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Detection, isolation, and characterization of human rotavirus (HRV) isolated from patients at two hospitals on Oahu

Siwak, Edward B., Ph.D.

University of Hawaii, 1990

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DETECTION, ISOLATION, AND CHARACTERIZATION OF HUMAN ROTAVIRUS (HRV) ISOLATED FROM PATIENTS AT TWO HOSPITALS ON OAHU

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF
THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN MICROBIOLOGY

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ABSTRACT

Stool samples obtained from patients with gastroenteritis at two Oahu hospitals were analyzed for the presence of rotavirus by Enzyme-linked immunosorbent assay (ELISA). Rotavirus was detected in 21/33 (64%) samples from the first hospital and 20/111 (18%) from the second. ELISA-positive stool samples were found predominantly in winter months (December through February).

The genomic RNA profiles of the rotaviruses detected in stool samples were determined by electrophoresis. RNA profiles for 25/41 (61%) stool samples were determined. Most, 22/25 (88%), were of the long RNA profiles.

The rotavirus stool isolates were adapted to growth in primary African Green monkey kidney (AGMK) cells from 10/21 (48%) KMC samples and from 4/20 (20%) TAMC samples. AGMK cells were determined to be superior to MA 104 cells (cell line of AGMK origin) in this regard.

Rotavirus isolate (TAMC 15) was adapted to cell culture and subsequently produced plaques, and was determined to be serotype 3 by plaque reduction neutralization.

Monoclonal antibody-based ELISA was used to determine the serotypes of rotavirus isolates. Of the 33 rotavirus isolates, 10 were serotype 1, 3 were serotype 4, one was serotype 2 and one was a dual serotype of 1 and 4. Eighteen isolates were not typable by this method.

Plaque forming efficiency was influenced by the use of agarose as the solidifying agent for the overlay. Inclusion of DEAE-dextran and proteolytic enzymes at a constant specific activity heightened plaque formation.

Anti-rotavirus serum was produced in guinea pigs and used to modify the nitrocellulose-enzyme immunoassay method of rotavirus detection. A sensitivity of 7×10^4 PFU was achieved.

Physical characterization of TAMC 15 was accomplished by electron microscopy (EM) and buoyant density determination. EM demonstrated that the typical morphological structures of rotavirus were present, and the buoyant density was determined to be 1.3614 g/ml and 1.3834 g/ml in cesium chloride.

The stability of rotavirus in environmental waters in the absence and presence of sunlight, to temperature, and chlorine were evaluated. The results demonstrated that SA11 (simian rotavirus) and TAMC 15 (human rotavirus) were more rotavirus strains suspended in environmental waters. Seawater was identified as a harsher environment than fresh stream water. Results of the addition of antibiotics to environmental waters suggest that endogenous bacteria have a deleterious effect on the stability of rotavirus strains. TAMC 15 was demonstrated to be more resistant to low level chlorination than SA11. Thus, TAMC 15 may be a better model system for studying rotaviruses than SA11.

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LIST OF ABBREVIATIONS

AGMK	African Green Monkey Kidney
BGMK	AGMK cell line
CFU	colony forming units
Cl-N	4-chloro-1-naphthol
CMK	Cynomolgus Monkey Kidney
CsCl	cesium chloride
CV	crystal violet stain
DAB	diaminobenzidine
DEAE-dextran	diethylaminoethyl-dextran
DS-1	prototype serotype 2 HRV
Earle's MEM	Eagle's MEM with Earle's salts
EDTA	ethylenediamine tetraacetic acid
ELISA	EnzymeLinkedImmunosorbentAssay
EM	electronmicroscopy
FBS	fetal bovine serum
FW	fresh stream water
HeLa	transformed human cervical cancer cell line
HRP	horseradish peroxidase
HRV	human rotavirus
IgG	immunoglobulin G
KMC	Kapiolani Womens and Childrens Medical Center
mA	milliamp
MAb	monoclonal antibody
MA-104	embryonic AGMK cell line (diploid)
MEM	minimal essential medium

mg	milligram
MRC-5	human embryonic lung cell line (diploid)
NC	nitrocellulose paper
NC-EIA	Nitrocellulose-Enzyme Immunoassay
NCS	newborn calf serum
NDM	non-fat dry milk
ng	nanogram
NR	neutral red stain
P	prototype serotype 3 HRV
PAGE	polyacrylamide gel electrophoresis
PBS	Dulbecco's phosphate buffered saline
PFU	plaque forming units
PMK	primary monkey kidney
PRN	plaque reduction neutralization
RNA	ribonucleic acid
SA11	simian rotavirus strain
ST3	prototype serotype 4 HRV
SW	seawater
TAMC	Tripler Army Medical Center
TAMC 15	HRV isolate 15
$TCID_{50}$	tissue culture infectious dose 50
VP3	viral protein 3 (rotavirus)
VP7	viral protein 7 (rotavirus)
Wa	prototype serotype 1 HRV
μg	microgram
μl	microliter

PART I. INTRODUCTORY INFORMATION

CHAPTER 1. LITERATURE REVIEW

CHAPTER 2. STATEMENT OF THE PROBLEM

CHAPTER 3. GOALS AND OBJECTIVES

CHAPTER 1

LITERATURE REVIEW

I. THE DISCOVERY OF ROTAVIRUSES

Rotavirus particles were first identified by electron microscopy (EM) in biopsy specimens taken from the mucosa of the small intestine of children with acute non-bacterial gastroenteritis in Australia (Bishop et al., 1973). The particles were found in the enterocytes which are the epithelial cells covering the intestinal villi lining the lumen of the intestinal tract.

This new virus was described as belonging to the orbivirus group, and resembling the virus causing epizootic diarrhoea of infant mice (E.D.I.M.) which is now known to be a murine rotavirus.

Using electron microscopy (EM), Middleton et al. (1974) identified the newly identified virus in stool specimens of patients in Canada, seven of which died. The authors also noted that the virus was recovered more often during the colder winter months.

Davidson et al. (1975) detected rotavirus in the cytoplasm of the epithelial cells by immunofluorescence (IF).

In 1975, Flewett et al. proposed the name rotavirus due to the characteristic wheel-like appearance (Gk. rota = wheel) of the double-capsid virus upon EM examination.

Rotavirus was first detected in the United States in 1976 by Kapikian et al. although they named this virus the human reovirus-like agent. They identified the virus as the major pathogen associated with winter gastroenteritis in hospitalized infants and children.

From this point and through the 1980's this new virus was called rotavirus and research on rotavirus increased dramatically, although efforts were restricted by the difficulty in cultivating rotavirus in cell culture systems.

II. ROTAVIRUS DISEASE

A. Clinical features of rotavirus gastroenteritis.

Several features of rotavirus gastroenteritis are characteristic of patients with the illness as compared to patients with acute diarrhea due to other agents (negative controls). El-Mougi et al. (1989) found that vomiting preceding diarrhea and respiratory symptoms are clinical signs with statistical significance for rotavirus diarrhea as compared to rotavirus negative controls. Clinical signs with high statistical significance for rotavirus diarrhea as compared to rotavirus negative diarrheal patients includes duration of watery stools (2.5±1.6 days vs. 3.2±2.0 days), percentage of patients with watery stools (92% vs. 60%), and cases with vomiting (85% vs. 65.5%).

Uhnoo, Olding-Stenkvist and Kreuger (1986) compared clinical features of gastroenteritis among patients infected with rotavirus, adenovirus, or bacteria. There was a high

correlation of diarrhea, vomiting and fever with rotavirus infection (Table 1). They noted that the mean duration for diarrhea was shortest for rotaviral diarrhea at 5.9 days, while it was longest for bacteria induced diarrhea at 14.1 days.

Table 1

Clinical signs and symptoms associated with rotavirus induced diarrhea

Features	No. cases(%) (n = 168)
Diarrhea	164(98)
Vomiting	146(87)
Fever	141(84)
Fever >39°C	71(42)
Admission to hospital	65(39)
Vomiting >5 times/day	62(37)
Respiratory symptoms	56(33)
Diarrhea >10 times/day	36(21)
Abdominal pain	31(18)
Mucus in stool	28(17)
Blood in stool	2(1)

Adapted from Uhnoo, Olding-Stenkvist and Kreuger (1986)

Clemens et al. (1983) reported that patients with watery diarrhea were younger (mean = 10 mo, range 4-12 mo) and vomiting occurred in 86% of these patients. In contrast, patients with non-watery diarrhea were older (mean = 24 mo, range 15-34 mo) and vomiting occurred in only 17% of the cases. Fecal blood and leukocytes were also more common in non-watery diarrhea as compared to watery diarrhea.

B. Epidemiology of rotavirus gastroenteritis.

1. Seasonal occurrence.

The first pattern to be recognized is that rotavirus gastroenteritis is seasonal. In temperate climates a winter epidemic occurs in the cool, dry months (December - March) of the year (Brandt et al., 1982; Brandt et al., 1983; Konno et al., 1978). On the American continent rotavirus gastroenteritis typically begins in central America early in the fall (September) and increased cases are observed through December (Mata et al., 1983). After the onset of this yearly epidemic in Central America, similar epidemics of rotavirus gastroenteritis are observed sequentially in time through central America, Mexico, southwestern U.S.A., southern U.S.A. and into the northeastern U.S.A. (Ho et al., 1988). The rotavirus epidemic season in the U.S.A. usually occurs from December to March.

A cyclical pattern of rotavirus epidemics is not as obvious in tropical climates. Some researchers have reported that an epidemic season occurs in the winter months whereas others have reported that rotavirus gastroenteritis appears to be endemic with low levels detected all year round (Chiba et al., 1984; Hanlon et al., 1987; Sitbon et al., 1985; Soenarto et al., 1983).

Winter epidemics of rotavirus gastroenteritis are believed to be a function of climatic conditions corresponding to temperature and rainfall (with high

relative humidity) since the epidemic observed in New Zealand each year is from May - October (Holdaway et al., 1985) which is the cool, dry (low relative humidity) winter season (Brandt et al., 1982).

Epidemics of rotavirus gastroenteritis are also periodic. Birch et al. (1988) reported that the severity of the epidemic may change from year-to-year. In England, minor outbreaks were reported in 1977, 1979, 1981, 1983, 1984, and 1985, whereas major epidemics were reported in 1975, 1978, 1980, 1982, and 1986.

In summary, three key epidemiological features of rotavirus gastroenteritis have been established: 1) Epidemics of this disease occurs throughout the world, 2) Epidemics of this disease have a correlation with cool, dry months of the year, and 3) Children 6-35 months of age are most susceptible (Ho et al., 1988).

2. Living conditions.

Yap et al. (1984) observed that large family size and crowded conditions are significant risk factors indicating that the mode of rotavirus transmission is via the fecal-oral route.

Koopman, Monto and Longini (1989) calculated that 80% of infections are community acquired whereas 20% are family acquired. They also proposed that effecting herd immunity through immunization of family members may be worth exploring.

Brandt et al. (1982) stated that there is a correlation between weather and higher rates of infection with rotavirus during cool, dry (low relative humidity) periods. These periods tend to keep people indoors with a greater chance of transmission due to more contact.

The tendency to cluster indoors may explain conditions observed in temperate climates, however it does not fully explain conditions in tropical/subtropical climates as exist in Hawaii. Based on clinical symptoms, Seto (personal communication) observed a winter epidemic of rotavirus gastroenteritis from late December through early March in Hawaii. Due to the yearly temperature that does not fluctuate by many degree and the relatively high annual relative humidity, there is not a significant "dry" season in this state. In addition, homes in Hawaii are unlike those in many other areas in that the houses tend to be more open to the outside environment vis-a-vis jalousie windows.

Some researchers (Lewis et al., 1979; Santosham et al., 1983; Holdaway et al.,1985; Foster et al., 1980) have suggested that the virus may be spread via the respiratory route. This is still controversial since rotavirus is generally not isolated from the respiratory tract in patients who are infected, and very rarely detected in respiratory tract washings. Respiratory isolation of rotavirus has not been reported in Hawaii.

3. Attack rate among hospitalized children.

During an epidemic season it has been observed that among hospitalized infants greater than 80% of the inpatients may be positive for the shedding of rotavirus particles as determined by EM examination (Albert, Bishop and Shann, 1983). Konno et al. (1978) have reported a figure of 79% positive samples by EM examination.

In temperate climates, several researchers have determined that the rate for rotavirus detection by EM and ELISA among infants hospitalized for diarrhea symptoms falls between 35% and 60% (Brandt et al., 1983; Hasegawa et al., 1984; Pickering, 1985). Similar results have been reported in tropical climates (Chiba et al., 1984; Hasegawa et al., 1984; Hanlon et al., 1987).

The previous studies were conducted hospitalized on children. The fact that they are hospitalized is indicative of the virulence of the strains of rotavirus involved.

4. Rotavirus illness in the community.

Koopman et al. (1984) compared the rate of rotavirus among children hospitalized for diarrheal symptoms versus similar children from two physicians' private practices, one being a single practice and the second a group practice. The positive rate based on ELISA examination of stools was 48% positive versus 30% for the single practice and 36% for the group practice. Thus it can be surmised that the general rate among non-hospitalized children may be lower

than the "captive" hospitalized group which generally have more severe symptoms.

Sethi et al. (1984) examined hospitalized infants, both diarrheal patients and nondiarrheal controls, and observed a positive rotavirus rate of 40.2% among the diarrheal cases and a positive rotavirus rate of 4.7% among the non-diarrheal cases. Therefore, not all rotavirus infections are symptomatic.

Keswick et al. (1983a) examined rotavirus infections among well children in a day care center. By ELISA examination of stool samples, detection of rotavirus occurred in 12.4% of children aged from 1 to 24 months. These children were identified as asymptomatic carriers of rotavirus.

5. Age factors among children for susceptibility to rotavirus gastroenteritis.

It is now accepted that rotavirus primarily causes gastroenteritis in children less than 2 years of age and is associated with winter epidemics in most areas of the world. The mean incubation period for rotavirus gastroenteritis ranges from 1-3 days as determined by Kapikian et al. (1983). Uhnoo and Svensson (1986) observed that the age group with the highest rate of gastroenteritis attributed to rotavirus was the 7 to 12 month old group. Uhnoo's work supports that of Lewis et al. (1979).

However, Clarke et al. (1981) reported that human rotavirus was responsible for approximately 25% of infant diarrhea cases for those less than 1 year of age, 60% for the 1-3 year old group, and 20-40% for the 4-6 year old group.

Clemens et al.(1983) examined 207 patients, 93 aged less than four years. 27/93 (29%) excreted rotavirus. For 13/93 (14%), rotavirus was the only isolated enteropathogen.

6. Rotavirus subgroups and rotavirus illness.

It was mentioned previously that rotaviruses may be classified into two serologic subgroups, I and II. Uhnoo and Svensson (1986b) compared the clinical picture involving these two subgroups of rotavirus and detected 36% subgroup I rotavirus and 64% subgroup II rotavirus. Fever was significantly more common with subgroup I infections. Diarrhea with greater than 10 stools/day were higher for subgroup II infections, and vomiting greater than 5 times/day were more common with subgroup II infections.

There is therefore no difference in the severity of gastroenteritis between the two subgroups such that hospital admissions are not more likely with one subgroup over the other. Spencer et al., (1983) reported that there was no relationship between short (subgroup I) and long (subgroup II) patterns and virulence in hospitalized and ambulatory patients.

The literature shows that subgroup I rotaviruses are recovered less frequently from rotavirus gastroenteritis patients. The lower recovery is repeated worldwide. The lowest incidence was reported by Nakagomi et al. (1985) for Japan with a percentage for 1981/1982 of 2.7%, while the percentage for 1982/1983 was 0.8%. The highest percentage was reported by Uhnoo and Svensson (1986) at 36%. This pattern of fewer subgroup I rotaviruses being detected than subgroup II is repeated worldwide, except for occasional epidemics specifically attributed to a subgroup I rotavirus strain.

7. Rotavirus illness and population size.

Albert, Bishop and Shann (1983) investigated an epidemic of rotavirus illness in Papua, New Guinea in isolated groups in the highlands. They have estimated that the minimum population size necessary for endemicity to be 5,000 persons. This is about the same population size that has been calculated as being necessary for the endemicity of measles virus, which is considered an "old" virus.

This might tend to refute the notion that rotavirus gastroenteritis is a "new" malady. When population centers reached the critical size rotaviruses might have become a permanent part of the viral burden. This would clearly lend support for the theory that better surveillance and detection methods have brought rotavirus under closer scientific scrutiny.

C. Impact on developed vs. developing nations.

Human rotavirus is now recognized as a major cause of acute gastroenteritis in children worldwide. Rotavirus gastroenteritis has been reported to be responsible for several million infant deaths per year in developing nations (Clarke and McCrae, 1982).

Guerrant et al. (1983) did a study of enteric pathogens producing diarrheal disease in children living in northeastern Brazil. The most frequently identified pathogen was enterotoxigenic *Escherichia coli*, followed by rotavirus. All pathogens identified are listed in Table 2.

The impact of rotavirus gastroenteritis on black infants residing in a periurban region (transitional between rural and urban) of Durban, Union of South Africa was considered (Loening, Coovadia and Van Den Ende, 1989). The results of pathogen identification were similar to Guerrant et al., except that rotavirus was the most frequently identified enteropathogen.

The role of rotavirus gastroenteritis in developed nations is one of morbidity, with acute gastroenteritis being the worst problem (Offit, Shaw and Greenberg, 1986).

Table 2

Distribution of pathogens causing gastroenteritis in Brazilian villagers

Pathogen	No. positive	No. samples	%
ETEC[a]	31	149	20.8
Rotavirus	24	124	19.4
Shigella spp.	12	149	8.0
Campylobacter jejuni	3	40	7.5
Giardia lamblia	10	150	6.7
Strongyloides stercoralis	8	150	5.3
EPEC[b]	6	130	4.6
Invasive *E. coli*	3	149	2.0
Entamoeba histolytica	3	150	2.0
Other[c]	3	150	2.0
No pathogen detected	70	150	46.7

a - enteropathogenic *Escherichia coli*
b - enterotoxigenic *Escherichia coli*
c - Includes one infection each with *Balantidium coli*, toxigenic *Aeromonas hydrophila*, and *Vibrio cholerae* non-O1.
Adapted from Guerrant et al. (1983)

III. TRANSMISSION OF ROTAVIRUS ILLNESS AND POSSIBLE RESERVOIRS

A. Most likely mode of transmission.

Rotavirus is grouped with those viruses that are transmitted by the fecal-oral route (Foster et al., 1980).

Since the transmission mode for rotavirus is fecal-oral, consideration must be given to stability of the rotavirus particles. Ansari et al. (1988) determined that rotavirus could still be recovered from experimentally infected fingertips four hours after inoculation. Their study demonstrates the role that contaminated human hands play in the distribution of rotavirus particles by persons attending to patients with rotaviral gastroenteritis. Contamination of the hands could occur in hospitals, or at the onset of disease in day care centers, and illustrates how easily the virus may be picked up on the hands and transmitted to another infant.

Neonates in hospital nurseries harbor many strains of rotavirus which are responsible for an asymptomatic rotavirus infection. The asymptomatic nature of neonatal infection could allow the dissemination of virus to the nursery staff with subsequent transmission outside the nursery. Due to the asymptomatic nature of the infection, more intensive precautions may not be taken to prevent transmission of the rotavirus particles.

Also, the neonates in a hospital nursery are responsible for the transmission of rotavirus among themselves due to the close contact. A similar situation is observed in children in day care centers. However, the epidemiology of illness among non-institutionalized children is not as well understood.

Keswick et al. (1983a) demonstrated that rotavirus is commonly found among children in day care centers, and rotavirus was isolated from various inanimate objects (fomites) (Keswick et al., 1983b) which aids in the transmission of rotavirus to uninfected infants.

Pickering et al. (1988) determined that 50% of the test population in a day care center shed rotavirus one day before the onset of diarrheal illness. Thus rotavirus is spread person-to-person either directly or through fomites.

There is a high probability of the adult population also transmitting rotavirus to susceptible individuals. In a community based study by Grimwood et al. (1983) the authors found a high incidence of intrafamilial infection. In families with a child with an active rotavirus infection, 46% of the family members developed rotavirus infections. In those families with an index case, 75% of the other children became infected but only 33% of the adults. However, adults did become infected. Since adults are more able to move within the community, these same adults could easily spread the virus to others.

It is now generally accepted that adults may become infected, however they are generally asymptomatic. Exceptions include immunocompromised individuals and the elderly that have weakened immune systems. This is not an absolute, however. Echeverria et al. (1983) examined gastroenteritis in adults induced by rotavirus. They

determined that 28/526 (5%) adults were infected and ranged in age from 16 to 72 years. It was also observed that there was a statistically significant difference in when the patient became ill. More patients were found to be infected in the cooler, dry months than in others. While the authors do not have a ready explanation for this severity observed in adults, they offer the suggestion that they were infected with serotypes to which they had no previous exposure. Thus, young parents may provide a link in the spread of the virus among children (Holdaway et al., 1985).

The transmission of rotavirus to adults is supported by von Bonsdorff et al. (1978). This study reported that adults may become infected during epidemics of rotavirus illness. Thus, infected individuals who are excreting rotavirus particles may be important in the spread of this enteric pathogen. Rotavirus infection in adults is usually acquired from five possible settings (Hrdy, 1987): 1) acquisition from contaminated environmental sources, especially during waterborne outbreaks, 2) as travelers diarrhea (Vollet et al., 1979; DuPont and Ericsson, 1989), 3) secondary contacts from pediatric cases, 4) epidemic spread, and 5) endemic or sporadic infections.

B. Transmission by the water route.

Epidemiological studies suggest that sewage polluted waters may be a vehicle involved in the transmission of rotavirus illness (Sattar, Raphael and Springthorpe, 1985;

Rao, Metcalf and Melnick, 1987). Rao, Metcalf and Melnick (1986) studied sewage in the Houston area and found rotavirus in chlorinated effluent in an activated sludge plant. They calculated that 4.8×10^7 infectious rotavirus particles per day were discharged.

Should a water source become sewage contaminated, a danger of infection becomes possible. Raphael, Sattar and Springthorpe (1985) reported that rotavirus particles can survive in raw river water for 10 days at 20°C with a two log loss in titer. The survival was even more pronounced at colder temperatures. Rotavirus has also been shown to be stable in conventionally treated drinking water. At 20°C the virus can survive 64 days with a two log drop in titer (Sattar, Raphael and Springthorpe, 1984). The virus could even withstand >100 mg/l of chlorine.

Thus it is clear that sewage-borne rotavirus introduced into a water supply which is subsequently ingested could become the source of infection. Epidemics of rotavirus gastroenteritis occur during the colder winter months in temperate climates. Considering the increased stability of rotavirus particles at colder temperatures, it is possible for sewage contaminated water sources to be a point source for gastroenteritis outbreaks.

While some attempts have been made to show a respiratory route of transmission for human rotavirus, these

attempts have not been successful (Lewis et al., 1979; Santosham et al., 1983; Holdaway et al.,1985).

C. Possible reservoirs.

Rotaviruses have been isolated from every mammalian species examined to date, but species specificity to severe diarrheal disease exists. While cross species infection may occur, clinically significant disease does not occur (Estes, Palmer an Obijeski, 1983).

Human patients may become infected with two strains of rotavirus at the same time. Mixed rotavirus infections have been reported to occur with a frequency of 10% (Spencer, Avendaño and Garcia, 1984; Buesa et al., 1987).

Rodriguez et al. (1983) did an electrophoresis study of rotavirus strains from a tertiary care nursery and found strains with more than the normal complement of 11 RNA genomic segments in 5/102 (4.9%) patients.

If a mixed infection is involved, reassortment of rotavirus RNA genomic segments within one individual could create a new rotavirus strain with properties different from the two "parent" strains and which potentially might have greater virulence. Reassortment is a common phenomenon among viruses with segmented genomes such as reovirus and influenza virus.

Some human viruses have non-human reservoirs. Influenza virus type A have bird, horse, and swine hosts, and rabies virus have feral mammal hosts (Davis et al.1980).

Infection of another animal species, even without disease production, may be the mechanism for evolution of new strains of rotavirus. The rotavirus genome is naturally segmented like that of influenza viruses and undergoes reassortment.

In this sense, animals other than humans may be considered reservoirs. However, an animal reservoir for human rotaviruses has not yet been demonstrated.

D. Susceptibility of human populations to rotavirus infection.

Rotavirus particles can be identified in stool from premature and neonatal babies. Renterghem, Borre and Tilleman (1980) examined stools from 199 premature babies in Belgium by EM and detected rotavirus in 24 (6.6%). It was found that 19 were asymptomatic.

Likewise, Perez-Schael et al. (1984) detected rotavirus by ELISA in the stools of 62/108 (57.4%) neonates. Of the 62 positive neonates only 9 of 62 (15%) had diarrhea, showing the generally asymptomatic nature of this situation. It is theorized that the enterocytes in the neonate's small intestine are not fully developed.

Hoshino et al. (1985) also examined neonates and could make no correlation between the specific serotype infecting neonates and the occurrence of asymptomatic or mild infection. It was observed that infection with "neonatal strains" of rotavirus did not confer protection against

reinfection but did modulate the severity of a subsequent rotavirus infection which occurred during the first three years of life.

IV. MOLECULAR CHARACTERISTICS OF ROTAVIRUSES

A. Characterization of rotavirus.

Newman et al. (1975) compared epizootic diarrhea of infant mice (E.D.I.M. agent) and calf rotavirus and reported that both rotavirus particles contained two capsids and 11-12 segments of double-stranded RNA. The infectious rotavirus particle has a double capsid and a buoyant density of 1.36 g/ml in cesium chloride. The complete infectious rotavirus particle has a molecular weight of 11-12 x 10^6 daltons. Non-infectious rotavirus particles have a single capsid and a buoyant density of 1.38 g/ml in cesium chloride.

It has now been established that the rotavirus group contains 11 double-stranded RNA segments ranging from 667 to 3,302 base pairs, each coding for a separate protein, 6 structural and 5 nonstructural (Estes and Cohen, 1989).

Infectivity remains stable at pH 3.0, is relatively heat stable, and is also resistant to ether (Meng et al., 1987). The morphology is icosahedral and virus particles are approximately 68 nm in diameter (Palmer, Martin and Murphy, 1977) although more recent studies indicate that the diameter is 70 to 75 nm (Madeley and Field, 1988). Replication takes place in the cytoplasm in the viroplasm

next to the rough endoplasmic reticulum (Schulze and Schumacher, 1984). The host range is mammalian and avian species and each virus is species specific for clinical diarrhea (Estes, Palmer and Obijeski, 1983).

B. Classification of rotavirus.

Davidson et al., in 1975, utilizing anti-reovirus antisera demonstrated that this newly detected virus was serologically different from reovirus. They called this new virus duovirus due to the double capsid and placed them in the family Reoviridae. The family Reoviridae now contains the genera reovirus, rotavirus, and orbivirus.

V. MOLECULAR EVENTS IN ROTAVIRUS INFECTIONS AND THE DISEASE PROCESS

A. Pathology of rotavirus infections.

The initial stage of infection occurs at the molecular level involving viral and cellular receptors. Rotaviruses contain two major neutralization proteins on the outer capsid, VP3 and VP7 (Hoshino et al., 1988).

When treated with trypsin, VP3 is partially cleaved thus increasing infectivity of rotavirus particles (Sato et al., 1987). Trypsin treated rotavirus enters cells by direct penetration of the enterocytes' membrane (Kaljot et al., 1988) whereas untreated virus, while non-infectious, is taken up into the cell by endocytosis (Suzuki et al., 1985; Suzuki et al., 1986). Therefore, a successful infection by rotavirus requires not only permissive cells, but the

proteolytic enzyme trypsin for primary cleavage of VP3. This condition is met in the small intestine where the pH is raised and trypsin is present to activate the virus particles.

Rotavirus generally invades enterocytes of the proximal small intestinal mucosa, but necropsy studies have shown that the infection may spread along the entire length of the small intestine and even invade the colon. Evidence exists for inflammation in the stomach, duodenum, and rectum. Thus the whole gastrointestinal tract may be affected (Walker-Smith, 1978). Blacklow and Cukor (1981), however, found the gastric and colonic mucosa to usually be normal in appearance. Following infection, the histological appearance of the gastrointestinal tract returns to a normal state in 4 - 8 weeks.

Davidson et al., 1975, by immunofluorescent examination of the duodenal mucosa of children observed fluorescence in a supranuclear position in the cytoplasm of duodenal epithelial cells and none was observed in the nuclei. Not all epithelial cells however, fluoresced in any one section nor was any fluorescence observed in the lamina propria.

B. Pathophysiology of rotavirus infections.

The diarrhea occurring from rotavirus infection is an osmotic type diarrhea resulting from primary carbohydrate malabsorption. Graham et al. (1984) found no evidence of adenylate cyclase mediating the diarrhea since c-AMP levels

in enterocytes were the same for virus infected piglets as well as the controls. Hamilton (1985) also reported an intact c-AMP-mediated secretory mechanism which is suggestive of producing a diarrhea due to undifferentiated crypt-cells lining the villi. This is in contrast to a disease such as cholera in which adenylate cyclase and c-AMP have a role due to the toxin affecting the adenylate cyclase and c-AMP.

Other changes noted (Snodgrass et al., 1979) include a reduced disaccharidase and thymidine kinase level which is indicative of immature cells, as well as a malfunctioning of the glucose-coupled sodium transport, and impaired D-xylose absorption. These effects could all lead to an impairment of the absorptive capacity of the intestine.

Carbohydrate malabsorption is generally regarded as the rule in rotavirus illness. Sack et al. (1982) stated that the hypothesized pathophysiological mechanism for the diarrhea is the decreased absorption of salt and water (particularly glucose-mediated absorption). While the fluid secreted into the small intestine contains normal levels of Na^+, K^+, Cl^-, and HCO^3, the bacterial fermentation that takes place using the malabsorbed carbohydrate consumes HCO^3 to yield water and carbon dioxide. Therefore the stool is acidic.

Uhnoo, Olding-Stenkvist and Kreuger (1986) stated that rotavirus induced diarrhea is associated with high levels of

carbohydrates. There is an osmotic shift of water into the gut lumen producing an osmotic diarrhea.

VI. METHODS OF ROTAVIRUS DETECTION AND CULTIVATION

A. Introduction to methodology.

Human rotaviruses grow to very high titers in the human intestinal tract. It was calculated that one gram of feces may typically contain between 10^8 and 10^{10} rotavirus particles (Albert et al., 1987). Rotaviruses have not been cultivated and plaqued directly from a fecal sample, therefore, any assay system for detection of rotavirus must be based on the detection of proteins contained in the capsid.

Techniques commonly used for rotavirus detection are listed in Table 3.

Nahmias et al. (1985) list the six S's of viral diagnosis as: 1) suitability - is the test right for what is sought, 2) specificity - will the test detect what you are trying to identify, 3) sensitivity - will the test detect low levels of the agent, 4) speed - is the test rapid, 5) simplicity - is the test easily performed, and 6) savings - is the test cost-effective. The most widely used tests at the outset of this project were EM, RIA, and ELISA (Blacklow and Cukor, 1981). As of 1989, the most popular techniques for detection of rotavirus are ELISA, latex agglutination, electrophoresis of viral genomic RNA, and EM.

