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REPLICATION OF HUMAN ROTAVIRUS IN TISSUE CULTURE: RECOVERY AND DETECTION IN FECAL, SEWAGE, AND NATURAL WATER SAMPLES

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ABSTRACT

Human rotavirus is the major cause of gastroenteritis among young children. To replicate this virus, sensitive methods using standard tissue culture systems are required. The project goal was to develop laboratory capability to recover and detect this infectious rotavirus in fecal, sewage, and natural water samples. Using simian rotavirus (SA-11) as a model system and an enzyme-linked immunosorption (ELISA) test capable of detecting high concentrations of rotavirus, the protamine sulfate method was determined as superior to the aluminum chloride precipitation and polymer two-phase methods for recovering rotavirus from sewage. The ELISA method was very effective in detecting rotavirus in stool samples of children. Stools from children not displaying clinical symptoms of rotavirus infection were negative for rotavirus, whereas 43 to 58% of stools from children displaying clinical symptoms of rotavirus infection was positive for rotavirus. The results suggested an association of increased rotavirus infections during the winter months in the state of Hawai'i. Stools positive for rotavirus by the ELISA test were used as inoculum to develop methods to replicate human rotavirus in the cell culture system. After many unsuccessful attempts, human rotavirus was cultured after two passages in primary cynomolgus monkey kidney cells. Human rotavirus which replicated in the primary monkey kidney cells was shown to be capable of replicating in continuous monkey kidney cell lines such as the MA-104.

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INTRODUCTION

Over 100 known pathogenic human enteric viruses (Table 1) can be transmitted to man via the water route. Based on recent virus isolation studies and on epidemiological data, Haley et al. (1980) concluded that the major waterborne illness in the United States continues to be gastroenteritis of suspected viral etiology. Although great improvements have been made during the past 15 years in the methods to detect human enteric viruses in sewage and natural waters, some of the medically most important viruses cannot be replicated using standard virological techniques. This group of viruses originally called the "non-cultivatable" viruses includes infectious Hepatitis A, the virus responsible for the greatest number of waterborne epidemics, and human rotavirus, the virus responsible for the greatest number of diarrheal infections in infants and young children. Also included in this group are some as yet unidentified viruses simply called non-A, non-B hepatitis virus, and gastroenteritis virus. However, results of studies conducted within the past four years indicate that Hepatitis A and human rotavirus can be replicated under specialized conditions and that immunological methods can be used to detect the growth of these viruses in cell culture. Based on recent results, this group of viruses is no longer referred to as "non-cultivatable" but, instead, as "difficult to cultivate" viruses.

During 15-16 November 1982, key experts from the United States were invited to a workshop sponsored by the University of Maryland Sea Grant College Program to assess the "problems and research needs of natural toxins and human pathogens in the marine environment." The virus working group concluded that the most significant problem and limitation in the current studies for the assessment of viruses in water is that the group of difficult to cultivate virus is not being measured. Thus, when water samples are analyzed and recorded as negative for virus, the water sample cannot be assumed as not containing low or even high concentrations of Hepatitis A virus, rotavirus, or other gastrointestinal viruses. In its final report (Sobsey 1983), the virus working group recommended that future research emphasis be placed on improving the methods to recover and detect the difficult to culture group of viruses from sewage and natural waters.

Based on the results of recent studies, the routine culture of human rotavirus appears to be more likely than any of the other difficult to cul-

TABLE 1. WATER-BORNE ENTERIC VIRUSES

Virus Group	No. of Types	Disease or Symptoms
Enterovirus		
Poliovirus	3	meningitis; paralysis, fever
Echovirus	34	meningitis, respiratory disease; rash, diarrhea, fever
Coxsackievirus A	24	herpangina, respiratory disease, meningitis; fever
Coxsackievirus B	6	myocarditis, respiratory disease, meningitis, pleurodynia; congenital heart anomalies, rash, fever
New enteroviruses	4	meningitis, encephalitis, respiratory disease, acute haemorrhagic conjunctivitis; fever
Hepatitis A (probably an enterovirus)	1	infectious hepatitis
Gastroenteritis A (probably an enterovirus)	2	epidemic vomiting and diarrhoea, fever
Rotavirus (gastroenteritis B)	1	epidemic vomiting and diarrhoea (chiefly in children)
Reovirus	3	not clearly established
Adenovirus	>30	respiratory disease; eye infection
Parvovirus		
Adeno-associated virus	3	associated with respiratory disease of children, but etiology not clearly established

tivate viruses. Three independent systems aid in the study to recover and replicate human rotavirus from sewage and natural waters. First, there is a tissue culture system to replicate a simian rotavirus. Although the simian rotavirus will not infect humans, it has many of the same properties as the human rotavirus and can be quantitated by the standard virus plaquing technique by using a special monkey cell line (MA-104). Thus, the expected stability and methods to recover human rotavirus can be predicted based on studies using the simian rotavirus model system. Second, the simian rotavirus is antigenically related to the human rotavirus and therefore the presence of human rotavirus can be immunologically tested using antiserum prepared against the simian rotavirus. In this regard, the Abbott Laboratories (Chicago, Illinois) have prepared a reliable enzyme-linked immunosorp-

tion assay (ELISA) test which can detect the presence of rotavirus antigen, including the human rotavirus which may be present in human feces, domestic sewage, or natural waters. Third, four recent and independent studies reported that under specified conditions, the replication of human rotavirus obtained from human feces and placed directly on enzyme-treated monkey cells resulted in the multiplication and subsequent recovery of human rotaviruses in these cell cultures. What is surprising and probably significant is that despite years of U.S. scientists trying to replicate human rotavirus in tissue culture, the first four laboratories to report the successful replication of human rotavirus were all in Japan: the first was reported by Urasawa, Urasawa, and Taniguchi (1981), then Sato et al. (1981), and more recently by Hasegawa et al. (1982) and Kutsuzawa et al. (1982). Using the techniques described by the Japanese scientists, recent attempts by western scientists (Birch et al. 1983; Wyatt et al. 1983; Ward, Knowlton, and Pierce 1984) have resulted in the replication of human rotaviruses. The techniques for the replication of rotavirus should be noted as requiring intricate manipulations and the virus as growing only under limited conditions. Thus, the replication of human rotavirus is still only in the research stage and a reliable technique which can be used by standard virological laboratories has not yet been developed.

