples were passed through a 30S Zeta Plus filter. (without any prefiltration) held in a 142 mm diameter Sartorius filter holder.

Elution with 100 mL of eluent was followed by hydroextraction. The material remaining after hydroextraction was resuspended in 10 mL of EBSS and membrane filtered. The concentrate was stored at -70°C till further testing could be done.

Virus Detection

Concentrates were centrifuged onto MA-104 cells in slide culture and incubated for 24 hr to 48 hr. They were then stained using the immunocytochemical technique under test.

RESULTS

VIRUS RECOVERY FROM SEEDED SAMPLES OF RAW WATER

Talc-Celite Layers

A 1-L volume of conditioned RW was contaminated with a known amount of infectious human rotavirus or SA-11. A 250 mL volume was then withdrawn and passed through a 47 mm diameter talc-Celite layer. The filtrate was collected and plaque assayed. As can be seen from the data presented in Table 10, the filtrate contained 6.1% (± 1.9) of the input virus PFU. This showed that by reducing the sample pH to 6.0 and adding EBSS at a final concentration of 1:100, talc-Celite layers could adsorb the rotavirus from seeded RW samples. A 10 mL amount of 1X TPB (pH 9.0) was subsequently passed through the layer in order to elute the absorbed virus. The eluate was plaque assayed to determine the amount of infectious virus present. From Table 10 it can also be seen that the mean virus recovery obtained was 47.3% (± 7.4).

Positively-Charged Filters

In the initial experiments Zeta Plus filters 5S and 10S were tested for their capacity to concentrate rotavirus SA-11. Table 11 summarizes the results of these experiments. There was no conditioning of the RW samples required. A 1-L volume of RW was contaminated with a known amount of SA-11. A 250 mL volume was passed through a 47 mm diameter filter. The filtrate was collected and plaque assayed. The mean loss of virus in the filtrate was 22.5% (± 2.5) for the 10S filter and 31.1% (± 10.2) for the 5S filter.

TABLE 9.

RELEVANT INFORMATION ON VIRUS-ELUTING AGENTS USED IN THIS STUDY

Item	Lot No.	Manufacturer	Concentration used	pH
Bovine albumin (BA) (product No. A-7030)	62F-0510	Sigma Chemical Co.	1% in deionized water	9.0
Beef extract (BE) in powder form (Lab-Lemco)	212-12344	Oxoid	3% in deionized water	9.0
Tryptose phosphate broth (TPB)	434820	Difco	1X in deionized water	9.0 or 9.5
Arginine (Arg) (product No. A-3006)	62F-0836	Sigma Chemical Co.	3% in deionized water	9.0
Glycine (Gly) (product No. G-7126)	42F-0339	Sigma Chemical co.	3% in deionized water	9.0

pH adjustment was done with 1N NaOH
Eluting agents were autoclave sterilized except for BA and Arg.

TABLE 10.

RECOVERY OF HUMAN ROTAVIRUS (STRAIN D) ADSORBED TO
TALC-CELITE LAYERS USING 1X TRYPTOSE PHOSPHATE BROTH (pH9.0)

EXPT. NO.	INPUT VIRUS PFU/mL IN RAW WATER	PFU/mL IN FILTRATE	% PFU LOST IN FILTRATE	ELUTED VIRUS PFU/mL	% RECOVERY
1	2.0×10^4	8.0×10^2	6.0	2.1×10^5	42.0
2	2.0×10^4	1.0×10^3	5.0	2.0×10^5	40.0
3	3.0×10^2	25	8.3	3.9×10^3	32.0
4	3.0×10^2	21	7.0	6.1×10^3	55.0
Mean ± S.D.			6.1 ± 1.9		47.3 ± 7.4

The pH of 1 L of experimentally contaminated raw water sample was adjusted to 6.0, and EBSS was added to a final concentration of 1:100. A 250 mL volume was passed through a layer of talc-Celite. For elution of adsorbed virus, a 10 mL volume of TPB was passed through the layer. Virus plaque assays were performed in MA-104 monolayers.

Tables 12 and 13 present the data for the comparison of different eluents in the recovery of human rotavirus adsorbed to Zeta Plus 30S filters. The procedure was the same as that used for the 5S and 10S filters. No virus was detected in any of the filtrates. In virus elution, 1% bovine albumin (pH 9.0) and 3% beef extract (pH 9.0) gave mean recoveries of 16.7% (± 5.2) and 14.1% (± 4.1), respectively (Table 12). 1X TPB (pH 9.5) 3% glycine + 3% arginine (pH 9.0) and 6% glycine + 6% arginine (pH 9.0) yielded virus recoveries of 44.1% (± 6.4), 46.1% (± 6.2) and 46.1% (± 3.1), respectively (Table 13).

In an attempt to improve the recovery rate, sequential elution was tried. Table 14, shows the data for sequential elution of rotavirus adsorbed to 30S filter using TPB in combination with mixtures of the basic amino acids glycine and arginine. In keeping with the reports of Sobsey and Glass (1980) and Guttman-Bass and Armon (1983) no virus was detected in the filtrates of the 30S filter. 1X TPB (pH 9.5) and 3% Arg + 3% Gly (pH 9.0), used sequentially, gave virus recovery of 70.0% (± 2.6) while 1X TPB (pH 9.5) and 6% Arg + 6% Gly (pH 9.0) resulted in the recovery of 70.3% (± 1.5) of the added virus.

Table 15 summarizes the data from experiments with large (20 L) volumes of experimentally-contaminated RW concentrated with the help of 30S Zeta Plus filters. Virus elution was carried out sequentially with 50 mL of 1X TPB (pH 9.5) and 50 mL of a mixture (pH 9.0) of 6% arginine + 6%

TABLE 11.

RECOVERY OF ROTAVIRUS SA-11 ADSORBED TO
ZETA PLUS 35 AND 105 FILTERS USING 1X TRYPTOSE PHOSPHATE BROTH (pH 9.0)

EXPT. NO.	INPUT VIRUS PFU/mL (X10 ⁶)	PFU/mL IN FILTRATE (X10 ⁶)	% PFU LOST IN FILTRATE	ELUTED VIRUS PFU/mL (X10 ⁶)	% RECOVERY
105					
1	4.0	0.8	20.0	1.0	14.0
2	4.0	1.0	25.0	2.2	22.0
3	4.0	0.9	22.5	1.0	30.0
Mean ± S.D.			22.5±2.5	22.0±1.0	
35					
1	4.0	1.6	40.0	1.8	18.0
2	4.0	1.2	33.3	2.0	20.0
3	3.0	1.0	20.0	1.1	11.0
Mean ± S.D.			31.1±10.2	16.3±4.7	

A 250 mL volume of RW experimentally contaminated with SA-11 was passed through a 47 mm diameter filter. The filtrate and the eluate were plaque assayed using MA-104 cells.

TABLE 12.

COMPARISON OF BOVINE ALBUMIN AND BEEF EXTRACT AS ELUENTS IN THE RECOVERY OF
HUMAN ROTAVIRUS (STRAIN D) ADSORBED TO ZETA PLUS 305 FILTERS

EXPT. NO.	INPUT VIRUS PFU/mL (X10 ⁶)	VIRUS IN FILTRATE	1% BOVINE ALBUMIN (pH 9.0)		3% BEEF EXTRACT (pH 9.0)	
			ELUATE PFU/mL (X10 ⁶)	% RECOVERY	ELUATE PFU/mL (X10 ⁶)	% RECOVERY
1	1.3	NVD ^a	7.3	22.3	6.1	18.8
2	1.3	NVD	5.0	15.4	4.0	12.3
3	1.3	NVD	4.0	12.3	3.6	11.1
Mean	1.3	-	5.4±1.69	16.7±5.2	4.6±1.34	14.1±4.1

A 250 mL volume of RW experimentally contaminated with the virus was passed through a 47 mm diameter filter. A 10 mL amount of eluent under test was used. The filtrate and the eluates were plaque assayed in MA-104 cells.

^aNVD = No Virus Detected

TABLE 13.

COMPARISON OF TRYPTOSE PHOSPHATE BROTH AND MIXTURES OF GLYCINE AND ARGININE AS ELUENTS IN THE RECOVERY OF HUMAN ROTAVIRUS (STRAIN D) ADSORBED TO ZETA PLUS 305 FILTERS

EXPT. NO.	INPUT VIRUS PFU/mL (X10 ⁶)	VIRUS IN FILTRATE	1X TRYPTOSE PHOSPHATE BROTH (pH 9.5)		3% GLYCINE + 3% ARGININE (pH 9.0)		6% GLYCINE + 6% ARGININE (pH 9.0)	
			ELUATE PFU/mL (X10 ⁵)	% RECOVERY	ELUATE PFU/mL (X10 ⁵)	% RECOVERY	ELUATE PFU/mL (X10 ⁵)	% RECOVERY
1	1.3	NVD ²	1.2	36.9	1.3	40.0	1.3	46.1
2	1.3	NVD	1.5	46.1	1.5	46.1	1.0	33.1
3	1.3	NVD	1.6	49.2	1.7	52.3	1.6	49.2
MEAN±S.D.		-	1.4±0.21	44.1±6.0	1.5±0.2	46.1±6.2	1.3±0.1	46.1±3.1

A 250 ml. volume of RW experimentally-contaminated with the virus was passed through a 47 mm diameter filter. A 10 ml. amount of eluent under test was used. The filtrate and the eluates were plaque assayed on MA-104 cells.

