DEVELOPMENT AND EVALUATION OF METHODS FOR THE ELUTION OF ENTERIC VIRUSES FROM MISSISSIPPI ESTUARINE SEDIMENTS

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CHAPTER I

INTRODUCTION

The contamination of estuarine waters by fecal wastes is regarded as a public health hazard. Enteric viruses are shed in the excretions of infected animals and humans and may enter estuarine waters by way of soil run-off or discharge of sewage. Estuarine sediments are thought to act as a reservoir for enteric viruses and protect them from environmental inactivation. Enteric viruses have been found to adsorb readily to estuarine sediments and are known to survive longer when attached to particulate matter than when suspended in seawater. Physical and chemical changes in the environment may be responsible for the release of virus from sediments which subsequently influence water and shellfish quality. Therefore, the study of sediment virology has epidemiological and economic significance.

It has been stated that past studies have greatly underestimated the level of natural sediment-associated virus and that the elution step is the critical defect. Only a few studies have concentrated on the detection of enteroviruses in marine sediments and none have estimated the efficiency of the methods they employed. At present, the lack of a reliable method to elute viruses from sediments

of different composition is a major handicap to the understanding of this epidemiological problem. The purpose of this investigation was to develop a suitable eluent for extraction of virus from sediments collected at Bayou Graveline, an estuarine system near Pascagoula, Mississippi.

CHAPTER II

REVIEW OF LITERATURE

A. The Enteric Viruses

In the late nineteen thirties, Trask et al. reported their finding of the presence of poliovirus in the feces of poliomyelitis patients (86). Later, as the development of virology and tissue culture techniques expanded, many viruses were isolated or identified in domestic sewage containing human urine and feces.

As many as 100 virus types have been found to be excreted through the human gut (81) and more are certain to be discovered. These viruses are grouped under the general heading of "enteric virus" and are frequently isolated from sewage and contaminated waters.

The term "enteric virus" is an epidemiological classification based on the oral-fecal route transmission of disease (5, 27). Enteric viruses enter the body via the oral route and are able to survive the acidic stomach contents and the adverse effect of bile in the duodenum (24). They multiply in the cells of the intestinal tract and can be excreted in large numbers in feces.

The enteric viruses consist of enteroviruses, reoviruses, adenoviruses and unclassified viruses such as

hepatitis and the Norwalk agent and related agents.

The enterovirus group (17) includes polioviruses (3 serotypes), coxsackieviruses A (24 serotypes), coxsackieviruses B (6 serotypes), echoviruses (34 serotypes) and additional serotypes designated enterovirus types 68-71 (63, 75). Enteroviruses inhabit the human alimentary tract. They share the basic properties of Picornaviridae including a genome of single-stranded RNA, small size (20-30 nm diameter), icosahedral capsid and lack of an envelop.

The genus Reovirus (78) is classified under the family Reoviridae (26). They are medium-sized (75-80 nm diameter), ether-resistant viruses which contain double-stranded RNA enclosed in a double capsid. The genus Rotavirus is also classified in the Reoviridae family (28).

The adenoviruses, classified under the family Adenoviridae, are medium-sized viruses (60-90 nm diameter) containing double-stranded DNA and exhibiting cubic symmetry. Thirty-one adenovirus serotypes are recognized.

Acute infection of the liver can be caused by the hepatitis viral agents. The hepatitis is the only common viral disease whose causative agent has still to be cultivated in the laboratory. It has been suggested that the hepatitis A virus could be considered either a parvovirus (25) or an enterovirus (43).

The Norwalk agent and related agents have been characterized as 27 nm diameter particles and are thought

to be parvoviruses with a DNA core (51). However, there is possibility that the Norwalk agent virus is a picornavirus with a RNA core.

B. Enteric Viruses and Public Health

Enteric viruses are transmitted by both direct and indirect contact; they can also be transmitted by the respiratory route. The enteric viruses in land disposed sewage can enter ground water used for drinking or agriculture purpose. In the marine ecosystem, potential human infection is usually associated with contact sports practiced in contaminated water or through the ingestion of raw shellfish which bioconcentrate such pathogens as they feed (33, 64, 89).

The infection caused by enteric viruses are common in children, especially those below ten years of age. Adults are generally less susceptible to enteric virus infection because of potential acquired immunity during previous contacts with the virus. The infection depends on the season, climate, geographic distribution and the socio-economic and hygienic levels of the population (63).