Rotaviruses are known to infect many animals. However, each animal species appears to have its own specific strain of rotavirus. Thus, human rotaviruses do not seem to infect other animals and, conversely, other animal rotaviruses do not infect humans. In humans, rotaviruses are the commonest cause of acute nonbacterial diarrhea in infancy and childhood, occasionally resulting in death (Flewett and Woode 1978). At any given time, 40 to 50% of the infants admitted to hospitals with acute diarrheal disease are associated with rotavirus infections (Kapikian et al. 1976) and this proportion may rise to 70 to 80% in winter months (Davidson et al. 1975). The peak incidences of rotavirus infections during the winter months in temperate zone countries have been well established. In tropical countries, rotavirus infections may occur throughout the year or follow some other variable pattern (Banatvala 1981). Adults can be infected with rotaviruses but appear to be generally more resistant to the pathological effects of the virus infection. Rotaviruses which are transmitted by ingestion have been detected by electron microscopy to be present in stools

of infected individuals at concentrations of 10^{10} to 10^{11} particles per gram of diarrheic stool. The major route of transmitting rotavirus in humans is believed to be by person-to-person contact, an explanation for the common epidemics of rotavirus infections in day-care centers, schools, and within families. However, evidence that rotavirus can also be transmitted in drinking and bathing water, and by shellfish consumption have been presented by Morens et al. (1979) and Cabelli (1981). Their data are supported by other studies which have presented evidence indicating that rotavirus can be expected to survive standard sewage treatment processes and to survive for long periods of time in fresh and marine waters (Hurst and Gerba 1980).

Rotavirus is a large (70 nm diameter), spherical, naked, double-capsid, and multisegmented, double-stranded RNA virus. Because of its wheel-like appearance it was given the name rota (wheel) virus. In 1974 this virus was first observed using electron microscopy (Bishop et al. 1974) in the stools of patients with diarrhea. For awhile, electron microscopy was the only means to detect the presence of this virus. Even when large numbers of virus particles were observed in the stools, numerous attempts to culture this virus were not productive. However, by 1976, immunological tests, such as the enzyme-linked immunosorbent assay (ELISA), were developed to detect the presence of these viruses in stools and for antibodies in people infected with this virus. These immunological methods are rapid, do not require expensive, specialized equipment, and have been very useful in characterizing this virus disease. Acute rotavirus disease is characterized by severe watery diarrhea with fever and vomiting, especially in young children.

GOALS AND OBJECTIVES

Our goal was to develop and strengthen the WRRC Environmental Virology Laboratory capability to recover and detect infectious human rotaviruses in fecal, sewage, and natural water samples. The human rotavirus was selected as the most promising member of the group of human enteropathogenic viruses which cannot be cultured using standard tissue culture methods. Clinical cases of rotavirus infections undoubtedly occur in Hawai'i. However, very little information is available with regard to the incidence of rotavirus infections in Hawai'i because no facility in the state of Hawai'i is capable

of recovering and identifying infectious human rotaviruses from sewage, natural waters or from human fecal samples. Thus, the success of this project will be beneficial to the state since our laboratory will then be Hawai'i's only facility with this capability.

EXPERIMENTAL DESIGN AND METHODOLOGY

Model Systems for Human Rotavirus

The model system for human rotavirus most often used is the simian rotavirus (SA-11) which can be cultured and quantitated using the plaque assay on a continuous African green monkey kidney cell line (MA-104). The SA-11 virus and the MA-104 cells were initially obtained from B.H. Keswick of Baylor College of Medicine in Houston, Texas. Because the source of MA-104 cells resulted in low yields and inconsistent plaquing of SA-11 virus, another MA-104 cell line was purchased from Microbiological Associates (Bethesda, Maryland), the original producer of the cell line. This source of MA-104 was satisfactory and was used in most of the experiments described in this study. The simian rotavirus does not infect humans but is structurally, antigenically, and biologically similar to human rotavirus and was therefore the model system used to predict the reaction of human rotaviruses under various conditions. Thus, to develop the best method to recover human rotavirus which may be present in feces, sewage, or natural waters contaminated with sewage, the simian rotavirus was used to seed water samples which were then processed by various techniques and the products assayed for simian rotavirus to evaluate the efficiency of the methods. For comparison, type 1 poliovirus (Sabin Lsc2ab) cultured and quantitated using another African green monkey kidney cell line (BGM) was used as the model system for a culturable human enteric virus. Standard methods and media used to maintain cell cultures, to plaque viruses, to purify virus, and to determine the buoyant density of viruses using CsCl gradients have been previously described by Loh, Fujioka, and Lau (1979).

The only available human rotavirus reported to be readily replicated in tissue culture is the "Wa" strain. This virus was recovered only after a human stool sample had undergone 11 serial passages through gnotobiotic piglet cells and 14 passages through primary monkey kidney cells. Because of the unusual conditions by which the Wa strain was recovered, this virus is

suspected as being an atypical virus and, thus, as not a good model system for human rotavirus. Recently available through the American Type Culture Collection, we purchased the Wa strain for comparative studies in early 1984.