²NVD = No Virus Detected

TABLE 14.

RECOVERY OF HUMAN ROTAVIRUS (STRAIN D) ADSORBED TO ZETA PLUS 305 FILTERS USING ELUENTS SEQUENTIALLY

EXPT NO.	INPUT VIRUS PFU/mL (X10 ⁶)	VIRUS IN FILTRATE PFU/mL	A. 3% Arg + 3% Gly (pH9.0) B. 1XTP3 (pH 9.5)		A. 6% Arg + Gly (pH9.0) B. 1X TPB (pH9.5)	
			ELUATE PFU/mL (X10 ⁵)	% RECOVERY	ELUATE PFU/mL (X10 ⁵)	% RECOVERY
1	4.0	NVD ²	6.8	68.0	6.9	69.0
2	4.0	NVD	7.3	73.0	7.0	70.0
3	4.0	NVD	6.9	69.0	7.2	72.0
MEAN±S.D.		-	7.0±0.26	70.0±2.6	7.0±0.15	70.3±1.5

250 ml. of RW experimentally contaminated with virus was passed through a 47 mm diameter filter. Elution was done with 10 ml. volume of eluent.

²NVD = No Virus Detected

glycine. The eluate from the filters was then subjected to second-step concentration by overnight PEG hydroextraction at 4°C. The concentrate was resuspended in 10 mL of EBSS and filter sterilized before plaque assay. Approximately 16% (± 3.04) of the input PFU could be recovered from these samples after a 2000-fold reduction in the sample volume.

TESTING OF FIELD SAMPLES FOR NATURALLY-OCCURRING ROTAVIRUSES

RW samples were taken from the Ottawa River at Britannia, Lemieux Island and Gatineau (CIP) during December, 1983. The samples were passed through a 30S Zeta Plus filter held in a 142 mm diameter Sartorius filter holder. Sequential virus elution was done with 50 mL of 1X TPB (pH 9.5) and 50 mL of 6% Arg + Gly (pH 9.0). The eluate was hydro-extracted in PEG overnight then resuspended in 10 mL of EBSS and membrane filtered.

MA-104 monolayers in slide chambers were inoculated with the sample concentrates and the cultures centrifuged (1000 Xg). Maintenance medium (without FCS) with trypsin (5 ug/mL) was added to the cultures and they were incubated for 24 hrs at 37°C. They were fixed and stained by the IF and IP techniques using mouse monoclonal antibodies against human rotavirus. Table 16 summarizes the data for immunoperoxidase focus forming units which ranged from 19.5 to 40.8/L while the sample concentration factor varied between 2000- and 4000- fold. Figures 19 (IF) and 20 (IP) show the immunocytochemical demonstration of rotaviruses concentrated from Ottawa River RW sampled at Gatineau (CIP).

TABLE 15.

RECOVERY OF HUMAN ROTAVIRUS (STRAIN D) FROM 20-LITRE VOLUMES OF SEEDED RAW WATER FROM THE OTTAWA RIVER

EXPT. NO.	INPUT VIRUS PFU/mL (x 10 ⁶)	VIRUS IN FILTRATE PFU/mL	CONC. FACTOR	ELUTED VIRUS PFU/mL (x 10 ⁷)	% RECOVERY
1	2.5	NVD ^a	2000X	1.0	20.0
2	2.5	NVD	2000X	0.52	14.0
3	2.5	NVD	2000X	0.81	16.2
Mean±S.D.	2.5	-	2000X	0.77±0.29	16.3±3.4

20L of RW was experimentally contaminated with human rotavirus and passed through a 305 Zeta Plus filter (142mm diameter). Eluant was 50 ml 1 X TPB (pH 9.3) and 50 ml 6% Arg 6% Gly (pH 9.0).

^aNVD, No Virus Detected.

TABLE 16.

ZETA PLUS (305) FILTERS IN THE DETECTION OF NATURALLY-OCCURRING ROTAVIRUSES IN FIELD SAMPLES OF RAW WATER FROM THE OTTAWA RIVER

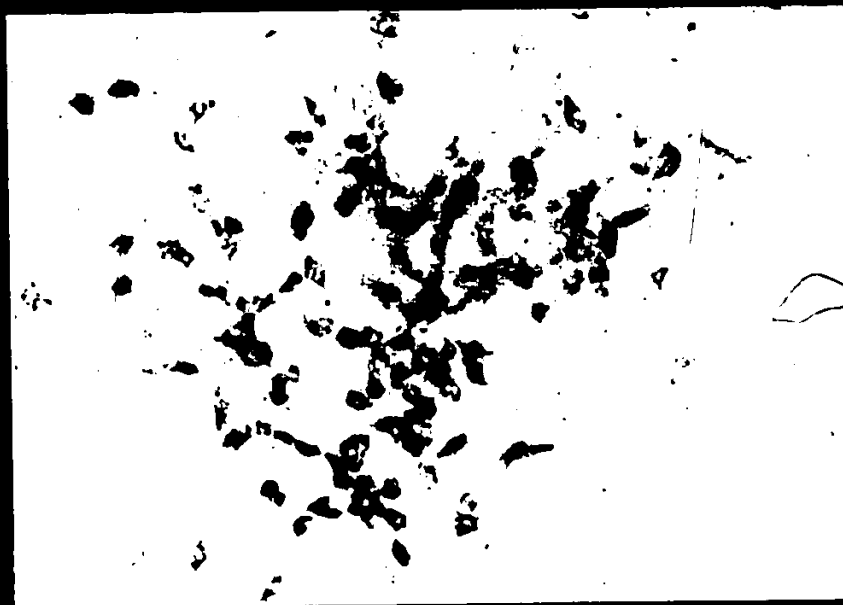
SITE	COLLECTION DATE (1983)	VOLUME CONC. (L)	CONC. FACTOR	IMMUNOPEROXIDASE FOCUS FORMING UNITS/L
Britania	Dec. 13	20	2000X	19.5
Lemieux Is	Dec. 14	40	4000X	22.0
Gatineau (CIP)	Dec. 15	35	3500X	34.9
Lemieux Is	Dec. 19	40	4000X	27.8
Gatineau (CIP)	Dec. 20	40	4000X	40.8

The samples were passed through a 142mm diameter filter under pressure. Virus elution was carried out by passing through the filter 50 mL of tryptose phosphate broth (pH 9.3) and then 50 mL of a mixture (pH 9.0) of 6% argen line and 6% glycine. The eluate was further concentrated by overnight hydroextraction at 4°C. the concentrate was resuspended in 10 mL of EBSS and filter sterilized before inoculation into MA-104 cultures.

Fig 19. Immunofluorescence Staining of MA-104 Cells
Infected with Rotavirus from the Ottawa
River Raw Water. (X 220)

Fig 20. Immunoperoxidase Staining of MA-104 Cells
Infected with Rotavirus from the Ottawa
River Raw Water. (X 220)

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DISCUSSION

RECOVERY OF ROTAVIRUSES FROM SEEDED SAMPLES OF RAW WATER

Earlier investigations have shown the suitability of the talc-Celite technique (Sattar and Ramia, 1979a, 1979b) and PEG hydroextraction (Ramia and Sattar, 1979b) from seeded samples of potable waters. In the talc-Celite method, the preconditioning of the water sample is necessary to maximize virus adsorption to the layer. However, when this technique was tested with seeded samples of RW the mean recovery of rotavirus was only 47.3%. This is much lower than the recovery of nearly 90% of rotavirus from seeded samples of potable water in previous experiments (Ramia and Sattar, 1980). The presence of a wide variety of organic and inorganic material in raw water may interfere with rotavirus retention as well as elution from the layer. The preconditioning requirement and the virus lost in the filtrate are two encumbrances of this technique not associated with the use of the positively-charged Zeta Plus 30S filters.

The 5S and 10S Zeta Plus filters were unable to retain all of the seeded virus and recovery of rotavirus from them was very low. Sobsey and Glass (1980), Guttman-Bass and Armon (1983) and Toranzos and Gerba (1983) using 50S and 60S Zeta Plus filters achieved greater than 99.99% adsorption of the seeded virus from tap water and secondarily treated sewage at ambient pH. In our study, using raw water, the 30S filter showed complete adsorption of the seeded rotavirus.

Guttman-Bass and Armon (1983) and Toranzos and Gerba (1983) tested different eluents and found that 3% beef extract (pH 9.0) and 3% beef extract in 0.05M glycine (pH 9.5) to be the most efficient eluents. In this study 3% beef extract (pH 9.0) was found to be a poor eluent of rotavirus adsorbed from RW. The highest recovery (65%) in the experiments of Guttman-Bass and Armon (1983) occurred when they recycled the eluted material through the filter a second time. In our experiments recycling the TPB gave a recovery of 46%. Using the amino acids glycine and arginine as eluents, the recovery of rotavirus was similar to that obtained for TPB. In order to increase the recovery, sequential elution was tried (TPB; Arg+Gly) and it brought the mean virus recovery to 70%.