Table 3

Methods used for rotavirus detection

detection method	virus component detected
Electron microscopy	whole rotavirus
Immune electron microscopy	whole rotavirus
Gel diffusion	whole rotavirus
Plaque assay	infectious rotavirus
Cytopathic effects	infectious rotavirus
Immunofluorescence	rotavirus antigen
Enzyme-Linked Immunosorbent Assay	rotavirus antigen
Radioimmunoassay	rotavirus antigen
Hemagglutination	rotavirus antigen
Peroxidase-anti-peroxidase	rotavirus antigen
Latex agglutination	rotavirus antigen
Complement fixation	Ab to antigen
Counterimmunoelectroosmophoresis	Ab to antigen
Electrophoresis	genomic RNA

Adapted from Estes, Palmer and Obijeski, 1983

The need for a reliable detection method that is simple to perform in the minimum amount of time are the prime considerations of any clinician since the exact therapy is dependent upon the specific etiologic agent which has induced the disease.

Advanced detection methods such as EM may not be feasible in developing nations. Small clinics and many large hospitals even in developed nations such as the United States may not have the financial ability to utilize this method of detection. Therefore, the optimal technique is one that is cheap to perform, does not require highly

trained personnel, and is stable even at ambient temperature.

Unfortunately, most of the easiest to perform tests are immunologically based and may be stable for long periods of time only at refrigerator temperature, which could pose problems in developing nations.

B. Electron microscopy (EM).

Human rotavirus has been detected in vivo and in vitro by many techniques. The first technique utilized for rotavirus detection was EM by Bishop in 1973. In these early studies, EM was the only tool available for the detection of rotavirus particles, and is still a good method since the morphological structure of rotavirus is diagnostic. Unfortunately the technique is expensive in terms of equipment and trained personnel and is time consuming.

C. Polyacrylamide gel electrophoresis (PAGE).

PAGE is a widely used method that is probably more suited for the research laboratory. This method is based on the extraction of the RNA from rotavirus particles and is electrophoresed through a polyacrylamide gel matrix.

The results obtained by this method have shown that the genome of rotavirus is comprised of three molecular weight species of RNA designated high, medium and low. When observed on the gel, rotavirus RNA yields a profile from the

gel top to bottom of (high to low m.w.) four groups of bands of four, two, three and two bands (Lourenco et al., 1981).

The gel may be stained with ethidium bromide and viewed under ultraviolet light (Clarke and McCrae, 1981; Taniguchi, Urasawa and Urasawa, 1982) or stained with silver nitrate and viewed directly (Herring et al., 1982). Follet and Desselberger (1983) estimated the silver-stain method to be 10-100 times more sensitive than ethidium bromide staining, although more time is required.

The main disadvantage of PAGE, at least for the diagnostic laboratory, is that the method requires special equipment, and takes two to three days for results. Highly trained personnel are required to perform the test, and ethidium bromide is potentially carcinogenic.

D. Enzyme-linked immunosorbent assay (ELISA).

The most widely used rotavirus detection method today is probably ELISA. Historically, ELISA's have been in existence since 1977 when Yolken et al. used an ELISA to detect the human reovirus-like agent which was what rotavirus was known as at that time.

This immunologically based method utilizes immune immunoglobulins to physically capture proteins or glycoproteins on the rotavirus particle's outer capsid. The immune globulins thus immobilize the rotavirus particles in wells of a plastic 96-well microtiter plate.

E. Assays using nitrocellulose (NC) paper.

NC paper is often used in a modification of the ELISA method. Basically this technique is the same as a solid-phase ELISA except that NC paper is used as the solid-phase for virus attachment instead of a plastic microtiter plate. NC paper has a high affinity for proteins.

F. Alternate methods not in common usage.

Many other methods have been reported but are used infrequently. Included in this group are reverse passive hemagglutination (Sanekata and Okada, 1983; Tsuchie et al., 1983), immune adherence hemagglutination (Matsuno and Nagayoshi, 1978; Kapikian et al., 1981), counterimmunoelectrophoresis (Hammond et al., 1984), staphylococcal co-agglutination (Skaug, Figenschau and Orstavik, 1983), and direct hemagglutination by goose and day-old chicken erythrocytes (Kitaoka et al., 1984).

G. Cultivation techniques.

One of the earliest reports of in vitro cultivation of a human rotavirus was by Wyatt et al. (1974) using human fetal intestinal organ culture. Then Purdham et al. (1975) reported that human rotaviruses could be cultivated using primary human embryonic gut cell monolayers. Wyatt et al. (1976) used stool filtrate from a different patient than in the first report and applied the sample to human embryonic cells and rotavirus was cultivated. Thus, the early attempts at adaptation of human rotavirus used cells that

were not readily available to all researchers and were poorly defined as to cell type (probably a mixed cell population).

In 1977, Wyatt et al. reported probable in vitro growth of a reovirus-like (rotavirus) agent obtained from stool of a 7 month old patient in a Washington, D.C. hospital.

Babiuk et al. (1977) reported that the presence of the proteolytic enzyme trypsin enhanced infectivity of bovine rotavirus isolates. Clark, Barnett and Spendlove (1979) also worked with a bovine rotavirus isolate and demonstrated that trypsin increased rotavirus yield 3,000 times. They also demonstrated that the trypsin had an effect on the rotavirus particles directly and not on the cells to increase rotavirus infectivity.

Smith et al. (1979) developed a plaque assay using the simian rotavirus isolate SA11. The plaque assay employed MA 104 cells which are of African Green monkey kidney origin. They also demonstrated that plaque production was possible using the plaque enhancing agents pancreatin or trypsin, and DEAE-dextran in the overlay.

An important event in the cultivation of human rotavirus was reported by Wyatt et al. (1980) with the in vitro cultivation of human rotavirus type 2 which was presumptively isolated in 1977. This was the first human rotavirus fully adapted to cell culture and capable of continuous passage in cell culture. Adaptation was only

possible by continuous passage of the rotavirus isolate in gnotobiotic piglets (11 passages) followed by 14 passages in MA 104 cells.

Shortly afterwards, isolation of human rotaviruses was achieved in primary AGMK cells which were subsequently demonstrated to be more sensitive to rotavirus than were MA 104 cells (Hasegawa et al., 1982).

It is now dogma that primary isolation should be done by pretrypsinizing the rotavirus isolate to increase infectivity. The rotavirus should be cultivated in primary AGMK cells in tubes, trypsin included in the overlay, and the rotavirus infected tubes should be continuously rolled at 37°C for 7 - 10 days.

CHAPTER 2

INTRODUCTION TO THE PROBLEM

I. OCCURRENCE OF ROTAVIRUS GASTROENTERITIS

At the start of this study (1982), little information existed regarding the prevalence of rotavirus illness in the state of Hawaii. Based on a limited EM study at Kapiolani Medical Center, Seto (1982) stated that rotavirus particles could be recovered from feces of selected patients with rotavirus symptoms in Hawaii. However, most rotavirus infections in Hawaii were routinely diagnosed based solely on the clinical signs and symptoms of the patient and were not confirmed by laboratory data.

Rotavirus infection has been reported to be responsible for much of the acute diarrhea among children. In the subtropical climate of Kuwait, Sethi et al., 1984 reported that 42.3% of the children with acute diarrhea aged 2 to 12 months of age were infected with rotavirus. In the temperate climate of Washington, D.C., the positive rotavirus detection rate in hospitalized infants with acute gastroenteritis averaged 34.5% over an eight year study (Brandt et al., 1983). Since patients in Hawaii are not routinely laboratory tested for rotavirus, the percentage of "true" rotavirus infections among children hospitalized for diarrhea symptoms in Hawaii is not known. This question is being evaluated in the present study.

II. SEASONALITY OF ROTAVIRAL INFECTIONS AND EPIDEMICS

A considerable body of epidemiological information has been amassed to date on rotavirus diarrhea and the first important pattern to be recognized is the seasonality of this illness. Every year it is observed that a winter epidemic occurs in the cool, dry months of the year (December - March) in temperate climates (Brandt et al., 1982; Konno et al., 1978). On continental America, rotavirus illness begins in central America early in the fall in September and cases are observed until December (Mata et al., 1983). After the onset of this yearly epidemic, the rotavirus infections are observed to move up through central America, through Mexico and into southwestern U.S. after which it spreads across the continental U.S. with a subsequent epidemic season that usually occurs from December to March. Brandt et al. (1983), working with patients in a Washington, D.C., reported that rotavirus is first detected between October 22 and December 22, with a peak in January to February of each year. Rotavirus was not detected in August, September and the first three weeks of October.

Sethi et al. (1984), working in a tropical climate, reported that the virus could be detected year round in Kuwait but was most frequent in months with low rainfall and humidity (March to May). The picture is not as clear cut in tropical climates since some researchers have reported that

an epidemic season occurs in the winter months whereas others have reported that the virus appears to be endemic with low levels detected all year round (Chiba et al., 1984; Hanlon et al., 1987; Sitbon et al., 1985; Soenarto et al., 1983). It would appear that these winter epidemics are a function of climatic conditions corresponding to temperature and rainfall since the epidemic observed in New Zealand each year is from May - October (Holdaway et al., 1985) which corresponds to the cool, dry winter season (Brandt et al., 1982). The pattern of rotavirus illness in Hawaii has not been determined and will be addressed in this study.

III. SEROTYPIC DIVERSITY

There are at least seven serotypes of rotavirus infecting humans based on differences of the protein composition of the viral capsids. Identification of different serotypes can be determined using immunological methods and is used to determine the epidemiology of different serotypes of rotavirus infection in one part of the state or country as compared to other parts. Classically, serotyping rotavirus isolates depended upon cell adaptation of the virus and subsequent plaque reduction neutralization. Since many rotavirus strains cannot be made to grow in cell culture, this approach has not been useful for rotavirus typing. As of 1987, monoclonal antibodies (MAb) used in an ELISA test became available for serotyping rotavirus even from stool specimens (Coulson et al., 1987).

Gerna et al. (1987b), claimed that of the immunologically based EM techniques, only solid-phase immunoelectron-microscopy (SPIEM) could be used for serotyping stool samples. Using this method the authors reported that from 1981 until 1985, in Italy, 70.5% (91/126) samples were serotype 1, 13.2% (17/126) were serotype 2, no serotype 3 samples identified, and 13.2% (17/126) were serotype 4. The prevalence in other European countries is 44.4% for serotype 1, 21.1% for serotypes 2 and 4, and 8.8% for serotype 3. The distribution of rotavirus serotypes in Hawaii has not been determined and will be addressed in this study.

IV. DIVERSITY OF ELECTROPHORETIC PROFILES

The genome of rotavirus is unusual in that it is comprised of 11 discrete RNA segments. Electrophoresis of rotaviral genomic RNA has yielded two major profiles (short and long) based on the differential mobility of segments 10 and 11 (Spencer, Avendano, and Araya, 1983). The majority of human rotavirus isolates yield a long pattern (Arista et al., 1983; Gomez et al., 1986), however, within the long pattern there seems to be great diversity: Buesa et al. (1987) found 14 subpatterns in 49 samples, Dolan et al. (1985) found 8 subpatterns in 22 samples, Gerna et al. (1987a) found 7 subpatterns in 38 samples, and Lourenco et al. (1981) found 29 subpatterns in 110 samples containing both short and long samples. Despite the many distinctive electrophoretic patterns demonstrated for the rotavirus

genome, Beards (1982) pointed out that there is no correlation between the electrophoretic patterns and serotypes of rotavirus. However, Beards (1982) concluded that the electrophoretic patterns of rotavirus can be used as epidemiological markers to assess the strain of rotavirus infecting a population. The distribution of the electrophoretic patterns of the RNA's of rotavirus in Hawaii has not been determined and will be addressed in this study.

V. CHARACTERIZATION OF PLAQUE-PRODUCING HUMAN ROTAVIRUS

Most disinfection and stability experiments use the two prototype rotavirus strains (SA11 and Wa) which are easily cultured and produce plaques. (Vaughn, Chen and Thomas, 1986; Ward, Knowlton and Winston, 1986; Winston and Ward, 1985; Berman and Hoff, 1984). The use of these two model rotaviruses to predict the characteristics of human rotavirus has not been widely accepted because SA11 is a nonhuman strain and the human Wa strain has an unusual history of being adapted to grow under laboratory conditions. Characterization of a newly isolated rotavirus strain from a human is a superior model virus to predict the properties of human rotaviruses. However, a plaque-producing rotavirus is a prerequisite for classical characterization of rotavirus. Plaque producing rotaviruses isolated from humans in Hawaii will be characterized and compared against the two model rotaviruses (SA11 and Wa).

CHAPTER 3

GOALS, OBJECTIVES AND EXPERIMENTAL DESIGN OF THIS STUDY

I. GOALS OF THIS STUDY

The initial goal of this study was to confirm by laboratory data the recovery of rotavirus from stool samples of hospital patients with diarrheal illness in Hawaii. The second goal of this study was to adapt these isolated rotavirus strains to grow in cell culture and to characterize these isolates to further understand the significance of this disease in Hawaii.

II. OBJECTIVES OF THIS STUDY

A. To confirm by detection and isolation of rotavirus in stool samples of patients that rotavirus is a cause of infections in diarrheal patients admitted to Kapiolani Medical Center for Women and Children, a civilian hospital, and to Tripler Army Medical Center.

B. To determine the electropherotypes of rotavirus recovered from the stools of diarrheal patients. This approach allows for determining whether one or several strains of rotavirus are circulating in a population at a given time, and to make comparisons temporally.

C. To adapt rotaviruses isolated from the stool specimens to cell culture, and to derive a plaque-forming isolate for full characterization. This information will allow for a comparison with the predominant human strain being used for experimentation (Wa) and an animal strains (SA11),

and for comparisons with previous work examining serotype category, electrophoretic subpatterns, physical characteristics, and stability under various conditions.

D. To determine the serotype(s) of the hospital isolates. Serotypic analysis may be used epidemiologically for tracking strains infecting a population.

E. To develop more efficient immunologically based detection systems for human rotaviruses as compared to the standard ELISA and EM.

III. OVERALL EXPERIMENTAL PLAN

A. Stool specimens were collected from two Oahu hospitals, Kapiolani Medical Center and Tripler Army Medical Center. The specimens were tested by two commercial ELISA kits for the presence of rotavirus antigen contained in stool specimens from these hospitals. Samples were collected from Tripler Hospital in all months of one year to determine what months rotavirus illness is observed. This information will aid in assessing whether a peak rotavirus gastroenteritis season occurs in the winter months in Hawaii as is observed in temperate climates.

B. Samples obtained from Kapiolani and Tripler Hospitals were electrophoresed in polyacrylamide gels to determine the electropherotypes of rotavirus RNA. This method is used epidemiologically to track the movement of rotavirus strains within a population. Taniguchi, Urasawa and Urasawa (1982) reported that the genomic electrophoretic

profile of rotavirus does not change even with high passage in cell culture.

C. Primary isolation of human rotavirus isolates from the two hospitals was made in primary AGMK cells and the AGMK derived continuous cell line MA 104. An ELISA was used for monitoring the growth of rotavirus in cell culture. Other cell lines were examined for their ability to support the growth of human rotavirus and possibly permit plaque formation.

D. Plaquing of rotavirus was done as has been reported previously in the literature. A determination of the optimal conditions for plaquing human rotaviruses was made. The parameters tested were the best gelling agent for the overlay (agar/agarose), concentration and type of trypsin, and enzyme kinetics of the trypsin types to determine whether a predictive optimal concentration can be made based on the kinetic data. An important part of this study was to adapt a human rotavirus isolated from a patient in Hawaii to produce plaques in a tissue culture system.

E. Characterization of strains of rotavirus adapted to growth in cell culture and to produce plaques was done. Electron microscopy was performed by a standard EM method using negative staining by phosphotungstic acid to determine the morphology. Determination of the buoyant density of rotavirus strains SA11 and an adapted, plaque

forming human isolate was done in cesium chloride. A plaque forming rotavirus strain was suspended in various diluents (PBS, seawater and fresh stream water) and exposed to sunlight to simulate survival under natural environmental conditions. Using the same diluents, the rotavirus was suspended and tested for the strain's stability at room temperature (26°C) vs. refrigerator temperature (4°C).

F. The stability of the plaque-forming strain of rotavirus exposed to a number of environmental parameters such as temperature, water types, sunlight and chlorine was evaluated. A plaque-forming rotavirus strain suspension in various diluents (PBS, seawater and fresh stream water) was exposed to sunlight to determine stability. Sunlight experiments simulate introduction of rotavirus to the environment via fecal contamination. Thus, an evaluation can be made of the degree of inactivation of rotavirus by sunlight's effects. Using the same diluents, stability of rotavirus at room (26°C) vs. refrigerator (4°C) temperatures were determined. Chlorination of wastewater treatments effluents is commonly practiced to inactivate human pathogens prior to discharge into an environmental water source. Comparison of chlorination of a plaque-producing human rotavirus strain with SA11 will allow assessment of SA11's suitability as a model system for rotaviruses.

PART II

DETECTION AND CHARACTERIZATION OF HOSPITAL ISOLATES

I. OBJECTIVES OF THIS PART OF THE STUDY

To confirm by the ELISA technique the presence of rotavirus in stool samples of patients of two Oahu hospitals.

To determine the electropherotypes of rotavirus recovered from the stools of diarrheal patients for the purpose of establishing the identity of the strains of rotavirus in Hawaii.

To adapt rotaviruses isolated from the stool specimens to cell culture, and to derive a plaque-forming isolate for full characterization.

To determine the serotype of the rotavirus isolated from hospital stool samples by immunologic methods.

II. INTRODUCTION

A. ELISA analysis of clinical specimens.

The most often used laboratory method to detect the presence of rotavirus in stool samples is enzyme-linked immunosorbent assay (ELISA) (Cukor et al., 1984). Occasionally EM is used, but has been calculated to be less sensitive than ELISA (Brandt et al., 1987) and much more expensive.

It is generally acknowledged that hospital admissions for rotavirus gastroenteritis represent the most severe cases. Rotavirus detection rates in these admissions may

range from 33 - 100% during epidemic seasons (Albert, Bishop and Shann, 1983; Greenberg et al., 1981; Koopman et al., 1984; Nakagomi et al., 1985; Santosham et al., 1985; Sethi et al., 1984; Szücs, Kende and Uj, 1987; Tufvesson, 1983; Kansouzidou et al., 1989). During non-epidemic seasons, patients with detectable rotavirus antigen may not ever be observed.

B. Electrophoresis of rotavirus genomic RNA.

Polyacrylamide gel electrophoresis (PAGE) as a technique to detect for the presence of rotavirus by its characteristic profile of 11 segments of RNA is as sensitive as ELISA. Kasempimolporn et al. (1988) compared PAGE with an ELISA assay and found of the 1,304 stool specimens tested, 325(24.9%) were positive by both methods, 936(71.8%) were negative by both methods, 26(2.0%) were ELISA positive-PAGE negative, and 17(1.3%) were ELISA negative-PAGE positive.

All rotaviruses have a segmented RNA genome. Different strains of rotavirus therefore may have distinctly different PAGE profiles of their RNA, short and long profiles being very characteristic. The two smallest RNA segments occurring in the bottom doublet migrate further in long profiles than in short profiles. Thus, this technique is of use for epidemiologic analysis of circulating strains in a given population (Clarke and McCrae, 1982; Gerna et al., 1987).

"Long" electrophoretic profiles are identified more frequently than "short" profiles. Gómez et al. (1986) noted that of 99 samples, 84(84.8%) were of the "long" RNA profile. Likewise, Arista et al. (1983) found 16(88.9%) of 18 samples to be of the "long" profile. Occasionally the ratio is higher for the "short" profile. Spencer, Avendaño and Araya (1983) identified 51(36%) out of 142 isolates as being of the "short" profile. Espejo et al. (1980) reported that 89% of the samples examined in Mexico City and Santiago (Chile) were "short" patterns.

Occasionally more than 11 RNA bands are visualized on a gel which is indicative of a mixed rotavirus infection involving more than one electropherotype (Lourenco et al., 1981; Buesa et al., 1987).

C. Adaptation of Human Rotavirus Isolates to Cell Culture.

Human rotavirus (HRV) was first cultured in human fetal intestinal organ culture (Wyatt et al. 1974). Two years later, Wyatt et al. (1976) again cultivated HRV in a human embryonic kidney-cell culture using a stool filtrate from another patient. Wyatt's first report dealt with organ culture while the second report dealt with primary cells of human origin. These cultures were not available to all researchers, had a finite life span and were moreover different from batch to batch.

Human rotavirus (HRV) was first adapted to serial growth in a continuous African Green monkey kidney cell line (MA 104) by Wyatt et al. (1980) using the serotype 1 HRV strain Wa. This was the first HRV adapted to grow and plaque in a continuous cell line. This breakthrough enabled many experiments with rotavirus which were not possible previously.

It was recognized early that successful adaptation of rotaviruses to cell culture required pretreatment of the virus particles with the proteolytic agent trypsin, and the inclusion of trypsin in the maintenance medium. Babiuk et al. (1977) showed that the inclusion of trypsin in the maintenance medium of a bovine rotavirus strain infected monkey kidney cell line (BSC-1) raised the titer by 1.5 logs over that without trypsin. Deletion of the trypsin resulted in decrease of the infectious titer and eventual loss of the rotavirus by the 9th passage. Clark et al. (1981) stated that trypsin treatment of rotavirus particles increased infectivity by 100-fold and growth in cell culture by 10,000-fold.

The mechanism of proteolytic enhancement of rotavirus infectivity was postulated to be the conversion of the noninfectious virus particles to an infectious state, which allows cell penetration and multiplication of rotavirus within the cell (Estes, Graham and Mason, 1981). It was subsequently found that trypsin cleaved the outer capsid

protein VP3, the product of gene segment 4. Two other enzymes, elastase and pancreatin were also shown to enhance infectivity, whereas chymotrypsin had no effect because it cleaved the VP3 protein at another site. Trypsin treatment affecting VP3 was also shown to raise the infectivity of the simian rotavirus strain SA11 by 8 fold (Espejo, López and Arias, 1981).

Kantharidis, Dyall-Smith and Holmes (1987) stated that trypsin enhancement of infectivity by cleavage of VP3 allows for efficient uncoating of the virus particles within the cell.

From 1981 to 1983 several attempts were made to adapt HRV to grow in various cell lines and under various growth conditions (Sato et al., 1981; Urasawa, Urasawa and Taniguchi, 1981; Hasegawa et al., 1982; Birch et al., 1983; Albert and Bishop, 1984; Wyatt et al., 1983). For optimal growth, the following requirements were found to be necessary: 1) pretreatment of rotavirus with trypsin to activate the virus, 2) inclusion of trypsin in the cell's growth medium, 3) continuous rolling of the tubes containing the rotavirus infected cells, and 4) use of primary cells being preferential over continuous cell lines such as MA 104.

Ward, Knowlton and Pierce (1984) reported that primary monkey kidney (PMK) cells supported HRV growth better than CV-1 or MA 104 cells, both of which are continuous cells of

monkey kidney origin. They reported that two passages in PMK cells, followed by propagation in MA 104 cells, allowed for adaptation to the continuous cell line. A significant result is their calculation that 1 out of every 46,000 rotavirus particles contained in stool are infectious whereas after 3 passages in primary cells the number becomes 1 infectious rotavirus particle out of every 6,600 particles. This suggests that competition between infectious and non-infectious particles may be a primary barrier that must be overcome in adaptation.

D. Serotyping of Human Rotavirus Isolates.

The reference strains of human rotavirus are Wa (serotype 1), DS-1 (serotype 2), P (serotype 3), and ST-3 (serotype 4). Their origins (animal-country-year) are human-U.S.A.-1974, human-U.S.A.-1976, human-U.S.A.-1974, and human(neonate)-England-1975, respectively (Green et al., 1989). In addition to the four serotypes found in humans, serotype 5 rotaviruses are porcine, serotype 6 rotaviruses are equine, serotype 7 rotaviruses are avian, and the presumptive two new human serotypes will probably be classified as serotypes 8 and 9 (Gerna et al., 1988; Green, Hoshino and Ikegami, 1989).

Human rotaviruses are categorized serologically by two ELISA methods. Both methods utilize antibody (Ab) generated against the serotype specific antigenic determinant on VP7 which is encoded by gene segments 8 or 9, depending on the

strain (Taniguchi et al., 1988). The protein encoded by segments 8 or 9 is an outer capsid structural component (Arias, López and Espejo, 1982).

The first method utilizes polyclonal Ab (PAb) usually produced in rabbits or guinea pigs. An advantage of this method is that Ab production is relatively quick and inexpensive and yields PAb with a neutralizing specificity of the immunizing virus. The major disadvantage is that the rotavirus strain to be tested must either be cell culture adapted to perform a tube neutralization, or capable of producing plaques in order to perform a plaque reduction neutralization (PRN) test.

To use PAbs in a quick assay such as an ELISA, the PAb must be extensively cross adsorbed with whole virus of the non-immunized serotypes to remove any Ab specific for group epitopes which could yield a positive ELISA result for all serotypes (Birch, Heath and Gust, 1988). An example of this protein determinant would be VP6 which is a component of the inner viral capsid found in all group A rotaviruses and is considered the group antigen for all group A rotaviruses. With an antibody present which recognizes this protein, all group A rotaviruses would react positively regardless of the serotype.

The second method utilizes monoclonal Ab (MAb). Advantages of this method are that the MAb is very specific for the epitope being tested, usually the neutralization

antigen on VP7 (Taniguchi et al., 1987), and testing of stool specimens may be done by standard ELISA using MAb. Disadvantages are that either the technological expertise for MAb production is not available in all laboratories, or the high expense.

Production of high titered antiserum against rotaviruses has been accomplished by inoculation of laboratory animals. For anti-rotavirus serum production the two animals of choice to date are rabbits (Gerna et al., 1988; Kitaoka et al., 1987; Knisley, Bednarz-Prashad and Pickering, 1986; Ward, Knowlton and Pierce, 1984) and guinea pigs (Beards et al., 1984; Bellamy et al., 1983; Pereira et al., 1985; Yolken et al., 1977), although other sources such as goats (Yolken and Leister, 1982), cows (Brüssow et al., 1988) and chicken's egg yolks (Yolken et al., 1988) have been used.

One significant problem of immunization is that the previously mentioned animal species develop rotavirus infections at an early age (Flewett and Woode, 1978) and by the time the animal is available for antibody production it has usually already had a primary rotavirus infection. A primary infection may interfere with subsequent immunizations especially since the serotype of the primary infecting virus is not known without time consuming testing. DiGiacomo and Thouless in 1984 reported that out of 199 New Zealand rabbits from a commercial rabbitry tested in the

U.S., 100% had Ab to rotavirus at the ages of <1 month and >2 months. From 1 to 2 months of age, only 25% had significant Ab titers. The same study looked at 91 young adult and adult rabbits in Canada and 98% had rotavirus titers, while in Japan, 82% of 39 adult rabbits had a titer. Thus, obtaining an immunologically naive rabbit of greater than 2 months of age can be difficult without special handling by the breeders. The same situation is presumably true for laboratory guinea pigs as well.

III. MATERIALS AND METHODS

A. Stool specimens.

Stool samples were collected from patients at two hospitals. Patients from Carter Ward at Kapiolani Medical Center (KMC) for Women and Children represented children from the general population with diarrheal symptoms severe enough to warrant hospitalization. Stools from patients at Tripler Army Medical Center (TAMC) came from either the Pediatric ward (inpatients) or the Pediatric clinic (outpatients). TAMC is a military hospital for members of the military and their dependents. All specimens were collected in plastic stool specimen cups by hospital staff members and stored in a refrigerator (4°C) until picked up.

B. ELISA testing of rotavirus containing samples.

Stool samples collected at Kapiolani Medical Center (KMC) were held on ice packs (4°C) and picked up the same day of collection. Stool samples from Tripler Army Medical

Center (TAMC) were stored in a refrigerator (4°C) at the hospital and picked up within 5 days of collection. All of the stool specimens from both hospitals were held in a refrigerator (4°C) for a maximum of one week. A weeks collection was processed in one batch.

Initially in 1982 the Rotazyme rotavirus diagnostic kit (Abbott Laboratories, North Chicago, Illinois) was used for ELISA analysis. A 10% stool suspension in phosphate buffered saline was recommended by the manufacturer. The assay was run as per manufacturers instructions. The complete protocol is listed in Appendix A.

In 1983, the ELISA method used was changed to Dakopatts rotavirus ELISA reagents (Dakopatts rotavirus ELISA kit; Lot No. 0386; Accurate Chemical, Westbury, N.Y.). The change of ELISA kit was made due to a reported dilution of capture antibody by Abbott Laboratories which resulted in lower positive ELISA readings, both visual (by eye) and spectrophotometrically. A Rotazyme kit contains a color chart for visual examination obviating the need for a spectrophotometer. The readings may be sufficient for clinical uses, but were too low for research purposes. The ELISA assay was run as per manufacturer's instructions. The complete protocol is listed in Appendix B. A preliminary study was conducted to determine the ideal plastic microtiter plate to be used for the Dakopatts reagents. Results of the study are listed in Appendix C. As a result

of this preliminary study, Immulon II 96-well microtiter plates (Catalog No. 011-010-3450; Dynatech Laboratories, Inc., Alexandria, Virginia) were selected for their high affinity for protein and the even dilution pattern obtained.

Color development was accomplished using o-phenylenediamine hydrochloride (OPD; Catalog No. P-1526; Lot No. 93F-5017; Sigma Chemical Co., St. Louis, Missouri) as instructed by the manufacturer.

Microtiter plates were read at 495 nm in a Titertek II microtiter plate reader (Flow Laboratories, McLean, Virginia).

C. Extraction of rotavirus RNA for electrophoresis.

A modification of the method of Croxson and Bellamy (1981) was used to extract rotavirus genomic RNA for electrophoresis. Briefly, stool samples positive for rotavirus (ELISA assay) were prepared as a 10% suspension in 0.01 M lithium acetate (lithium acetate dihydrate; Catalog No. L-6883; Sigma Chemical Co., St. Louis, Missouri), pH 5.0. The vortexed suspension was centrifuged in a Beckman TY 65 fixed angle rotor with 8 slots (Spinco Division of Beckman Instruments, Inc., Palo Alto, California) in a Beckman model L Ultracentrifuge (Spinco Division of Beckman Instruments, Inc., Palo Alto, California) at 6,000 x g for 10 minutes.

The supernatant was collected and brought to a concentration of 1% with lithium dodecyl sulfate (lauryl

sulfate, lithium salt; Catalog No. L-4632; Sigma Chemical Co., St. Louis, Missouri). This suspension was heated at 65°C for 5 minutes, and then extracted twice with phenol (liquefied U.S.P.; Matheson, Coleman & Bell, Norwood, Ohio) and chloroform (chloroform, N.F.; Mallinckrodt, Inc., Paris, Kentucky) at a 2:1 ratio. After centrifugation for 5 minutes at 850 x g in an IEC model PR-2 centrifuge with 12 slot swinging bucket rotor (International Equipment Co., Needham Heights, Massachusetts), the aqueous phase was collected then extracted once with ethyl ether (Fisher Scientific, Fair Lawn, New Jersey). This mixture was centrifuged as above and the ether phase was removed with a Pasteur pipette. The aqueous phase was exposed to gently blown air for 1 hour to remove residual ether.