Rotavirus Particle Detection Using ELISA Technique

Rotavirus particles, or antigens, in samples can be detected by immunological techniques, such as the enzyme-linked immuno-sorbent-assay (ELISA). This type of assay procedure is especially useful in detecting rotaviruses which cannot be detected by a routine culturing method. Major advantages of the ELISA method are its reliability, reproducibility, and feasibility to analyze many samples for rotavirus. The limitations of this methodology are its inability to differentiate between infectious and non-infectious viruses, cross reaction between human and non-human rotaviruses, and its relative insensitivity. It has been estimated that 10^7 virus particles per ml must be present before a positive reaction is obtained using this technique. Rotazyme, a rotavirus diagnostic kit produced by Abbott Laboratories (Chicago, Illinois) was initially used in our studies. The ELISA whose antigenic base is the simian SA-11 is the most used kit for rotavirus detection. Toward the end of our studies, the rotavirus ELISA kit produced by DAKOPATTS and distributed by Accurate Chemical and Scientific Corp. (Westbury, New York) was used. This rotavirus ELISA kit uses human rotavirus as the antigenic base.

Human Rotavirus Replication Systems

At the start of this project, only four laboratories, all in Japan, had reported some success in culturing human rotavirus by using tissue culture systems. Most of the laboratories used MA-104 cells as one of their cell culture system to replicate human rotavirus. Other monkey cells which were successful include Vero, CV-1 and primary monkey kidney cells. The procedure which appeared to allow the replication of human rotavirus in cell culture systems was the pretreatment of the virus and/or the cell system with the enzyme trypsin, presumably to partially cleave a peptide on the virus capsid and thereby allow the virus to become uncoated, an essential step in the infection of cells. In some experiments DEAE-dextran or some other chemical means of enhancing cell penetration was sometimes used. Use of the

roller tube technique as opposed to the standard stationary incubation method was also reported to enhance rotavirus replication in cell cultures. Moreover, in most of these studies, replication of rotavirus in cell culture systems was difficult to detect since the formation of cytopathogenic effect (CPE) or production of plaques in these cell cultures did not occur or occurred only sporadically. Finally, evidence of rotavirus infection in cell cultures was often observed only after several blind passages of the inoculated cells. Although several laboratories have reported the successful replication of human rotavirus in cell culture systems, the methodology required is still in the developmental stage and must be simplified and made more efficient before this technology can be used by most virological laboratories.

The experimental approach to replicate human rotavirus in our laboratory will be to evaluate the techniques which have been reported to enhance rotavirus replication in cell cultures. Initially, infected cell cultures will be monitored for CPE and plaque formation since these are the most sensitive indicators of virus replication in cells. However, since rotavirus have been reported to replicate in cells in the absence of CPE and plaque formation, infected cells will also be analyzed for the presence of rotavirus antigens (virus particles) by the ELISA assay method. A positive ELISA test, especially after several cell passages, is a good indicator of virus replication since 10^7 rotavirus particles per ml must be present before a positive response is measured by the ELISA test. The presence of high concentrations of rotavirus particles in cell cultures can also be determined by examination of the cell extracts by electron microscopy or by banding and determining the buoyant density of these particles in CsCl gradients.

Source of Human Rotavirus

The source of human rotavirus is known to be the stools of infected humans, primarily young children. Other likely sources, but expected at low concentrations, are sewage effluent and sewage-contaminated waters. All three sources were analyzed for presence of rotavirus. However, for obvious reasons, most of the samples processed were stool samples from children. Arrangements were made to obtain stool samples of children from three separate agencies. Stools from children ill for various reasons and visiting an

outpatient clinic at Pearl Harbor were obtained. No attempt was made to preselect stools from children with symptoms resembling rotavirus infections from this clinic. Stools preselected from young children displaying symptons associated with rotavirus infections were obtained from Kapiolani/ Children's Medical Center and from the Pediatric Clinic at Tripler Army Medical Center. All of these samples were initially screened for the presence of rotavirus particles by the ELISA assay. Those samples which were positive by the ELISA assay were then used to inoculate cell cultures (MA-104 or primary monkey kidney cells) to culture the virus.

RESULTS AND DISCUSSION

Simian Rotavirus Model System

The simian rotavirus (SA-11) which can be plaqued in MA-104 cells is the model system for human rotavirus. The SA-11 virus and MA-104 cells were donated by B.H. Keswick (Baylor College of Medicine, Houston, Texas). Trypsin and procedures as described by Estes, Palmer, and Obijeski (1983) were used to establish our laboratory's capability of growing and quantitating SA-11 virus in MA-104 cells. However, plaque production and total yield of SA-11 virus were erratic and generally low (10^4-10^6 PFU/ml) even when several concentrations and other sources of trypsin as well as pancreatin, another proteolytic enzyme, were used to pretreat the virus. Since SA-11 was subsequently shown to plaque in BGM cells, our initial source of MA-104 cell line was concluded as an unreliable cell line to culture SA-11 virus. Thus, another line of MA-104 cells was obtained from Microbiological Associates of Bethesda, Maryland, the originator of this cell line. When this cell line was used to grow and plaque SA-11 virus, high yields and reproducible plaques were obtained. For example, the yield of SA-11 virus from four different experiments using this cell line were 4.7×10^8, 4.8×10^7, 2.3×10^8 and 1.7×10^7 PFU/ml. The SA-11 grown in this cell line was then purified of cellular components by differential centrifugation and isopycnic centrifugation and isopycnic centrifugation in CsCl gradient. The virus banded at the buoyant density of 1.36 gm/ml, a density reported for rotaviruses indicating that the virus produced was truly a rotavirus. This purified SA-11 virus with a titer of approximately 2×10^8 PFU/ml was subsequently used in evaluating the methods to concentrate rotavirus from sewage.