In the use of 30S Zeta Plus filters in large volume concentration experiments, there was a noticeable reduction in the mean recovery of rotavirus (16%). Virus loss could have occurred during the membrane filtration step, elution or hydroextraction. It is unlikely that there was a reduced adsorption efficiency because no virus was detected in the filtrates. Inefficient elution is a more likely possibility. The build-up of layers of material on the filter may prevent access of the eluent to the adsorbed virus (Guttman-Bass and Armon, 1983). Another possibility is that the accumulation of organic matter of certain types on the filters may lead to rotavirus inactivation.

Rao and Melnick (1983), using the Viradel method and magnetic iron oxide adsorption as a second-step concentration method, were able to efficiently concentrate rotavirus from seeded samples of estuarine water. Toranzos and Gerba (1983) found that the Virosorb 1-MDS filters

(positively-charged) exhibited higher rates of recovery of seeded rotavirus from tap water and wastewater than the Viradel or 50S Zeta Plus filters. Melnick et al. (1984) completed a round robin investigation of methods for the recovery of poliovirus from drinking water. The Viradel-organic flocculation procedure was recommended as a provisional method for the recovery of human enteroviruses from drinking water although the recovery ranged from 8% to 198%. Other methods tested included magnetic iron oxide, Virozorb filters in combination with membrane filters and membrane disks alone. The average virus recoveries for the four methods were 36, 20, 0.4 and 5%, respectively. The Viradel method does have some major disadvantages which are the need for the reduction of the input water pH to 3.5, the addition of aluminum chloride to the water, and the sensitivity of the filters to interference by contaminants in the water (Sobsey and Glass, 1980).

DETECTION AND QUANTITATION OF WATERBORNE ROTAVIRUSES

Using 30S filter concentration of RW combined with immunocytochemical methods, we were able to detect and quantitate naturally-occurring rotaviruses in RW from the Ottawa River. The number of IP focus forming units ranged from 19.5 to 40.8/L. Rao and Melnick (1984), used Viradel, magnetic iron oxide absorption in second-step concentration and IF focus counts to detect 119 to 4980 IF rotavirus foci/100 gallons of estuarine water from Galveston Bay, Texas (1.2 to 49.8/4:2L). Deetz et al. (1984) detected rotaviruses in raw and drinking water samples using the Viradel method. They recovered from 26 to 210 IF foci/20L. In our experiments, the numbers are higher than those for Rao and Melnick (1984) and Deetz

et al. (1984) but this may be due to either higher levels of sewage-pollution in the Ottawa River or the higher rotavirus recovering efficiency of the sample concentration methodology used in this study.

The 24 hr incubation of concentrates in cell culture prior to immunocytochemical staining was necessary. When 14-15 hr incubation periods were tried, no rotavirus antigens could be detected in the inoculated cultures. The concentrates were not cytotoxic to the cells in culture. Centrifugation of the concentrates onto monolayers of MA-104 cells was done to enhance adsorption of the viruses to the cells. Inoculated cultures were incubated in the presence of trypsin (and the absence of FCS) to promote virus focus formation. Substances present in concentrates of field samples are known to interfere with the detection of enteroviruses (Sattar, 1979). No information is as yet available on their effects on rotaviruses.

Larger sample volumes, more samples at other sites along the Ottawa River and sampling at other times during the year are needed to give a better picture of the presence of waterborne rotaviruses in the Ottawa River. As yet no reports exist of gastroenteritis due to waterborne rotaviruses in the Ottawa River.

GENERAL DISCUSSION AND CONCLUDING REMARKS

There were 4 main objectives in this study. (1) the investigation of rotavirus survival in raw and treated water, (2) the comparison of the suitability of IF and IP techniques for the detection and quantitation of rotaviruses in concentrates of water samples, (3) the testing of the talc-Celite technique and positively-charged Zeta Plus filters in the concentration of rotaviruses from seeded samples of sewage-polluted surface waters, (4) to examine field samples of sewage-polluted surface waters for naturally-occurring rotaviruses.

The short-term virus survival experiments demonstrate the resistance of rotavirus to levels of chlorine found in the terminal stages of water treatment and emphasizes the possible danger to human health of post-treatment contamination of a water system with sewage. The ability of rotaviruses to survive in the surface water environment on a long-term basis has a bearing on the quality of recreational waters as well as those used for the cultivation of shell-fish and the irrigation of certain types of crops. However, no definitive data are as yet available to indicate the level of risk to human health that such rotaviral pollution may represent. The positively-charged 30S Zeta Plus filter and the talc-Celite technique show potential for further study in the recovery of indigenous rotaviruses from raw water. In this study a limited number of field samples (5) of river water was tested and found to contain naturally-occurring rotaviruses. Whether they are of animal or human origin is not known.

The methodology developed in this study will be helpful in the (a) detection of rotaviruses in waters incriminated in outbreaks of non-bacterial gastroenteritis, (b) assessment of rotavirus levels and survival in waters used for recreational purposes and drinking, (c) determination of the rotavirus eliminating efficiency of wastewater and drinking water treatment systems and (d) the development of virus standards for recreational and potable waters.

APPENDIX I

IMMUNOCYTOCHEMICAL TECHNIQUES

Immunofluorescence Staining

- (1) Pour off liquid medium into ethanol, remove chamber from slide
- (2) PBS bath-2 min, 5 min
- (3) Acetone fixative-30 sec, 8 min
- (4) PBS bath-2 min, 5 min
- (5) Primary antiserum-30 min, room temp
- (6) PBS bath-2 min, 5 min
- (7) FITC-conjugated antiserum (or Pr.A-FITC) - 30 min, room temp.
- (8) Distilled water-5 min

Immunoperoxidase Staining

- (1)-(2) Same as IF staining steps
- (3) Acetone/Methanol fixative (1:1, H₂O₂ drops)-30 sec, 8 min
- (4) PBS bath-2 min, 5 min
- (5) Primary Antiserum-30 min, room temp
- (6) PBS bath-2 min, 5 min
- (7) Peroxidase-conjugated antiserum (or Pr:A-peroxidase)-30 min, room temp
- (8) PBS bath-2 min, 5 min
- (9) Reaction substrate-15 min, room temp

(10) PBS bath-2 min, 5 min

(11) Distilled water-5 min

Reaction Substrate

2 mg AEC/0.5 mL dimethylformamide

9.5 mL sodium acetate buffer (pH 5.0)

0.1 mL 3% hydrogen peroxide

Sodium acetate buffer (pH 5.0)

sodium acetate 2.72 gm

succinic acid 0.54 gm

merthiolate 0.10 gm

distilled water 500 mL

Foci Counting

The microscope used was a Reichert Binolux model. The microscope has a custom built stage with a grid which permits the counting of foci in 50 randomly distributed microscope fields in a 20 mm X 20 mm square. In this area, at 100X magnification, there are 316 such fields. This is a modification of the system used by Sattar and Westwood (1967). An average count of 50 fields was multiplied by the total number of fields to get the total number of foci per grid (per 0.1 mL of inoculum).

REFERENCES

- Adams, W.R. and Kraft L.M. 1967. Electron microscope study of the intestinal epithelium of mice infected with the agent of epizootic diarrhea of infant mice (EDIM) virus, *Am. J. Pathol.* 51: 39-60.
- Altenburg, B.C., Graham, D.Y. and Estes, M.K. 1979. Ultrastructural immunocytochemistry of rotavirus infected cells. In: Proc 37th annual meeting of the Electron Microscopy Society of America. p. 40-41.
- Babiuk, L.A., Mohammed, K.A., Spence, L., Fauvel, M. and Petro, R. 1977. Rotavirus isolation and cultivation in the presence of trypsin. *J. Clin. Microbiol.* 6:610-617.
- Banatvala, J.E., Totterdel, B., Christie, I.L. and Woode, G.N. 1975. In-vitro detection of human rotaviruses. *Lancet* 1:821.
- Banatvala, J.E. and Chrystie, I.L. 1978. Rotaviral infections in human neonates. *J. Am. Vet. Med. Assoc.* 173:527-530.
- Banfield, W.G., Kasnic, G. and Blackwell, J.H. 1968. Further observations on the virus of epizootic diarrhea of infant mice: an electron microscopic study. *Virology* 36:411-421.
- Barnett, B.B., Egbert, L.N. and Spendlove, R.S. 1978. Characteristics of neonatal calf diarrhea virus ribonucleic acid. *Can. J. Comp. Med.* 42:46-53.
- Barrio, R.V. and O'Brien, R.T. 1984. Inactivation of rotavirus SA-11 by chlorine, chlorine dioxide and iodine. Abste. No. Q106. Ann. Meeting Am. Soc. Microbiol., St. Louis, Mo. p. 222.
- Bastardo, J.W. and Holmes, I.H. 1980. Attachment of SA-11 rotavirus to erythrocyte receptors. *Infect. Immun.* 29:1134-1140.
- Benjamin, D.R. 1977. Use of immunoperoxidase for rapid diagnosis of mucocutaneous herpes simplex virus infection. *J. Clin. Microbiol.* 6:571-573.
- Bican, P., Cohen, J., Charpilienne, A. and Scherrer, R. 1982. Purification and characterization of bovine rotavirus cores. *J. Virol.* 43:113-117.
- Birch, C.J., Rodger, S.M., Marshall, J.A. and Gust, I.D. 1983. Replication of human rotavirus in cell culture. *J. Med. Virol.* 11:241-250.
- Bishai, F.R., Blaskovic, P. and Goodwin, D. 1978. Physicochemical properties of Nebraska calf diarrhea virus hemagglutinin. *Can. J. Microbiol.* 24:1425-1430.
- Bishop, R.F., Davidson, G.P., Holmes, I.H. and Ruck, B.J. 1973. Virus particles in the epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. *Lancet* II:1281-1283.