The ether-free RNA solution was precipitated with 2 volumes of 90% ethanol (Fisher Scientific, Pittsburgh, Pennsylvania) at -20°C overnight. The RNA was pelleted in a Beckman TY 65 rotor at 6,000 x g for 15 minutes, the supernatant was decanted, and the pellet was allowed to air dry for 2 hours. The dry RNA was finally suspended in electrophoresis running buffer (Appendix D) and stored at -20°C until used.

Purified RNA was quantitated by spectrophotometry at 260 nm. One mg of rotavirus genomic RNA has been reported to equal to 5.1 A_{260} units (Espejo et al., 1980b; Offit et

al., 1983). Therefore, an absorbance reading of 1 is equal to 196 μg of genomic RNA.

D. Electrophoresis of rotavirus genomic RNA.

Electrophoresis was done in 0.75 mm, 14 cm x 16 cm polyacrylamide gels with a 4% stacking gel and a 10% resolving gel by Laemmli's method (1970). The running buffer was modified for this study (Ward, 1983, personal communication; see Appendix D). The elimination of sodium dodecyl sulfate from the running buffer was suggested by Espejo and Puerto (1984). A gel was preelectrophoresed at 20 mA for 1 hour and run with sample at 15 mA for 16 hours in a vertical electrophoresis apparatus (Model SE 400; Hoefer Scientific Instruments, San Francisco, California).

The gel was silver-stained using a modification of the method of Herring et al. (1982). Briefly, the gel was soaked for 30 minutes in 10% ethanol and rinsed twice for 1 minute in 400 ml of distilled water. The gel was then flooded with 200 ml of 0.011 M silver nitrate and incubated at 26°C for approximately 1.67 hour in the dark with gently shaking. Approximately 20 minutes before the end of the 2-hour incubation, the gel was exposed to the light from a 75 watt incandescent bulb held six inches from the gel's surface. The gel was rinsed in distilled water as before. Two hundred ml of reducing solution (0.75 M sodium hydroxide-0.1 M formaldehyde-0.0023 M sodium borohydride) was added to the gel. The gel was shaken gently for 5

seconds, the liquid decanted, and 200 ml of new reducing solution was added and gently shaken until the bands appeared. The reducing solution was decanted and the gel was rinsed with distilled water as before. Development was stopped by soaking the gel in 5% acetic acid. Gels were successfully stored in the acid solution in a covered plastic food tray at 4°C for six months.

E. General cell culturing methods.

MA 104 cells for the cultivation of rotavirus were the gift of Dr. R.L. Ward, Cincinnati, Ohio. MA 104 cells were grown in either Sani-Glas 32 oz. glass prescription bottles (Brockway Glass Co., Piscataway, New Jersey) or 150 cm^2 tissue culture flasks (Catalog No. 25126; Corning Glass Works, Corning, New York).

Cells were subcultured by washing twice with GKN (Appendix E), and adding GKN supplemented with 0.1% EDTA (Ethylenediamine tetraacetic acid, tetrasodium salt; Catalog No. ED-4SS; Sigma Chemical Co., St.Louis, Missouri) and 0.25% tissue culture grade trypsin (Difco 1:250 tissue culture grade trypsin (Difco Laboratories, Detroit, Michigan). When the cells rounded and began streaming from the glass, the monolayer was triturated, using a 10 ml pipette, from the glass with 10 ml of MEM + 8% FBS. Cells were split 1:4 into new flasks every week.

Media for cell growth was Minimum Essential Medium (Eagle-Modified) with Earle's salts (Catalog No. 10-121-22;

Flow Laboratories, McLean, Virginia) containing 8% Fetal Bovine Serum (FBS; Catalog No. A-1111-L; Flow Laboratories, McLean, Virginia). Cells were grown for three to four days at 37°C in a CO_2 incubator (National Model 3331/3321; National Appliance Company, Portland, Oregon) and fed with the above medium supplemented with 5% FBS.

F. Primary isolation of human rotavirus in cell culture.

Primary AGMK cells were purchased from Microbiological Associates. Primary Cynomolgus monkey kidney cells (CMK) were a gift of Tripler Army Medical Center. MA 104 A and B cells were a gift of Dr. R. L. Ward. MRC-5 cells were a gift of Flow Laboratories.

AGMK and CMK cells were seeded into 13mm x 125mm glass screw-cap tubes (Corning Glass Works, Corning, New York). These cells were grown in MEM-L15 medium (formula in Appendix E) plus 10% FBS. MEM-L15 medium was described by Simmonds et al. (1985). MA 104 cells were cultured in the same tubes and grown in MEM(Eagles) plus 10% FBS.

Primary isolation was done by the method of Urasawa, Urasawa and Taniguchi (1981). Briefly, stool samples containing rotavirus were made up to a 10% suspension in PBS (Appendix E). To this suspension, tissue culture grade trypsin (Gibco, gift of Dr. R. L. Ward) was added to a final concentration of 10 μg/ml. The mixture was allowed to react at 37°C for 1 hour.

The cells in screw-cap tubes (roller tubes) were washed twice with PBS, and 0.25 ml of the virus was inoculated onto the monolayer and incubated for 1 hour at 37°C. The inoculum was redistributed at 10-minute intervals. Following adsorption, the tubes were washed twice with PBS and maintenance medium consisting of serum free MEM-L15 medium for primary cells or serum free MEM(Earles) containing 3 μg/ml of Gibco trypsin.

The tubes were incubated in a roller apparatus (Wyble Engineering Development Corporation, Silver Spring, Maryland) at 37°C for 5 to 7 days on the initial passage and 3 to 5 days thereafter.

Detection of rotavirus growth was determined by ELISA analysis of 3X freeze-thawed cell lysates.

G. Plaque reduction neutralization.

The plaque reduction neutralization assay used was a modification of Wyatt et al. (1982). Briefly, a rotavirus stock was diluted to 100 - 150 PFU/0.1 ml. An equal volume of 3μg Gibco trypsin/ml was added to the rotavirus stock and incubated at 37°C for 1 hour. After this incubation, the virus suspension was mixed with an equal volume of 2-fold dilutions of rotavirus immune serum.

Cell culture plates containing monolayers of MA 104 cells were washed twice with Dulbecco's PBS (Appendix E) and 0.4 ml of the rotavirus-antiserum mixture was added per plate of MA 104 cells and incubated at 37°C for 1 hour with

intermittant redistribution of the inoculum. After the incubation, the monolayers were overlayed with medium containing 0.18% ME agarose, 3 μg Gibco trypsin/ml, 50 μg DEAE-dextran/ml, and 1:500 fetal bovine serum.

Antibody titers were expressed as a dilution of antiserum which yielded a 60% reduction in plaque counts. A 20-fold or greater difference in titer distinguished between serotypes.

CHAPTER 4

ELISA ANALYSIS OF STOOL SAMPLES FOR ROTAVIRUS ANTIGEN FROM PATIENTS AT TWO OAHU HOSPITALS

I. OBJECTIVES OF THE CHAPTER

To obtain stool samples from two Oahu hospitals from the fall of 1982 until the spring of 1986, and assay for the presence of rotavirus by commercial ELISA.

II. RESULTS

A total of 33 stool samples from KMC and 111 stool samples from TAMC were analyzed. The results summarized in Table 4 show that 64% (21/33) of KMC samples and 18% (20/111) of TAMC samples were positive for rotavirus antigen.

Analysis of rotavirus positive samples for distribution of positive samples over time (Figure 4) shows that the appearance of positive samples corresponded with the winter months of December to March of each year. When samples from only these months were considered, the number of ELISA-positive samples was 64% (21/33) for KMC and 28% (20/71) for TAMC. Samples from Kapiolani Hospital were collected from November until March of each year because this was the only time during the year the physicians made multiple diagnosis of rotavirus gastroenteritis based on clinical symptoms. The same applies for Tripler Hospital except for 1984 when samples were collected in all months of that year except for February through April.

Table 4

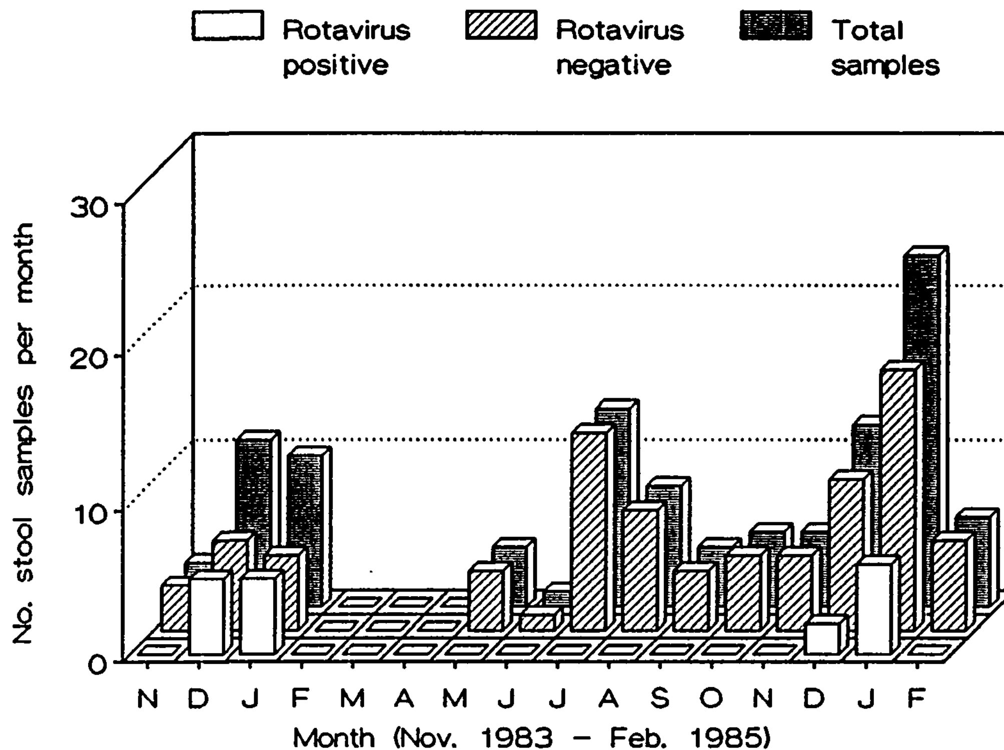
ELISA positive stool samples by year from Kapiolani Medical Center for Women and Children (KMC) and Tripler Army Medical Center (TAMC)

	KMC		TAMC	
Year	No. Positive/ No. Tested	% Pos.	No. Positive/ No. Tested	% Pos.
1982-83	11/17	65	N.D.[a]	N.D.
1983-84	4/7	57	10/64	16
1984-85	N.D.	N.D.	8/45	18
1985-86	4/5	80	N.D.	N.D.
1986-87	2/4	50	N.D.	N.D.
1987-88	N.D.	N.D.	2/2	100
Total	21/33	64	20/111	18

a = not done

An outbreak of gastroenteritis which affected both adult and child dependents of military members as well as active military members housed at Barbers Point Military Housing Facility was monitored from July until August of 1984. Stool samples as well as water samples were examined by Rotazyme ELISA for the presence of rotavirus antigen. The result of ELISA analysis of a total of 33 stool specimens were and 3 water samples were negative.

Figure 1. Distribution of stool samples suspected of containing rotavirus obtained from Tripler Army Medical Center by month



III. DISCUSSION

Using commercial ELISA kits, rotavirus antigen was detected in the stool of hospitalized infants/children at the two Oahu hospitals (KMC and TAMC). These results represent the first set of laboratory data to demonstrate the presence of rotavirus in Hawaii.

The one year survey at TAMC showed that rotavirus was detected only from December through March of 1984-85. While samples may have been few in number, there were nonetheless no positive samples outside of the winter months. This is suggestive that rotavirus gastroenteritis has a winter epidemic season such as is seen in temperate climates.

It should be pointed out that any case of rotavirus illness observed at Tripler Hospital represents a case contracted in Hawaii and was not directly imported from elsewhere since the infant was in residence in Hawaii for a longer period of time than is observed for the incubation period of rotavirus (72 hours). However, the initial rotavirus infection could have been contracted outside of Hawaii and be imported by a traveler to Hawaii. This imported rotavirus could infect residents in Hawaii. This study cannot address this hypothesis directly. However, evidence for this can be obtained by characterizing the rotavirus isolated from patients in Hawaii.

The stool samples from patients at the Barber's Point Military Housing Complex were consistently negative for

rotavirus and indicate that rotavirus was not the cause of the gastroenteritis outbreak. Two other important features of this epidemic support this statement. First, the outbreak occurred in the summer months. Based on ELISA testing of hospital cases in Hawaii, rotaviruses were detected most frequently in the winter months. Second, the gastroenteritis observed occurred in both adults and children.

Tao et al. (1984) reported on a new rotavirus type that infected adults and caused gastroenteritis, and was responsible for an epidemic affecting 12,000 adults in China in late 1982 - early 1983. However, these rotaviruses are novel, and represent newly recognized group B and C rotaviruses (Bridger, 1987). These new groups are morphologically similar to group A rotaviruses but are serologically dissimilar and can not be detected by the standard immunologically based methods (Eiden et al., 1986).

Thus, the possibility exists that a novel rotavirus strain may have been responsible for the outbreak at the Barbers Point facility but this is speculation. Reports of new groups of rotavirus are becoming more frequent (Von Bonsdorff and Svensson, 1988; Yolken et al., 1988; Matsumoto et al., 1989; Ushijima et al., 1989; Peñaranda et al., 1989), and with better detection methods the frequency of detection of novel rotavirus strains will surely increase.

CHAPTER 5

ELECTROPHORESIS AND VISUALIZATION OF ROTAVIRUS dsRNA

I. OBJECTIVES OF CHAPTER

To determine the genomic RNA profiles of the hospital isolates by polyacrylamide gel electrophoresis (PAGE).

To adapt the silver-staining method of Herring et al. (1982) for visualizing the genomic RNA bands of rotavirus.

II. RESULTS

Preliminary work was done to determine the quantity of genomic RNA necessary for visualization by silver-staining. Espejo et al. (1980b) reported that ≤ 250 ng of rotaviral RNA is needed to visualize the full complement of 11 bands by ethidium bromide staining.

The SA11 genomic RNA was extracted by the method of Croxson and Bellamy (1981) as described in Methods and Materials (Part II). A dilution series of SA11 genomic RNA demonstrated that 150 - 200 ng was necessary for visualization of the bands in the gel following silver-staining.

Results of the ELISA positive specimens showed that 16/21 (76.2%) stool samples from Kapiolani Medical Center (KMC) and 9/20 (45%) from Tripler Army Medical Center (TAMC) contained enough sample to extract RNA for visualization by PAGE and silver-staining. Table 5 summarizes the samples visualized by year of collection.

Electropherograms observed in this study were all group A rotavirus profiles. The direction of RNA migration on all polyacrylamide gels was from the top to the bottom, and the 11 bands were segregated into four major subgroups comprised of four, two, three, and two bands from top to bottom. In many cases, less than 11 bands were seen but this is indicative of comigration of two bands which causes the two bands to be superimposed. This is supported by the

Table 5

Distribution by year of hospital stool samples detected by ELISA and visualized by PAGE

Year	ELISA positive (no./total)	ELISA positive PAGE negative (no./total)	ELISA positive PAGE positive (no./total)
1982-83	11/17	2/11	9/11
1983-84	14/71	4/14	10/14
1984-85	8/45	8/8	0/8
1985-86	4/5	0/4	4/4
1986-87	2/4	1/2	1/2
1987-88	2/2	0/2	2/2
Total No.	41/144(28.5%)	15/41(36.6%)	26/41(63.4%)

observation that one band stained more intensely than the others in that region.

Results of electrophoresis of KMC stool samples revealed that the majority of the profiles were of the

"long" type. Figure 2A shows the RNA profile of KMC isolates 1, 2, 5, 7, 8 and 9. Isolates 5 and 7 were determined to be similar as were isolates 8 and 9. Lane 5 (KMC 5) was difficult to visualize, so a second gel was run with more RNA from isolates KMC 5 and KMC 7 than were used previously (Figure 2B). Isolates KMC 5 and 7 displayed the same RNA profile, and 18 bands were observed.

PAGE results showed that all KMC profiles were "long" except for KMC 24 which was "short".

Figure 3 shows a representative gel containing TAMC samples. Both TAMC 16 and TAMC 20 are "long" profiles while TAMC 23 was a "short profile. All TAMC profiles were determined to be "long" except for TAMC 12 and TAMC 23 which were "short".

Figure 4 shows isolates KMC 24 and TAMC 23 electrophoresed on the same gel. It was determined that both isolates were similar, and came from the same rotavirus season. An insufficient number of samples were available from TAMC 12 for comparison, however, the profile seen in a separate gel indicates a similar profile.

Genomic RNA profiles of the 16th passage (primary AGMK cells) of cell-adapted isolates was determined for several KMC (Figure 5A) and TAMC isolates (Figure 5B).

In all, for the 25 isolates observed by PAGE, 17 separate RNA profiles were observed.

Figure 2. Electrophoretic profiles of KMC isolates directly isolated from stool samples

A - lane 1 = SA11 lane 2 = KMC 9 lane 3 = KMC 8 lane 4 = KMC 7
lane 5 = KMC 5 lane 6 = KMC 2 lane 7 = KMC 1 lane 8 = Wa

B - lane 1 = KMC 5 lane 2 = KMC 7

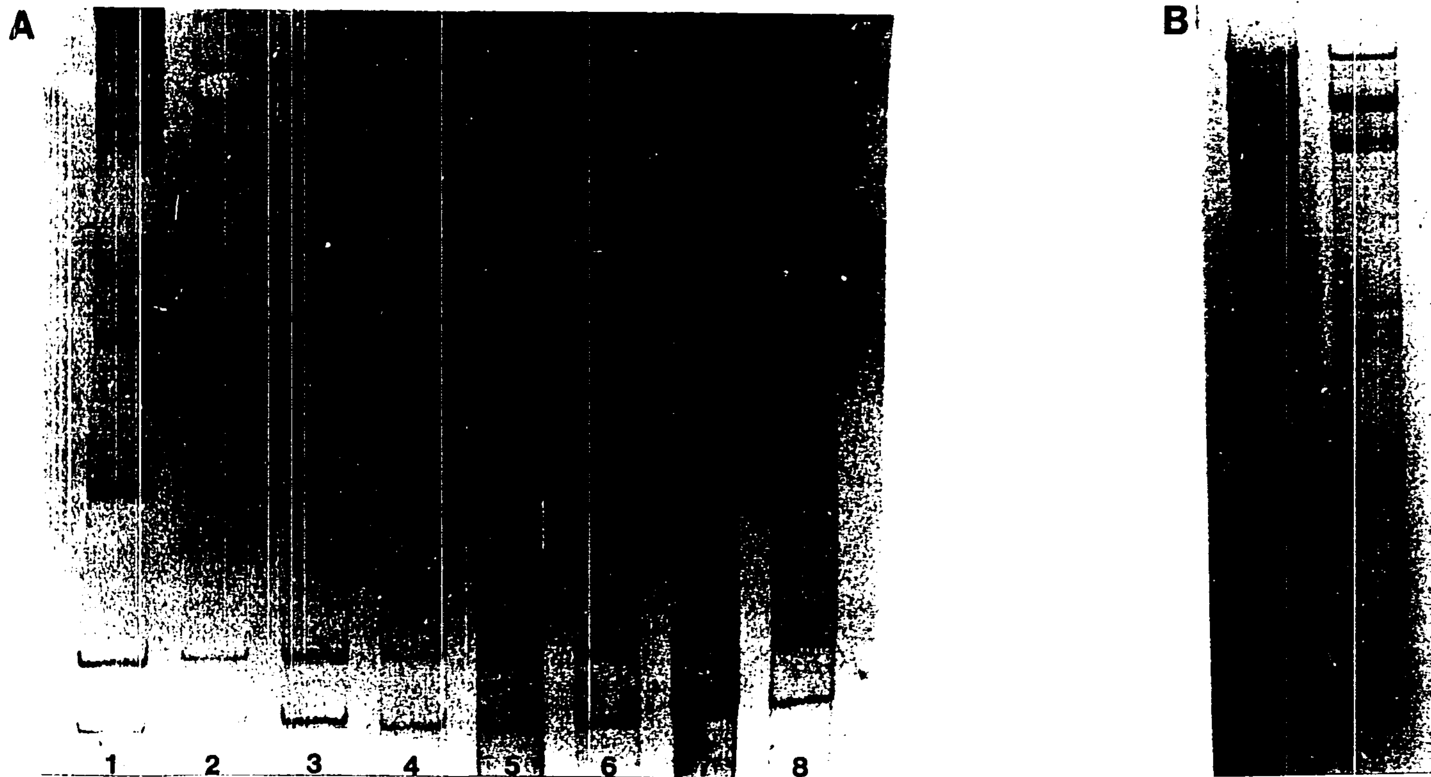


Figure 3. Electrophoretic profiles of TAMC isolates directly isolated from stool samples

lane 1 = SA11
lane 2 = TAMC 16
lane 3 = TAMC 20
lane 4 = TAMC 23

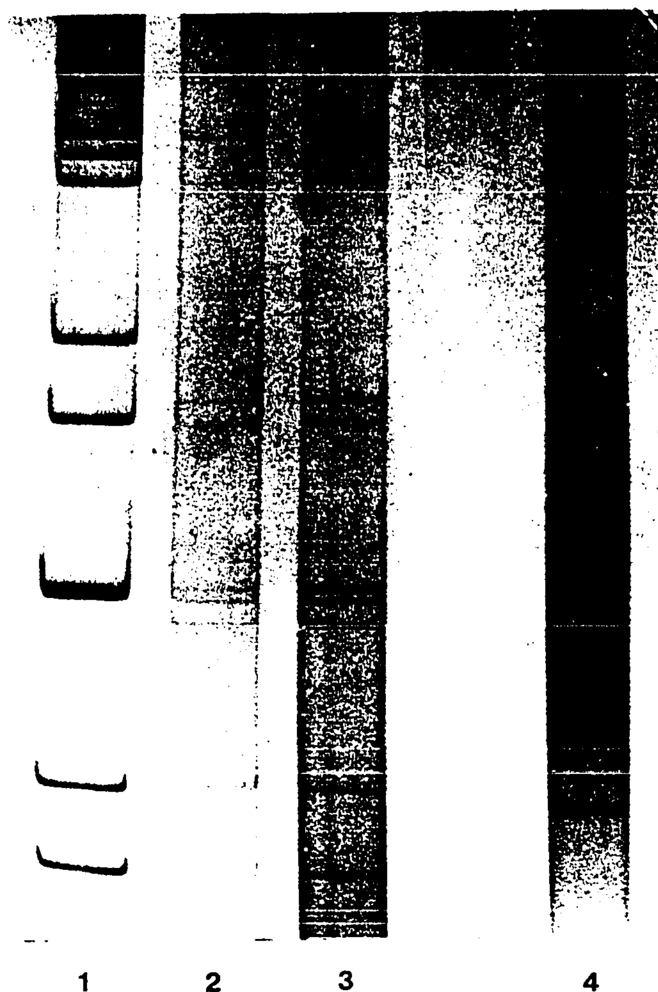


Figure 4. Short electrophoretic profiles of isolates KMC 24 and TAMC 23 directly isolated from stool samples

lane 1 = SA11
lane 2 = KMC 24
lane 3 = TAMC 23

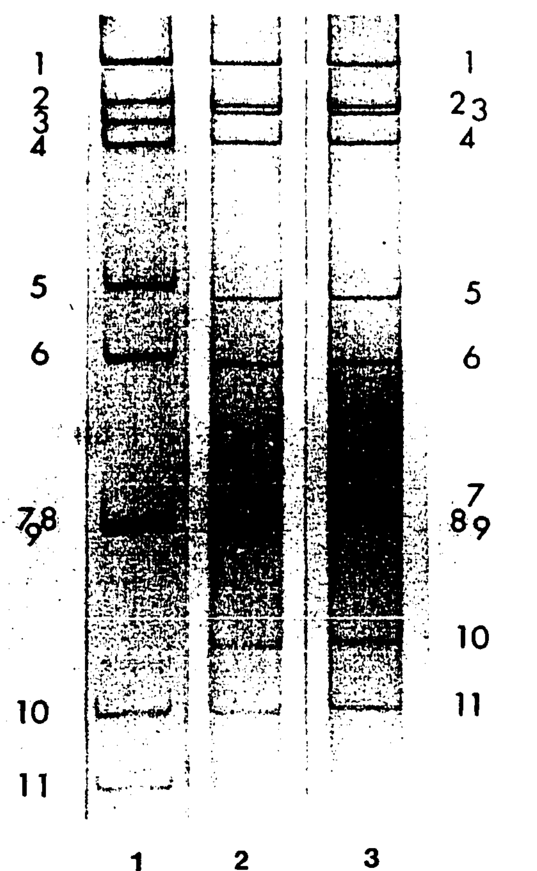
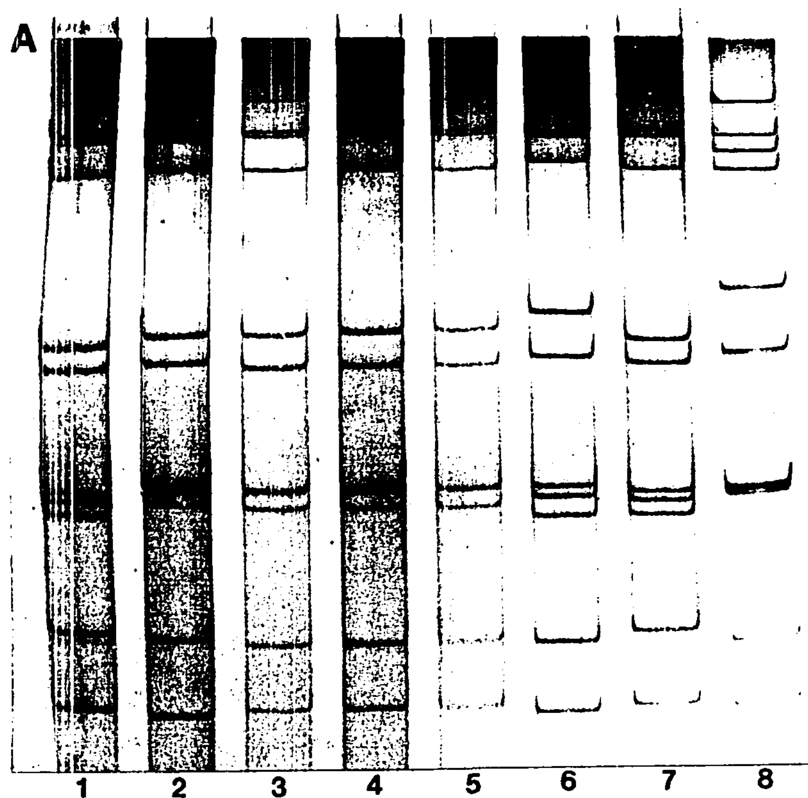


Figure 5. Electrophoretic profiles of cell adapted KMC and TAMC isolates

A - lane 1 = KMC 18 lane 2 = KMC 15 lane 3 = KMC 9 lane 4 = KMC 9 + 8
lane 5 = KMC 8 lane 6 = KMC 2 lane 7 = KMC 1 lane 8 = SA11

B - lane 1 = TAMC 18 lane 2 = TAMC 18 + 11 lane 3 = TAMC 11
lane 4 = TAMC 15 lane 5 = TAMC 7 lane 6 = SA11



DISCUSSION

Silver-staining was chosen over ethidium bromide staining since silver has been reported to be more sensitive than ethidium bromide. Follett and Desselberger (1983) have stated that PAGE with ethidium bromide staining is less sensitive than silver staining by one order of magnitude.

The majority of the RNA profiles observed in this study were of the "long" profile (22/25 - 88%) while 3 (12%) were of the "short" profile. Similar results were found in a previous study. Albert, Soenarto and Bishop (1982), observed 68/85(80%) "long" and 17/85(20%) "short" profiles among stool samples acquired from Indonesia.

Rotavirus stool isolates TAMC 12, TAMC 23, and KMC 24 were all obtained in the same year, which is highly suggestive that they are all the same strain. Gómez et al. (1986) have stated that "short" profiles are genetically more stable than "long" profiles, therefore less variation of profiles were observed. The current work supports this statement.

Many of the stool samples collected from TAMC were less than 1 ml in volume which may account for negative PAGE results. Theil et al. (1981) stated that 6 ml of stool sample will yield sufficient RNA for ≥ 15 PAGE assays. A volume of 0.1 ml of sample was required for ELISA and 0.1 ml was required for cell culture adaptation attempts. That left less than 1 ml for RNA extraction, and not all of the

sample was extracted at once. In some cases, the spectrophotometric reading indicated that sufficient RNA was present but interference from contaminants present in the preparation, such as phenol, may have accounted for an overestimation of the RNA content.

Many diverse RNA profiles were observed in this study. This is consistent with other researchers' reports from different geographical locations around the world. Steele and Alexander (1988) in Africa found 14 distinct RNA profiles, Chiba et al. (1984) also in Africa found 16 RNA profiles, and Gerna et al. (1987a) working in Italy found seven different PAGE profiles.

Two isolates, KMC 5 and KMC 7, displayed RNA profiles of more than 11 bands. Spencer, Avendaño and García (1983) determined that 10% of their 149 samples were mixed. The gel therefore displayed more than the usual 11 bands following electrophoresis and staining. Extra bands may be due to minor mutations which change the molecular weight and hence the mobility of the band, or concatemers of low M.W. segments may be formed (Tam et al., 1986). This phenomenon was observed twice in two separate gels. However, when a third gel was prepared with the same RNA extracts, both isolates displayed the usual 11 bands. The reason for the discrepancy is unknown. It may be that the extra bands were unstable and degraded, or else there was concatemerization with dissolution later.

A comparison of genomic RNA extracted from stool with genomic RNA extracted from cell culture suggests that no changes occurred. Taniguchi, Urasawa and Urasawa (1982) observed the RNA profile of cultivated rotavirus isolates to be identical to the profiles observed for RNA extracted directly from stool samples. Birch et al. (1983) also reported that cultivation of rotavirus in cell culture did not affect the electrophoretic profile.

PAGE has epidemiologic applications for following the spread of a rotavirus strain in a gastroenteritis outbreak (Chanock, Wenske and Fields, 1983). PAGE is also a useful method for detecting non-group A rotaviruses (Kasempimolporn et al., 1988) which do not react with present ELISA assay systems (Peñaranda et al., 1989), but whose RNA profile may be visualized on a polyacrylamide gel.

It should be noted stated that the electropherotype of a rotavirus isolate may not necessarily relate to other known biologic or antigenic properties of rotaviruses (Tam et al., 1988).