Three Methods to Recover Rotavirus from Sewage

Rotavirus can be expected in sewage effluent but at such low concentrations that the sewage effluent will probably have to be concentrated before the virus can be detected. Three methods, the $AlCl_3$ precipitation, polymer two phase, and protamine sulfate, have been successfully used to recover other enteric viruses from sewage by our laboratory (Fujioka and Loh 1978). To determine the expected efficiency by which these three methods will recover and concentrate rotavirus suspended in sewage approximately 10^5 PFU/ml of SA-11 virus were seeded into activated sludge treated sewage effluent obtained from the Wahiawa Wastewater Treatment Plant (WWTP) and processed by these three methods. By comparing the PFU/ml of SA-11 virus in the sewage effluent before and after treatment with these three methods, the ability to remove the virus in the sewage effluent to a smaller volume such as the precipitate in the protamine sulfate or aluminum chloride ($AlCl_3$) method or the dextran sulfate phase in the two phase method can be determined. The results of three separate experiments (Table 2) show that the polymer two phase method is the least effective and has the greatest variability from experiment to experiment. Although the $AlCl_3$ precipitation and the protamine sulfate methods appeared to be effective in concentrating rotavirus from the sewage, the latter method was selected because it was more effective and reliable.

In our previous study, we determined that the concentration of fetal calf serum added to the sewage effluent often determines the effectiveness by which the protamine sulfate will precipitate out poliovirus from the sewage. To determine the optimum concentration of serum required to precipitate out rotavirus from the sewage effluent, poliovirus and rotavirus were added to sewage effluent and portions of this mixture dosed with various

TABLE 2. ANALYSIS OF THREE METHODS TO CONCENTRATE ROTAVIRUS (SA-11) FROM ACTIVATED SLUDGE TREATED SEWAGE

METHOD	% ROTAVIRUS REMOVAL FROM SEWAGE			MEAN	STD. DEV.
	Expt. 1	Expt. 2	Expt. 3		
Protamine Sulfate	99.99	99.96	99.99	99.98	0.017
Aluminum Chloride (ppt)	97.01	99.93	96.91	97.95	1.715
Two-Phase	79.87	99.63	96.44	91.98	10.608

TABLE 3. EFFECT OF SERUM CONCENTRATION ON EFFICIENCY OF CONCENTRATING POLIOVIRUS VS. ROTAVIRUS (SA-11) FROM SEWAGE BY PROTAMINE SULFATE METHOD

SERUM ADDED (ml/250 ml)	% POLIOVIRUS REMOVAL FROM SEWAGE				% SA-11 REMOVAL FROM SEWAGE			
	Experiment				Experiment			
	1	2	3	4	1	2	3	4
0	63	0	0	52	99	99	100	100
0.1	68	43	36	31	99	100	100	100
0.2	90	60	66	79	100	100	100	100
0.3	N.D.	24	32	61	N.D.	82	76	78

concentrations of serum before being treated with protamine sulfate. By determining the PFU/ml of both viruses in the sewage effluent before and after treatment, the efficiency of virus precipitation under various conditions could be determined. The results of four different experiments (Table 3) show that the protamine sulfate method is much more effective in precipitating out rotavirus than poliovirus from the sewage effluent. This is consistent with England's (1972) earlier study in which she determined that protamine sulfate is more effective in precipitating out the larger viruses, such as reovirus, as compared to the smaller viruses such as poliovirus. Moreover, the addition of various concentrations of serum did not appear to markedly increase the efficiency of the protamine sulfate method, especially for rotavirus.

Although the results indicated that protamine sulfate was effective in removing or precipitating rotavirus from sewage effluent, the actual recovery of virus is dependent on the effectiveness by which the virus in the precipitate can be recovered. With regard to the protamine sulfate precipitate, several reagents have been used to elute the virus from the precipitate. Two experiments in which 2 ml of three different reagents (3% beef extract, 1 NaCl; 10% tryptose phosphate broth) were used to elute the virus from the precipitate trapped on a 47-mm AP20 filter (Millipore Corp.) were compared. The results (Table 4) show that elution of rotavirus from the precipitate was relatively inefficient with mean recoveries of 12.5% using beef extract, 14% with 1 NaCl and 17% using 10% tryptose phosphate broth. The ineffective elution of virus in the precipitate has always been a problem in virus recovery methodology. In these experiments, the small volume of 2 ml used to elute the precipitate trapped on a 47-mm filter may have

TABLE 4. COMPARATIVE EFFICIENCY OF ELUTING ROTAVIRUS (SA-11) FROM PROTAMINE SULFATE PRECIPITATES USING THREE ELUANTS

ELUANT	% ROTAVIRUS RECOVERED IN ELUANT Experiment 1	2	MEAN (%)	STD. DEV.
3% Beef Extract*	18	7	12.50	7.78
1*N* NaCl	22	5	14.00	11.31
10% Tryptose Phosphate Broth*	20	14	17.00	4.24

*pH 9.5

been a limiting factor. A larger volume of eluate would undoubtedly increase the yield of virus recovered from the precipitate. Based on the data at hand, elution of the protamine sulfate with larger volumes of 10% tryptose phosphate broth is recommended.

Recovery of Rotavirus from Sewage and Drinking Water

Sewage is a source of human rotavirus. To determine whether rotavirus can be recovered from sewage, raw and primary treated sewage obtained from the Kailua and Mililani WWTPs were concentrated by the $AlCl_3$ precipitation method and by the protamine sulfate methods as described earlier, and the precipitate eluted with 1*N* NaCl or with 10% tryptose phosphate broth. The final concentrate was then assayed for the presence of human rotavirus by inoculation onto MA-104 cells and by the ELISA (rotazyme) method. All results were negative. The results supported our prediction that the ELISA method is too insensitive to detect the presence of low concentrations of virus. The cell culture technique for culturing human rotavirus has yet to be optimized in our laboratory.