- Bishop, R.F., Davidson, G.P., Holmes, I.H. and Ruck, B.J. 1974. Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. *Lancet* 1:149-151.
- Bishop, R.F., Cameron, D.J.S., Veenstra, A.A. and Barnes, G.L. 1979. Diarrhea and rotavirus associated with differing regimens for postnatal care of newborn babies. *J. Clin. Microbiol.* 9:525-529.
- Bitton, G. and Mitchell, R. 1974. Removal of Escherichia coli bacteriophage T7 by magnetic filtration. *Water Res.* 8:549-551.
- Black, R.E., Huq, I., Merson, M.H., Rahman, A.S.M.M., Yunus, M., Alim, A.R.M.A., Huq, I., Yolken, R.H. and Curlin, G.T. 1980. A two-year study of bacterial, viral, and parasitic agents associated with diarrhea in rural Bangladesh. *J. Infect. Dis.* 142:660-664.
- Black, R.E., Huq, I., Merson, M.H., Alim, A.R.M.A. and Yunus, M. 1981. Incidence and severity of rotavirus and Escherichia coli diarrhea in rural Bangladesh. *Lancet* 1:141-143.
- Blacklow, N.R. and Cukor, G. 1981. Viral gastroenteritis. *N. Engl. J. Med.* 304:397-406.
- Bolivar, R., Conklin, R.H., Vollet, J.J., Pickering, L.K., DuPont, H.L., Walters, D.L. and Kohl, S. 1978. Rotavirus in travelers' diarrhea: study of an adult student population in Mexico. *J. Infect. Dis.* 137:324-327.
- von Bonsdorff, C.H., Hovi, T., Makela, A.P., Hovi, L. and Tevalvoto-Aarnio, M. 1976. Rotavirus associated with acute gastroenteritis in adults. *Lancet* 2:423.
- Brade, L. and Schmidt, W.A.K. 1979. Simian rotavirus (SA-11) in serodiagnosis of human infections. *Med. Microbiol. Immunol.* 167:55-60.
- Brade, L., Schmidt, W.A.K. and Gattert, I. 1981. Zur relativen Wirksamkeit von Desinfektion-mitteln gegenüber Rotaviren. *Zentralbl. Bakteriol. (Orig B)* 174:151-159.
- Bridger, J.C. and Woode, G.N. 1976. Characterization of two particle types of calf rotavirus. *J. Gen. Virol.* 31:245-250.
- Butler, M. and Harakeh, M.S. 1982. Inactivation of rotavirus in wastewater effluents by chemical disinfection. In Viruses and Disinfection of Water and Wastewater. Butler, M., Medlen, A.R. and Morris, R. (eds.). University of Surrey Press, Guilford, U.K.
- Carleson, J.A.K., Middleton, P.J., Szymanski, M.T., Huber, J. and Petric, M. 1978. Fatal gastroenteritis: an analysis of 21 cases. *Am. J. Dis. Child.* 132:477-479.
- Cevenini, R., Rumpianesi, F., Mazzarcchio, R., Donati, M., Falcieri, E. and Sarov, I. 1984. A simple immunoperoxidase method for detecting enteric adenovirus and rotavirus in cell culture. *J. Infect.* 8:22-27.

Chanock, R.M. 1981. Strategy for development of respiratory and gastrointestinal tract viral vaccines in the 1980's. *J. Infect. Dis.* 143:364-374.

Chasey, D. 1977. Different particle types in tissue culture and intestinal epithelium infected with rotavirus. *J. Gen. Virol.* 37:443-451.

Chasey, D. 1980. Investigations of immunoperoxidase-labeled rotavirus in tissue culture by light and electron microscopy. *J. Gen. Virol.* 50:195-200.

Cheever, F.S. and Mueller, J.H. 1947. Epidemic diarrheal disease of suckling mice. I. Manifestation, epidemiology and attempts to transmit the disease. *J. Exp. Med.* 85:405-416.

Chrystie, I.L., Totterdel, B.M. and Banatvala, J.E. 1978. A symptomatic endemic rotavirus infections in the newborn. *Lancet* II:79.

Chrystie, I.L., Totterdel, B.M. and Banatvala, J.E. 1978. A symptomatic endemic rotavirus infections in the newborn. *Lancet* II:1176-1178.

Clark, S.M., Rothe, J.R., Clark, M.L., Barnett, B.B. and Spendlove, R.S. 1981. Trypsin enhancement of rotavirus infectivity: mechanisms of enhancement. *J. Virol.* 39:816-822.

Cohen, J. 1977. Ribonucleic acid polymerase activity associated with purified calf rotavirus. *J. Gen. Virol.* 36:395-402.

Cohen, J., Laporte, J., Charpilienne, A. and Scherrer, R. 1979. Activation of rotavirus RNA polymerase by calcium chelation. *Arch. Virol.* 60:177-186.

Craun, G.F. 1979a. Waterborne disease outbreaks in the United States. *J. Environ. Health* 41:259-265.

Craun, G.F. 1979b. Disease outbreaks caused by drinking water. *J. Water Pollut. Control Fed.* 51:1751-1760.

Crewe, E. and Murphy, A.M. 1980. Further studies on neonatal rotavirus infections. *Med. J. Australia* 1:61-63.

Cubitt, N.D. and Holzel, H. 1980. An outbreak of rotavirus infection in a long-stay ward of geriatric hospital. *J. Clin. Pathol.* 33:306-308.

Davidson, G.P., Bishop, R.F., Townley, R.R., Holmes, I.H. and Ruck, B.J. 1975. Importance of a new virus in acute sporadic enteritis in children. *Lancet* I:242-245.

Deetz, T., Smith, E.M., Vollet, J., Goyal, S. and Dupont, H.L. 1981. Recovery of rotavirus and enterovirus in tap and environmental waters in Mexico. Abstr. No. Q49, Ann. Meeting of the Am. Soc. Microbiol., Dallas, Texas, p. 208.

Deetz, T.R., Smith, E.M., Goyal, S.M., Gerba, C.P., Vollet, J.J., Tsai, L., DuPont, H.L. and Keswick, B.H. 1984. Occurrence of rota- and enteroviruses in drinking and environmental water in a developing nation. *Water Res.* 18:567-571.

Desmond, E.P., Schmidt, N.J. and Lennette, E.H. 1979. Immunoperoxidase staining for detection of Colorado Tick fever virus and a study of congenital infection in the mouse. *Am. J. Trop. Med. Hyg.* 28:729-732.

Drozdov, S.G. Shekoyan, L.A., Korolev, M.B. and Andzhaparidze, A.G. 1979. Human rotavirus in cell culture: isolation and passaging. *Vopr. Virusol.* 4:389-392.

Esparza, J. and Gil, F. 1978. A study on the ultrastructure of human rotavirus. *Virology* 91:141-150.

Espejo, R.T., Lopez, S. and Arias, C. 1981. Structural polypeptides of simian rotavirus SA-11 and the effect of trypsin. *J. Virol.* 37:156-160.

Estes, M.K., Graham, D.Y., Smith, E.M. and Gerba, C.P. 1979. Rotavirus stability and inactivation. *J. Gen. Virol.* 43:187-192.

Estes, M.K., Palmer, E.L. and Obijeski, J.F. 1983. Rotaviruses: a review. *Current Topics in Microbiology* 105:123-184.

Falini, B. and Taylor, C.R. 1983. New developments in immunoperoxidase techniques and their application. *Arch. Pathol. Lab. Med.* 107:105-117.

Farmilo, A.J. 1983. Peroxidase substrates gain in popularity in pathology lab. *Canadian Clinical Laboratory* 2:33.

Farr, A.G. and Nakane, P.K. 1981. Immunohistochemistry with enzyme-labeled antibodies: a brief review. *J. Immunol. Methods* 47:129-144.

Farrar, S.R., Goyal, S.M., Gerba, C.P., Conklin, R.H. and Smith, E.M. 1978. Comparison between adsorption of poliovirus and rotavirus by aluminum hydroxide and activated sludge flocs. *Appl. and Environ. Microbiol.* 35:360-363.

Farrar, S.R., Gerba, C.P., Wallis, C. and Melnick, J.L. 1976. Concentration of viruses from large volumes of tap water using pleated membrane filters. *Appl. Environ. Microbiol.* 31:221-226.

Flewett, T.H., Bryden, A.S. and Davies, H. 1973. Virus particles in gastroenteritis. *Lancet* II:1497.