CHAPTER 6

ADAPTATION OF HUMAN ROTAVIRUS ISOLATES TO CELL CULTURE

I. OBJECTIVES FOR THIS CHAPTER

To adapt human rotavirus isolates from hospital stool samples to growth in cell culture using MA 104 cells and primary AGMK cells.

II. RESULTS

Stool samples were collected from Kapiolani Women's and Children's Medical Center and Tripler Army Medical Center and assayed by either Rotazyme or Dakopatt rotavirus ELISA kits. Samples which were ELISA positive were clarified, pretreated with trypsin, and initially inoculated onto monolayers of MA 104 cells. Three different sources of MA 104 cells (lines UH, A and B) were tested for sensitivity to HRV. Selected rotavirus isolates were tested for continuous propagation in cell culture (Table 6) and the results indicate that no major difference existed between cell lines UH and B. TAMC 10 rotavirus was weakly positive by passage three in cell line UH, but negative in cell line UH at the fourth passage and thereafter. One significant advantage of cell line B over UH is that it had a higher degree of resistance to trypsin in the maintenance medium and the cells could be incubated for over 1 week as compared to 4 to 5 days for cell line UH. For this reason, cell line B was preferentially used for all further work.

Later in the study, primary monkey kidney (PMK) cells were available for comparison with MA 104 cells for HRV

Table 6

Efficiency of MA 104 cells for the cultivation of HRV isolates after three passages (detection of growth by ELISA)

	MA 104 line		
HRV Isolate	UH[a]	A[b]	B[b]
TAMC 7	+	+	+
TAMC 8	+	+	+
TAMC 10	±	-	-
TAMC 12	-	-	-
TAMC 16	+	-	+
TAMC 19	-	-	-
TAMC 20	-	-	-

a = purchased from Flow Laboratories
b = gift from Dr. R.L. Ward, Thomas N. Gamble Institute, Cincinnati, Ohio.

isolation. Results of ELISA detection of rotavirus growth presented in Table 7 showed that PMK cells were superior to the continuous cell line MA 104 for the cultivation of HRV strains. Another observation was that MA 104 cells were erratic in their ability to adapt HRV. The results listed in Table 7 showed that isolates TAMC 7 and TAMC 8 were successfully adapted to MA 104 cells whereas in the experimental results listed in Table 7, the same isolates did not grow. For this reason, primary AGMK cells were determined to be the best cell for adaptation of rotavirus isolates.

The isolation and primary AGMK passage results of KMC and TAMC HRV strains are summarized in Table 8. Not all stool samples positive for HRV based on ELISA testing (e.g.,

Table 7

Efficiency of three cell types for the cultivation of HRV isolates after three passages (detection of growth by ELISA).

HRV Isolate I.D.	Cell line		
	MA 104	*Cynomolgus* monkey kidney	African Green monkey kidney
TAMC 5	-	-	-
TAMC 7	-	+	+
TAMC 8	-	-	+
TAMC 10	-	-	-
TAMC 11	-	+	+
TAMC 12	-	-	±

KMC 10 and TAMC 20) could be passed in primary AGMK cells. Some samples (KMC 24, TAMC 19, TAMC 23) were positive for rotavirus only through three passages in primary AGMK cells. ELISA reactivity stopped at the fifth passage for strains KMC 12, KMC 21, TAMC 10, TAMC 12, and TAMC 16. ELISA reactivity stopped at the seventh passage for strains KMC 7, and TAMC 8. ELISA reactivity stopped at the tenth passage for strain TAMC 5.

Successful adaptation for this study was defined as a positive ELISA result after 5 passages in primary or secondary AGMK cells in roller tubes (Table 9).

Table 8

Efficiency of primary AGMK cells for the cultivation of HRV isolates after multiple passages (detection of growth by ELISA).

HRV isolate I.D.	Passage Number 1	3	5	7
KMC 1	+	+	+	+
KMC 2	+	+	+	+
KMC 7	+	+	+	-
KMC 8	+	+	+	+
KMC 9	+	+	+	+
KMC 10	-	-		
KMC 12	+	+	-	-
KMC 15	+	+	+	+
KMC 18	+	+	+	+
KMC 21	+	+	-	-
KMC 24	+	-	-	
KMC 26	+	+	+	+
KMC 27	+	+	+	+
KMC 29	+	+	+	+
KMC 30	+	+	+	+
TAMC 5	-	±	+	+
TAMC 7	+	-	+	+
TAMC 8	+	+	+	-
TAMC 10	+	+	-	-
TAMC 11	+	+	+	+
TAMC 12	-	+	-	-
TAMC 15	+	+	+	+
TAMC 16	+	+	-	-
TAMC 18	-	+	-	+
TAMC 19	+	-	-	
TAMC 20	-	-		
TAMC 23	+	-	-	
TAMC 68	-	-		
TAMC 69	-	-		
TAMC 71	-	-		
TAMC 79	-	-		
TAMC 83	-	-		
TAMC 86	-	-		
TAMC 94	-	-		
TAMC 109	-	-		
TAMC 110	-	-		
TAMC 111	-	-		

TABLE 9

Distribution by year of rotavirus isolates from Kapiolani (KMC) and Tripler (TAMC) Medical Centers adapted to cell culture for 5 passages

	KMC		TAMC	
Year	No. Positive/ No. Tested	% Pos.	No. Positive/ No. Tested	% Pos.
1982-83	6/11	55	N.D.[a]	N.D.
1983-84	1/4	25	5/10	50
1984-85	N.D.	N.D.	0/8	0
1985-86	3/4	75	N.D.	N.D.
1986-87	1/2	50	N.D.	N.D.
1987-88	N.D.	N.D.	0/2	0
Total	11/21	52	5/20	25

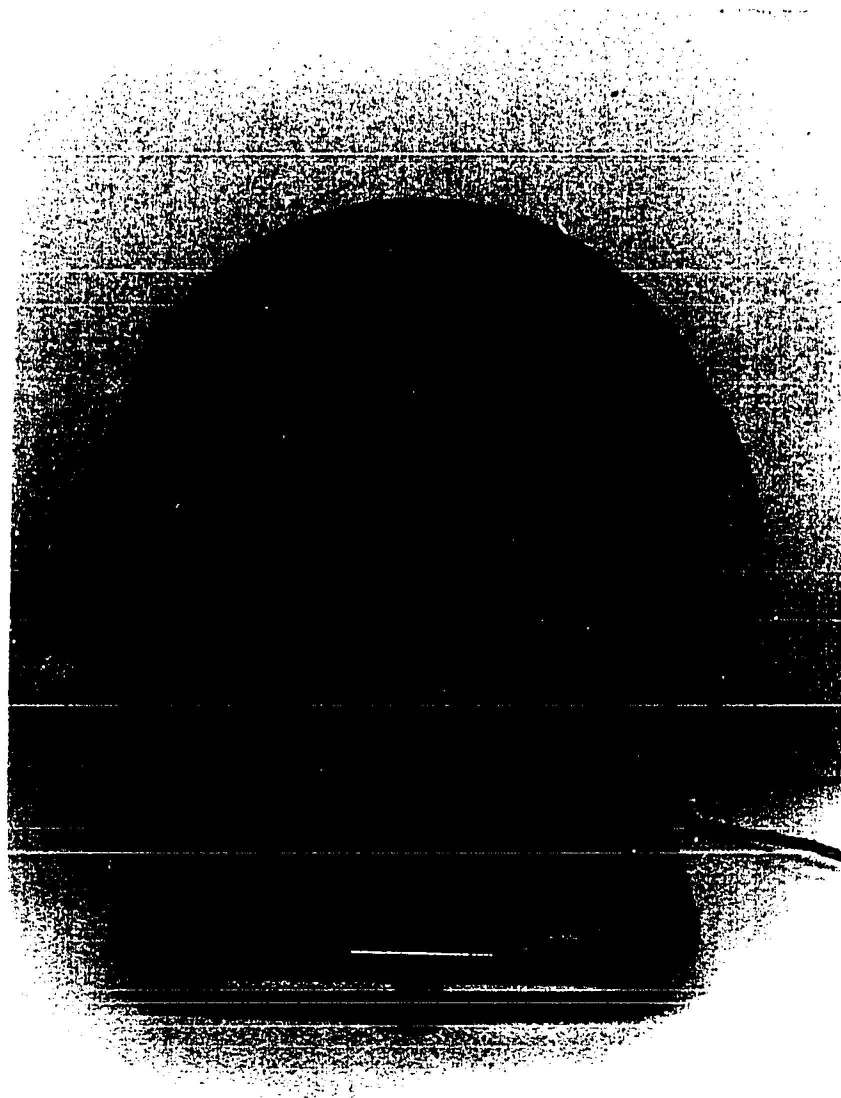
a = not done

The roller apparatus used throughout this study is shown in Figure 6.

Adaptation results for Kapiolani stool samples showed isolates KMC 1,2,8,9 and 15 from the 1982-83 season, isolates KMC 18 from 1983-84, isolates KMC 26, 27, and 29 from 1985-86, and isolates KMC 30 from 1986-87 were all successfully adapted to continuous growth in primary AGMK cells.

Adaptation results for Tripler stool samples showed that isolates TAMC 7,11,15, and 18 were adapted from the 1983-84 season. No 1984-85 stool samples were adapted.

Figure 6. Roller apparatus for the continuous rolling of tubes containing rotavirus-infected cell monolayers



ELISA negative samples from both hospitals were also inoculated onto primary AGMK cells but no specimen yielded an adaptable rotavirus strain after three passages as determined by ELISA.

Results for initial attempts at adapting HRV isolates to MA 104 cells were less successful than in primary AGMK cells. Some isolates could be passaged for three to five times before ELISA detection of rotavirus antigen stopped.

Optimal results were obtained when the rotavirus stool isolates were continuously passaged in primary AGMK cells. Upon sequential passage in MA 104 cells, some of the isolates would become ELISA negative after 2 passages. Therefore, the isolates were passed only in primary AGMK cells. For the purposes of this study, a positive adaptation is defined as the ability to sequentially pass the isolate in primary AGMK cell culture for 5 passages.

Ward, Knowlton and Pierce (1984) calculated that 1 rotavirus particle out of every 46,000 rotavirus particles contained in stool is infectious. To determine whether a high noninfectious/infectious stool contained rotavirus particle ratio could interfere with successful cell adaptation, selected rotavirus isolates were purified and banded by cesium chloride buoyant density centrifugation to separate infectious from noninfectious rotavirus particles. The selected rotavirus isolates included all three short electrophoretic profile rotavirus isolates (KMC 24, TAMC 12,

TAMC 23) and long profile isolates KMC 12, KMC 32, and TAMC 5. These isolates were selected on the basis of results obtained under the standard adaptation conditions in which these isolates did not adapt to primary AGMK cells, or adapted poorly and were eventually lost.

The purified and potentially infectious rotavirus particles were pretreated with trypsin, inoculated onto primary AGMK cells, and maintained for 7 days in roller tubes at 37°C and supplemented with trypsin. None of the banded isolates yielded a positive ELISA reaction after any of the five passages made in primary AGMK cells. The results suggest that the ability to adapt an isolate to cell culture growth is not solely due to the presence of interfering (noninfectious) rotavirus particles which were removed after cesium chloride banding.

III. DISCUSSION

A total of 52% of the ELISA positive samples from Kapiolani hospital, and 25% of the ELISA positive samples from TAMC adapted to primary AGMK cells. The disparity between the adaptation rates between the samples from the two hospitals may be that the majority of the positive samples from Tripler hospital were obtained in a year in which no samples were collected from Kapiolani hospital. The rotavirus strains in circulation in the different years may have been different enough to preclude adaptation to

cell culture. The small sampling size does not allow for meaningful statistical analysis.

However, there is a different makeup of patients in the two hospitals. Since KMC is a public hospital and charges for services, an infant may be more acutely ill when the parents seek medical attention than would an infant that is admitted to TAMC where services for military dependents are free. Stool samples from KMC were screened for presumptive rotavirus containing samples, whereas TAMC samples were not as carefully pre-screened.

After five passages in AGMK cells, the ELISA positive Kapiolani hospital stool samples yielded 11/14 (78.57%) adapted HRV strains whereas Tripler hospital samples yielded 5/20 (25%). The majority of the samples adapted were from two different years (1982-83 for Kapiolani and 1983-84 for Tripler). It was noted that the total load of rotaviral antigen in the later stool samples from Tripler gave very low positive/negative ratios in general, as compared to earlier samples from both hospitals.

CHAPTER 7

SEROTYPING OF HUMAN ROTAVIRUS ISOLATES

I. OBJECTIVES OF THIS CHAPTER

To produce anti-rotavirus immune serum in guinea pigs for serotyping plaque-producing rotavirus isolates.

To serotype hospital isolates from this study by MAb-based ELISA.

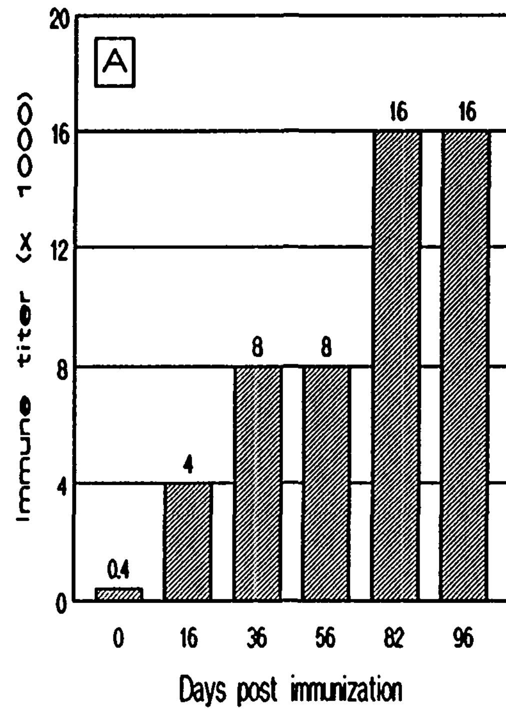
II. RESULTS

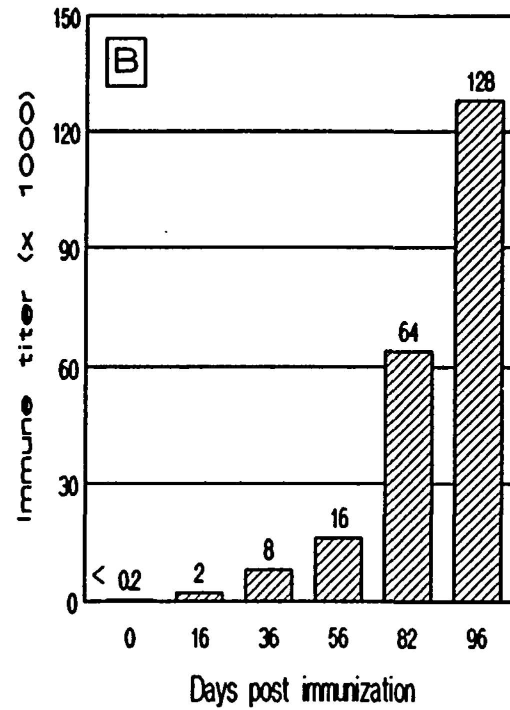
A. Production of Hyperimmune Guinea Pig Antiserum

Hyperimmune antiserum was produced in Hartley guinea pigs using purified, cesium chloride banded isolate TAMC 15 (serotype undetermined) and strain P (serotype 3) infectious rotavirus particles (buoyant density: 1.36 g/ml). Suspensions of these rotavirus isolates was inoculated into the flanks of guinea pigs (see infection schedule, Part II, Materials and Methods). Serum from the guinea pigs was monitored for Ab to rotavirus by plaque reduction neutralization (PRN) using isolate TAMC 15. The preimmune reciprocal titer of the guinea pig serum immunized with TAMC 15 was < 200. The titer at 16 days was 2,000, 8,000 at 36 days, 16,000 at 56 days, 64,000 at 82 days and 128,000 at 96 days (Figure 7A). The preimmune reciprocal titer of the guinea pig serum immunized with serotype 3, strain P was 400. The titer at 16 days was 4,000, 8,000 at 36 days, 8,000 at 56 days, 16,000 at 82 days and 16,000 at 96 days (Figure 7B).

Figure 7. Increase in titer with time of anti-TAMC 15 and anti-P immune sera in guinea pigs

A - anti-P B - anti-TAMC 15





The anti-rotavirus serum made in this study was used for rotavirus serotyping by PRN and tube neutralization. Anti-rotavirus serum (anti-WA, serotype 1; anti-DS-1, serotype 2; anti-P, serotype 3; anti-ST3, serotype 4) specific for the four human rotavirus serotypes was donated by Dr. Richard L. Ward (James N. Gamble Institute of Medical Research, Cincinnati, Ohio) and used as standards.

B. Serotypic Analysis of Human Rotavirus Isolates

Three methods were used for serotyping rotavirus isolates. These were PRN, tube neutralization, and monoclonal antibody-based ELISA (MAb-ELISA).

Thirty-one rotavirus hospital isolates from stool samples and rotavirus grown in cell culture were assayed by MAb-ELISA for the determination of serotype. All samples were sent to Baylor College of Medicine (Texas Medical Center, Houston, Texas). The MAb-ELISA assay was done courtesy of Dr. David Matson in Texas.

Results are summarized in Table 10. Out of seventeen isolates from KMC, 10 (58.82%) were serotyped by MAb-ELISA. Six (60%) were serotype 1, one (10%) was serotype 2, none were serotype 3, and two (20%) were serotype 4. KMC 12 was serotyped as both serotypes 1 and 4 (10%).

Out of fifteen isolates from TAMC, 5 (31.3%) were serotyped by MAb-ELISA. Four (80%) were serotype 1, one (20%) was serotype 4, and none belonged to serotypes 2 or 3.

Of the fifteen serotyped isolates from both hospitals, the two predominant serotypes were 1 (62.5%) and 4 (18.75%).

TABLE 10

Serotypic analysis of human rotavirus hospital isolates by monoclonal antibody ELISA[a]

KMC samples		TAMC samples	
Sample I.D.	Serotype	Sample I.D.	Serotype
KMC-1	1	TAMC-5	4
KMC-2	1	TAMC-7	1
KMC-7	1	TAMC-10	N.I.[B]
KMC-8	4	TAMC-11	N.I.
KMC-9	N.I.	TAMC-16	1
KMC-10	N.I.	TAMC-18	N.I.
KMC-12	1 & 4	TAMC-20	1
KMC-15	N.I.	TAMC-22	N.I.
KMC-16	1	TAMC-23	N.I.
KMC-18	N.I.	TAMC-68	N.I.
KMC-24	2	TAMC-71	N.I.
KMC-25	N.I.	TAMC-79	1
KMC-26	1	TAMC-86	N.I.
KMC-27	1	TAMC-94	N.I.
KMC-29	4	TAMC-109	N.I.
KMC-30	N.I.	TAMC-110	N.I.
KMC-32	N.I.		

a - study performed by Dr. D.O. Matson, Baylor College of Medicine, Houston, Texas
b = not identified by MAb-ELISA

In addition, one serotype 2 (6.25%) and one mixed serotypes 1 and 4 (6.25%) were identified.

The serologic relationships among rotavirus isolates using guinea pig anti-rotavirus antisera are summarized in Table 11. All values were obtained by PRN.

Isolate TAMC-15 and strain P rotaviruses (serotype 3) have two-way neutralization which indicates similarity. The simian rotavirus SA11, also a serotype 3 rotavirus, neutralized to a dilution 1:16,000 with anti-P serum that was made in this study, but 1:8,000 with anti-TAMC 15 serum. When Ward's anti-P serum was used to neutralize strain P and isolate TAMC 15, both neutralized to a dilution of 1:8,000.

Isolate TAMC 15, which produces plaques, was assayed by PRN to determine the serotype. The method of Wyatt et al. (1982) was used for the PRN. TAMC 15 was neutralized by anti-TAMC 15 (1:128,000) and anti-P (1:8,000) sera but not anti-Wa, anti-DS-1, and anti-ST3 (all < 1:10). TAMC 15 was determined to be serotype 3 by PRN (Table 11).

TABLE 11

Neutralizing titers of guinea pig anti-rotavirus sera for strains of human and simian rotavirus

	Serum Titer					
Virus	α-Wa	α-DS-1	α-P	α-P	α-TAMC15	α-ST3
Wa(1)*	**10,000**	< 10	< 10	< 100	< 100	< 10
DS-1(2)	< 10	**4,800**	< 10	< 100	400	< 10
P(3)	< 10	< 10	**8,000**	16,000	128,000	< 10
TAMC15(3)	< 10	< 10	8,000	16,000	**128,000**	< 10
SA11(3)	N.D.[a]	N.D.	N.D.	16,000	8,000	N.D.
ST3(4)	< 10	< 10	< 10	< 100	< 100	**35,000**

* - serotype of strain in parentheses a = not done
Homologous titers listed in bold-face

Cell culture grown rotavirus isolates were assayed by tube neutralization using the method of Wyatt et al. (1983). Briefly, two-fold dilutions of rotavirus antiserum (Ward's antiserum) were mixed with 100 $TCID_{50}$ units of all cell adapted rotavirus isolates, incubated for one hour at 37°C and inoculated onto roller tubes of MA 104 cells. Each dilution was run with five replicates, with inconclusive results obtained. The dilution series for each virus was either all ELISA positive or all ELISA negative for rotavirus growth. The experiment was repeated three times with the same results.

Results of MAb-ELISA using commercially purchased (RV-4, Silenus Laboratories Pty., Ltd., Hawthorn, Victoria, Australia) anti-rotavirus serotype 1 MAb done in this study were inconclusive. All rotavirus samples reacted positive. When the assay was done on nitrocellulose paper by the NC-EIA method of Loh, Dow and Fujioka (1985) all rotavirus samples were negative.

II. DISCUSSION

Results summarized in Table 10 show that the predominant serotype from stool samples were identified as serotype 1 followed by serotype 4. One serotype 2 was successfully identified. Compared with the electrophoresis data, three strains were demonstrated to be the "short" profile. One of the "short" profiles, KMC 24, was typed by MAb-ELISA.

Ten out of 17 (58.8%) samples from KMC were successfully typed by MAb-ELISA. Five out of 17 (31.3%) samples from TAMC were successfully typed by MAb-ELISA. Gouvea et al. (1990) reported that 20 to 30% of strains are incapable of being typed by this method. Stool samples were from 1982 to 1986 with the majority from the 1982 - 1983 seasons. Therefore, a possible explanation is that some subtle change in capsid integrity could account for the low yield of the present results. However, Williams (1989) reported that rotavirus particles retained their characteristic morphology as determined by EM for as long as 10 years at -70°C. Thus, the stool samples sent to Houston for MAb-ELISA analysis were most likely to contain intact rotavirus particles.

One serotype 3 strain was identified. This strain was adapted to cell culture and produced plaques. This finding was confirmed by two different anti-3 antisera.

Birch, Heath and Gust (1988) in the United States reported a sample distribution of 28.8% serotype 1, 14.8% serotype 2, 16.5% serotype 3, and 2.4% serotype 4 tested by MAb-ELISA. Nakagomi et al. (1988) reported a distribution of 47.3%, 2.9%, 2.9%, and 17.7% for serotypes 1 to 4, respectively, in Japan. In Central Africa, Georges-Courbot et al. (1988) reported a distribution of 69.9%, 15%, and 15.3% for serotypes 1 through 3, respectively, with 2.4% being non-1,2, or 3. Therefore, there is the possibility

that the strains which could not be typed, were in fact serotype 4.

Unicomb and Bishop (1989) in Australia examined samples from the winters of 1986 and 1987. By MAb-ELISA, 95%, 4%, 0.5% and 0.5% were serotypes 1 through 4, respectively. Thus it is clear that the prevalence of serotypes 2 through 4 may fluctuate between locales and with time.

The predominant serotype isolated worldwide is serotype 1 which is the same as this study based in Hawaii.

Results summarized in Table 11 demonstrates that an anti-rotavirus antibody increase occurred in response to the antigens used to immunize the guinea pigs used in this study. This is indicated by both the increase in titer after immunization and the high anti-TAMC 15 serum neutralizing titer against the homologous rotavirus as opposed to the heterologous rotavirus strains tested. This conclusion is also supported by the use of antisera from another source (Ward, Cincinnati). Similar results using Ward's antisera, as compared to the anti-rotavirus antiserum made in this present study, also listed in Table 11, were obtained.

In the present study the majority of the rotavirus strains adapted to cell culture and successfully serotyped were isolated from stool samples obtained in 1982-83 and 1983-84. The significance of this relationship is not known at this time. Since a major epidemic occurred in the 1982-

83 winter season (Birch, Heath and Gust, 1988), it is possible that the strains circulating during that period were more virulent, and hence more infectious in cell culture.

One isolate, KMC 12, was found to have dual serotypic specificities, both 1 and 4. While dual serotypic specificities are not a common occurrence, the phenomenon has been reported previously. Yasutaka et al. (1985) reported a new Venezuelan isolate (M37) which contained viral protein 3 (outer capsid protein) similar to prototype strain ST3 (serotype 4) and viral protein 7 (outer capsid protein) similar to prototype strain Wa (serotype 1). M37 also was serotyped as 1 and 4.

No one has ever reported that one rotavirus serotype is more pathogenic than the others.

PART III: IMPROVING METHODS FOR THE DETECTION AND ANALYSIS OF ROTAVIRUS

I. OBJECTIVES OF THIS PART OF THE STUDY

To determine optimal conditions for plaquing rotavirus strains used in this study. Factors evaluated will include cell types, gelling agents (agar/agarose) for solid overlay, and the plaque enhancing agents trypsin and diethylaminoethyl (DEAE)-dextran.

To determine optimal conditions for the detection of rotavirus using the nitrocellulose-enzyme immunoassay (NC-EIA) technique.

II. INTRODUCTION

A. Detection of rotavirus infectivity by plaque assay.

The first plaque assays involving rotaviruses were developed for rotavirus strains infecting animals other than humans.

A necessary component for the plaque technique is a solid overlay, solidified with either agar or a derivative of agar (agarose), although methyl cellulose has been used. Agar is a polymer containing the agarobiose structure 1,3-linked ß-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose. This structure may be substituted with half-ester sulfate groups, pyruvic acid ketal, and methyl esters. Purification of agar yields a product called agarose which does not contain many of these substituted side-chain structures (FMC, 1988).

Liebhaber and Takemoto (1961) reported that addition of DEAE-dextran to the overlay will bind sulfated polysaccharides thereby removing the inhibitory effect. Takemoto and Spicer (1965) reported that polyanions present in agar used for overlays are responsible for the inhibition of virus (encephalomyocarditis virus and poliovirus 1). This effect can be modulated by the addition of polycations such as dextran sulfate.

Feorino and Hannon (1966) used DEAE-dextran to enhance the size of ECHO virus plaques. Williams (1970) reported on using magnesium chloride to enhance adenovirus plaque formation. Williams also reported that calcium enhances poliovirus plaque formation on rabbit embryo kidney cells, and calcium plus magnesium in the overlay enhances certain rhinoviruses in HeLa cells.

Borden, Gary, and Murphy (1970) reported on the problems of the agar used to make the overlay. They stated that agar contains a polysulfated polysaccharide which is inhibitory to many viruses (e.g., mengo encephalomyelitis virus, Newcastle disease virus, group A arboviruses). On the other hand, a purified agar preparation (agarose), does not contain this component and did not inhibit these viruses. The inhibitory activity of agar could be overcome by the presence of supplements such as DEAE-dextran and protamine sulfate. However, DEAE-dextran has been shown to

enhance interferon production, and protamine sulfate will interfere with virus-cell interactions.

Green and Baron (1975) used 5-iododeoxyuridine (5-IDU) to increase the *in vitro* replication of vesicular stomatitis virus, Sindbis virus, mouse encephalitis virus, and vaccinia virus. Benton and Hurst (1986) included 5-IDU in overlays and demonstrated a two- to threefold increase in plaque titer of various enteroviruses.

Loh, Achong and Epstein (1977) further demonstrated that inclusion of DEAE-dextran into the overlay enhanced the formation of plaques of a human syncytium-forming virus. The reason given for this effect was that both cells and virus particles are negatively charged and the positively charged DEAE-dextran helped to overcome electrostatic repulsion. Thus, adsorption of virus to cell was aided.

The first report of a plaque assay for rotaviruses was made by Matsuno, Inouye and Kono (1977). Using MA 104 cells, the authors successfully plaqued Nebraska calf diarrhea virus (NCDV). Maximum titers were achieved by including both trypsin and DEAE-dextran in their overlay. The titer measured was lower if either trypsin or DEAE-dextran was eliminated, and no plaques formed when both enhancing agents were deleted.

In 1979 Smith et al. reported on a method to plaque a simian rotavirus (SA11). Since simians are phylogenetically related to humans, SA11 was established as the model

rotavirus system, especially since no human rotavirus was available at that time.

The plaque assay for the simian rotavirus SA11 included the plaque enhancing agents pancreatin and DEAE-dextran. A maximum yield of virus based on PFU/ml, was obtained by using either a 1:50 dilution of pancreatin stock from Oxoid Laboratories (Oxford, U.K.), 25 μg/ml of tissue culture grade trypsin (Difco Laboratories, Detroit, Michigan), or 10 μg/ml 2 x crystallized trypsin. Also included was 100 μg/ml DEAE-dextran. The enhancing agents were included in an overlay consisting of Eagle's MEM containing glutamine, sodium bicarbonate, antibiotics and 1.5% Bacto agar (Difco Laboratories.

From 1979 on, many papers described plaque assays for rotavirus. Each group of researchers used their own combination of gelling agent, proteolytic enzyme and DEAE-dextran in their solid overlay (Table 12).

B. Detection of rotavirus by physical measurement.

Historically, the first methods used for rotavirus detection have been electron microscopy (EM) (Bishop et al., 1973), radioimmunoassay (RIA) (Middleton et al., 1977), immunofluorescence (Moosai et al., 1979) and enzyme-linked immunoassay (ELISA) (Yolken et al., 1977). Today the most often used methods are EM, electrophoresis of genomic RNA, and the immunologically based methods such as ELISA, latex agglutination, radioimmunoassay, and immunofluorescence.

Table 12

Concentrations of trypsin, DEAE-dextran, agar, and agarose reported in the literature for the plaque assay of rotavirus

Rotavirus strain	Cell line	Gelling agent (concn)	Proteolytic enzyme (concn)	Concn DEAE-dextran
NCDV[a]	MA 104	0.8% purified agar	2 μg/ml p. trypsin[i]	100 μg/ml
SA11[b]	MA 104	0.7% ionagar 2	5 μg/ml trypsin	100 μg/ml
SA11[c]	MA 104	1.5% Bacto agar	15 μg/ml t.c. trypsin[j] 7.5 μg/ml p. trypsin 1:50 dil'n of Oxoid stock pancreatin	100 μg/ml
HRV[d]	MA 104	0.6% purified agar	3 μg/ml a. trypsin[k]	50 μg/ml
Wa[e]	MA 104	0.5% agarose	13 μg/ml t.c. trypsin	N.U.[l]
HRV[f]	MA 104	0.6% purified	2 μg/ml p. trypsin	N.U.
RRV[g]	MA 104	0.55% agarose	0.5 μg/ml p. trypsin	N.U.
SA11[h]	BSC-1	0.8% agarose	5 μg/ml trypsin	N.U.

a = Matsuno, Inouye and Kono, 1977; b = Ramia and Sattar, 1979
c = Smith et al., 1979; d = Urasawa, Urasawa and Taniguchi, 1981
e = Offit et al., 1983; f = Albert and Bishop, 1984
g = Twist, Kolonich and Rubin, 1984; h = Kaljot et al., 1988
i = purified trypsin; j = tissue culture grade trypsin; k = acetyl-trypsin
l = Not used

Two of the most practical methods for clinical samples are electrophoresis and ELISA.