In the summer of 1983, an outbreak of gastroenteritis occurred in U.S. Navy personnel stationed at Barbers Point. Drinking water was one of the suspected sources of the pathogen. Five drinking water samples provided by the U.S. Navy were pooled to a total of 480 ml concentrated onto 1 MDS filter (AMF Cuno Corp.), and the 47-mm filter eluted with 5 ml of 1*N* NaCl. The concentrate was analyzed by the ELISA method and determined to be negative. Since gastroenteritis at Barbers Point involved personnel of all ages and

rotavirus rarely cause disease in adults, it was improbable that the etiological agent was rotavirus.

ELISA Test for Rotavirus Infections in Children

Rotavirus infections in humans is primarily an early childhood disease which can be detected worldwide wherever proper diagnostic tests have been used. Currently, the most practical way to confirm that a child has a rotavirus infection is to analyze the feces for the presence of rotavirus particles (antigens) by using immunological tests, such as the ELISA. Since no such studies have been conducted in Hawai'i, a good estimate of rotavirus infection in Hawai'i is not available.

The ELISA diagnostic test kit for rotavirus (Abbott Laboratories) was used for the following reasons: (1) to determine the incidence of rotavirus infections in sick children in Hawai'i, (2) to select the fecal samples to be used to culture human rotavirus in tissue culture, (3) to determine the presence and replication of rotavirus in tissue culture systems. The rehability and sensitivity of the diagnostic test kit was evaluated against two separate pools of simian rotavirus (SA-11). Ten-fold dilutions of the two virus preparations were analyzed and the results (Table 5) confirm the reliability of the ELISA for the detection of rotavirus. Moreover, the sensitivity of the ELISA method appears to be approximately 10^5 infectious units or PFU/ml of rotavirus. Based on the estimate that there are usually 100 times more physical particles than infectious particles, the sensitivity of the ELISA method was calculated as actually 10^7 virus particles per ml, which is the lower limit of sensitivity as reported by the Abbott Laboratories.

PEARL HARBOR OUTPATIENT CLINIC. Arrangements were made with Lt. John Wallace (Environmental Preventive Medicine Unit 6, Pearl Harbor) to obtain fecal samples from children who visited the outpatient clinic at Pearl Harbor. These children were ill and displayed various symptoms, mostly diarrhea or stomach ache, but none were ill enough to be hospitalized. Also, many of these children were older (>6 yr) and were no longer in the acute phase of their illness. A total of 33 fecal samples from 27 children were tested for the presence of rotavirus by using the ELISA method. All samples were negative, indicating that either rotavirus was not present in the feces of these children or that the time of sampling was late.

TABLE 5. ROTAVIRUS (SA-11) CONCENTRATION CORRELATION VS. ELISA REACTIVITY

ROTAVIRUS PREPA-RATION	ROTAVIRUS (SA-11) CONCENTRATION			ELISA REAC-TIVITY	SPECTRO-PHOTOMETRIC ABSORBANCE (492 nm)
	Dilu-tion	PFU/ml	Calculated Particles per ml		
Stock A	10^{-2}	1×10^{7}	1×10^{8}	4+	2.0
	10^{-3}	1×10^{6}	1×10^{8}	4+	1.6
	10^{-4}	1×10^{5}	1×10^{7}	2+	0.4
	10^{-5}	1×10^{4}	1×10^{6}	±	0.1
	10^{-6}	1×10^{3}	1×10^{5}	0	0.04
	10^{-7}	1×10^{2}	1×10^{4}	0	0.02
	10^{-8}	1×10^{1}	1×10^{3}	0	0.01
Stock B	10^{-1}	6×10^{7}	6×20^{8}	4+	1.58
	10^{-2}	6×10^{6}	6×10^{8}	3+	1.24
	10^{-3}	6×10^{5}	6×10^{7}	3+	0.94
	10^{-4}	6×10^{4}	6×10^{6}	±	0.09
	10^{-5}	6×10^{3}	6×10^{5}	0	0.002
	10^{-6}	6×10^{2}	6×10^{4}	0	0.000
	10^{-7}	6×10^{1}	6×10^{3}	0	0.000

KAPIOLANI/CHILDREN'S MEDICAL CENTER. Arrangements were made with Dr. Dexter Seto to obtain diarrheal stool samples from children hospitalized in the gastroenteritis ward of the Kapiolani/Children's Medical Center. These children were suffering from gastroenteritis symptoms which were severe enough to require hospitalization. Stool samples were not obtained from all children in this ward but were obtained when it was practical for the hospital nursing staff to collect the samples. Samples were obtained during the winter months (January-March) of 1983 and in 1984. The samples were analyzed for rotavirus using the ELISA method. The results (Table 6) show that during the winter of 1983, 8 of 17 (47%) stool samples were positive for rotavirus. These results indicate that a significant percent (43-47%) of the children who are hospitalized for gastroenteritis at Kapiolani/Children's Medical Center are probably due to rotavirus infection.