Flewett, T.H., Bryden, A.S., Davies, H., Woode, G.N., Bridger, J.C. and Derrick, J.M. 1974. Relation between viruses from acute gastroenteritis of children and newborn calves. *Lancet* II:61-63.

Flewett, T.H. and Woode, G.N. 1978. The rotaviruses. Brief review. *Arch. Virol.* 57:1-23.

Forsgren, A. and Sjoquist, J. 1966. Protein A from Staphylococcus aureus. J. Immunol. 97:822-827.

Foster, S.O., Palmer, E.L., Gary, G.W., Martin, M.L., Herrmann, K.L., Beasley, P. and Sampson, J. 1980. Gastroenteritis due to rotavirus in an isolated Pacific island group: an epidemic of 3,439 cases. J. Infect. Dis. 141:32-39.

Gaul, S.K., Simpson, T.F., Woode, G.N. and Fulton, R.W. 1982. Antigenic relationships among some animal rotaviruses: virus neutralization in vitro and cross-protection in piglets. J. Clin. Microbiol. 16:495-503.

Gerba, C.P., Keswick, B.H., DuPont, H.L. and Fields, H.A. 1983. Isolation of rotavirus and hepatitis A from drinking water. In Proc. International Symposium on Enteric Viruses in Water. Melnick, J.L. and Karger, S. (eds.). New York.

Gerba, C.P., Singh, S.N. and Rose, J.B. 1984. Waterborne gastroenteritis and viral hepatitis. personal communication.

Ghose, L.H., Schnagl, R.D. and Holmes, I.H. 1978. Comparison of an enzyme-linked immunosorbent assay for quantitation of rotavirus antibodies with complement fixation in an epidemiological survey. J. Clin. Microbiol. 8:268-276.

Goding, J.W. 1978. Use of staphylococcal protein A as an immunological reagent. J. Immunol. Methods 20:241-253.

Goldwater, P.N., Chrystie, I.L. and Banatvala, J.E. 1979. Rotaviruses and the respiratory tract. Br. Med. J. 4:1551.

Gordon, A.G. 1982. Rotavirus infection and the SOMPE syndrome. J. Inf. Dis. 146:117-118.

Goyal, S.M. and Gerba, C.P. 1979. Comparative adsorption of human enteroviruses, simian rotavirus and selected bacteriophage of soils. Appl. Environ. Microbiol. 38:241-247.

Goyal, S.M. and Gerba, C.P. 1983. Viradel method for detection of rotavirus from sea water. J. Virol. Meth. 7:279-285.

Grabow, W.O.K., Gauss-muller, V., Prozesky, O.W. and Deinhardt, F. 1983. Inactivation of hepatitis A virus and indicator organisms in water by free chlorine residuals. Appl. Environ. Microbiol. 46:619-624.

Graham, D.Y. and Estes, M.K. 1979. Comparison of methods for immunocytochemical detection of rotavirus infections. Infect. Immun. 26:686-689.

Graham, D.Y. and Estes, M.K. 1980. Proteolytic enhancement of rotavirus infectivity: biologic mechanisms. Virology 101:432-439.

Graubelle, P.C., Vestergaard, B.F., Meyling, A. and Genner, J. 1981. Optimized enzyme-linked immunosorbent assay for detection of human and bovine rotavirus in stools: comparison with electron-microscopy, immunoelectro-osmophoresis, and fluorescent antibody techniques. *J. Med. Virol.* 7:29-40.

Gurwith, M., Wenman, W., Hindle, D., Feltham, S. and Greenberg, H. 1981. A prospective study of rotavirus infection in infants and young children. *J. Inf. Dis.* 144:218-224.

Guttman-Bass, N. and Armon, R. 1983. Concentration of simian rotavirus SA-11 from tap water by membrane filtration and organic flocculation. *Appl. Environ. Microbiol.* 45:850-855.

Halvorsrud, J. and Orstavik, I. 1980. An epidemic of rotavirus-associated gastroenteritis in a nursing home for the elderly. *Scand. J. Infect. Dis.* 12:161-164.

Hancock, K., Gary, G.W. and Palmer, E.L. 1983. Adaptation of two avian rotaviruses to mammalian cells and characterization by hemmagglutination and RNA electrophoresis. *J. Gen. Virol.* 64:853-861.

Hara, M., Mukoyama, J., Tsuruhara, T., Ashiura, Y., Saito, Y. and Tagaya, I. 1978. Acute gastroenteritis among school children associated with reovirus-like agent. *Am. J. Epidemiol.* 107:161-169.

Harris, J.R., Cohen, M.L. and Lippy, E.C. 1983. Water-related disease outbreaks in the United States - 1981. *J. Inf. Dis.* 148:759-762.

Hejkal, T.W., Keswick, B.H., LaBelle, R.L., Gerba, C.P., Sanchez, Y., Dreesman, G. and Hafkin, B. 1982. Viruses in a community water supply associated with an outbreak of gastroenteritis and infectious hepatitis. *J. Am. Water Works Assn.* 74:318-324.

Hejkal, T.W., Smith, E.M. and Gerba, C.P. 1984. Seasonal occurrence of rotavirus in sewage. *Appl. Environ. Microbiol.* 47:588-590.

Hildreth, C., Thomas, M. and Ridgway, G.L. 1981. Rotavirus infection in an obstetric unit. *Br. Med. J.* 282:231.

Holmes, I.H., Mathan, M., Bhat, P., Albert, M.J., Swaminathan, S.P., Maiya, P.P., Pereira, S.M. and Baker, S.J. 1974. Orbiviruses and gastroenteritis. *Lancet* II:658-659.

Holmes, I.H. 1979. Viral gastroenteritis. *Prog. Med. Virol.* 25:1-36.

Holzel, H., Cubitt, D.W., McSwiggan, D.A., Sanderson, P.J. and Church, J. 1980. An outbreak of rotavirus infection among adults in a cardiology ward. *J. Infect.* 2:33-37.

Hopley, J.F.A. and Doane, F.W. 1983. Comparison of immunoassays for the detection of rotavirus in cell culture. Abstract No. RA-31. Conjoint Meeting on Infectious Diseases, Montreal, Quebec. November 29 - December 2.

- Hoshino, Y., Baldwin, C.A. and Scott, F.W. 1981. Isolation and characterization of feline rotavirus. *J. Gen. Virol.* 54:313-323.
- Hrdy, D., Rosen, L. and Fields, B.N. 1979. Polymorphism of the migration of double-stranded RNA genome segments of reovirus isolated from humans, cattle and mice. *J. Virol.* 31:104-123.
- Hurst, C.J. and Gerba, C.P. 1980. Stability of simian rotavirus in fresh and estuarine water. *Appl. Environ. Microbiol.* 39:1-5.
- Hurst, C.J., Gerba, C.P. and Cech, I. 1980. Effects of environmental variables and soil characteristics on virus survival in soil. *Appl. Environ. Microbiol.* 40:1067-1079.
- Johnson, G.D. and de Nogueira-Araujo, G.M. 1981. A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods* 43:349-350.
- Kalica, A.R., Garon, C.F., Wyatt, R.G., Mebus, C.A., Van Kirk, D.H., Chanock, R.M. and Kapikian, A.Z. 1976. Differentiation of human and calf reovirus-like agents associated with diarrhea using polyacrylamide gel electrophoresis of RNA. *Virology* 74:86-92.
- Kapikian, A.Z., Kim, H.W., Wyatt, R.G., Rodriguez, W.J., Ross, S., Cline, W.L., Parrott, R.H. and Chanock, R.M. 1974. Reovirus-like agent in stools: association with infantile diarrhea and development of serologic tests. *Science* 185:1049-1053.
- Kapikian, A.Z., Kim, H.W., Wyatt, R.G., Cline, W.L., Arrobia, J.O., Brandt, C.D., Rodriguez, W.J., Sack, D.A., Chanock, R.M. and Parrott, R.H. 1976. Human reovirus-like agent as the major pathogen associated with winter gastroenteritis in hospitalized infants and gastroenteritis in hospitalized infants and young children. *N. Engl. J. Med.* 244:965-972.
- Kapikian, A.Z., Yolken, R.H., Greenberg, H.B., Wyatt, R.G., Kalica, A.R., Chanock, R.M. and Kim, H.W. 1979. Gastroenteritis viruses. In, Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette and Schmidt (eds.). *Am. Publ. Hlth. Associ.* pp.927-995.
- Kapikian, A.Z., Greenberg, H.B., Kalica, A.R., Wyatt, R.G., Kim, H.W., Brandt, C.D., Rodriguez, W.J., Floreo, J., Singh, N., Parrott, R.H. and Chanock, R.M. 1981. New developments in viral gastroenteritis. In, Acute enteric infections in children. New prospects for the treatment and prevention. Holme, T., Holmgren, J., Merson, M.H. and Mollby, R. (eds.). Elsevier/North-Holland Biomedical, Amsterdam, pp. 9-57.
- Kapikian, A.Z., Wyatt, R.G., Levine, M.M., Yolken, R.H., Van Kirk, D., Dolin, R., Greenberg, H.B. and Chanock, R.M. 1983. Oral administration of human rotavirus to volunteers: Induction of illness and correlates of resistance. *J. Inf. Dis.* 147:95-106.
- Keswick, B.H. and Gerba, C.P. 1980. Viruses in groundwater. *Environ. Sci. Tech.* 14:1290-1297.