The ELISA technique utilizes an enzyme labeled immunoglobulin that specifically recognizes an antigen on the rotavirus capsid. Briefly, the ELISA test may be conducted (Voller, Bidwell and Bartlett, 1980) by the "sandwich" method where anti-rotavirus immunoglobulins are adsorbed to the surface of wells in a plastic microtiter plate. The sample presumed to contain rotavirus particles is added to the wells of the microtiter plate enabling an antibody-virus complex to form. The primary antibody-virus complex is then incubated with a second anti-rotavirus immunoglobulin conjugated to an enzyme (e.g., horseradish peroxidase). When this complex is incubated with a chromogenic substrate and hydrogen peroxide, the chromogenic substrate becomes oxidized by the transfer of electrons to the hydrogen peroxide which is reduced by the horseradish peroxidase. Once oxidized, the chromogenic substrate appears colored and can be read by eye or in a spectrophotometer.

Early uses of ELISA demonstrated that the method was as sensitive as EM and RIA (Yolken et al., 1977). Beards and Bryden (1981) compared EM against a newly released commercial ELISA, Rotazyme by Abbott Laboratories. Rotazyme would become the industry standard for years. Beards and Bryden reported that the Rotazyme kit detected rotavirus

antigen in 8% more samples than did EM, but they claimed that the kit left much room for improvement in sensitivity.

Briefly, the Rotazyme assay is based on rabbit anti-rotavirus immunoglobulins bound to plastic beads. The beads are placed in plastic tubes included in the kit, and the complete assay is done in a tube, thus microtiter plates are not needed. After introduction of the Rotazyme assay, the company was reported to decrease the antibody concentration bound to the beads to save money, and the sensitivity decreased by approximately 60%.

Krause et al. (1983) demonstrated that only 4/61 (7%) of the Rotazyme positive samples from neonates could be substantiated by a confirmatory test (ELISA, EM, RNA hybridization). The authors theorized that disrupted particles could account for negative EM results, and nonspecific binding of rotavirus antibody to bacteria and staphylococcal protein A in neonatal stool could account for negative ELISA and RNA hybridization results. In contrast, 10/13 (77%) of Rotazyme-positive samples from children and adults were in concordance with confirmatory test. Therefore, the efficacy of using, specifically, the Rotazyme ELISA test for identifying rotavirus in the stools of neonates is contraindicated.

In 1984, Cukor et al. described a monoclonal antibody based ELISA test. Results indicate that the monoclonal test has promise as a detection method since it was slightly more

sensitive than EM or the Rotazyme test, but this was not statistically significant.

Miotti, Eiden and Yolken (1985) compared three commercial ELISA assays systems to their own reference ELISA assay. They determined that the Rotazyme assay was statistically less sensitive than the other two commercial ELISA or their reference ELISA assay when samples were taken more than 3 days following the onset of recognized gastroenteritis, but was equivalent to the others before 3 days.

After objections to the loss of sensitivity of the Rotazyme assay, Abbott Laboratories released a second generation kit called Rotazyme II which was supposed to have heightened sensitivity and comparable to the original Rotazyme assay.

More recent reports have conflicting results regarding polyclonal and monoclonal antibody based ELISA's. Dennehy, Gauntlett and Tente (1988) determined monoclonal ELISA's to have greater sensitivity than polyclonal ELISA's while Thomas et al. (1988) determined the polyclonal based Rotazyme II to be the most sensitive. However, both groups reported that Rotazyme II was poor as regards specificity.

Rotazyme II was demonstrated to be less sensitive to other ELISA tests and to latex agglutination tests for rotavirus detection due to the high rate of false positive results (Dennehy, Gauntlett and Tente, 1988). This

emphasizes the problem with the Rotazyme II ELISA test which is still widely used.

Coulson et al. (1987) reported on a new monoclonal antibody based ELISA test that not only detected rotavirus antigen but could also be used for direct serotyping directly from a stool sample. They determined that the sensitivity of the monoclonal test was almost as sensitive as EM, but more sensitive than a polyclonal antibody based ELISA. The MAbs are now commercially available from Silenus Laboratories, Australia.

Other solid phases are constantly being sought for immunologically based virus detection systems. Salonen and Vaheri (1979) reported that only about 2 - 10% of the total IgG added to polystyrene microtiter plates is left attached by the enzyme step. The detachment of IgG indicates that a lot of wasted reagents are involved in standard microtiter plate based tests. Thus, antibody originally bound to the microtiter plate which captured viral antigen may become detached by the enzyme step. This would underestimate the quantity of virus present.

A method used for virus detection appearing in the early 1980's is the nitrocellulose-enzyme immunoassay (NC-EIA). This technique takes advantage of the high affinity of nitrocellulose paper for proteins (Bode et al., 1984), and has been used for detection of viral antigen. Loh, Dow and Fujioka (1985) reported that the NC-EIA was 10-100 times

more sensitive than a conventional ELISA. Also, this method could be used to detect a much lower level of enteroviruses. Added advantages are that no capture antibody is necessary, smaller volumes of sample may be tested, and smaller reagent volumes may be used.

Reynolds and Hughes, in 1985 compared the Rotazyme kit against a dot-immunobinding assay (dot-blot) and determined the dot-blot to have greater sensitivity by comparison to the Rotazyme assay (25% detection at the greatest dilution and 92% respectively). Gouvea, de Castro and Pereira (1987) applied the method to rotavirus detection and found it to be a feasible method equal to a conventional assay in sensitivity.

III. MATERIALS AND METHODS

Glassware, disposable plastics and sterilization.

All glassware was washed in tap water using Alconox detergent (Alconox, Inc., New York, New York). The glassware was rinsed 10 times with tap water and 7 times with distilled water and sterilized in an AMSCO model 2021 autoclave (American Sterilizer Company, Erie, Pennsylvania) under standard conditions (15 min-15 p.s.i.-121°C).

Cell culture growth and passage.

Outlined in Part II.

Viruses.

Human rotavirus stocks of Wa (serotype 1), DS-1 (serotype 2), P (serotype 3), and ST3 (serotype) were kindly

donated by Dr. Richard Ward (Cincinnati, Ohio). Simian strain SA11 was kindly donated by Dr. Bruce Keswick (Baylor College of Medicine, Houston, Texas).

Production and purification of rotavirus stocks.

Virus stocks were treated with 10 µg trypsin/ml at 37°C for 30 minutes to 1 hour. Flasks with confluent monolayers of MA 104 cells were washed twice with PBS and inoculated with 5 ml of trypsin-treated virus stocks. The flasks were incubated at 37°C for 1 hour with periodic rocking to redistribute the inoculum. The flasks were washed twice with PBS following incubation, overlayed with serum-free MEM Earle's medium containing 3 µg/ml trypsin, and incubated at 37°C until 90% of the monolayer displayed signs of CPE.

Infected cells in cell-culture flasks were frozen and thawed 3 times. Cell lysates were centrifuged at 850 x g for 5 minutes at 26°C in an IEC PR-2 centrifuge with 12 slot swinging bucket rotor (International Equipment Co., Needham Heights, Massachusetts) to remove gross cellular material. The supernatant was then centrifuged at 8,500 x g for 10 minutes at 4°C in an IEC HR-1 centrifuge with 8 hole angle rotor (International Equipment Co., Boston, Massachusetts) to remove soluble cellular material. The supernatant was then centrifuged at 35,000 x g for 3 hours at 4°C in a Beckman Angle 30 fixed angle rotor with 12 slots (Spinco Division of Beckman Instruments, Inc., Palo Alto, California) in a Beckman model L ultracentrifuge (Spinco

Division of Beckman Instruments, Inc., Palo Alto, California) to pellet the virus. The supernatant was discarded, and the pellet was resuspended in PBS. The supernatant was centrifuged at 8,500 x g for 10 minutes at 4°C in an IEC HR-1 centrifuge with an 8 slot angle head rotor to remove any particulate material and the supernatant was collected and used for experiments. Short term storage was at 4°C and long term storage was done at -70°C.

Plaque production of rotaviruses.

A modification of the method of Urasawa, Urasawa and Taniguchi (1981) was used to plaque rotavirus. Briefly, a rotavirus stock was treated with 10µg/ml Gibco trypsin (tissue culture grade; Grand Island Biological Company, Grand Island, New York) and incubated at 37°C for 30-60 minutes. Monolayers of MA 104 cells growing in 16 mm tissue culture plates (Falcon 3002; Catalog No. 4-3002-3; Becton Dickinson) were washed twice with PBS and 0.20 - 0.25 ml of trypsin-treated virus was added per plate. Plates were incubated at 37°C for 1 hour with redistribution of inoculum every 10 minutes. Following incubation, the plates were washed twice with PBS and 3 ml of equal volumes of Complete medium (Appendix E) and 0.36% Seakem agarose ME (Seakem ME agarose; Catalog No. 50012, Lot No. 26884; FMC Corporation, Rockland, Maine) was added per plate. The plates were incubated at 37°C for 3 - 5 days, then stained for visualization of plaques.

Enhancing agents for plaque production.

Agarose types used in this study included Seakem agarose ME (Catalog No. 50012, Lot No. 26884), Seakem LE agarose (Catalog No. 50001, Lot No. 61024), Seakem HGT(P) agarose, (Catalog No. 50053, Lot No. 10594), Seakem HEEO agarose, (Catalog No. 50031, Lot No. 02584) all from FMC Corporation, Rockland, Maine. In addition, electrophoresis grade agarose (Catalog No. 120000; Lot No. 19112; Grand Island Biological Co. (GIBCO), Grand Island, New York) was used. Agarose was made up in triple-distilled water to 0.36% and sterilized by autoclaving.

Agar types used in this study included Ionagar No. 2S (Lot No. 1859; Wilson Diagnostics, Inc. Glenwood, Illinois), Bacto agar (Difco Laboratories, Detroit, Michigan), and Noble agar (Catalog No. 0142-01; Difco Laboratories, Detroit, Michigan). Agars were made up in triple-distilled water to 0.70% and sterilized by autoclaving.

Proteolytic agents selected included were pancreatin (grade III; Catalog No. P-1625; Lot No. 99C-0256; Sigma Chemical Co., St. Louis, Missouri), trypsin (crystallized, type IX; Catalog No. T-0134; Lot No. 95F-0673; Sigma Chemical Co., St. Louis, Missouri), Gibco trypsin (tissue culture grade 1:250; gift of Dr. R. Ward, Cincinnati, Ohio), and acetyl-trypsin (Catalog No. T-6763; Lot No. 124F-8125; Sigma Chemical Co., St. Louis, Missouri). Proteolytic enzyme stocks were filtered through a 0.45 μm pore-size

membrane to remove bacteria and stored at 4°C. This stock was added to the overlay to the desired concentration.

DEAE-dextran (diethyl-aminoethyl-dextran; Catalog No. 7.0350.0; Lot No. KD96458; Pharmacia AB Laboratory Separation, Uppsala, Sweden) was suspended in MEM Earle's to a concentration of 10,000 μg/ml. This solution was diluted in Complete medium (Appendix E) to the required concentration, filtered through a 0.45 μm pore-size membrane and stored at 4°C. This stock was added to the overlay to the desired concentration.

Kinetic measurements of proteolytic enzymes.

Enzyme kinetic measurements for specific activity of enzyme stocks were done as described by Rick (1977). Calculation of specific activity was determined by the equation outlined in the Worthington Manual (1988).

The substrate used for enzyme calculations was Nα-p-tosyl-L-arginine methyl ester (TAME) (Catalog No. T-4626; Lot No. 36F-0667; Sigma Chemical Co., St. Louis, Missouri) as described by Smith et al. (1979).

The kinetic reaction was read in a Gilford Response II UV-visible spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) using proprietary software.

Staining of tissue culture plates for plaque visualization.

Tissue culture plates were stained by one of two methods.

Method 1 was to add 2 ml of a 3.33 mg/ml neutral red solution (Flow Laboratories, McLean, Virginia) to 100 ml of overlay composed of equal volumes of 2X Earle's solution (Appendix E) and 0.36% ME agarose. Three ml of the second overlay was added per plate 3 days after infection, and the plates were checked 6 hours later and daily thereafter for plaques.

Method 2 was to gently decant the agarose overlay from the plates, rinse once with PBS, and add 5 ml of crystal violet staining solution (Appendix E) per plate. The plate were left at room temperature for 5 minutes then quickly rinsed once with PBS. The plates were scored immediately.

Antiserum Production in Guinea Pigs.

Two white Hartley guinea pigs were obtained for the production of antisera against two human rotavirus strains. Purified strain P (serotype 3) antigen and the plaquing rotavirus isolate TAMC 15 (serotype unknown) antigen were selected for immunization. The immunization schedule is listed in Table 13. Preimmune sera was drawn on the same day as the first immunization.

Table 13

Immunization schedule of guinea pigs inoculated with rotavirus strain P and isolate TAMC 15

Date	Antigen Concn	Diluent and Additives	Site of Inoculation
9/18/87	10 µg	PBS[a] + FCA[b]	Flanks & Neck[c]
10/4/87	10 µg	PBS + FIA[d]	Flanks & Neck
10/24/87	10 µg	PBS	Flanks & Neck
11/20/87	10 µg	PBS	I.P.[e]
12/4/87	10 µg	PBS	I.P.

a = Dulbecco's phosphate buffered saline (Appendix E)
b = Freund's complete adjuvant (Catalog No. F-4258; Sigma Chemical Co., St. Louis, Missouri)
c = Flanks of legs and nape of neck
d = Freund's incomplete adjuvant (Catalog No. F-5506; Sigma Chemical Co., St. Louis, Missouri)
e = intraperitoneal

Purification of antiserum by affinity chromatography.

Hyperimmune guinea pig immunoglobulin was purified in a GammaBind G prepack (Catalog No. GBP020001; Genex Corporation, Gaithersburg, Maryland) as per manufacturer's instructions.

Nitrocellulose-enzyme immunoassay (NC-EIA) method.

Methodology for the NC-EIA is described in Chapter 9.

CHAPTER 8

OPTIMAL CONDITIONS FOR PLAQUING ROTAVIRUS

I. OBJECTIVES OF THE CHAPTER

To determine the effect of proteolytic enzymes and DEAE-dextran on plaque efficiency.

To determine the effect of overlay medium using agar, noble agar, ionagar, electrophoresis grade agarose, and ME, LE, HEEO, and HGT(P) agaroses on plaque efficiency.

To determine the optimum conditions for plaque production of rotavirus strains and isolates used in this study.

II. RESULTS

Ward (personal communication) reported that optimum plaquing conditions in his laboratory were 3 μg Gibco tissue-culture-grade trypsin/ml and 0.18% ME agarose (FMC Corporation) in the overlay. To determine the optimum conditions to plaque rotaviruses in this study. Several parameters were evaluated.

A. Effect of gelling agents on plaquing efficiency

The first parameter evaluated was the gelling agent for the solid overlay. Agar, Noble agar, ionagar, electrophoresis-grade agarose, and more highly purified agaroses ME, LE, HEEO, and HGT(P) were evaluated.

The results (Table 14) show that ME agarose was the best gelling agent for two HRV strains (TAMC 15 and C-1) and for simian rotavirus SA 11. However, Wa plaqued poorly in

Table 14

Effect of agar and agarose on the plaquing efficiency of rotavirus

	% plaque formation							
	agarose					agar		
Virus	ME	LE	HGT(P)	HEEO	Ag	A	NA	IA
TAMC 15	**100**	48	N.D.[a]	80	56	< 2	< 2	15
TAMC 15	**100**	71	N.D.	74	39	< 1	< 1	4
Ward-1	**100**	70	N.D.	58	41	5	3	7
Ward-1	**100**	57	N.D.	41	93	11	23	16
Wa	42	34	40	29	62	71	**100**	33
SA 11	**100**	42	69	N.D.	63	23	49	40
SA 11	**100**	95	79	71	79	32	37	81

a = not done

this agarose as compared to noble agar and Bacto agar. The two HRV strains TAMC 15 and C-1 plaqued poorly in all of the agars in contrast to strain Wa, with a better yield occurring in the more highly purified agaroses.

The concentrations found to be optimal for use in an overlay were 0.18% and 0.70% for agarose and agar, respectively. These concentrations ensured sufficient gelling. At lower concentrations the overlay is too liquid and the virus progeny can easily diffuse through the matrix thus causing an overestimation of the concentration of virus assayed. A soft overlay often resulted in plaque "smearing" when the cell culture plates were handled daily for evidence of plaque formation.

B. Enzyme kinetics measurement of proteolytic enzymes

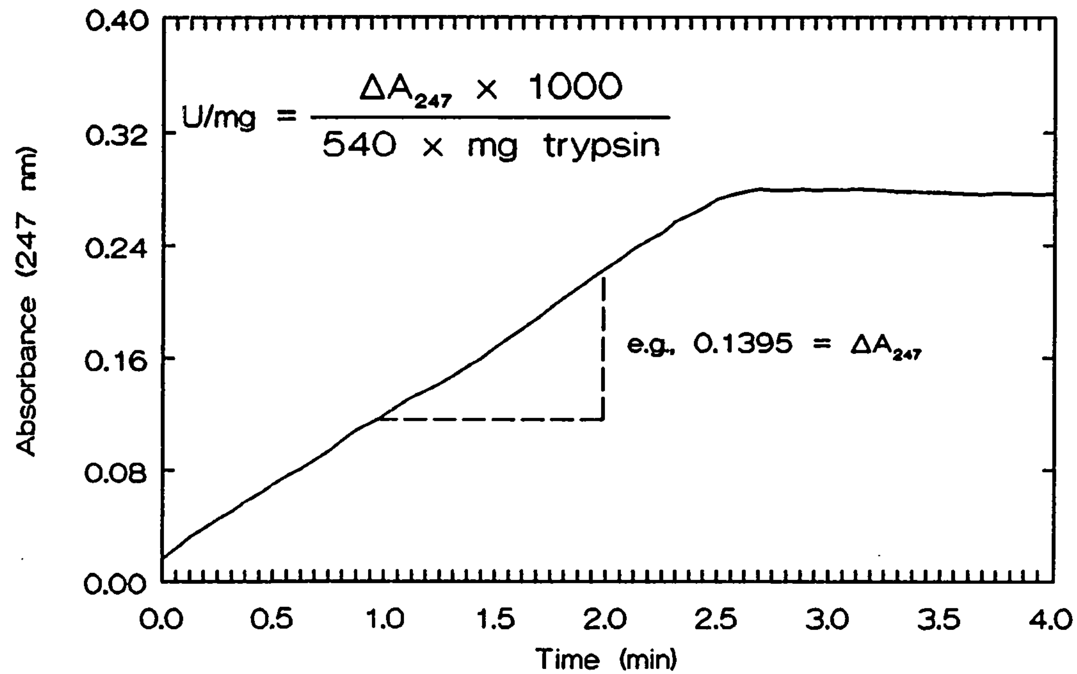
The second parameter to be examined was the optimum concentration of Gibco trypsin (tissue culture grade), Sigma trypsin (crystallized), acetyl-trypsin, and pancreatin. It was recognized early that not all of the enzymes could be used at the same weight per volume (w/v) since they have different specific activities (S.A. in TAME units/μg). Pancreatin has been used previously as well as trypsin for plaquing rotavirus. However, pancreatin is not a single enzyme but a mixture of many enzymes (amylase, trypsin, lipase, ribonuclease, and protease).

To determine the S.A. of any enzyme preparation, the enzyme was exposed to a specific substrate and the kinetics of substrate utilization determined (see Figure 8).

Preliminary experiments were done with Gibco trypsin and Sigma pancreatin. Both the trypsin and pancreatin induced optimal plaque production at 3 μg/ml. It was observed that purified Sigma trypsin at the same concentration (w/v) caused the MA 104 monolayer to degrade rapidly within two days. Enzyme kinetic measurements were made to determine the S.A. of the proteolytic enhancement agents and to determine their effect on cell monolayers as regarded the observed toxicity to cell monolayers.

Calculations showed that the Sigma trypsin stock had 16.5 times the S.A. of Gibco trypsin while the Sigma acetyl-trypsin stock had 13.3 times the S.A. of Gibco trypsin. Subsequent stocks of all proteolytic enzymes used in this study were made to a concentration of 1 mg/ml and dilutions of the stock enzymes were made to achieve the desired final S.A. Pancreatin was found to have a lower S.A. than Gibco trypsin. Optimal plaque formation with pancreatin occurred, however, at the same concentration as was used for Gibco trypsin (w/v basis).

Figure 8. Determination of specific activity of proteolytic enzymes by measurement of enzyme kinetics



The results summarized in Table 15 show that the purified, crystallized Sigma trypsin and Sigma acetylated trypsin had a higher S.A. than the cruder enzyme preparations of Gibco trypsin and Sigma pancreatin. While pancreatin had a lower S.A. than the trypsins, it gave similar results to Gibco trypsin at the same concentration (w/v).

Table 15

Calculated specific activity of proteolytic enzymes

Enzyme	Specific activity units (TAME U/mg[a])
Crude trypsin (Gibco)	3.290
Pure trypsin (Sigma)	54.280
Acetyl-trypsin (Sigma)	43.760
Pancreatin (Sigma)	0.435

a = based on the cleaving the substrate N_{α}-p-toluenesulphonyl-L-arginine methyl ester

C. Effect of proteolytic enzymes on plaquing efficiency

For the HRV strain Wa, the optimal concentration of tissue culture grade trypsin, crystallized trypsin, acetyl-trypsin, and pancreatin resulting in highest plaque titer was 3 μg/ml of a stock solution (Table 16). The optimal S.A. for the three trypsins equaled 9.87×10^{-3} TAME units/ml while the optimal S.A. for pancreatin was 1.31×10^{-3} TAME units/ml.

The results were similar for HRV TAMC 15. It was determined that 9.87×10^{-3} TAME units/ml was the optimal S.A. for both trypsin and crystallized trypsin, however, when pancreatin was added, no plaques formed (Table 16).

For SA 11 the results between enzymes were more variable. Pancreatin was optimal at an S.A. of 8.7×10^{-4} TAME units/ml, trypsin at 9.87×10^{-3} TAME units/ml, and crystallized trypsin at 2.17×10^{-1} TAME units/ml (Table 16).

D. Effect of DEAE-dextran on plaquing efficiency

The inclusion of DEAE-dextran in the overlay also influenced plaque formation. While plaques formed without the inclusion of DEAE-dextran, the yields were lower. The results were not as consistent between the rotavirus strains for this enhancing agent. The optimal concentrations were determined to be 25 μg/ml, 50 μg/ml, and 75 μg/ml for TAMC 15, Wa and SA 11 respectively (Table 17). The optimal concentrations of DEAE-dextran listed above for TAMC 15 and SA11 are averages of 3 - 4 trials. DEAE-dextran in the overlay of strain Wa was 50 μg/ml in both trials.

It was observed that when DEAE-dextran was excluded from the overlay when plaquing Wa, plaque formation was unpredictable. When DEAE-dextran was excluded from the overlay plaque yields for TAMC 15 and SA11 were consistently lower. Moreover, Wa did not produce plaques in some experiments even with DEAE-dextran added to the overlay.

Table 16

Effect of proteolytic enzymes on the plaque formation of rotavirus

Proteolytic enzyme	virus	Plaques formed (%) at various specific activities of enzyme in medium					
		0	3.29#	6.58	9.87	13.16	16.45
Crude trypsin (Gibco)	TAMC 15(2)*	0	58	92	100	92	70
	Wa(3)	0	63	76	100	97	89
	SA11(3)	13	78	92	100	86	80
Pure trypsin (Sigma)	TAMC 15(2)	0	40	62	100	77	69
	Wa(2)	28	87	81	100	99	99
	SA11(1)	0	67	58	96	100	89
Acetyl-trypsin (Sigma)	Wa(1)	38	68	78	100	93	89
Pancreatin (Sigma)	TAMC 15(2)	0	0	0	0	0	0
	Wa(2)	19	66	85	100	88	92
	SA11(2)	14	48	100	89	88	81

- x 10^{-3} TAME U/ml medium
* - number of trials

Table 17

Effect of DEAE-dextran on the plaque formation of rotavirus

Virus	No. trials	Plaque formation (%) at various DEAE-dextran concn (μg/ml)				
		0	25	50	75	100
TAMC 15	4	76	**95**	84	71	48
Wa	2	47	76	**100**	95	79
SA11	3	62	77	78	**91**	43

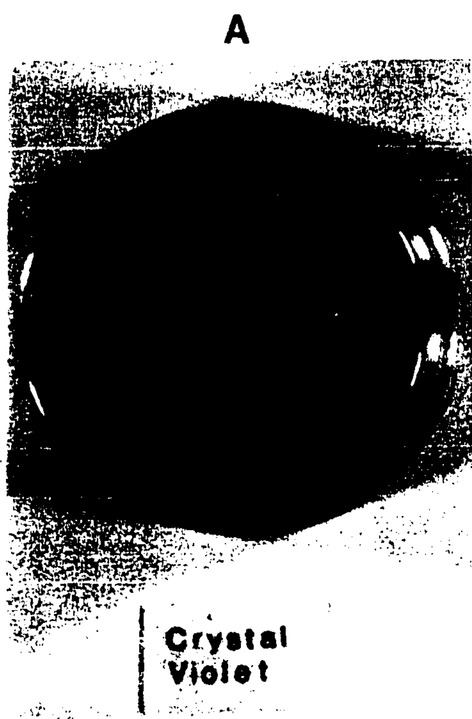
E. Visualization of plaques by staining cell monolayers

Visualization of plaques was accomplished by dyeing the monolayer with one of two stains. Either the monolayer was flooded with crystal violet solution after decanting the primary agarose overlay or the vital stain neutral red was included in a second overlay applied three days after infection (Figure 9).

Results indicated that the neutral red stain added 3 days post infection in a second overlay was more sensitive for the detection of plaques. Plaques counted by both methods 3 days post infection were more numerous by 2.6 to 3.6 fold in the neutral red stained plates as compared to the crystal violet stained plates (Figure 10). Plaques at day 5 were only about 1.1 fold more numerous in the neutral red-stained plates as compared to the crystal violet-stained plates.

Figure 9. Rotavirus-infected MA 104 cells stained with crystal violet (CV) and neutral red (NR)

A - SA 11 stained with CV B - TAMC 15 stained with NR



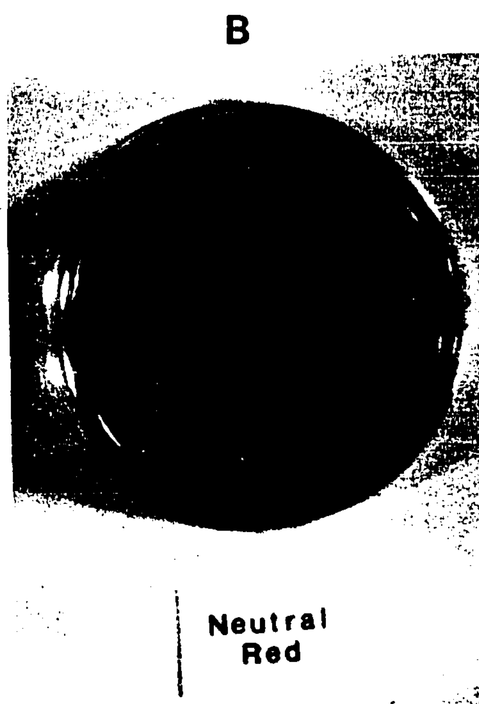
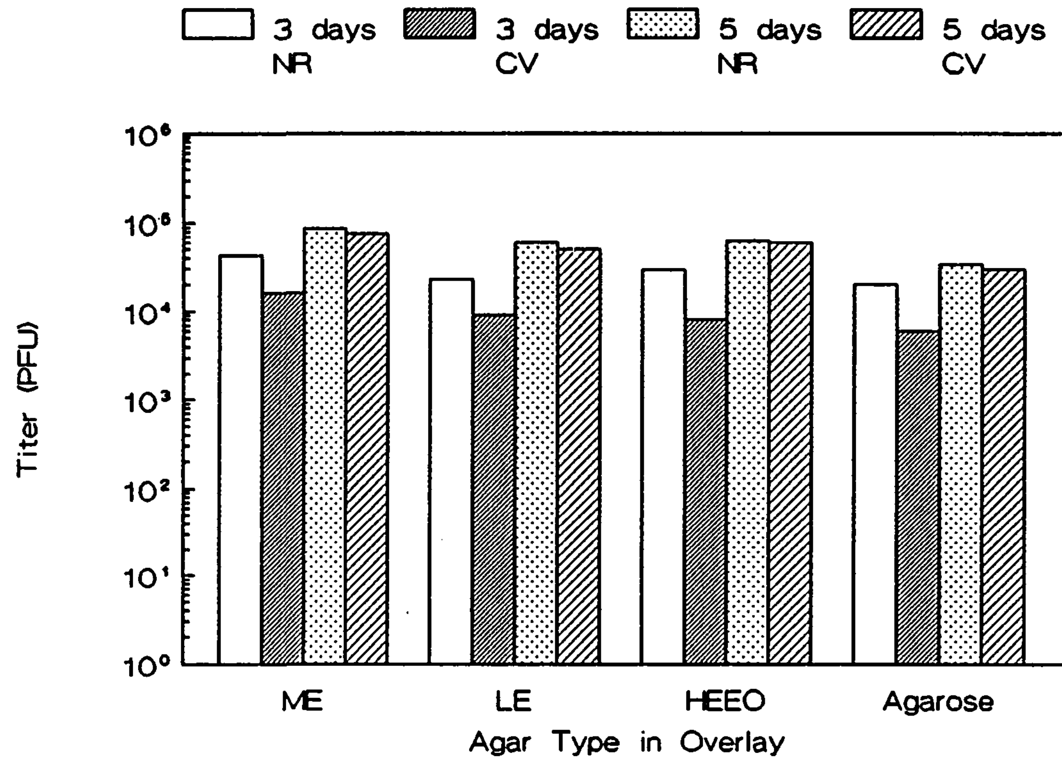


Figure 10. Comparison of neutral red (NR) and crystal violet (CV) stains for enumerating rotavirus plaques using four different agarose types in the overlay



F. Cultivation and plaquing of rotavirus in 6 cell types

Six cell types were tested for growth and plaque production of rotavirus. SA11, Wa, and TAMC 15 rotavirus strains were cultivated in PMK cells, MA 104, BGMK, all of monkey origin, and HeLa and MRC-5 cells, both of human origin (Table 18). However, the viruses would produce plaques only in Ma 104 and BGMK cells. The plaque yield in BGMK cells was determined to be approximately 90% less than in MA 104.