TRIPLER ARMY MEDICAL CENTER. Arrangements were made with Dr. James W. Bass (Pediatric Clinic, Tripler Army Medical Center) to obtain stool samples during the winter of 1983 and 1984 from children with gastroenteritis symptoms, many of whom were specifically selected based on a clinical symptom profile which suggested rotavirus infections. Eleven of the 19 stool samples (58%) were positive for rotavirus by ELISA (Table 7). The high percentage of rotavirus detected in these stool samples reflect the close cor-

TABLE 6. ROTAVIRUS (ELISA REACTIVITY) IN STOOL SAMPLES OBTAINED FROM KAPIOLANI/ CHILDREN'S MEDICAL CENTER

Sample Date	Sample Stool	ELISA Reactivity
11/01/83	K-1	+
11/01/83	K-2	+
13/01/83	K-3	-
18/01/83	K-4	-
18/01/83	K-5	-
18/01/83	K-6	+
18/01/83	K-7	+
26/01/83	K-8	+
26/01/83	K-9	+
26/01/83	K-10	+
26/01/83	K-11	-
01/02/83	K-12	+
01/02/83	K-13	-
01/02/83	K-14	-
01/02/83	K-15	+
08/02/83	K-16	-
24/02/83	K-17	-
13/01/83	Normal Stool*	-
09/01/84	K-18	+
18/01/84	K-19	-
18/01/84	K-20	-
18/01/84	K-21	+
04/02/84	K-22	-
04/02/84	K-23	-
06/03/84	K-24	+

*Stool from healthy child.

relation between typical clinical symptoms of this disease and the confirmation of infection based on the recovery of rotavirus from the stools of these suspected patients. Although preliminary, these results also suggest that there may be a peak of rotavirus infections associated with the winter

TABLE 7. ROTAVIRUS (ELISA REACTIVITY) IN STOOL SAMPLES OBTAINED FROM TRIPLER ARMY MEDICAL CENTER

Stool Sample	Sample Date	ELISA Reactivity
28/11/83	T-1	-
28/11/83	T-2	-
28/11/83	T-3	-
12/12/83	T-4	-
12/12/83	T-5	+
12/12/83	T-6	-
12/12/83	T-7	+
12/12/83	T-8	+
27/12/83	T-9	+
27/12/83	T-10	+
30/12/83	T-11	+
11/01/84	T-12	+
11/01/84	T-13	+
16/01/84	T-14	-
16/01/84	T-15	+
18/01/84	T-16	-
18/01/84	T-17	+
18/01/84	T-18	+
18/01/84	T-19	-

months even in a subtropical region such as here in Hawai'i. The peak incidences of rotavirus infections during winter months in temperate zone countries is well established (Kapikian et al. 1976).

Rotavirus In Stool Samples

Based on ELISA analysis of stool samples, evidence was obtained that rotavirus particles at concentrations of 10^7 to 10^9 per ml were present in some of the stool samples. At this level of concentration, the virus particles can be banded on a CsCl gradient, visualized, and the buoyant density determined. The buoyant density of intact, infectious rotavirus in CsCl has been reported to be 1.36 mg/ml; therefore, this information can be taken as independent evidence that rotavirus particles are present in the stool samples. To obtain this evidence, a stool sample, which reacted strongly positive (3+) in the ELISA test, was selected and purified by two separate techniques: the aqueous two-phase method and the differential centrifugation method. Preparations from the two methods as well as purified simian rotavirus (SA-11) were then processed by isopycnic centrifugation in a CsCl gradient and the bands formed in all the samples compared. The results (Table 8) show that purified SA-11 virus and the stool sample purified by

TABLE 8. CORRELATION OF BUOYANT DENSITY FRACTIONATION OF SAMPLES WITH ELISA REACTIVITY

Sample	Band No.	Buoyant Density (g/ml)	Degree ELISA Reaction
Purified SA-11	1	1.3859	-
	2	1.3603	4+
Stool K-1 purified by 2 phase	1	1.3895	-
	2	1.3635	3+
Stool K-1 purified by differential centrifugation	1	1.3899	-
	2	1.3780	4+
	3	1.3710	4+
	4	1.3640	4+

the two-phase method resulted in two very similar bands. The virus band for the SA-11 preparation was at a buoyant density of 1.3603, whereas the virus band for the human stool preparation had a buoyant density of 1.3635 g/ml. Both bands reacted strongly with the ELISA test. The second band in the SA-11 preparation had a buoyant density of 1.3859 g/ml while the second in the human stool sample had a buoyant density of 1.3895 g/ml. Both bands did not react with the ELISA test. Based on previous reports that a rotavirus which has lost its outer capsid will band at a density of 1.38 g/ml, this second band was concluded as representing the rotavirus with only its inner capsid. Also, since this fraction did not react with the ELISA test, the antigenicity associated with the ELISA test is probably part of the outer capsid of the virus. In contrast to these two samples, the stool sample purified by only differential centrifugation resulted in four bands, of which three bands were closely associated. The heaviest band had a density of 1.3899 g/ml and did not react with the ELISA test. This band was concluded to correlate with rotavirus without its outer shell as observed in the other two samples. The other three bands reacted strongly with the ELISA test, thus indicating that these particles contained outer capsid proteins. The lightest of these bands with a density of 1.3640 g/ml most closely correlated with the virus band as observed in the two-phase treated preparation and was concluded to represent the intact, human rotavirus. The other two bands with closely related buoyant densities of 1.3710 and 1.3780 g/ml were concluded to be virus particles which were in intermediate

stages of losing their outer capsid. These structures were preserved when the stool sample was purified by differential centrifugation but was lost when the same sample was purified using the two-phase method.

Production of Antisera to Human Rotavirus

The purified and concentrated preparation of human rotavirus obtained from stool K-1 should be an excellent source of antigen. To prepare specific antisera to human rotavirus, this virus preparation was emulsified with Freund's complete adjuvant and 0.5 ml injected intramuscularly into each hind leg of two New Zealand white rabbits, approximately 5 months in age. This was followed by two similar booster inoculations at 2-wk intervals. Blood serum was obtained from the rabbits before and after the series of inoculations. These sera will be tested for precipitating and neutralizing antibodies against the various isolates of human rotavirus which we anticipate to culture. The results of the analyses will be used to determine differences in the various human rotavirus isolates.