Keswick, B.H. 1983. Survival of enteric viruses absorbed on electropositive filters. *Appl. Environ. Microbiol.* 46:501-502.

Keswick, B.H., Pickering, L.K., DuPont, H.L. and Woodward, W.E. 1983. Survival and detection of rotaviruses on environmental surfaces in daycare centers. *Appl. Environ. Microbiol.* 46:813-816.

Keswick, B.H., Gerba, C.P., Rose, J.B. and Toranzos, G.A. 1984. Detection of rotavirus in treated drinking water. In *Proc. International Association for Water Pollution Research and Control*. Elsevier/North Holland, Amsterdam.

Konno, T., Kutsuzawa, T., Imai, A., Katsushima, N., Sakamoto, M., Kitoaka, S., Tsuboi, R. and Adachi, M. 1978. Human rotavirus infection in infants and young children with intussusception. *J. Med. Virol.* 2:265-269.

Kraft, L.M. 1957. Studies on the aetiology and transmission of epidemic diarrhoea of infant mice. *J. Exp. Med.* 101:743-755.

La Bonardiére, C., Cohen, J., Contrepois, M., De Vaureix, C., Laude, H. and Scherrer, R. 1980. Interferon-like activity in rotavirus infected newborn calves. In *Proc. Third International symposium on Neonatal Diarrhoea*. Saskatoon, Sask.; Acres, S.D., Forman, A.J. and Fast, H.H. (eds.). pp.149-160.

Lam, S.K. 1982. National overview on the incidence of major viral diseases in Malaysia. In *Viral Diseases in South-East Asia and the Western Pacific*. MacKenzie, J.S. (ed.), Academic Press, Toronto, Ontario. pp. 224-228.

Lancet editorial. 1979. Rotavirus syndrome. *Lancet* II:186.

Lewis, H.M., Parry, J.V., Davies, H.A., Parry, R.P., Mott, A., Dourmashkin, R.R., Sanderson, P.J., Tyrrell, D.A. and Valman, H.B. 1979. A year's experience of the rotavirus syndrome and its association with respiratory illness. *Arch. Dis. Child.* 54:339-346.

Light, J.S. and Hodes, H.L. 1943. Studies on epidemic diarrhoea of the newborn: isolation of a filtrable agent causing diarrhoea in calves. *Am. J. Public Health.* 33:1451-1454.

Light, J.S. and Hodes, H.L. 1949. Isolation from cases of infantile diarrhoea of a filtrable agent causing diarrhoea in calves. *J. Exp. Med.* 90:113-135.

Linhares, A.C., Pinheiro, F.P., Freitas, R.B., Gabbay, Y.B., Shirley, J.A. and Beards, G.M. 1981. An outbreak of rotavirus diarrhoea among a non-immune, isolated South American Indian community. *Am. J. Epidemiol.* 113:703-710.

Loh, P.C., Fujoika, R.S. and Lau, R.S. 1979. Recovery, survival and dissemination of human enteric viruses in ocean waters receiving sewage in Hawaii. *Water, Air, and Soil Pollution.* 12:197-217.

- Lourenco, M.H., Nicolas, J.C., Cohen, J., Scherrer, R. and Bricout, F. 1981. Study of human rotavirus genome by electrophoresis: attempt of classification among strains isolated in France. *Ann. Virol.* 32:161-173.
- Lyke, E., Blomberg, J., Berg, G., Eriksen, A. and Madsen, L. 1978. Epidemic acute diarrhea in adults associated with infantile gastroenteritis virus. *Lancet* 2:1056-1057.
- Magnusson, S., Hedstrom, C.E. and Lycke, E. 1966. The virus inactivating capacity of sea water. *Acta Path. Microbiol. Scand.* 66:551-559.
- Magnusson, S., Gundersen, K., Brandenberg, A. and Lycke, E. 1967. Marine bacteria and their possible relation to the virus inactivating capacity of sea water. *Acta Path. Microbiol. Scand.* 71:274-280.
- Malherbe, H.H. and Strickland-Cholmley, M. 1967. Simian virus SA-11 and the related "O" agent. *Arch. Gesamte Virusforsch* 22:235-245.
- Marcollet, M., Morin, J. and Lecher, P. 1980. Comparison between two chromogenic substrates for revealing an immunoperoxidase reaction of human metaphase chromosomes. *Stain Tech.* 55:35-38.
- Marrie, T.J., Lee, S.H.S., Faulkner, R.S., Ethier, J. and Young, C.H. 1982. Rotavirus infection in a geriatric population. *Arch. Intern. Med.* 142:313-316.
- Matsuno, S., Inouye, S. and Konno, R. 1977. Plaque assay of neonatal calf diarrhea virus and the neutralizing antibody in human sera. *J. Clin. Microbiol.* 5:1-4.
- Matsuno, S., Utagawa, E. and Sugiura, A. 1983. Association of rotavirus infection with Kawasaki syndrome. *J. Inf. Dis.* 148:177.
- Matthews, R.E.F. 1979. The classification and nomenclature of viruses. Summary of results of meetings of the International Committee on Taxonomy of Viruses, The Hague (Netherlands), September, 1978. *Intervirology* 11:133-135.
- McCulloch, B.G., Phipps, P.H. and Rossier, E. 1978. Rotavirus, adenovirus and Norwalk virus in seasonal gastroenteritis. *Can. J. Pub. Hlth.* 69:69.
- McDaniels, A.E., Cochran, K.W., Gannon, J.J. and Williams, G.W. 1983. Rotavirus and reovirus stability in microorganism-free distilled and wastewaters. *Water Res.* 17:1349-1353.
- McCrae, M.A. and Faulkner-Valle, G.R. 1981. Molecular biology of rotaviruses: I. Characterization of basic growth parameters and pattern of macromolecular synthesis. *J. Virol.* 39:490-496.
- McNulty, M.S. 1978. Rotaviruses. *J. Gen. Virol.* 40:1-18.

- McNulty, M.S., Allan, G.M., Todd, D., McFerran, J.B., McKillop, E.R., Collins, D.S. and McCracken, R.M. 1980. Isolation of rotaviruses from turkeys and chickens: demonstration of distinct serotypes and RNA electropherotypes. *Avian Pathol.* 9:363-376.
- Mebus, C.A., Underdahl, N.R., Rhodes, M.B. and Twiehaus, M.J. 1969. Calf diarrhea (scours): reproduced with a virus from a field outbreak. *Bull. Neb Agric Exp. Station* 233:1-6.
- Mebus, C.A., Konno, M., Underdahl, N.R. and Twiehaus, M.J. 1971. Cell culture propagation of neonatal calf diarrhea (scours) virus. *Can. Vet. J.* 12:69-72.
- Melnick, J.L., Safferman, R., Rao, V.C., Goyal, S., Berg, G., Dahling, D.R., Wright, B.A., Akin, E., Stetler, R., Sorber, G., Moore, B., Sobsey, M.D., Moore, R., Lewis, A.L. and Wellings, F.M. 1984. Round robin investigation of methods for the recovery of poliovirus from drinking water. *Appl. Environ. Microbiol.* 47:144-150.
- Middleton, P.J., Szymanski, M.T., Abbott, G.D., Bortolussi, R. and Hamilton, J.R. 1974. Orbivirus acute gastroenteritis of infancy. *Lancet* I:1242-1244.
- Middleton, P.J., Szymanski, M.T. and Petric, M. 1977. Viruses associated with acute gastroenteritis in young children. *Am. J. Dis. Child.* 131:733-737.
- Moosai, B.B., Gardner, P.S., Almeida, J.D. and Greenway, M.A. 1978. A simple technique for the detection of human rotavirus. *J. Med. Virol.* 4:189-194.
- Morens, D.M., Zweighaft, R.M., Vernon, T.M., Gary, G.W., Eslien, J.J., Wood, B.T., Holman, R.C. and Dolin, R. 1979. A waterborne outbreak of gastroenteritis with secondary person-to-person spread. *Lancet* I: 964-966.
- Murphy, D., Todd, J.K., Chao, R.K., Orr, I. and McIntosh, K. 1981. The use of gowns and masks to control respiratory illness in pediatric hospital personnel. *J. Pediatr.* 99:746-750.
- Murphy, A.M., Grohman, G.S. and Sexton, M.F. 1983. Infectious gastroenteritis in Norfolk Island and recovery of viruses from drinking water. *J. Hyg.* 91:139-146.
- Newman, J.F.E., Brown, F., Bridger, J.C. and Woode, G.N. 1975. Characterization of a rotavirus. *Nature* 258:631-633.
- Nicolas, J.C., Ingrand, D., Fortier, B. and Bricout, F. 1982. A one-year virological survey of acute itussusception in childhood. *J. Med. Virol.* 9:267-271.
- Notani, G.W., Parsons, J.A. and Erlandsen, S.L. 1979. Versatility of Staphylococcus aureus protein A in immunocytochemistry use in unlabeled antibody enzyme system and fluoroescnet methods. *J. Histochem. Cytochem.* 27:1438-1444.