Table 18

Efficiency of six cell types for the cultivation of rotavirus and production of plaques

Cell Line		SA11	Wa	TAMC 15
Primary AGMK	growth	+	+	+
	plaques	-	-	-
Primary CMK	growth	+	+	+
	plaques	-	-	-
MA 104	growth	+	+	+
	plaques	+	+	+
BGMK	growth	+	+	+
	plaques	+	+	+
MCR-5	growth	+	+	+
	plaques	-	-	-
HeLa	growth	+	+	+
	plaques	-	-	-

A last condition not empirically tested was that the pH of the growth medium used to cultivate MA 104 cells prior to seeding in tissue culture plates for the plaque assay influenced rotavirus plaque production. Cells grown in medium maintained at pH 7.0 to 7.4 consistently yielded reproducible plaque titers. If the cells were cultivated below pH 7.0, as determined by the color of the growth medium, plaques may not form.

II. DISCUSSION

Consistent with all previously published studies, MA 104 cells were determined to be the best cell line for plaque production of rotavirus strains. If that cell line is not available, BGMK cells may be used for the plaque assay of rotavirus, although the yield is approximately one log lower. MRC-5 (diploid human embryonic lung cell line) cells were determined to be as good as MA 104 or PMK cells in the cultivation of rotavirus. Infectious titers of the rotaviruses grown in MRC-5 cells were as high as MA 104 grown rotaviruses, however, the cells were more sensitive to the toxic effects of trypsin used in the overlay of the plaque assay. This is the first report of this cell line used for the cultivation of rotavirus strains.

It was observed that all of the parameters evaluated had an effect on the ability of a rotavirus strain to produce plaques. The use of agarose is highly recommended as a standard plaque gelling agent, however, the role of

agarose as the gelling agent for the overlay when plaquing HRV strain Wa should be checked further. The reason for the enhanced plaquing of Wa in Noble agar is not known at this time. The lower efficiency of agarose as the overlay gelling agent is suggestive that HRV strain Wa may not be a good representative of human rotaviruses.

Use of a proteolytic enzyme is essential for plaque production. Determining the specific activity of an enzyme preparation may not always be possible for a research laboratory. While it was demonstrated that the optimal S.A. for a proteolytic enzyme was 9.87×10^{-3} TAME units/ml, tissue culture grade trypsin may be used at a concentration of 2-4 μg/ml or a purer, crystallized preparation may be used at a concentration of 125-300 ng/ml. These S.A.'s will result in efficient plaque formation.

DEAE-dextran has an effect on plaque formation. It was demonstrated that DEAE-dextran is essential to ensure plaque formation. It is recommended that 25-50 μg/ml DEAE-dextran be included in the overlay for HRV strains, 50-75 μg/ml for SA 11, and 50 μg/ml for Wa. While the yield of plaques is influenced in all strains, it was observed that Wa was influenced more than other strains tested.

It was demonstrated that the neutral red-stain method used to visualize the rotavirus plaques yielded higher plaque counts at 3 days than at 5 days post-infection. The advantages of using neutral red outweigh those of crystal

violet. Neutral red-stained plates can be reincubated and enumerated again at a later time while the crystal violet-staining procedure fixes the monolayer at the time of staining.

Since the plaque enhancement qualities of DEAE-dextran was not known during the early experiments, it was not included in the overlay. However, in subsequent plaque titration of rotavirus, DEAE-dextran was routinely included as a component of the overlay.

It was finally recognized that there was a strict requirement of DEAE-dextran for efficient plaque production of Wa, and a narrow pH range of tolerance. These observations probably account for the variable plaque production of Wa when this rotavirus strain was first used. A more definitive study examining the pH range for cell culture and its effect on plaque production should be done.

Preston, Bitton and Farrah (1990) have recently reported on the use of the cationic polymer polyethylene-imine (PEI) to enhance enterovirus infectivity. The increase in infectious titer induced by PEI ranged from 1.2 to 5.5 fold for poliovirus 1, echovirus types 1 and 5 and coxsackievirus type B5. The authors also state that PEI is more effective than DEAE-dextran in enhancing plaque formation. No reports on the use of this enhancing agent in a plaque assay involving rotavirus are known. This agent should be examined for use in a rotavirus plaque system.

CHAPTER 9

MODIFICATION OF A NITROCELLULOSE-ENZYME (NC-EIA) IMMUNOASSAY FOR THE DETECTION OF HUMAN ROTAVIRUS

I. OBJECTIVES FOR THIS CHAPTER

To modify the NC-EIA method of Loh, Dow and Fujioka (1985) for the detection of rotavirus.

To evaluate the various anti-rotavirus serum (anti-TAMC 15 and anti-Wa) described in Chapter 7 for use in the NC-EIA assay.

II. RESULTS

A. Preliminary reagent characterization

Anti-rotavirus serum included in the Dakopatt rotavirus ELISA kit was evaluated as a detection antiserum. This antiserum was ineffective for this assay at dilutions from 1:5 to 1:1,000. No further work was done with this antiserum in the NC-EIA.

A serious limitation of the NC-EIA is background color following color development. Background may occur due to nonspecific reactions due to components contained in buffers, blocking agents, and sera which can react with the nitrocellulose paper or the antigen that is assayed. Each of the reagents (diluent, blocking agent and chromogenic substrate) were pretested with regard to background color development.

The results showed that phosphate-buffered saline, as a diluent, yielded a lower background than Tris-buffered saline.

Blocking with normal goat serum, bovine serum albumin, and 5% non-fat dry milk (NDM) were then compared for their ability to reduce background in the blocking step. Best results were obtained using 5% NDM in the blocking step and the inclusion of 1% NDM in all subsequent steps yielded the lowest background.

The chromogenic substrate used for visualization of positive results may also affect background color. A soluble substrate, which is used in conventional ELISA, assays is not suitable for the NC-EIA assay therefore a substrate which will precipitate on the NC paper must be used. Diamino benzidine (DAB) was compared to 4-chloro-1-naphthol (Cl-N). DAB was slightly more sensitive by visual examination than Cl-N, however, the background was higher for DAB. Therefore Cl-N was selected as the substrate.

Under the optimized conditions determined previously, anti-rotavirus immune serum produced against human rotavirus isolate TAMC 15 (made in the present study), and anti-Wa immune serum (commercially available from the American Type Culture Collection) were evaluated for use in the NC-EIA. By checkerboard titration using the method of Loh, Dow and Fujioka (1985), the optimum dilution of both antisera was determined to be a 1:3000 dilution in PBS. The optimum

dilution of the detection serum (goat anti-guinea pig IgG-HRP conjugate) was determined to be a 1:200 dilution in PBS.

B. Methodology

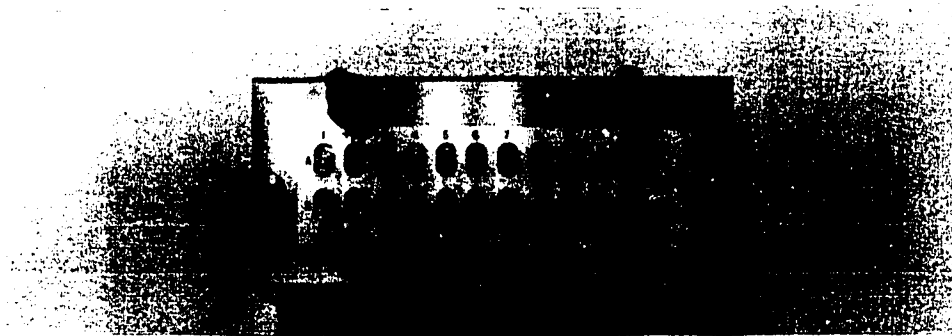
The steps involved in the NC-EIA are a modification of Loh, Dow and Fujioka, and include the following:

1) Schleicher & Schuell nitrocellulose paper, 0.1 μm porosity (Catalog No. PH 79, Schleicher & Schuell, Inc., Keene, New Hampshire) was cut into 12 cm x 3.5 cm strips. The strips were soaked in 0.01M PBS until wet, heated in a microwave oven just to boiling, then blotted dry between pieces of Whatman filter paper (Whatman International, Ltd., Maidstone, England). The strips were air dried at least 10 minutes before use, or stored overnight at 4°C.

2) For use the NC strips were placed on top of two PBS wetted filters (Bio-Dot slot format; catalog No. 162-0161; Bio-Rad Laboratories, Richmond, California) placed on the bottom Plexiglass template of a Hybri-Slot Manifold apparatus (Catalog No. 1052MM; Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Maryland) (Figure 11). The NC strips were placed on top of the filter pads and both sandwiched between the top and bottom templates of the manifold.

3) 100 μl volumes of rotavirus sample dilutions in 0.85% saline were placed in each well. For an unknown sample, a positive rotavirus control was included. The sample

Figure 11. Hybri-Slot Manifold used in the NC-EIA rotavirus assay



was allowed to soak through the NC paper by gravity and capillary action. When no visible signs of any saline solution was left in the well, the well was washed and filled with an additional 100 μl of PBS. When the wash buffer soaked through, gentle vacuum was applied to remove any liquid left on the NC paper. The application of vacuum eliminated streaking between slots after color development.

4) The NC strips were removed from the template and blotted dry from the bottom and allowed to air dry. Air drying was done for a minimum of 15 minutes before assay, or the dried NC strip was stored at 4°C overnight.
5) Blocking step: the NC paper with adsorbed rotavirus antigen was immersed in a 5% NDM solution made in distilled water and gently rocked at room temperature for 2 hours on a Belly Dancer rocking platform.
6) Wash step 1: the NC paper was washed four times with PBS containing 1% NDM and 0.05% Tween-20 (PBS-M-T), 1 minute each, with gentle rotation.
7) Primary antibody: the NC strip was covered with 2 ml of a 1:3,000 dilution of guinea-pig anti-rotavirus antiserum in PBS-M-T and incubated at 37°C for 2 hours.
8) Wash step 2: as in step 6 above.
9) Detection antibody: the NC strip was covered with 2 ml of a 1:200 dilution of goat anti-guinea pig IgG-HRP conjugate (peroxidase labeled goat anti guinea pig

IgG(H+L); Catalog No. 141706; Lot. No. KB 45-5; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) in PBS-M-T and incubated at 37°C for 1 hour.

10) Wash step 3: as in step 6 above.

11) Substrate solution: ten minutes before the end of the incubation step in 9 above, a 3 mg Cl-N/ml methanol solution was prepared. Just before use, the Cl-N solution was mixed 1:5 in PBS plus 0.05% Tween-20 and 0.018% of a 30% H_2O_2 stock. Color development of the NC strip was accomplished by flooding the NC strip with 25 ml of the freshly prepared Cl-N-PBS-Tween-20-H_2O_2 solution.

12) The NC strip was incubated for 1 hour at room temperature (≈26°C) and protected from room lighting by covering the plate containing the NC strip. At the end of the 1 hour incubation the strip was washed four times with 50 ml of distilled water to stop the reaction.

13) The NC strip was finally blotted dry and allowed to air dry completely at room temperature or at 4°C in the refrigerator. The strip was usually held at 4°C overnight before reading on the densitometer.

A typical NC strip developed for rotavirus by the NC-EIA assay is shown in Figure 12.

Both Loh, Dow and Fujioka's punched-dot method and the modification of applying the sample to NC paper strips contained in the Hybri-Slot apparatus were found to yield

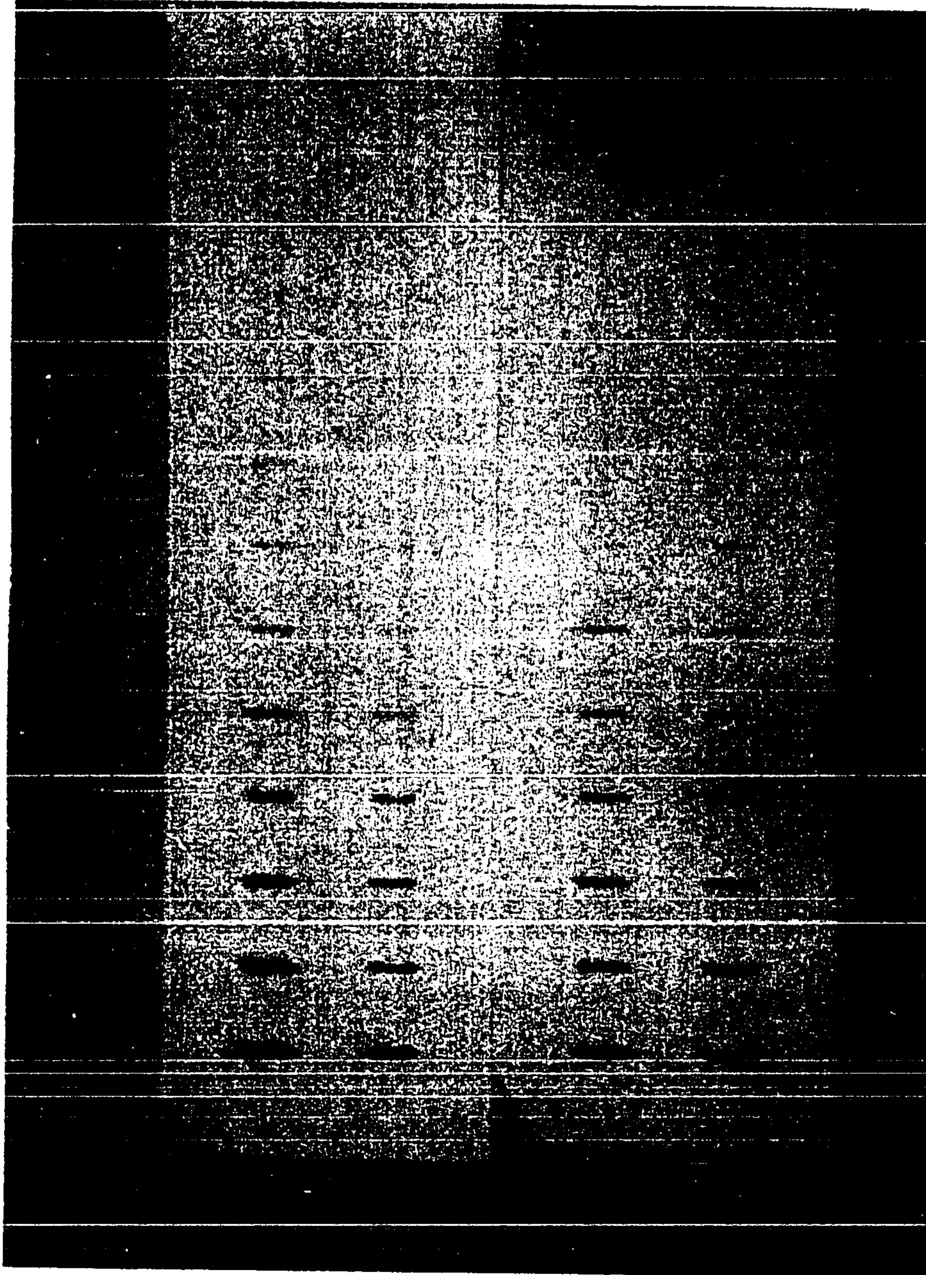


Figure 12. Rotavirus detected by the NC-EIA assay

equivalent results. For densitometric reading, the Hybri-Slot method was prefered. The NC punched-dots created an uneven distribution of the precipitated substrate. In contrast the former method produced a narrow band of more concentrated, precipitated substrate which gave a darker densitometric reading.

C. Application of NC-EIA assay to detect human rotaviruses

The previously described anti-rotavirus immune sera were evaluated for their ability to detect human rotavirus TAMC 15 and rotavirus strain Wa antigen by the NC-EIA assay. When anti-TAMC 15 immune serum was used for the detection of TAMC 15, it was visually observed when the sample was determined to contain 16.120 ng rotavirus protein. This level of antigen is equivalent to 7.8×10^4 PFU of purified rotavirus particles. The lowest level detected by densitometric reading was 1.56×10^5 PFU of rotavirus or 32.24 ng rotavirus protein (Table 19).

When commercial guinea pig anti-Wa antiserum was used for TAMC 15 detection, the lowest visual limit was found to be 6.95×10^4 PFU of rotavirus TAMC 15 (16.120 ng rotavirus protein). The densitometer was again not as sensitive and detected 1.39×10^5 PFU of rotavirus TAMC 15 (32.24 ng rotavirus protein) (Table 20).

Both antisera were determined to have the same sensitivity and able to detect approximately 7×10^4 PFU of

purified rotavirus TAMC 15 antigen as determined by visual inspection. The limit for detection of TAMC 15 by densitometer reading was approximately 2 fold higher at 1.4 x 10^5 PFU of TAMC 15 for both antisera.

It was determined that both antisera (anti-TAMC 15 and the commercial anti-Wa) were capable of detecting all four serotypes of rotavirus by this assay.

Table 19

Sensitivity of NC-EIA detection of human rotavirus strain TAMC 15 based on virus (PFU) and protein content of samples using guinea pig anti-TAMC 15 immune serum made in this study

		NC-EIA measured by	
Total PFU in sample	Protein content in sample	visually (eye)	densitometer reading
2.00 x 10^8	41.20 µg	+	191
1.00 x 10^7	2.06 µg	+	191
5.00 x 10^6	1.03 µg	+	175
2.50 x 10^6	515.00 ng	+	141
1.25 x 10^6	257.95 ng	+	118
6.25 x 10^5	128.98 ng	+	79
3.13 x 10^5	64.49 ng	+	54
1.56 x 10^5	32.24 ng	+	11
7.80 x 10^4	16.12 ng	+	1
3.90 x 10^4	8.06 ng	-	1
1.95 X 10^4	4.03 ng	-	1
0(PBS control)	0(PBS control)	-	1

While both anti-rotavirus sera used as primary antibodies in the NC-EIA rotavirus detection assay yielded a background-free assay, one further purification step was evaluated. Anti-TAMC 15 immune serum was passed through an affinity chromatography column (GammaBind protein G prepack

Table 20

Sensitivity of NC-EIA detection of human rotavirus strain TAMC 15 based on virus (PFU) and protein content of samples using commercial guinea pig anti-Wa immune serum

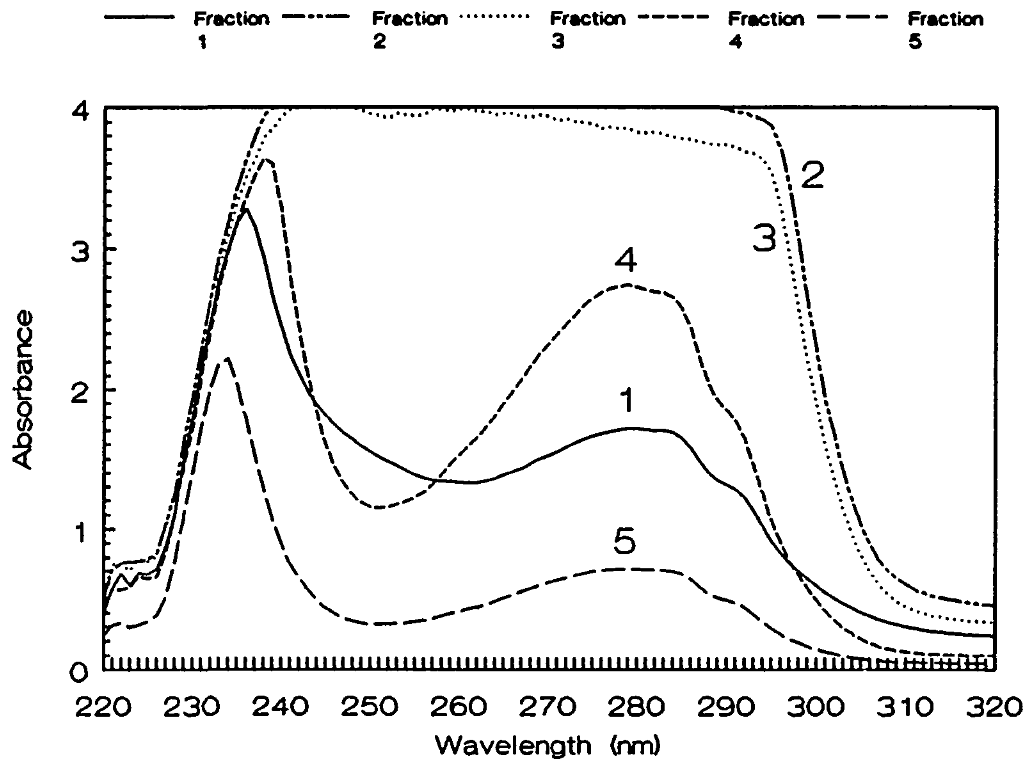
		NC-EIA measured by	
Total PFU in sample	Protein content in sample	visually (eye)	densitometer reading
1.78×10^7	4.127 μg	+	187
8.90×10^6	2.060 μg	+	149
4.45×10^6	1.030 μg	+	129
2.23×10^6	515.000 ng	+	108
1.11×10^6	257.950 ng	+	75
5.56×10^5	128.980 ng	+	57
2.78×10^5	64.490 ng	+	35
1.39×10^5	32.240 ng	+	18
6.95×10^4	16.120 ng	+	1
3.48×10^4	8.060 ng	-	1
0(PBS control)	0(PBS control)	-	1

column) to determine whether higher sensitivity could be achieved through further purification. A volume of 1 ml of

serum was passed through the column and 0.5 ml fractions were collected and assayed by UV absorbance to determine their absorbance at 280 nm (peak absorbance of proteins) (Figure 13). The manufacturer has reported that all of the IgG should elute from the column in the first 2 ml of eluate. The majority of the IgG was determined to elute in the second and third fractions, with the first, fourth and fifth containing the rest. From the sixth fraction on, minor quantities were eluted. It was determined that no increase in sensitivity was achieved for the anti-TAMC 15 immune serum by.

The total volume before purification was 1 ml and was determined to be optimal at a dilution of 1:3,000. Following affinity chromatographic purification the volume was 2.5 ml and was found to be optimal at a dilution of 1:2,000. Therefore, anti-TAMC 15 immune serum was used directly without further affinity chromatographic purification.

Figure 13. Fractionation of anti-TAMC 15 immune serum using a GammaBind recombinant protein G column



III. DISCUSSION

Densitometric measurement of rotavirus adsorbed to NC-EIA strips and developed with Cl-N substrate was not as sensitive as visual scoring of the precipitated dye. It is therefore recommended that a reading of 10 densitometer units and above be considered a positive result for rotavirus detection using the NC-EIA method and reading results by densitometer. However, it should be stressed that the densitometer reading results in relative numbers which can change depending on the region of the NC paper chosen for the zeroing step. It is therefore not advisable to compare densitometer readings between experiments unless a rotavirus standard is included and the densitometer adjusted for this standard.

Previous reports have shown that the sensitivity of a conventional ELISA is about 10^6 particles. The NC-EIA assay was determined capable of consistently detecting approximately 7×10^4 PFU of rotavirus particles visually, and approximately 1.4×10^5 PFU of rotavirus particles by densitometry. The reagent savings were about 50% as compared to the Dakopatt rotavirus ELISA assay.

If Loh, Dow and Fujioka's method of applying sample to NC punched-dots is used, the savings of antibody reagents were 75%. The punched-dot NC-EIA has the added advantage of permitting application of different reagents to each individual dot. However, a disadvantage is the occurrence

of an edge effect due to capillary action during the drying step which causes the precipitation of the substrate to be uneven.

A disadvantage of the Hybri-Slot NC-EIA method is that if a volume of reagent sufficient to cover the NC strip is applied, more reagents are used. Excessive use of reagents could be eliminated by leaving the NC strip in the template and applying the reagents directly to the wells, however, when this is done a halo appears around the substrate slit which is not as easily read densitometrically. An advantage of the Hybri-Slot NC-EIA method is the even precipitation of substrate resulting in narrow bands on the NC paper which are easier to read densitometrically than the punched-dots.

Affinity chromatography as it was performed in this study did not increase the sensitivity of rotavirus detection. The loading volume of anti-TAMC 15 was 1 ml and the collected volume was 2.5 ml for a 150% volume increase in the antiserum stock. The optimal antiserum dilutions noted were 1:3,000 for the unfractionated antiserum and 1:2,000 for the fractionated antiserum which is a 50% reduction in concentration. Thus, approximately 1.65 times as many assay may be done after chromatography as before.

Background color was not a problem even before the antiserum was fractionated, so no further purification was necessary. However, it should be mentioned that if a very dilute antiserum stock is purified on this column, then

concentration of IgG fractions is possible. If unacceptable background color is a problem, then this method may be valuable in eliminating serum components present in the antiserum which may cause nonspecific interactions leading to increased background.

PART IV: PHYSICAL AND BIOLOGICAL CHARACTERIZATION OF A HAWAIIAN HUMAN ROTAVIRUS ISOLATE

I. OBJECTIVES OF THIS PART OF THE STUDY

To characterize the physical properties of viruses recovered from human stool samples and identified as rotavirus by ELISA, an immunological test.

To characterize the biological properties of the viruses recovered from human stool samples and identified as rotavirus by ELISA, an immunological test.

II. INTRODUCTION

A. Physical properties of rotavirus.

Based on electron microscopy, rotavirus particles have been determined to be a double-shelled icosahedral virus with a diameter of 70 nm. Suzuki et al. (1981) calculated that double-shelled Wa strain rotavirus particles were 75 - 85 nm in diameter, while single-shelled particles were 64 - 68 nm in diameter. In 1984, Schulze and Schumacher estimated that the simian rotavirus strain SA11 to have 70 nm double-shelled particles while single-shelled particles were measured at 65 nm, when grown in MA 104 cells. Also in 1984, Suzuki et al. examined a newly isolated HRV grown in MA 104 cells. The double-shelled particles were calculated to be approximately 70 nm in diameter while the single-shelled particles were calculated to be approximately 60 nm in diameter.

Buoyant density ultracentrifugation of HRV in CsCl normally yields two major bands (Shirley, et al., 1981). The 1.36 g/ml top band contains the infectious double-shelled particles while the 1.38 g/ml bottom band contains noninfectious single-shelled particles.

B. Biological properties of rotavirus.

The role of sunlight in the inactivation of rotavirus in environmental situations has not been investigated previously. Previous work with other systems has demonstrated the role of sunlight in the inactivation of microbial agents (Fujioka et al., 1981; Fujioka and Siwak, 1985).

Raphael, Sattar and Springthorpe (1985a) stated that microbial activity in fresh water is deleterious to virus survival. The bacterial activity is modulated by the temperature at which the sample is held. The authors determined that greater than 64 days was required for a 99% loss in HRV infectious titers when suspended in filtered and treated water held at 4 and 20°C. For raw river water, a 99% loss in infectivity required 10 days at 20°C and 32 days at 4°C. Winston and Ward (1985) reported that the infectivity of SA11 decreased 99% in $\leq$ 7 days at 27±1°C in all fresh waters tested.

Ward, Knowlton and Winston (1986) stated that previous studies suggested that inactivation of rotavirus in natural fresh waters is most likely due to bacterial enzymes,

turbidity, light, and temperature, which is the dominant factor. Ward, Knowlton and Winston demonstrated that the stability of SA11 was dependent on the temperature, with increasing stability at lower temperatures (16°C vs. 4°C). They identified the growth of microorganisms in stream water as resulting in a concomitant increase in virucidal activity, and the mechanism may be a proteolytic bacterial enzyme.

Chlorine inactivation of rotaviruses has been well documented. Berman and Hoff (1984) found that > 99.99% of SA11 infectivity occurred in < 15 seconds at 5°C using 0.5, 0.25, and 0.1 mg/l chlorine in potassium phosphate buffer at pH 6. However, these rates were obtained using a free virus preparation. Cell-associated virus inactivated much slower. The mechanism of chlorine inactivation is by removing the outer capsid (Rodgers et al., 1985) which may occur within 1 minute.

III. MATERIALS AND METHODS

Electron microscopy (EM).

Copper grids (200 mesh; Ted Pella, Inc., Tustin, California) were coated with formvar resin (Catalog No. 19221; Ted Pella, Inc., Tustin, California). Samples containing rotavirus particles were applied to the formvar coated grids by the method of Bridger and Woode (1976). Briefly, purified and concentrated rotavirus preparations were suspended in PBS. Single drops of the suspension were

placed on Parafilm and a formvar-coated grid was floated on the surface for 1 minute. The grid was blotted, then air dried.

Grids containing rotavirus particles were stained with 2% phosphotungstic acid, pH 7.00, (PTA; Catalog No. 19402; Ted Pella, Inc., Tustin, California) according to the method of Hayat (1986).

Electronmicroscopy of grids holding rotavirus particles was done with a Zeiss 10/A transmission electron microscope (Carl Zeiss, Inc., Thornwood, New York).

Virus purification and buoyant density determination.

For cesium chloride banding, the virus pellet from above was suspended in 0.01 M Tris (tris[hydroxy-methyl] aminomethane base; Catalog No. T-1503; Sigma Chemical Co., St. Louis, Missouri) buffer. 3.2 ml of the rotavirus suspension in 0.01 M Tris was added to 1.8 ml of a sterile cesium chloride (Catalog No. 340303; Beckman Instruments, Inc., Palo Alto, California) solution made in distilled water, and added to a 5 ml Ultra-Clear centrifuge tube (13mm x 51 mm; Catalog No. 344057; Beckman Instruments, Inc., Spinco Division, Palo Alto, California). The tubes were placed in either a Beckman SW 50L swinging bucket rotor with 3 buckets, or a Beckman SW 50.1 swinging bucket with 6 buckets (Spinco Division of Beckman Instruments, Inc., Palo Alto, California), and centrifuged at 100,000 x *g* for 16-24 hours at 4°C in a Beckman model L2-65B or model L

ultracentrifuge (Spinco Division of Beckman Instruments, Inc., Palo Alto, California).

Following centrifugation, the tubes were punctured from the bottom and 0.20 ml fractions were collected. Samples containing rotavirus particles were placed in dialysis tubing (Spectrapore, m.w. cutoff 12,000-14,000, 10mm x 25 m; Catalog No. D1615-4; Scientific Products, McGaw Park, Illinois) and dialyzed against 1l of PBS for 1 hour at 28°C, then twice more against 1l of PBS at 4°C for 12 hours each. After dialysis, the virus stocks were removed from the dialysis tubing with a sterile tuberculin needle and placed in a screw-cap tubes and frozen at -20°C until needed.

Measurement of refractive index.

Refractive indices of CsCl banded rotavirus were determined with an Abbe Refractometer (American Optical Corporation, Buffalo, New York).

Calculation of density of banded rotavirus.

Calculation of buoyant density was made using the following equation described by Ifft, Voet and Vinograd (1961) where P = density and R.I. = refractive index.

$$P = 10.8601 \times R.I. - 13.4974$$

Protein determinations and spectrophotometry.

All protein assays were done using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond California) and performed as per manufacturers instructions. Standards were made using bovine albumin (bovine fraction V; Catalog No.

A-8022; Lot 114F-0016; Sigma Chemical Co., St. Louis, Missouri).

Assays were read in a Gilford Response II, UV-visible spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) using proprietary software.