Rotavirus Replication In Cell Cultures

STATIONARY CULTURES OF MA-104 CELLS. MA-104 cells are the single most-used cell line to culture human rotavirus. In the first series of experiments standard, stationary cultures of MA-104 cells were used in our attempts to culture human rotavirus. Stool samples which reacted strongly with the ELISA test were selected as sources most likely to contain high concentrations of infectious human rotavirus. Three of these stool samples were purified and the virus extract treated with trypsin before they were used to inoculate stationary cultures of MA-104. The cells were then incubated at 37°C and evidence for virus replication in the cells monitored for the appearance of CPE or plaques. CPE or plaque formation was never observed in the cells which suggested that rotavirus replication had not occurred. However, after the experiments were completed, other reports (Birch et al. 1983; Wyatt et al. 1983) indicated that human rotavirus may replicate in cell cultures in the absence of CPE or plaque formation. Thus, the results of the first series of experiments were concluded as inconclusive with regard to the ability to culture human rotavirus in cell cultures.

ROLLER CULTURE TECHNIQUE AND PRIMARY MONKEY KIDNEY CELLS. In March 1984, R. Ward presented the results of their study on the cultivation of

human rotavirus in cell culture systems (Ward, Knowlton, and Pierce 1984) at the 84th Annual Meeting of the American Society for Microbiology. He indicated that the use of roller culture technique and the passage of the sample through several cultures of primary monkey kidney cells were essential for the successful replication of human rotavirus in cell culture. He also demonstrated that CPE and plaque formation were unreliable indicators of rotavirus replication in cell culture and that the ELISA test can be used to demonstrate the presence of high concentrations of rotavirus in cell cultures. A second series of experiments utilizing the above recommendations to replicate rotavirus in cell cultures was conducted in our laboratory. Initially various purified stool cultures were trypsinized and inoculated onto roller cultures of either primary African green monkey kidney cells which had been stored in liquid nitrogen or onto MA-104 cells. The inoculated cells were incubated for 5 days at 37°C (passage 1), after which time the cells were frozen and thawed and 0.1 ml used to inoculate a fresh set of cells (passage 2) which was incubated for 3 days at 37°C. Samples from passage 1 and 2 were then assayed for presence of rotavirus by the ELISA test. The results (Table 9) show that two samples (T-13, T-16) of the seven samples at the passage 1 level in the AGMK and the MA-104 cell system yielded a positive ELISA response, whereas all second passage samples resulted in a negative ELISA response. Based on these results, the concentration of human rotavirus was concluded as being so high in samples T-13 and T-16 that the residual inoculum was responsible for the positive ELISA reaction in passage level 1. At passage level 2, the inoculum was diluted beyond detec-

TABLE 9. ATTEMPT TO REPLICATE HUMAN ROTAVIRUS IN CELL CULTURES

HUMAN STOOL	PRESENCE OF 10^7 ROTAVIRUS/ml (ELISA REACTIVITY)			
	SET A		SET B	
	Pass No. 1 (MA-104)	Pass No. 2 (MA-104)	Pass No. 1 (Primary AGMK)	Pass No. 2 (MA-104)
K-18	-	-	-	-
K-19	-	-	-	-
K-21	-	-	-	-
T-13	+	-	+	-
T-16	+	-	+	-
T-18	-	-	-	-
T-19	-	-	-	-

tion and replication of rotavirus was insufficient to result in a positive ELISA test in passage level 2.

Another experiment was set up utilizing the same procedure as the previous set of experiments, but initially passing the inoculum through two passages of primary Cynomolgus Monkey kidney (CMK) cells as well as MA-104 cells. The results in Table 10 indicate again that several samples reacted positively with the ELISA test at passage level 1 in the primary CMK cells and in the MA-104 cell systems. However, all stool samples of passage levels 2 and 3 in MA-104 cells were negative by the ELISA test indicating that extensive replication of human rotavirus had not occurred. In passage level 2 of the primary CMK cells, 3 of the 7 stool samples remained positive and remained positive at passage level 3 in which MA-104 cells were used. As a positive control, the Wa strain of human rotavirus was also used as an inoculum in this experiment. The results show that when Wa was used as an inoculum, all passage levels 1, 2, and 3 were positive for rotavirus based on the ELISA test in the primary CMK and the MA-104 cells. This response was expected since the Wa strain of human rotavirus has been adapted to grow under tissue culture conditions. Based on these results, human rotavirus replication are concluded to have taken place in the second passage in the primary CMK cell but not in the MA-104 cells. However, once the human rotavirus was able to replicate substantially in primary CMK, the viruses were then able to replicate in MA-104 cells.

TABLE 10. ROTAVIRUS REPLICATION FOLLOWING SERIAL PASSAGES OF FECAL EXTRACT INTO PRIMARY CYNOMOLGUS MONKEY KIDNEY (CMK) CELLS

HUMAN STOOL	PRESENCE OF 10^7 ROTAVIRUS/ml (ELISA REACTIVITY)					
	SET A			SET B		
	(Primary CMK)		(MA-104)		(MA-104)	
	Pass 1	Pass 2	Pass 3	Pass 1	Pass 2	Pass 3
K-18	+	+	+	+	-	-
T-05	-	-	-	-	-	-
T-07	+	+	+	-	-	-
T-08	+	-	-	+	-	-
T-10	-	-	-	-	-	-
T-11	+	+	+	+	-	-
T-12	+	-	-	+	-	-
Wa Human Rotavirus	+	+	+	+	+	+

SUMMARY AND CONCLUSIONS

Most of the over 100 known pathogenic human enteric viruses which may be transmitted by the water route can be replicated using tissue culture systems. Thus, the absence or presence of these viruses in water can be determined using standard virological methods. However, some medically significant human enteric viruses such as rotavirus, Hepatitis A virus, and Norwalk virus cannot be detected using standard virological methods and therefore there is no feasible test to determine that water or any other suspected medium is or is not contaminated with these viruses. Currently, improving the methods to recover and detect these difficult to culture viruses is one of the greatest challenges and needs in water virology. The purpose of this study was to develop methods to concentrate and culture human rotavirus from feces, sewage, and sewage-contaminated waters. When this project began in 1982, there were only two reports from Japan which indicated that human rotavirus could be cultured using tissue culture systems. Most of the studies to predict how human rotaviruses would behave under specified conditions were carried out using a simian rotavirus (SA-11) which was physically and biologically similar to the human rotavirus and which could be readily cultured in a continuous monkey kidney cell line (MA-104).