- O'Brien, R.T. and Newman, J.S. 1977. Inactivation of polioviruses and coxsackieviruses in surface water. *Appl. Environ. Microbiol.* 33:332-340.
- Otnaess, A.B. and Orstvik, I. 1980. The effect of human milk fractions on rotavirus- in relation to the secretory IgA content. *Acta. Pathol. Microbiol. Scand. (C)* 88:15-21.
- Palese, P. 1977. The genes of influenza virus. *Cell* 10:1-10.
- Palmer, E.L. Martin, M.L. and Murphy, F.A. 1977. Morphology and stability of infantile gastroenteritis virus: comparison with reovirus and bluetongue virus. *J. Gen. Virol.* 35:403-414.
- Palmer, E.L. Gary, G.W., Black, R. and Martin, M.L. 1980. Antiviral activity of colostrum and serum immunoglobulins A and G. *J. Med. Virol.* 5:123-129.
- Petralli, J.P., Hinton, D.M., Moriarty, G.C. and Sternberger, L.A. 1974. The unlabeled antibody enzyme method of immunohistochemistry. Quantitative comparison of sensitivities with and without peroxidase-antiperoxidase complex. *J. Histochem. Cytochem.* 22:782-801.
- Petric, M., Szymanski, M.T. and Middleton, P.J. 1975. Purification and preliminary characterization of infantile gastroenteritis virus (orbivirus group). *Intervirology.* 5:233-238.
- Petrie, B.L., Graham, D.Y. and Estes, M.K. 1981. Identification of rotavirus particle types. *Intervirology.* 16:20-28.
- Petrie, B.L., Graham, D.Y., Hanssen, H. and Estes, M.K. 1982. Localization of rotavirus antigens in infected cells by ultrastructural immunocytochemistry. *J. Gen. Virol.* 63:457-467.
- Primrose, S.B. and Day, M. 1977. Rapid concentration of bacteriophages from aquatic habitats. *J. Appl. Bacteriol.* 42:417-421.
- Primrose, S.B., Seeley, N.D. and Logan, K. 1981. The recovery of viruses from water. In, *Viruses and Wastewater Treatment*. Goddard, M. and Butler, M. (eds.). Pergamon Press, Toronto, Ontario. pp. 212-231.
- Quan, C.M. and Doane, F.W. 1983. Ultrastructural evidence for the cellular uptake of rotavirus by endocytosis. *Intervirology.* 20:223-231.
- Ramia, S. and Sattar, S.A. 1979a. Simian rotavirus SA-11 plaque formation in the presence of trypsin. *J. Clin. Microbiol.* 10:609-614.
- Ramia, S. and Sattar, S.A. 1979b. Second-step concentration of viruses in drinking and surface waters using polyethylene glycol hydroextraction. *Can. J. Microbiol.* 25:587-592.
- Ramia, S. and Sattar, S.A. 1980. Concentration of seeded simian rotavirus SA-11 from potable waters by using talc-Celite layers and hydroextraction. *Appl. Environ. Microbiol.* 39:493-499.

Rao, V.C., Sullivan, R., Read, R.B. and Clarke, N.A. 1968. A simple method for concentrating and detecting viruses in water. *J. Am. Water Works Ass.* 60:1288-1299.

Rao, V.C. and Melnick, J.L. 1984. Recovery of rotaviruses from estuarine water of Galveston Bay. Abstr. No. Q64. Ann. Meeting Am. Soc. Microbiol., St. Louis, Mo. p. 215.

Riepenhoff-Talty, M., Bogger-Goren, S., Li, P., Carmody, P.J., Barrett, H.J. and Ogra, P.L. 1981. Development of serum and intestinal acquired response to rotavirus after naturally acquired infection in man. *J. Med. Virol.* 8:215-222.

Rodger, S.M., Bishop, R.F., Birch, C., McLean, B. and Holmes, I.H. 1981. Molecular epidemiology of human rotaviruses in Melbourne, Australia, from 1973 to 1979 by electrophoresis of genome ribonucleic acid. *J. Clin. Microbiol.* 13:272-278.

Rodger, S.M., Bishop, R.F. and Holmes, I.H. 1982. Detection of a rotavirus-like agent associated with diarrhea in an infant. *J. Clin. Microbiol.* 16:724-726.

Rodriguez, W.J., Kim, H.W., Brandt, C.D., Bise, B., Kapikian, A.Z., Chanock, R.M., Curlin, G. and Parrott, R.H. 1980. Rotavirus gastroenteritis in Washington, D.C. area. *Am. J. Dis. Child.* 134:777-779.

Rotbart, H.A., Levin, M.J., Yolken, R.H., Manchester, D.K. and Kahsnitz, E. 1983. An outbreak of rotavirus-associated neonatal necrotizing enterocolitis. *J. Pediatr.* 103:454-463.

Ryder, R.W., McGowan, J.E., Hatch, M.H. and Palmer, E.L. 1977. Reovirus-like agent as a cause of nosocomial diarrhea in infants. *J. Pediatr.* 90:698-702.

Ryder, R.W., Oquist, C.A., Greenberg, H., Taylor, D.N., Orskov, F., Orskov, I., Kapikian, A.Z. and Sack, R.B. 1981. Travelers' diarrhea in Panamanian tourists in Mexico. *J. Infect. Dis.* 144:442-448.

Salmi, T.T., Arstilla, P. and Koivikko, A. 1978. Central nervous system involvement in patients with rotavirus gastroenteritis. *Scand. J. Inf. Dis.* 10:29-31.

Samadi, A.R., Huq, M.I. and Ahmed, Q.S. 1983. Detection of rotavirus in handwashings of attendants of children with diarrhea. *Br. Med. J.* 286:188-190.

Santosham, M., Yolken, R.H., Quiroz, E., Dillman, L., Oro, G., Reeves, W.C. and Sack, R.B. 1983. Detection of rotavirus in the respiratory secretions of children with pneumonia. *J. Pediatr.* 103:583-585.

Sarrette, B.A., Danglot, C.D. and Villagines, R. 1977. A new and simple method of recuperation of enteroviruses from water. *Water Res.* 11:355-358.

Sato, K., Inaba, Y., Shinozaki, T., Fugii, R. and Matumoto, M. 1981. Isolation of human rotavirus in cell cultures. *Arch. Virol.* 69:155-160.

Sattar, S.A. and Westwood, J.C.N. 1967. Immunofluorescence in the study of viruses in tissue culture. I. Development of an immunofluorescent focus assay for herpes simplex virus. *Can. J. Microbiol.* 13:1489-1497.

Sattar, S.A. and Westwood, J.C.N. 1976. Comparison of talc-Celite and the polyelectrolyte PE60 in virus recovery from sewage: development of techniques and experiments with poliovirus (type 1, Sabin)-contaminated multilitre samples. *Can. J. Microbiol.* 22:1620-1627.

Sattar, S.A. 1979. Viral pollution of the Ottawa River and its possible impact on the quality of potable and recreational waters in the Ottawa area-Phase II. Final Report for research study contract no. 77-004-11, Ontario Ministry of the Environment.

Sattar, S.A. and Ramia, S. 1979a. Use of talc-Celite layers in the concentration of enteroviruses from large volumes of potable waters. *Water Res.* 13:637-643.

Sattar, S.A. and Ramia, S. 1979b. Talc-Celite layers in virus recovery from potable waters experimentally contaminated with field isolates and sewage. *Water Res.* 13:1351-1353.

Sattar, S.A. 1981. Virus survival in receiving waters. In Viruses and Wastewater Treatment. Goddard, M. and Butler, M. (eds.). Pergamon Press, Toronto, Ontario. pp. 91-108.

Sattar, S.A., Raphael, R.A. and Springthorpe, V.S. 1983. Rotaviruses and chemical disinfectants. In Proc. Fourth International Symposium on Neonatal Diarrhea, Saskatoon, Sask.; Acres, S.D., Forman, A.J. and Fast, H.H. (eds.) p. 90-99.

Saulsbury, F.T., Winkelstein, J.A. and Yolken, R.H. 1980. chronic rotavirus infection in immunodeficiency. *J. Pediatr.* 97:61-65.

Schoub, B.D., Lecatsas, G. and Prozesky, O.W. 1977. Antigenic relationships between human and simian rotaviruses. *J. Med. Microbiol.* 10:1-6.

Sheridan, J.F., Awrelian, L., Barbour, G., Santosham, M., Sack, R.B. and Ryder, R.W. 1981. Travelers' diarrhea associated with rotavirus infection: analysis of virus-specific immunoglobulin classes. *Infect. Immun.* 31:419-429.

Shuval, H.I., Thompson, A., Fattal, B., Cymbalista, S. and Weiner, Y. 1971. Natural virus inactivation processes in sea water. *J. Sanit. Eng. Div., ASCE* 97:505-506.

Smith, E.M., Estes, M.K., Graham, D.Y. and Gerba, C.P. 1979. A plaque assay for the simian rotavirus SA-11. *J. Gen. Virol.* 43:513-519.

Smith, E.M. and Gerba, C.P. 1980a. Survival and detection of rotaviruses in the environment. In Proc. Third International Symposium on Neonatal Diarrhea, Saskatoon, Sask.; Acres, S.D., Forman, A.J. and Fast, H.H. (eds.) pp. 67-81.