Collection of environmental water sources.

Seawater (SW) was collected from Black Point beach and fresh stream water (FW) was collected from Nuuanu stream as previously described by Fujioka et al. (1981).

Experimental design: stability in sunlight.

Rotavirus stocks were diluted into SW to a final concentration of 10^4 to 10^6 PFU/ml. Two hundred fifty mls of the rotavirus-SW suspension were poured into 250 ml beakers containing a teflon-coated stir-bar. Equivalent beakers were set-up and left in the laboratory under an inverted cardboard box as dark controls. The beaker was placed into a specially constructed styrofoam box with holes in the lid. The beaker's rim was even with the styrofoam box lid's top surface to exclude all sunlight except that entering from the top of the beaker. The inside of the box was filled with tapwater and ice to maintain a constant temperature of 20°C. Water or ice was added as needed to adjust the temperature. The box was placed on stir plates which were on the roof of Snyder Hall, and the rotavirus suspension was constantly kept stirring for the duration of the experiment (1100 until 1500 hours). Every hour, five ml

samples were taken and placed into a sterile 10 ml screw-cap tube and immediately transferred to the laboratory under dark conditions. The samples were either titrated immediately under standard plaquing assay conditions or held in the refrigerator (4°C) overnight before assaying.

Experimental design: temperature and environmental water stability.

Rotavirus stocks were diluted into SW, FW, or PBS to a final concentration of 10^4 to 10^6 PFU/ml. One hundred mls of the rotavirus suspension was put into 100 ml serum bottles containing a teflon-coated stir-bar. Two sets of the bottles were set-up and one set was left at ambient room temperature (26°C) and the second was placed into a refrigerator (4°C). An initial sample and subsequent samples were taken at five day intervals (0, 4, 9, and 15 days for the first experiment). The bottles were stirred for 1 - 2 minutes and a 5 ml sample was immediately taken. The samples were either titrated immediately under standard plaquing assay conditions or held in the refrigerator (4°C) overnight before assaying.

For the antibiotic phase of the experiments, 100 μg/ml streptomycin sulfate and 100 units/ml penicillin were added to the rotavirus-diluent suspension at the beginning of the experiment.

Assay of bacteria.

Counts of *Streptococcus faecalis* in the sunlight inactivation experiment was made by growing the bacteria on m-Enterococcus agar (Difco Laboratories, Detroit, Michigan) and incubated at 37°C, and colonies counted after 2 days.

Enumeration of total bacteria was done by growing endogenous bacteria on either marine agar for SW or total plate count agar (Difco Laboratories, Detroit, Michigan). Plates were incubated at room temperature (26°C), and colonies enumerated after 5 days.

Chlorine inactivation

The chlorine experiment was performed as described by Vaughn, Chen and Thomas (1986). Chlorine was obtained as a standard solution of 57.5 ±0.2 mg/l (Hach Company, Ames, Iowa). This solution was diluted to 2 mg/l as measured in a Hach spectrophotometer (Model DR/3000, Hach Company, Ames, Iowa). Briefly, the 2 mg/l solution was diluted 1:100 in the diluent, containing 10^5 to 10^6 PFU/ml rotavirus, to achieve a final concentration of 0.02 mg/l. Rotavirus samples were taken at time 0 and after 30 minutes of exposure, and chlorine reactivity stopped with 0.1 ml of 0.5 M sodium thiosulfate. The samples were held at 4°C overnight and titrated in MA 104 cells the next day by the standard plaque assay.

CHAPTER 10

PHYSICAL CHARACTERIZATION OF A HAWAIIAN HUMAN ROTAVIRUS ISOLATE (TAMC 15)

I. OBJECTIVES OF THE CHAPTER

The ELISA assay performed on stool samples indicated that antigens of rotavirus were present in the samples. To confirm that these antigens were rotavirus particles, the physical characteristics (buoyant density, structure by electron microscopy) of the particles isolated from the ELISA-positive samples were determined and compared against those of model rotavirus strains.

II. RESULTS

Buoyant density ultracentrifugation of the four prototypic human rotavirus serotypes, the simian rotavirus SA11, and two human stool isolates from this study was done in cesium chloride (CsCl). Calculated densities from this study were compared against reported densities for the two major bands reported in other studies. In this study, the density of the infectious (1.36 g/ml) band ranged from 1.3581-1.3657 g/ml (mean = 1.3614 g/ml) and the non-infectious (1.38 g/ml) band ranged from 1.3799-1.3896 g/ml (mean = 1.3834 g/ml) (Table 21). Typical centrifuge tubes containing rotavirus banded in CsCl are shown in Figure 14.

Electron microscopy (EM) was done by a direct method with negative staining to show TAMC 15 rotavirus particles (Figure 15). Both single- and double-shelled particles, as

well as empty particles were observed which is consistent with previous reports. The estimated diameter of double-shelled particles was 75 nm.

Table 21

Buoyant density determination of purified rotavirus strains after isopycnic centrifugation in cesium chloride

Virus	Serotype	Top Band (Infectious)	Bottom Band (Non-infectious)
Wa	1	1.3625	1.3929
KH 1	1	1.3603	1.3799
DS-1	2	1.3603	1.3820
DS-1	2	1.3657	1.3896
P	3	1.3603	1.3820
P	3	1.3614	1.3831
TAMC 15	3	1.3592	1.3820
TAMC 15	3	1.3581	1.3799
TAMC 15	3	1.3625	1.3831
SA11	3	1.3657	1.3831
SA11	3	1.3603	1.3809
ST3	4	1.3592	1.3820
ST3	4	1.3625	1.3843
Mean		1.3614	1.3834
SEM[a]		0.0006	0.0010

a = standard error of the mean
Calculations made from the equation of Ifft, Voet and Vinograd (1961): $Den._{25^\circ C} = 10.8601(R.I.) - 13.4974$

Figure 14. Cesium chloride banding of rotaviruses

A) TAMC 15 human rotavirus isolate and simian rotavirus SA11
B) Left to right: human rotavirus strains Wa, DS-1, and ST3

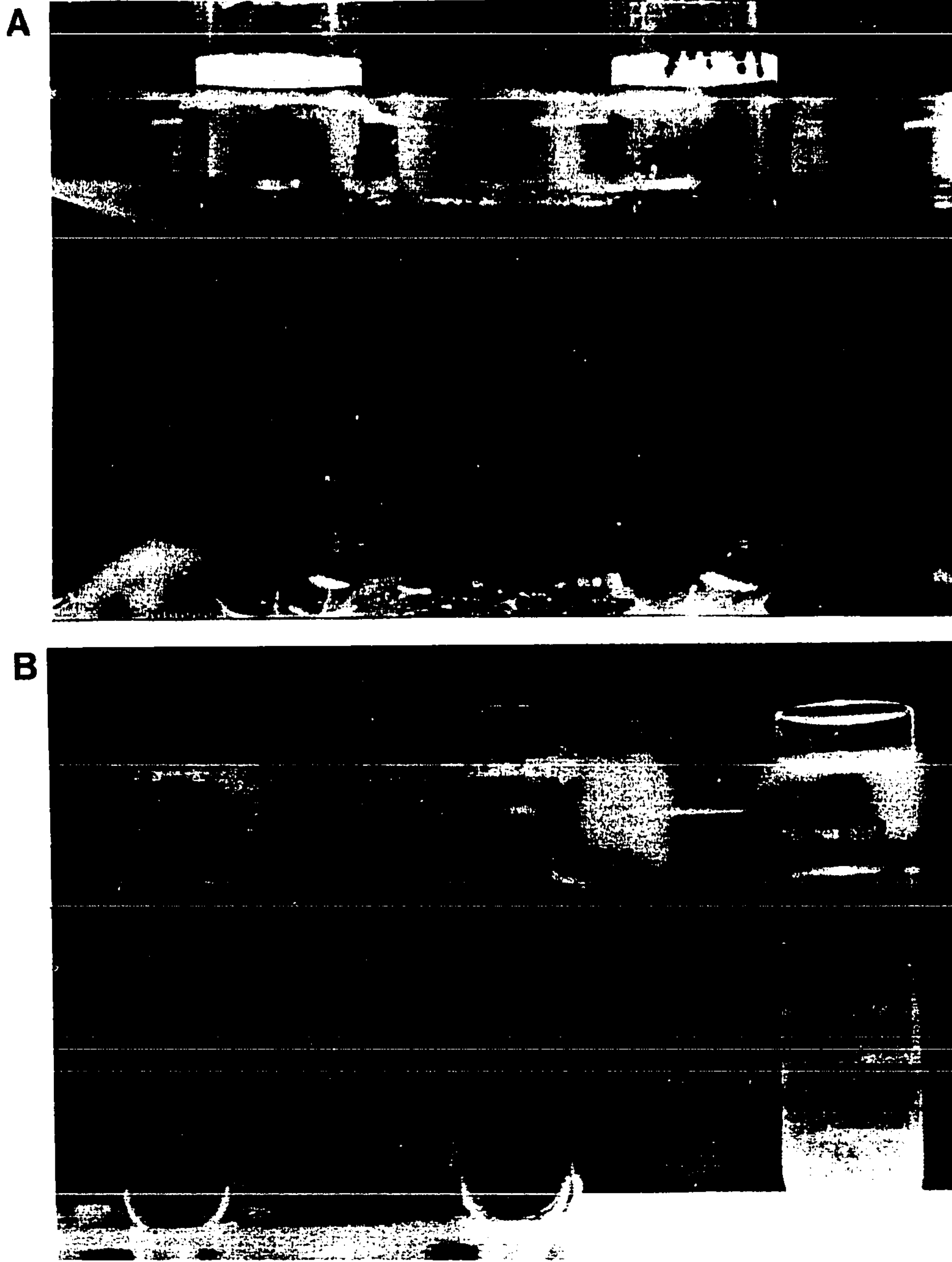
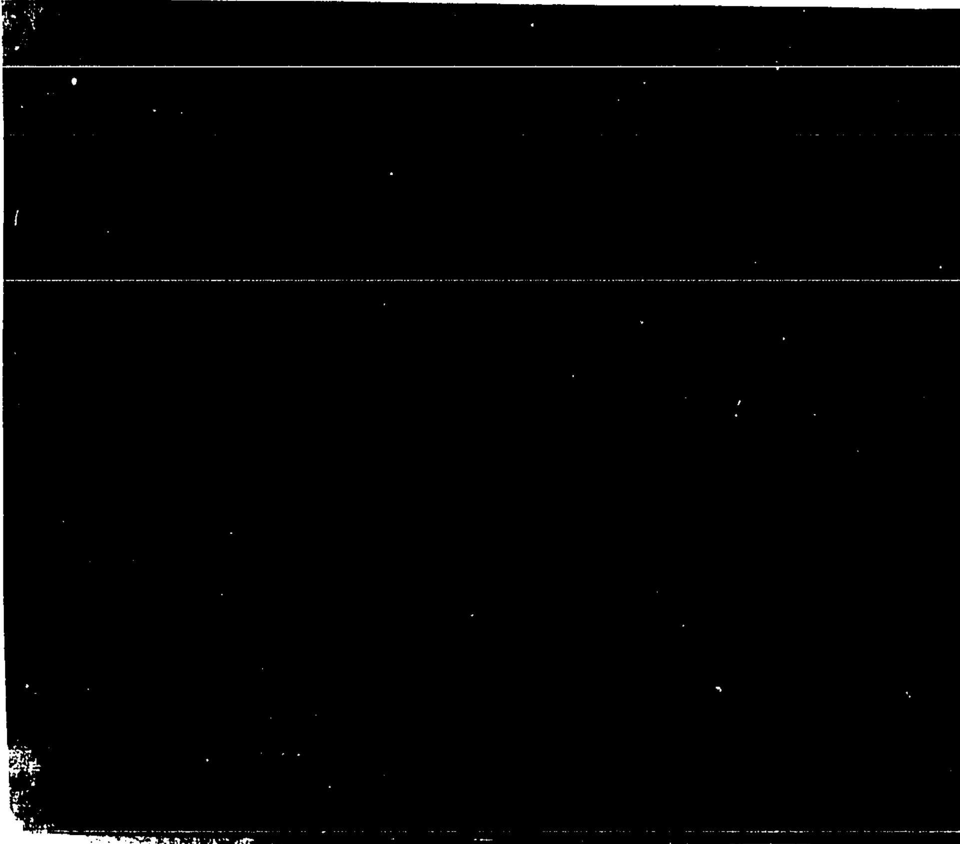
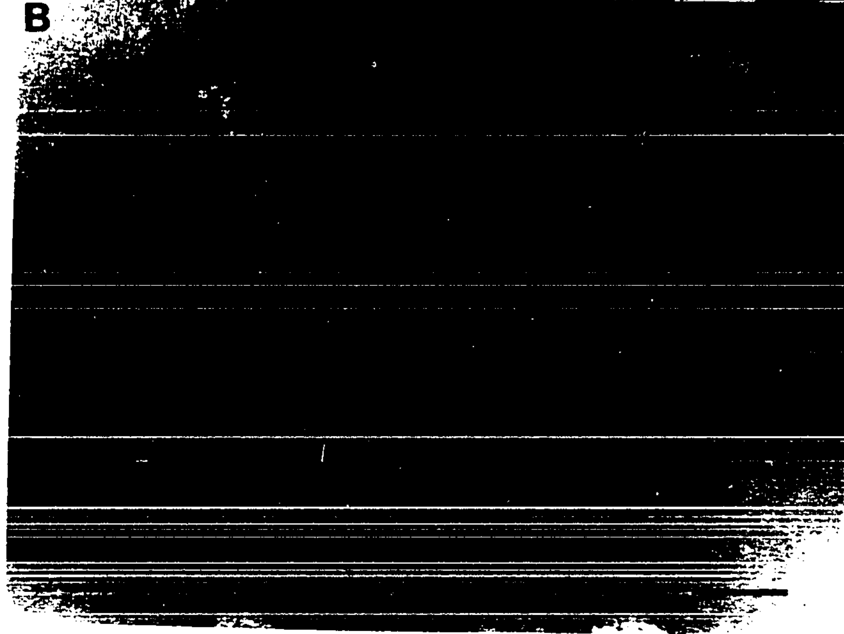


Figure 15. Electron micrographs of TAMC 15

A) Bar = 100nm; complete particles
B) Bar = 100nm; incomplete and empty particles





III. DISCUSSION

Buoyant density calculations from previous studies report the density as 1.36 g/ml for double-shelled infectious virus particles (top band in tube), and 1.38 g/ml for single-shelled noninfectious virus particles (bottom band in tube) in cesium chloride. Thirteen samples were tested and each sample revealed a particle density of 1.36 g/ml as well as another fraction with a particle density of 1.38 g/ml. The results of this study conclusively show that rotaviruses were present in the hospital stool samples which were ELISA positive for rotavirus.

Electron microscopic examination of TAMC 15 revealed three main structures: a) double-shelled particles, b) single-shelled particles, and c) empty particles (Figure 15). The presence, size and structure of these particles are consistent with that of known rotaviruses.

CHAPTER 11

BIOLOGICAL CHARACTERIZATION OF A HAWAIIAN HUMAN ROTAVIRUS ISOLATE (TAMC 15)

I. OBJECTIVES OF THE CHAPTER

To compare the environmental stability of a human rotavirus isolate and of poliovirus 2 when exposed to natural sunlight.

To determine the stability of rotavirus in seawater and fresh stream water under environmental simulation.

To determine the relative stability of SA11 (simian rotavirus) and TAMC 15 (human rotavirus) in the presence of chlorine.

II. RESULTS

A. Sunlight stability.

To determine the stability of rotavirus when exposed to sunlight, rotavirus strains SA11 and TAMC 15 were added to seawater and incubated at 22°C in the presence and absence of sunlight for four hours. The results (Figure 16-A) show that the infectious titer of SA11 decreased 1.34 logs in four hours, TAMC 15 decreased 1.88 logs, and poliovirus 2 decreased 2.54 logs. In contrast, controls incubated in the dark changed by -0.11 logs, +0.09 logs and -0.03 logs respectively.

These results indicate that TAMC 15 and SA11 were more resistant to the effects of sunlight than poliovirus 2.

The experiment was repeated (Figure 16-B) using the same three viruses in addition to the bacterium S. faecalis. Similar results were observed in both cases. TAMC 15 decreased 1.35 logs, SA11 decreased 1.77 logs, poliovirus 2 decreased > 4.05 logs and S. faecalis decreased 4.43 logs in four hours. The four-hour dark controls yielded a titer change of -0.07 logs, +0.03 logs, -0.18 logs and -0.01 logs respectively.

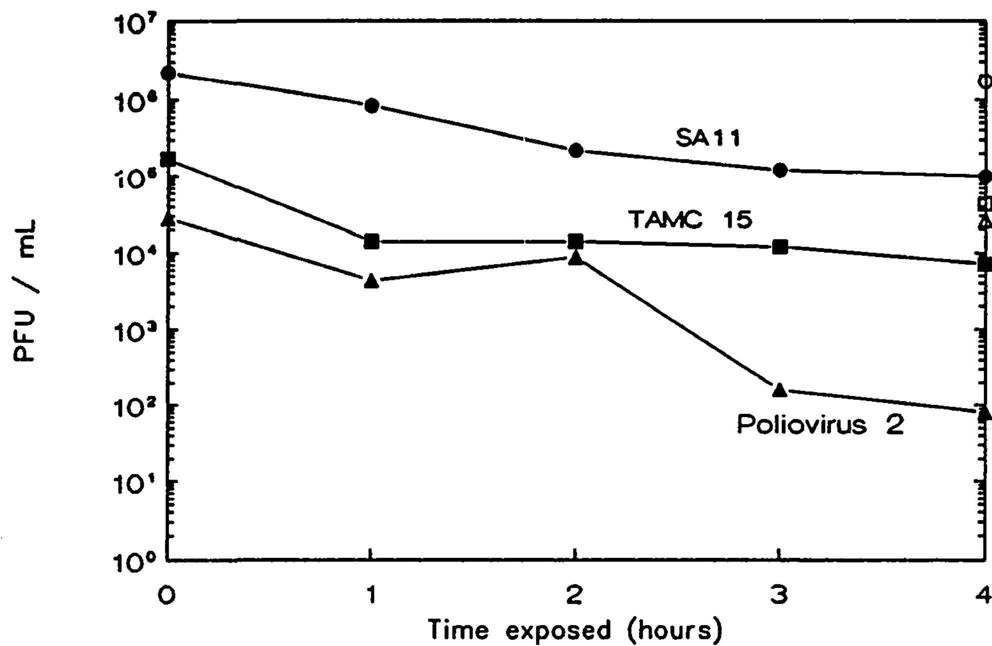
These results indicate that the two rotavirus strains were more resistant to the effects of sunlight than are poliovirus 2 and S. faecalis.

B. Stability of rotavirus in seawater (SW) and fresh stream water (FW): effects of salinity and temperature.

To compare the stability of rotavirus in SW and FW free of endogenous microorganisms, rotavirus strains SA11 and TAMC 15 were suspended in SW and FW filtered through a 0.45 μm size membrane. Two 100 ml suspensions of rotavirus were incubated at 4°C (refrigerator) and 26°C (room temperature) for 15 days.

The results of this experiment (Table 22) shows that the two rotavirus strains were more stable, with less decrease in infectious titer, for 15 days at 4°C than at 26°C. The inactivation of the rotavirus strains suspended in SW was greater than those suspended in FW. The decrease in infectious titer after 15 days of incubation in SW-4°C

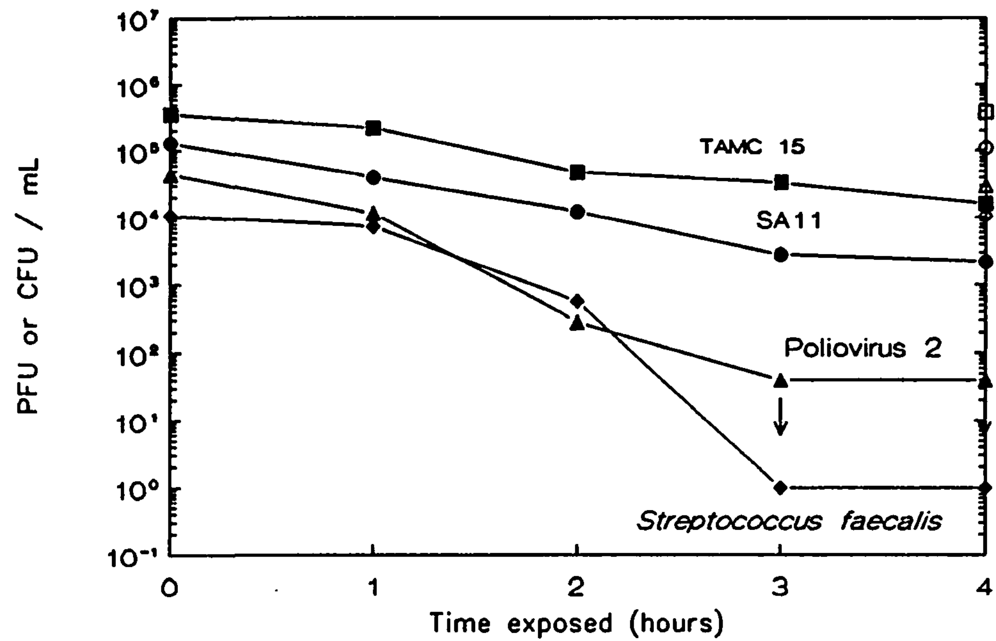
Figure 16-A. Effect of sunlight on the stability of rotavirus SA11, TAMC 15, poliovirus 2, and _Streptococcus faecalis_



Date of experiment: July 13, 1988

Filled symbols = sunlight exposure; open symbols = dark laboratory controls

Figure 16-B



Date of experiment: July 29, 1988 Arrows = below detection limit

Filled symbols = sunlight exposure; open symbols = dark laboratory controls

Table 22

Comparative stability of SA11 and TAMC 15 suspended in 0.45 μm filtered seawater and fresh stream water and incubated at room temperature (26°C) and 4° C.

suspending medium	virus	temp (°C)	survival ($\log N/N_o$) after day 4	9	15
seawater	SA11	26	-0.528	-1.712	> -3.716
	SA11	4	+0.025	+0.112	-1.060
	TAMC 15	26	-0.164	-0.451	-1.226
	TAMC 15	4	-0.027	-0.019	-0.238
stream water	SA11	26	+0.073	-2.087	> -3.643
	SA11	4	+0.220	-0.095	-1.927
	TAMC 15	26	+0.122	-0.244	-1.705
	TAMC 15	4	+0.106	-0.122	-0.845

was 1.060 logs for SA11 and 1.927 logs for TAMC 15. This titer change is in contrast to FW-4°C where the decrease was determined to be 0.238 log for SA11 and 0.845 log for TAMC 15. The decrease in infectious titer after 15 days of incubation in SW-26°C was > 3.580 logs for SA11 and > 3.643 logs for TAMC 15. The decrease in infectious titer in FW-26°C was 1.226 logs for SA11 and 1.705 logs for TAMC. Thus, these results indicate that SW is a harsher environment than FW under the experimental conditions.

To compare the stability of rotavirus in SW, FW and phosphate buffered saline (PBS) rotaviruses SA11 and TAMC 15 were suspended in natural SW, FW and PBS and incubated at 4°C and 26°C for 10 days. The results (Table 23) show that a greater decrease in infectious titer occurred at 26°C than at 4°C. Inactivation was greater in SW-26°C than in FW-26°C. The infectious titer of both rotaviruses decreased approximately 2.7 logs in FW-26°C, whereas the infectious titer of SA11 decreased 3.434 logs in SW-26°C while TAMC 15 decreased > 4.342 logs in 10 days. SA11 in PBS at both temperatures was stable for 10 days whereas the infectious titer of TAMC 15 decreased 1.188 logs in PBS-4°C and 2.012 logs in PBS-26°C. These results indicate that SW is a harsher environment than FW for both rotaviruses, and the HRV TAMC 15 is not as stable in PBS as SA11.

Table 23

Comparative stability of SA11 and TAMC 15 suspended in unaltered seawater and fresh stream water and incubated at room temperature (26°C) and 4°C

suspending medium	virus	temp (°C)	survival (log N/N_o) after day	
			5	10
seawater	SA11	26	+0.092	-3.434
	SA11	4	+0.848	-0.839
	TAMC 15	26	-0.644	> -4.342
	TAMC 15	4	+1.534	+0.055
stream water	SA11	26	-1.089	-2.732
	SA11	4	-0.008	-1.207
	TAMC 15	26	-1.291	-2.747
	TAMC 15	4	+0.115	-1.678
phosphate buffered saline	SA11	26	+0.880	-0.259
	SA11	4	+0.802	+0.070
	TAMC 15	26	-0.016	-2.012
	TAMC 15	4	+0.199	-1.188

To assess the role of endogenous bacteria in the natural water sources on the stability of rotaviruses, the two antibiotics (ATB) penicillin (100 I.U./ml) and streptomycin (100 μg/ml) were added to SW and FW. Rotaviruses SA11 and TAMC 15 were suspended in these ATB treated waters and incubated at 4°C and 26°C for 10 days. Fujioka, Loh and Lau (1980) reported that endogenous bacteria were responsible for viral inactivation in Hawaiian SW.

The results (Table 24) showed that both rotavirus strains were stable for 10 days in treated and untreated FW at 4°C, except that SA11 in FW+ATB decreased 1.503 logs.

When ATB's were added to FW-26°C, the infectious titer decrease was determined to be 1.845 logs for SA11 and 2.376 logs for TAMC 15 after 10 days. Without an ATB supplement, the infectious titer decrease was 1.343 logs for SA11 and 1.003 logs for TAMC 15 after 10 days.

When ATB's were added to SW-26°C (Table 25), the infectious titer decrease was determined to be 0.645 log for SA11 and 0.422 log for TAMC 15 after 10 days. Without an ATB supplement, the infectious titer decrease was 1.968 logs for SA11 and 2.970 logs for TAMC 15 after 10 days.

The results indicate that the mode of action of the ATB supplement is different for SW and FW. ATB's added to SW suppressed anti-rotaviral activity whereas in FW the activity was enhanced.

Table 24

Comparative stability of SA11 and TAMC 15 rotavirus suspended in environmental waters (seawater and fresh stream water) in the absence and presence of antibiotics at 4° C

suspending medium	virus system	survival (log N/N_o) after day	
		5	10
seawater	SA11 - antibiotics	-0.079	-0.259
	SA11 + antibiotics	+0.027	-0.185
	TAMC 15 - antibiotics	-0.138	-0.383
	TAMC 15 + antibiotics	-1.047	-0.173
stream water	SA11 - antibiotics	-0.026	-0.295
	SA11 + antibiotics	-0.046	-1.503
	TAMC 15 - antibiotics	-0.031	-0.184
	TAMC 15 + antibiotics	-0.076	-0.160

Table 25

Comparative stability of SA11 and TAMC 15 rotavirus suspended in environmental waters (seawater and fresh stream water) in the absence and presence of antibiotics at 26° C

suspending medium	virus system	survival (log N/N_o) after day	
		5	10
seawater	SA11 - antibiotics	-0.430	-1.968
	SA11 + antibiotics	-0.079	-0.645
	TAMC 15 - antibiotics	-1.414	-2.970
	TAMC 15 + antibiotics	-0.113	-0.422
stream water	SA11 - antibiotics	-0.354	-1.343
	SA11 + antibiotics	-0.456	-1.845
	TAMC 15 - antibiotics	-0.262	-1.004
	TAMC 15 + antibiotics	-1.293	-2.336

To confirm the role of endogenous bacteria in natural waters as regards anti-rotavirus activity, the previous experiment was repeated. The stability of SA11 and TAMC 15 suspended in SW and FW, both supplemented with ATB's and unaltered, was assessed. In addition, total bacteria counts were determined by plating samples on Difco's Total Plate Count agar (FW) and Difco's Marine agar (SW) to evaluate the effect of ATB's on endogenous bacterial populations.

The results (Table 26) showed that both SA11 and TAMC 15 were stable in SW and FW at 4°C, except, the infectious titer of SA11 in FW-4°C+ATB decreased 1.503 logs. At 26°C, greater decreases in infectious titer were observed in SW-ATB's as compared to SW+26°C. A greater decrease in infectious titer was noted in FW-26°C+ATB's than FW-26°C-ATB's (Table 27).

Bacterial concentrations increased in both ATB supplemented and unsupplemented SW (except for TAMC 15 in SW-26°C+ATB's). Bacterial concentrations in FW under both ATB conditions increased by > 1 log.

Results of the change in bacterial concentrations for SW and FW unsupplemented with ATB's at 4°C shows variability. The concentration of bacteria varied from a 0.449 log increase to a 1.136 log decrease. The bacterial titer of both diluents at 4°C supplemented with ATB's decreased by ≥ 1.659 logs.

The results indicated that bacteria were suppressed in both SW and FW at 4°C and any observed anti-rotaviral effects were not due to bacterial activity. However, ATB's added to SW-26°C suppressed bacterial strains deleterious to the virus strains used. The situation was reversed for FW at 26°C where the addition of ATB's increased anti-rotavirus properties of the water as compared to ATB free FW.

Table 26

Comparative stability of SA11, TAMC 15 rotavirus and total bacteria (TB) suspended in environmental waters (seawater and fresh stream water) in the absence and presence of antibiotics, and phosphate buffered saline at 26° C

suspending medium	virus system	survival (log N/N_o) after day 5		survival (log N/N_o) after day 10	
		rotavirus	TB	rotavirus	TB
PBS	SA11 - antibiotics	-0.118	N.D.	-0.268	N.D.
	SA11 + antibiotics	N.D.	N.D.	N.D.	N.D.
	TAMC 15 - antibiotics	-0.015	N.D.	-0.341	N.D.
	TAMC 15 + antibiotics	N.D.	N.D.	N.D.	N.D.
seawater	SA11 - antibiotics	-0.430	+2.319	-1.970	+1.417
	SA11 + antibiotics	-0.079	-0.455	-0.645	+1.788
	TAMC 15 - antibiotics	-1.410	+2.097	-2.970	+1.161
	TAMC 15 + antibiotics	-0.113	+0.174	-0.422	> -1.659
stream water	SA11 - antibiotics	-0.354	+0.550	-1.340	+1.664
	SA11 + antibiotics	-1.317	+2.575	-1.973	+3.196
	TAMC 15 - antibiotics	-0.262	-0.035	-1.007	+1.120
	TAMC 15 + antibiotics	-1.603	+2.454	-2.336	+2.750

Table 27

Comparative stability of SA11, TAMC 15 rotavirus and total bacteria (TB) suspended in environmental waters (seawater and fresh stream water) in the absence and presence of antibiotics, and phosphate buffered saline at 4° C

suspending medium	virus system	survival (log N/N_o) after day 5 rotavirus	5 TB	10 rotavirus	10 TB
PBS	SA11 - antibiotics	-0.047	N.D.	-0.761	N.D.
	SA11 + antibiotics	N.D.	N.D.	N.D.	N.D.
	TAMC 15 - antibiotics	-0.154	N.D.	-0.835	N.D.
	TAMC 15 + antibiotics	N.D.	N.D.	N.D.	N.D.
seawater	SA11 - antibiotics	-0.079	+0.860	-0.259	-0.960
	SA11 + antibiotics	-0.027	> -1.659	-0.185	> -1.659
	TAMC 15 - antibiotics	-0.138	+0.835	-0.383	-1.057
	TAMC 15 + antibiotics	-0.105	> -1.659	-0.173	> -1.659
stream water	SA11 - antibiotics	-0.026	+0.235	-0.295	+0.449
	SA11 + antibiotics	-0.046	> -3.223	-0.503	-2.922
	TAMC 15 - antibiotics	-0.031	+0.485	-0.184	-1.136
	TAMC 15 + antibiotics	-0.075	> -3.223	-0.159	-2.922

B. Chlorine inactivation

To determine the sensitivity of rotavirus strains SA11 and TAMC 15 to chlorine, the viruses were suspended in untreated tap water (TW) and phosphate buffered saline (PBS) and exposed to 0.02 mg sodium hypochlorite/l for 30 minutes.