Using the SA-11 rotavirus and the MA-104 cell line, we determined that the protamine sulfate method was superior to the $AlCl_3$ precipitation method and the polymer two-phase method to concentrate and recover rotavirus suspended in sewage. Although the protamine sulfate method was effective in precipitating out the rotavirus from the sewage, the efficiency of eluting or recovering the rotavirus from the precipitate was relatively inefficient (12-17%); therefore, this phase of the concentration technique remains to be optimized. Four raw and primary treated sewage samples were obtained from the Kailua WWTP and the Mililani WWTP. The samples were concentrated by protamine sulfate or $AlCl_3$ precipitation methods and the final concentrates were assayed for the presence of rotavirus by inoculation onto MA-104 cells and by using an immunological (ELISA) test to detect rotavirus particles (antigens). All results were negative indicating the insensitivity of the ELISA to detect low concentrations of rotavirus and the need to optimize conditions to culture human rotavirus in cell culture systems. Drinking

water samples obtained from a U.S. Navy facility at Barbers Point, O'ahu, where an outbreak of gastroenteritis had occurred were similarly concentrated, assayed, and determined to be negative for the presence of rotavirus.

Because rotavirus is primarily a disease of early childhood and the virus can be expected to be found in high concentrations in stools of infected children, arrangements were made to obtain stools of young children and to screen these stools for the presence of human rotavirus by using the ELISA test. The ELISA method was evaluated using the SA-11 rotavirus. Our data confirms that the ELISA method is reliable but relatively insensitive because 10^7 particles per ml of rotavirus must be present before a positive ELISA reaction (2+) is observed. The first group of stools was from an outpatient clinic and were primarily from children although some patients were older children or even young adults. These patients were not ill enough to require hospitalization and presumably were suffering from some symptoms related to gastroenteritis, although the symptoms may no longer have been acute at the time the stools were sampled. Thirty-three stool samples from 27 patients were tested and all samples were determined to be negative for rotavirus using the ELISA test. Based on these results we conclude that it is unlikely to detect rotavirus in stools from patients not displaying clinical symptoms of rotavirus infection. Stool samples were then obtained from Kapiolani/Children's Medical Center from young children seriously ill and requiring hospitalization for gastroenteritis symptoms. During the winter of 1983, 8 of 17 (47%) of the stool samples analyzed by ELISA were positive for rotavirus while 3 of 7 (43%) of the stool samples collected in the winter of 1984 were similarly positive for rotavirus. The results indicate that a significant percent (43-47%) of the children hospitalized for gastroenteritis at Kapiolani Children's Hospital are probably due to rotavirus infection. Stool samples were also obtained from Tripler Army Medical Center from young children displaying clinical symptoms of rotavirus infection. Of 19 stool samples analyzed by ELISA, 11 or 58% were positive for rotavirus, indicating a close correlation between clinical symptoms of rotavirus infection and confirmation of rotavirus in stools of these patients. In summary, the ELISA test for rotavirus was shown to be effective in detecting rotavirus in stools of infected individuals. Thus, there was a strong correlation between the presence of rotavirus in stools

and children with clinical symptoms of rotavirus infections. Based on the data collected, there appears to be a peak of rotavirus disease associated with the winter months in Hawai'i. The results demonstrate for the first time the incidences of rotavirus infections in Hawai'i based on laboratory confirmation of the virus in stool samples. However, due to the relatively small number of samples tested, more samples should be analyzed to substantiate the preliminary conclusions of this study.

The ELISA is an immunological test for the presence of the antigens associated with rotavirus. The limitations of this test are that it does not differentiate between infectious and noninfectious viruses in the sample, and high concentrations (10^7/ml) of virus particles must be present before a positive ELISA reaction is observed. The advantages of the ELISA are its reliability and practicality which simplifies the analysis of many samples for rotavirus. The ELISA test was used to determine which stool samples contained large concentrations of rotavirus. This allowed us to select samples containing rotavirus to develop methods to culture the virus in cell cultures. The ELISA test was also used to demonstrate that extensive replication of rotavirus in cell cultures had occurred. Moreover, a stool sample which reacted strongly with the ELISA was subject to isopycnic centrifugation in a CsCl gradient. The banding of particles at a 1.36 g/ml density which reacted positively with the ELISA test was taken as independent evidence for the presence of rotavirus in the stool samples. This purified and concentrated rotavirus preparation was used to immunize rabbits and to produce antisera specific for human rotavirus.

The ELISA positive stool samples were used as an inoculum to develop techniques to culture human rotavirus in cell cultures. The difficulty of culturing human rotavirus in cell cultures became apparent as many attempts resulted in negative findings. However, successful replication of human rotavirus in cell culture was finally achieved in 3 of 7 samples by treating the inoculum with trypsin, and passing this inoculum through at least two consecutive transfers in primary cynomulgus monkey kidney cells which were incubated under roller culture conditions at 37°C. This was the first time that human rotavirus was replicated in cell cultures in Hawai'i and was only the third such success in the United States. These results demonstrate that the WRRC Environmental Virology Laboratory has developed the capability of detecting and replicating human rotavirus.

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