Smith, E.M. and Gerba, C.P. 1980b. Detection of rotavirus in raw and treated wastewater. *Abstr. Div. Environ. Chem., 179th National Meeting Am. Chem. Soc.* Vol. 20:167-169.

Smith, E.M. and Gerba, C.P. 1982. Development of a method for detection of human rotavirus in water and sewage. *Appl. Environ. Microbiol.* 43:1440-1450.

Snodgrass, D.R. and Herring, J.A. 1977. The action of disinfectants on lamb rotavirus. *Vet. Record* 101:81.

Sobsey, M.D. and Jones, B.L. 1979. Concentration of poliovirus from tap water using positively charged microporous filters. *Appl. Environ. Microbiol.* 37:588-595.

Sobsey, M.D. and Glass, J.S. 1980. Poliovirus concentration from tap water with electropositive absorbent filters. *Appl. Environ. Microbiol.* 40:201-210.

Soenarto, Y., Sebodo, T., Ridho, R., Alrasjid, H., Rhode, J.E., Bugg, H.C., Barners, G.L. and Bishop, R.F. 1981. Acute diarrhoea and rotavirus infection in newborn babies and young children in Yogyakarta, Indonesia, from June 1978 to June 1979. *J. Clin. Microbiol.* 14:123-129.

Spence, L.; Fauvel, M., Petro, R. and Babiuk, L.A. 1978. Comparison of rotavirus strains by hemagglutination inhibition. *Can. J. Microbiol.* 24:353-362.

Standard Methods For The Examination Of Water And Wastewater. 1980. 15th Edition, Am. Pub. Hlth. Assoc. Washington, D.C.

Steinhoff, M.C. 1980. Rotaviruses: the first five years. *J. Pediatr.* 96:611-622.

Steinmann, J. 1981. Detection of rotavirus in sewage. *Appl. Environ. Microbiol.* 41:1043-1045.

Sternberger, L.A. 1979. Immunocytochemistry. 2nd edition. J. Wiley & Sons. New York.

Straus, W. 1971. Inhibition of peroxidase by methanol and by methanol-nitroferricyanide for use in immunoperoxidase procedures. *J. Histochem. Cytochem.* 19:682-688.

Straus, W. 1979. Peroxidase procedures - technical problems encountered during their application. *J. Histochem. Cytochem.* 27:1349-1351.

Sutmoller, F., Azeredo, R.S., Lacerda, M.D., Barth, O.M., Pereira, H.G., Hoffer, E. and Schatzmayr, H.G. 1982. An outbreak of gastroenteritis caused by both rotavirus and Schigella sonnei in a private school in Rio de Janeiro. *J. Hyg.* 88:285-293.

Tallet, S., MacKenzie, C., Middleton, P., Kerzner, B. and Hamilton, R. 1977. Clinical, laboratory and epidemiologic features of viral gastroenteritis in infants and children. *Pediatrics* 60:217-222.

Tam, J.S., Szymanski, M.T., Middleton, P.J. and Petrie, M. 1976. Studies on the particles of infantile gastroenteritis virus (orbivirus group). *Intervirology* 7:181-191.

Tan, J.A. and Schnagl, R.D. 1981. Inactivation of a rotavirus by disinfectants. *Med. J. Australia* 1:19-23.

Theil, K.W., Bohl, E.H. and Agnes, A.G. 1977. Cell culture propagation of porcine rotavirus (reovirus-like agent). *Am. J. Vet. Res.* 38:1765-1768.

Thomson, H.A., Dearlove, J., Lewis, I., Latham, P., Birkin, N. and Keogh, A. 1981. Outbreak of rotavirus gastroenteritis among premature infants. *Br. Med. J.* 183:1405.

Toranzo, A.E., Barja, J.L. and Hetrick, F.M. 1983. Mechanism of poliovirus inactivation by cell-free filtrates of marine bacteria. *Can. J. Microbiol.* 29:1481-1486.

Toranzos, G.A. and Gerba, C.P. 1983. Concentration of rotavirus from water and sewage on microporous filters. Abstr. No. Q57, Ann. Meeting Am. Soc. Microbiol., New Orleans. p. 269.

Torres, B.V., Ilya, R.M. and Esparza, J. 1978. Epidemiological aspects of rotavirus infection in hospitalized Venezuelan children with gastroenteritis. *Am. J. Trop. Med. Hyg.* 27:567-575.

Tulchinsky, T.H., Levine, I. and Schwartz, T.A. 1983. Waterborne enteric disease outbreaks in Israel 1976-1980. In Proc. International Symposium on Enteric Viruses in Water. Melnick, J.L and Kager, S. (eds.), New York.

Urasawa, S., Urasawa, T. and Taniguchi, K. 1981. Sequential passages of human rotavirus in MA-104 cells. *Microbiol. Immunol.* 25:1025-1035.

Van Renterghem, L., Borre, P. and Tilleman, J. 1980. Rotavirus and other viruses in the stool of premature babies. *J. Med. Virol.* 5:137-142.

Vesikari, T., Isolauri, E., Delem, A., D'Hondt, E., Andre, F.E. and Zissis, G. 1983. Immunogenicity and safety of live oral attenuated bovine rotavirus vaccine strain RIT 4237 in adults and young children. *Lancet* II:807-811.

Voogd, C.E., Van der Stel, J.J. and Coombs, J.J. 1980. On the mutagenic action of some enzyme immunoassay substrates. *J. Imm. Methods* 36: 55-61.

Wallis, C. and Melnick, J.L. 1967. Concentration of viruses on aluminum and calcium salts. *Am. J. Epidemiol.* 85:459-468.

Wallis, C., Melnick, J.L. and Fields, J.E. 1970. Detection of viruses in large volumes of natural waters by concentration on insoluble polyelectrolytes. *Water Res.* 4:787-796.

Walsh, J.A. and Warren, K.S. 1979. Selective primary health care: an interim strategy for disease control in developing countries. *N. Engl. J. Med.* 301:967-974.

Walter, R.A. 1983. A virological study about the health hazards associated with indirect reuse of water. In Viral Disinfection. Pergamon Press, New York.

Welch, A.B. 1971. Purification, morphology and partial characterization of a reovirus-like agent associated with neonatal calf diarrhea. *Can. J. Comp. Med.* 35:195-202.

Welch, A.B. and Thompson, T.L. 1973. Physicochemical characterization of a neonatal calf diarrhea virus. *Can. J. Comp. Med.* 37:295-301.

Woode, G.N. and Bridger, J.C. 1975. Viral enteritis of calves. *Vet. Rec.* 96:85-88.

Woode, G.N. 1978. Epizootiology of bovine rotavirus infection. *Vet. Rec.* 103:44-46.

World Health Organization. 1980. Rotavirus and other viral diarrheas. *Bull W.H.O.* 58:183-198.

Wyatt, R.G., James, W.D., Bohl, E.H., Theil, K.W., Saif, L.J., Kalica, A.R., Greenberg, H.B., Kapikian, A.Z. and Chanock, R.M. 1980. Human rotavirus type 2: cultivation in vitro. *Science* 207:189-191.

Wyatt, R.G., Greenberg, H.B., James, W.D., Pittman, A.L., Kalica, A.R., Flores, J., Chanock, R.M. and Kapikian, A.Z. 1982. Definition of human rotavirus serotypes by plaque reduction assay. *Infect. Immun.* 37:110-115.

Yolken, R. and Murphy, M. 1982. Sudden infant death syndrome associated with rotavirus infection. *J. Med. Virol.* 10:291-296.

Yolken, R.H., Bishop, C.A., Townsend, T.R., Bolyard, E.A., Bartlett, J., Santos, G.W. and Saral, R. 1982. Infectious gastroenteritis in bone-marrow-transplant recipients. *N. Engl. J. Med.* 299:1156-1161.

Yolken, R. 1983. Avidin-biotin radioimmunoassay for human rotavirus. *J. Infect. Dis.* 148:942.



Zamotin, B.A., Libiyainen, L.T., Bortnik, F.L., Chernitskaya, E.P., Enina, Z.I., Rossikhin, N.F., Veselov, V.I., Kharyutkin, S.A., Egerev, V.A., Nikulin, V.A., Dorozhkina, Z.D., Kostyukova, K.P., Dorozhkin, G.V., Shironina, V.G., Chernyi, V.S., Agapov, E.I. and Vlasov, V.I. 1981. Waterborne group infection of rotavirus etiology. *Microbiol. Epidemiol. Immunol.* 11:99-101.

Zehr, D.R. 1978. Use of hydrogen peroxide-egg albumin to eliminate non-specific staining in immunoperoxidase techniques. *J. Histochem. Cytochem.* 26:415-416.

Zissis, G., Lambert, J.P., Fonteyne, J. and De Kegel, D. 1976. Child-Mother transmission of rotavirus? *Lancet* I:96.

Zissis, G., Lambert, J.P., Marbehant, P., Marissens, D., Lobmann, M., Charlier, P., Delem, A. and Zygraieh, N. 1983. Protection studies in colostrum-deprived piglets of a bovine rotavirus vaccine candidate using human rotavirus strains for challenge. *J. Infect. Dis.* 148:1061-1068.