Results summarized in Table 28 show that TAMC 15 was more resistant to the disinfecting effect of chlorine than was SA11. After 30 minutes, the infectious titer of SA11 in TW decreased by 4.176 logs and in PBS decreased by 3.868 logs. In contrast, TAMC 15 decreased by 0.701 log in TW and 0.434 log in PBS.

The results indicated that the HRV TAMC 15 was more stable than the current model simian rotavirus SA11 at low level chlorine concentration.

Table 28

Effect of chlorine on human rotavirus TAMC 15, simian rotavirus SA11, and poliovirus 2

Virus	Free chlorine concn	Time exposed (min)	Titer	Titer change
SA11 in tapwater	0.020 mg/l	0	6.00×10^{5}	
SA11	0.017 mg/l	30	4.00×10^{1}	-4.176
SA11 in PBS	0.020 mg/l	0	5.90×10^{5}	
SA11	0.016 mg/l	30	8.00×10^{1}	-3.868
TAMC 15 in tapwater	0.020 mg/l	0	4.56×10^{6}	
TAMC 15	0.019 mg/l	30	9.08×10^{5}	-0.701
TAMC 15 in PBS	0.020 mg/l	0	4.75×10^{6}	
TAMC 15	0.016 mg/l	30	1.75×10^{6}	-0.434

III. DISCUSSION

The environmental simulation of suspending rotavirus in natural SW and exposing the mixtures to natural sunlight has not been investigated previously. This type of study is significant for an island community like Hawaii where the ocean environment is of such importance.

Fujioka, Loh and Lau (1980) identified marine bacteria in SW as the inactivating agent of enteroviruses. In 1981, Fujioka et al. demonstrated that sunlight plays a major role in the inactivation of bacteria used as indicator organisms for human fecal contamination.

The results of this study graphically displayed in Figure 16 demonstrate these effects. The rotavirus suspensions show a slow, steady inactivation rate while the bacterium *Streptococcus faecalis* has a very slight initial inactivation rate which increases with time. The rotaviruses were less sensitive to the effects of sunlight than were poliovirus 2 and *S. faecalis*. This is significant since *S. faecalis* is a standard bacterial indicator organism of human fecal contamination. Bacteriologically "clean" water may not necessarily indicate that the water is free of human enteric viruses.

These experiments demonstrated that infectious virus have the capacity to persist in the ocean environment for a period of time sufficiently long to pose a potential problem for the human population. Viruses often are stabilized in

particulate environments such as in ocean sediments, and may persist for a long time. Jofre et al. (1989) found rotaviruses in the same concentrations as human enteroviruses in sediments off the coast of Spain.

The initial experiment examining the stability of rotaviruses suspended in SW and FW filtered through a 0.45 μm pore-size membrane demonstrated that SW at 26°C had a more deleterious effect than FW. The decrease in infectious titer when rotavirus is suspended in FW could be due to the ionic makeup of the water. It has been reported that the presence of chelating agents reduces rotavirus stability through the loss of Ca^{2+} ions and the subsequent loss of the outer capsid (Ward and Ashley, 1980; Shahrabadi and Lee, 1986; Ward, Knowlton and Winston, 1986).

The experiment which included PBS, in addition to SW and FW, as a control demonstrated that the infectious titer of TAMC 15 decreased after 15 days by approximately 2 logs. Shirley et al. (1981) reported that HRV's may be less stable in PBS due to the precipitation of free Ca^{2+} ions.

This experiment also examined SW and FW which had not been prefiltered. Any endogenous particulate matter including native microorganisms were left in the water. This experiment showed that the infectious titers of both SA11 and TAMC 15 were greatly reduced in both SW and FW after 10 days, with a greater decrease observed for SW.

The role of microbiological activity was examined in

the next experiment. The presence of the antibiotics penicillin and streptomycin clearly retarded any inactivation events in SW, but increased the effects in FW. This may be indicative of the bacterial makeup of the two water types. Apparently the bacteria responsible for inactivation of viruses in SW are being repressed. This effect was also noted by Fujioka, Loh and Lau (1980).

However, decreases in infectious titers of rotavirus in FW was enhanced with ATB supplementation. This indicates that the bacteria responsible for inactivation in FW are not affected by the particular ATB's used. The bacteria suppressed allowed the growth of those bacteria which are deleterious to rotavirus. This may be accomplished by eliminating competition for nutrients. Fujioka, Loh and Lau (1980) reported that Nuuanu stream water, the same source as the one used in this study, had no inactivating effect on infectious titers of poliovirus 1, coxsackievirus B5, or echo virus 7. This could be due to either different ionic tolerances, an actual change in the composition of the stream water over the years, or inherent properties of the viruses used.

The last environmental simulation experiment examined the endogenous bacterial population. The results of virus stability were similar to the previous experiment. However, the total bacteria counts were seen to increase with time in FW in the presence of ATB's at 26°C whereas in SW at 26°C,

ATB's either slowed or prevented the growth of bacteria. The decrease of titer in SW indicates that most if not all endogenous strains of bacteria were sensitive to the supplemented penicillin and streptomycin. In FW, one or more strains of bacteria were not inhibited and most probably were able to grow to high titers by utilizing nutrients available due to the lack of competition by other bacterial strains. The ATB's in both media incubated at 4°C inactivated the viable bacterial counts essentially completely.

Chlorine treatment of rotavirus strains SA11 and TAMC 15 demonstrated that TAMC 15 is more stable than SA11 at a chlorine concentration of 0.02 mg/l. Sattar, Raphael and Springthorpe (1984) reported that some rotaviruses have been able to withstand chlorine concentrations of > 100 mg/l.

While only one concentration was tested in this study, it indicates that there is a significant difference in the stabilities of the simian rotavirus SA11 and the human isolate TAMC 15. At higher concentrations this effect might not be quite so pronounced. Vaughn, Chen and Thomas (1986) reported that concentrations of chlorine above 0.20 mg/l caused total inactivation of both SA11 and the human rotavirus strain Wa, although the rate of inactivation for Wa was slower. The current practice of using SA11 as a model system to describe the stability of human rotaviruses must be reexamined.

Grabow et al. (1984) reported that the residual titer of SA11 treated with 0.1 mg/l chlorine for 60 minutes was higher than that of hepatitis A virus, an important human pathogen, along with other viruses including poliovirus 2, coxsackie virus B5, as well as standard bacteria used to indicate the degree of pollution in a water source. If newly isolated human rotaviruses should prove to be more stable to the effects of chlorine inactivation than SA11, the inadequacies of using the current model system must be dealt with.

In conclusion, the environmental simulations in this study demonstrates that some differences exist between the simian rotavirus (SA11) and the human rotavirus (TAMC 15). A human rotavirus isolate used as a model system would be preferable to estimate the stability of human rotaviruses released into environmental waters.

PART V: CONCLUSIONS OF THIS STUDY

Chapter 12. Summary and conclusions

CHAPTER 12

SUMMARY AND CONCLUSIONS OF THIS STUDY

Stool samples obtained from the two hospital were prescreened for the greatest likelihood of detecting rotavirus antigen by ELISA in human stool samples. The presence of human rotaviruses from these samples was confirmed by molecular and biological characterization of the virus particles in these samples.

The monthly distribution study of stool samples from Tripler Army Medical Center is suggestive that rotavirus gastroenteritis is seasonally transmitted in Hawaii, occurring in the winter months as has been reported in temperate climates. The number of stool samples obtained during 1983-84 came from all months, except for February through April of 1984. No samples were made available by the physician in charge due to cessation of gastroenteritis. The only ELISA positive rotavirus samples encountered were during the months of December through March which are winter months in Hawaii.

The prevailing evidence shows that HRV is transmitted by the fecal-oral route rather than the respiratory route. However, the incidence of viral infections spread by the respiratory route usually increase during the fall-winter months. This time distribution has been linked to the increased contact of people, such as children returning to school and the clustering of people indoors during cold

weather. The lifestyle of people in Hawaii is such that people are active outdoors in every month of the year. Yet, rotavirus gastroenteritis was observed in the winter months. Thus, there are factors that have yet to identified that lead to the occurrence of rotavirus gastroenteritis in Hawaii.

HRV's from humans have been demonstrated to infect other species of animals. Thus animals may serve as reservoir hosts. HRV is similar to influenza virus in that HRV may infect other animals and reassort within that animal to give rise to new strains. Reassortant HRV strains may have different properties for human infection and illness.

Studies have shown that a particular strain of HRV may be found in two separate epidemic seasons. Therefore a given strain must persist elsewhere for several months until a new rotavirus epidemic season. The exact mechanism by which HRV accomplishes this is unknown, but HRV may persist in the population until the specific conditions arise which favors an outbreak of rotavirus gastroenteritis.

Commercial enzyme-linked immunosorbent assays (ELISA) have been demonstrated to detect rotavirus antigen in stool specimens. The Rotazyme rotavirus ELISA kit used at the outset of this study had the ability to detect between 10^6 and 10^7 rotavirus particles. The NC-EIA modified in this study could detect 7×10^4 PFU of rotavirus. Since this was a freshly cesium banded preparation of virus, it should have

contained mostly infectious virus particles. Therefore, a modification of an existing immunologically based detection method for rotavirus was achieved which raised the sensitivity of detection.

Analysis of the RNA in stool samples by electrophoresis demonstrated that 88% of the rotavirus RNA profiles observed were of the "long" profile and 12% resulted in "short" profiles. Many different electrophoretic patterns were observed. Since the state of Hawaii is a popular tourist area, importation of many new strains of rotavirus is probable.

The occurrence of two samples with more than the characteristic 11 bands of genomic RNA suggests that multiple infections are occurring in Hawaii which may result in new strains of HRV.

Serologic analysis yielded results consistent with previous work. The majority of isolates typed were of serotype 1. TMC 12 gave a dual serotype 1 and 4 response by MAb-ELISA. The electrophoretic profile of this rotavirus was of the "long" type, and no extra band beyond the standard 11 were observed. This indicates that this isolate was not a mixture of two serotypes. Most likely, this HRV isolate carried two different serologic epitopes, which has been noted in previous literature and is considered a new phenomenon.

Adaptation of several isolates of HRV to cell culture was accomplished in primary AGMK cells. The most success at adaptation to cell culture was achieved with the isolates from KMC.

Optimization of conditions for plaquing HRV was successfully accomplished. Every researcher uses their own recipe for the overlay used in plaquing rotaviruses. Dissemination of the information from this study should help other researchers to establish the highest titer of a rotavirus preparation overlay method.

The stability of rotavirus in natural sunlight has not been previously reported. Rotavirus strains TAMC 15 and SA11 suspended in seawater were demonstrated to be more resistant to the effect of natural sunlight than was poliovirus type 2 which is often used as an enteric virus model.

Other environmental simulation experiments demonstrated that while the ocean and stream environments were hostile environments for rotaviruses. Therefore, the rotaviruses may persist long enough for human exposure to occur. Fujioka, Loh and Lau (1980) showed that several enteroviruses were inactivated after days of exposure to seawater. They also showed that stream water had little effect. Rotavirus was equally inactivated in seawater, however, fresh stream water was also a hostile environment to rotaviruses after many days.

The theory that endogenous bacteria in environmental waters is a major factor in the inactivation of viruses in these waters was addressed. This study demonstrated that endogenous bacteria in seawater have an inactivating effect on rotavirus suspended in this water. The same result was observed for fresh stream water, but the effect was not as pronounced as was the seawater results. All of these experiments helped to stress the point that rotaviruses must be considered in their own right, rather than extrapolating information from experiments utilizing enteroviruses.

The environmental simulation experiments showed that rotavirus, like other viruses, persist longer in the environment than indicator bacteria such as *Streptococcus faecalis*. Therefore environmental sources may be a potential source of human infection.

Most of the work on understanding the role of rotaviruses has been based on animal strains of rotavirus. HRV isolate TAMC 15 was successfully adapted to cell culture and moreover produced plaques on MA 104 cells. TAMC 15 is as easy to cultivate and plaque as SA11. HRV isolate TAMC 15 would be a likely candidate for an alternate human model system. Further work with human isolates like TAMC 15 is needed.

A rotavirus vaccine has been demonstrated to be efficacious in developed nations. However, these vaccines have not been shown to be as useful in developing nations.

Since the standard of living conditions in developing nations is lower than in developed nations, more diarrheal disease occurs due to poorer sanitary conditions. In developing nations diarrheal disease often results in death due to malnutrition and other factors. Therefore, the population of developing nations would benefit the most from a vaccine. A vaccine is available for rotavirus, however, trials to test the efficacy of the vaccine have not been as successful as in developed nations. Not only is there a problem with vaccine development, but recently new rotavirus groups are being detected which are immunologically distinct from the classical group A rotaviruses. A large epidemic was reported in China which was responsible for illness in adults and was attributed to a new group of HRV. The high attack rate in adults is consistent with a completely new variety of rotavirus (Flewett et al., 1987). Many new rotaviruses appear to exist but remain to be detected.

Thus it can be concluded that rotavirus gastroenteritis in Hawaii appears to occur in the winter months. The hospital isolates examined are similar to those examined elsewhere in the world. The information that is of significance for Hawaii in particular is the environmental studies. While the virus may persist for hours in natural waters when exposed to sunlight, this must be viewed as an ideal situation for inactivation of the rotavirus under the experimental parameters used.

The situation in the environment is probably much different. The temperature and water stability experiments demonstrated that the rotaviruses used in this present study may persist for many days in the environment. Since Hawaiian waters are a highly utilized resource, precautions must be taken to protect these waters from contamination with viruses such as rotavirus. Without proper water management, dissemination of rotavirus and subsequent rotavirus gastroenteritis is possible.

APPENDICES

APPENDIX A

PROTOCOL FOR USING THE ROTAZYME ROTAVIRUS ELISA ASSAY

1. Adjust temperature of a waterbath to 45° ± 1°C.
2. Pipette 200 μl of each diluted specimen (1:5 dilution of stool specimen w/v or v/v in sample diluent), sample diluent for diluent control, and positive control into separate labeled tubes.
3. Dispense one guinea pig anti-rotavirus antibody coated bead into each tube containing sample or control.
4. Mix the contents of each tube for 3 to 5 seconds.
5. Apply cover sealer to each tube and incubate the tubes in the water bath for 3 ± 0.5 hours.
6. Remove the tubes from the water bath. Uncover and aspirate the contents and wash each bead four times with distilled water.
7. Pipette 200 μl of rabbit anti-rotavirus-horseradish peroxidase conjugate into each tube and mix for 3 to 5 seconds.
8. Apply cover sealer to each tube and incubate the tubes in the water bath for 60 ± 5 minutes.
9. During the last 5 to 10 minutes of the incubation, prepare orthophenylenediamine chloride (OPD) substrate solution by placing 1 OPD tablet into 5 ml of OPD solution in a clean tube. Swirl gently to dissolve tablet, protect from light, and use within 20 minutes.
10. At the end of the one-hour incubation, remove the tubes from the waterbath and wash the beads six times with 4 to 5 ml of distilled water.
11. Transfer beads to clean, properly labeled tubes.
12. Pipette 200 μl of the OPD solution into each tube and also into one empty tube as a substrate blank.
13. Incubate at room temperature for 15 ± 2 minutes. Avoid bright sunlight, and shield from light during incubation.

14. After 15 ± 2 minutes, read the results of the assay either by the visual method (within 5 minutes) and comparing the colors against then included color chart, or add 1 ml of 1 N HCl to each tube, mix well and read in a spectrophotometer at 492 nm.

APPENDIX B

PROTOCOL FOR USING THE DAKOPATTS ROTAVIRUS ELISA KIT

1. Dilute rabbit anti-rotavirus (Dakopatt # B218) 1:50 by pipetting 100 µl into 5 ml of coating buffer A. Mix gently. Pipette 100 µl of the dilution into each well in columns numbered 1,2,5,6,9, and 10 on the microtiter plate. Repeat for rows A through H. Use Dynatech's Immulon II microtiter plates.

2. Dilute rotavirus rabbit negative control serum (Dakopatt # X904) 1:50 by pipetting 100 µl into 5 ml of coating buffer A. Mix gently. Pipette 100 µl of the dilution into each well in columns numbered 3,4,7,8,11, and 12 on the microtiter plate. Repeat for rows A through H. Allow plate to incubate at room temperature for 1 hour.

3. Empty the plate into a sink. Tap the plate, upside down, on paper towels. Fill the wells with washing solution C by immersing in the liquid. Allow the plate to sit for 1 minute. Repeat the wash 4 times. After the last wash, empty the wells and tap, upside down on paper towels.

4. Prepare fecal specimens by homogenizing feces in 0.15 M sodium chloride solution. Made up a 10-20% suspension. Vortex vigorously, and clarify the suspension by low speed centrifugation (1000 x g, 10 min). Also dilute the supernatant 1:10 by pipetting 100 µl into 1 ml of dilution buffer D for a 10^{-1} dilution.

5. Pipette 100 µl of each fecal supernatant plus 10^{-1} dilution into each of 4 wells, 2 test wells and 2 negative control wells starting with row A, columns 1 to 4. The next patients sample into B1 to B4, etc. until the plate is filled. Allow the plate to incubate at room temperature for 1 hour.

6. Aspirate the samples from all wells. Fill all wells with washing solution C and aspirate after 1 minute. Repeat washing six times.

7. Block non-specific reactions by diluting normal rabbit immunoglobulins (Dakopatt # X903) 1:25 by pipetting 400 µl into 10 ml of dilution buffer D. Pipette 100 µl of the diluted antibody dilution into all wells and incubate the plate at room temperature for 30 minutes. Do not empty wells !

8. Dilute rabbit peroxidase-conjugated anti-rotavirus (Dakopatt # P219) 1:250 by pipetting 40 μl into 10 ml of dilution buffer D. Pipette 100 μl of the diluted antibody dilution into each well. Allow to incubate at room temperature for 30 minutes.

9. Wash as described in step 3. Wash for 1 minute in 0.1 M citric acid-phosphate buffer E. Empty all wells.

10. Prepare substrate solution by dissolving 8 mg orthophenylene diamine·2 HCl (OPD) in 15 ml of 0.1 M citric acid-phosphate buffer E. Add 5 μl of 30% H_2O_2. Pipette 100 μl of enzyme substrate into all wells. Allow the plate to stand for 15 minutes. Do not empty the wells!

11. Stop the color development by pipetting 150 μl of 1 M H_2SO_4 into each well. Read the plate in a spectrophotometer at 492 nm.

FORMULAE

A) Coating Buffer (0.05 M Carbonate-Bicarbonate Buffer, pH 9.6)

Na_2CO_3 ....1.59 g (0.015M)
$NaHCO_3$....................................2.93 g (0.035M)
H_2O..q.s. to 1 liter
Store at 4°C. Stability: 14 days.

B) 0.15M Sodium Chloride Solution

NaCl.......................................8.78 g (0.15M)
H_2O..q.s. to 1 liter

C) Washing Solution, pH 7.2

NaCl.......................................20.20 g (0.50M)
KH_2PO_4.................................0.20 g (0.0015M)
$Na_2HPO_4 \cdot 2H_2O$.........................1.15 g (0.0065M)
Tween 20...................................0.5 ml
H_2O..q.s. to 1 liter
Store at 4°C. Stability: 2 months.

D) Dilution Buffer, pH 7.2

Dissolve 5g bovine albumin in 1 liter of washing solution C. Store at 4°C. Stability 1 week, or store at -20°C.

E) 0.1 M Citric Acid-Phosphate Buffer, pH 5

citric acid·H_2O..........................7.30 g (0.0347M)
$Na_2HPO_4 \cdot 2H_2O$.........................11.86 g (0.0667M)
H_2O...................................q.s. to 1 liter
Store at 4° C. Stability: 2 months.

APPENDIX C

COMPARISON OF SELECTED SOURCES OF PLASTIC MICROTITER PLATES FOR THE DETECTION OF ROTAVIRUS ANTIGEN: P/N RATIOS

Plate	Virus (PFU)	Mean Pos.[a]	Mean Neg.[a]	P/N Ratio[b]
Costar[c]	1.88×10^{7}	1.1090	0.0815	13.61
	9.38×10^{6}	0.7390	0.0755	9.79
	4.69×10^{6}	**0.4450**	**0.0430**	**10.35**
	2.34×10^{6}	0.3320	0.0625	5.31
	1.17×10^{6}	0.1570	0.0345	4.55
	5.86×10^{5}	0.1210	0.0455	2.66
Linbro[d]	1.88×10^{7}	0.3735	0.0260	14.37
	9.38×10^{6}	0.2855	0.0300	9.52
	4.69×10^{6}	0.1920	0.0165	11.64
	2.34×10^{6}	**0.1470**	**0.0205**	**7.17**
	1.17×10^{6}	0.0695	0.0180	3.86
	5.86×10^{5}	0.0525	0.0145	3.62
Falcon[e]	1.88×10^{7}	0.3250	< 0.0010	> 325.00
	9.38×10^{6}	0.2140	< 0.0010	> 214.00
	4.69×10^{6}	0.1890	0.0030	63.00
	2.34×10^{6}	**0.1060**	**< 0.0010**	**> 106.00**
	1.17×10^{6}	0.0735	0.0020	36.75
	5.86×10^{5}	0.0440	0.0085	5.18
Nunc[f]	1.88×10^{7}	1.2170	0.0820	14.84
	9.38×10^{6}	0.8710	0.0950	9.17
	4.69×10^{6}	**0.6030**	**0.0790**	**7.63**
	2.34×10^{6}	0.4000	0.0825	4.85
	1.17×10^{6}	0.2395	0.0635	3.77
	5.86×10^{5}	0.2065	0.0650	3.18
Nunc[g]	1.88×10^{7}	1.0110	0.0055	183.82
	9.38×10^{6}	0.6265	< 0.0010	> 626.50
	4.69×10^{6}	0.4005	0.0055	72.82
	2.34×10^{6}	0.2320	< 0.0010	> 232.00
	1.17×10^{6}	0.1260	0.0065	19.38
	5.86×10^{5}	**0.1080**	**0.0145**	**7.45**
Dynatech[h]	1.88×10^{7}	0.7910	< 0.0010	> 791.00
	9.38×10^{6}	0.4905	< 0.0010	> 490.50
	4.69×10^{6}	0.2695	< 0.0010	> 269.50
	2.34×10^{6}	**0.1770**	**< 0.0010**	**> 177.00**
	1.17×10^{6}	0.1025	0.0385	2.66
	5.86×10^{5}	0.0725	0.0245	2.96

APPENDIX C continued

Plate	Virus(PFU)	Mean Pos.	Mean Neg.	P/N Ratio[a]
Dynatech[i]	1.88×10^7	1.1660	0.0385	30.29
	9.38×10^6	0.7345	0.0275	26.71
	4.69×10^6	0.4425	0.0460	9.62
	2.34×10^6	0.3425	0.0405	8.46
	1.17×10^6	**0.1690**	**0.0280**	**6.04**
	5.86×10^5	0.1075	0.0280	3.84

Boldface type is lowest dilution detected according to Dakopatt criteria.

a = mean positive and negative based on two replicates
b = Mean Pos./Mean Neg.
c = Costar flat bottom; Lot 2585
d = Linbro flat bottom; Lot 76381001; Flow Laboratories
e = Falcon; Lot 22912053; Becton Dickinson
f = NUNC flat bottom;
g = NUNC round bottom;
h = Dynatech PVC round bottom; Lot 45-824-M-168
i = Dynatech Immulon II flat bottom; Lot CB52074

APPENDIX D

Formulae and Recipes for Polyacrylamide Gels

I. Stock solutions for electrophoresis

A. Monomer acrylamide solution (30%T, 2.7%C bis-acrylamide)

Acrylamide	58.4 g
Bis-acrylamide	1.6 g
H_2O	to 200 ml

Store at 4° C in the dark. Discard after 1 month.

B. Resolving gel buffer (1.5 M tris-Cl pH 8.8)

Tris base	36.3 g
H_2O	to 200 ml

Adjust to pH 8.8 with 6 N HCl.

C. Stacking gel buffer (0.5 M tris-Cl pH 6.8)

Tris base	3.0 g
H_2O	to 50 ml

Adjust to pH 6.8 with 6 N HCl.

D. Initiator (10% ammonium persulfate)

Ammonium persulfate	0.5 g
H_2O	to 5.0 ml

Prepare solution before each use, or keep frozen at -20°C.

E. Tank buffer (0.05 M tris-0.38 M glycine-0.005 M EDTA)

Tris base	12.0 g
glycine	57.6 g
Na-EDTA	7.44 g
H_2O	to 4 liters

II. Gel Recipes for a 0.75 mm thick slab gel

	Separating gel	Stacking gel
Solution A.	10 ml	1.33 ml
Solution B.	7.5 ml	-------
Solution C.	------	2.50 ml
H_2O	12 ml	6.10 ml
Solution D.	150 µl	50 µl
TEMED	10 µl	5 µl.
Final vol.	30 ml	10 ml

APPENDIX E

FORMULAE FOR REAGENTS USED IN THIS STUDY

1) GKN 10X stock solution - Ca^{2+} and Mg^{2+} free.

	g/liter
NaCl	80.0
KCl	4.0
Glucose	10.0

Dissolve above in 900 ml of triple distilled water ($dddH_2O$), add 10 ml of a 2.0% phenol red solution, and add 90 ml of $dddH_2O$ water to q.s. to 1 liter. Filter through Whatman filter of any porosity, add 3-4 ml of chloroform, and store at 4°C. Dilute 1:10 for working solution and sterilize.

2) Phosphate-buffered saline (PBS) 10X stock solution.

Solution A	g/liter	Solution B	g/liter
NaCl	80.0	$CaCl_2$	1.0
KCl	2.0	$MgCl_2 \cdot 6H_2O$	1.0
Na_2HPO_4	11.5		
KH_2PO_4	2.0		

Dissolve Solution A above in 800 ml of $dddH_2O$ and Solution B above in 200 ml of $dddH_2O$, and store separately. Filter through a Whatman filter of any porosity and refrigerate. For a working solution sterlize Solutions A and B separately at a 1:10 dilution. Upon cooling, mix both solutions together. The final concentration is A:B at 4:1 respectively. Store at 4°C.

3) Earle's Saline 10X stock solution.

Solution A	g/liter	Solution B	g/liter
NaCl	68.0	$CaCl_2 \cdot 2H_2O$	2.66
$MgSO_4 \cdot 7H_2O$	2.0		
KCl	4.0	Solution C	
$NaH_2PO_4 \cdot H_2O$	1.4	32 ml of a 0.5% stock solution of phenol red	

Dissolve the components of Solution A together in the order listed in 800 ml of $dddH_2O$. Do the same with Solution B in 200 ml of $dddH_2O$. Slowly mix solutions A and B together. Filter through any porosity of Whatman filter paper, add Solution C to A and B, and q.s. to 1 liter. Add 1-2 ml chloroform and store at 4°C. Dilute 1:5 with $dddH_2O$ and sterilize for use.

4) Complete medium for overlay, 10X stock solution.

Component	To make 1 liter
Earle's 10X stock	200 ml
dddH_2O	800 ml
10% glucose	20 ml
100X nonessential amino acids[a]	20 ml
100X vitamins[b]	20 ml
100X glutamine[c]	20 ml
200X penicillin[d]-streptomycin[e]	20 ml

Dilute 10X Earle's stock 1:5 with dddH_2O. Sterilize and let cool. Add the remaining (sterile) ingredients, aliquot in 100 ml serum bottles, and store at 4°C. For use of the 2X solution for plaque analysis, add 1 ml of 1M HEPES buffer (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid; Catalog No. H9138; Lot 37F-56515; Sigma Chemical Co., St. Louis, Missouri). and 4.5 ml of a 6.5% stock of $NaHCO_3$ per 100 ml of 2X Complete medium. Mix 1:2 with molten agar or agarose.

a = Catalog No. 16-810-49; Flow Laboratories, McLean, Virginia.
b = 100X BME vitamin solution; Catalog No. 104; Grand Island Biological Co. (GIBCO), Grand Island, New York.
c = 200mM; Catalog No. 16-801-49; Flow Laboratories, McLean, Virginia.
d = Catalog No. PEN-K; Sigma Chemical Co., St. Louis, Missouri.
e = Catalog No. S6501; Sigma Chemical Co., St. Louis, Missouri.

5) MEM-L15 growth medium for primary cells.

Component	To make 1 liter
MEM (Hanks)[a] + L-glutamine[b]	5.3 g
L15 (modified) + L-glutamine	6.9 g
$NaHCO_3$ (0.1%)	1.0 g
nonessential amino acids[c] (0.1%)	10.0 ml
HEPES buffer[d] (0.4%)	4.0 g
penicillin[e] (100 IU/ml)	100,000 IU
streptomycin[f] (100 μg/ml)	100,000 μg
distilled water	890.0 ml

a = Catalog No. 410-1200; Grand Island Biological Company (Gibco), Grand Island, NY.
b = 200mM; Catalog No. 16-801-49; Flow Laboratories, McLean, Virginia.
c = Catalog No. 16-810-49; Flow Laboratories, McLean, Virginia.
d = N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid; Catalog No. H9138; Lot 37F-56515; Sigma Chemical Co., St. Louis, Missouri.
e = Catalog No. PEN-K; Sigma Chemical Co., St. Louis, Missouri.
f = Catalog No. S6501; Sigma Chemical Co., St. Louis, Missouri.

6) Nitrocellulose-enzyme-immunoassay (NC-EIA) reagents.

a) PBS (0.01 M)

$NaH_2PO_4 \cdot H_2O$	2.55 g
Na_2HPO_4	19.96 g
NaCl	86.65 g
q.s. with $dddH_2O$	1 l
Adjust to pH 7.2 with NaOH	

b) Horseradish peroxidase substrate (HRP)

Dissolve 3 mg/ml HRP substrate reagent ([4-chloro-1-naphthol]; Catalog No. 170-6534; Bio-Rad Laboratories, Richmond, California) in methanol. Mix 5 parts NC-PBS plus 1 part HRP substrate. Add 0.018% of 30% H_2O_2. Add to NC paper and incubate 1 hour.

7) Crystal violet stain. (0.5% crystal violet - 2% citric acid).

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