Most cases of infections due to enteric viruses are mild or silent episode and do not result in overt clinical syndromes. However. waterborne outbreaks of non-bacterial gastroenteritis have been observed (39). Outbreaks of infectious hepatitis and non-bacterial gastroenteritis also have been traced to the consumption of shellfish from polluted

marine water (21, 60, 74). There is also suitable epidemiological evidence for the existence of hepatitis A virus in polluted water since at least 50 waterborne outbreaks of this disease have been described (13, 70, 71).

Although enteric viruses usually produce subclinical infections, certain clinical symptoms can be observed in severe cases. The clinical symptoms caused by the polioviruses included paralytic poliomyelitis and aseptic meningitis. Coxsackie type A viruses can produce herpangina, respiratory illness, fever, aseptic meningitis and paralysis. Type B coxsackieviruses have the ability to cause aseptic meningitis, pericarditis and myocarditis. Echoviruses are often responsible for respiratory infection and rashes. The new enterovirus type 71 has been reported to cause aseptic meningitis and enterovirus type 70 is the cause of acute hemorrhagic conjunctivitis. Enterovirus type 68 has been associated with lower respiratory illness in children (63).

Although the role of reoviruses as causative agents in human disease is unclear, they have been isolated from children with minor febrile illness and patients with a variety of illness affecting the liver, alimentary, respiratory and central nervous system (48, 76, 77). The infectious agent of infantile gastroenteritis is believed to be a reovirus or rotavirus-like agent (44, 49, 50, 52). Recent studies have indicated that rotaviruses may be the causative agents in nearly one-half of all hospitalized cases of

acute diarrheal illness in infants and young children (19, 41). Rotaviruses have also been reported in epidemics of acute gastroenteritis in adults (66) and in a recent water-borne outbreak (59).

Most adenoviruses are associated with respiratory infections and conjunctivitis. They are also known for their oncogenic properties in newborn hamsters (24).

The Norwalk agent and related agents are responsible for causes of non-bacterial gastroenteritis in adults and children (51, 62).

C. Enteric Viruses in Sewage

The discharge of untreated or partially-treated domestic sewage effluents into coastal water is regarded as a public health hazard (36). Pathogenic enteric viruses enter estuarine waters through water contaminated with human and animal feces. Viruses are common in sewage due to enteric viral infections or to the use of vaccines. Clarke and Kabler reported a theoretical average number of enteric viruses in raw sewage of 100 to 5,000 plaque-forming units per liter (7).

At present, over 8 billion gallons of municipal sewage are discharged each day into the coastal waters of the United States, about one-half of the total discharge receiving secondary treatment (32). Furthermore, conventional sewage treatment does not completely remove enteric viruses. Investigators estimate that there are approxima-

tely 50 plaque-forming units of enteric viruses per liter present in the effluent of a well-functioning sewage treatment plant (7).

Disinfection of sewage effluent by chlorine, ozone or other virucidal additions does reduce the numbers of viruses entering receiving water. Virus inactivation also occurs in seawater by processes still unexplained.

Although relatively few virus can survive sewage treatment and environmental inactivation, only a few virus particles may be required to produce an infectious dose. Thus, even a low level of virus survival could contribute to viral dissemination and contamination and possibly constitute a public threat.

D. Enteric Viruses Versus Estuarine Environmental Factors

Viruses that infect humans are incapable of multiplying in the environment but are resistant to many environmental factors and could survive and contaminate seafood or coastal waters. Also viruses appear to survive longer than coliform bacteria which are presently used to judge water quality (82). Therefore, an understanding of the fate of enteric virus in the estuarine environment is an important aspect of environmental virology.

Since viruses multiply only in living cells, the duration of viral exposure to environment and environmental factors could play an important role in virus survival. Recently, the survival of viruses in marine and fresh waters

has been examined by several laboratories (1, 8, 35, 61, 65, 72). The most decisive factor involved in virus inactivation is temperature (8, 22, 31, 57, 65). Temperature affects the virus in two ways: high temperature causes rapid denaturation of viral proteins while low temperature allows faster denaturation of RNA than of proteins (22, 31, 73).

Solar radiation is believed to be involved in viral inactivation (8). It has been demonstrated that solar radiation plays a significant role in the die-off of coliforms in seawater (3). However, the actual mechanism of virus inactivation in seawater remains unexplained. Turbidity, as one variable characteristic of surface waters, is thought to protect viruses by decreasing light intensity below the surface. Pollutants and colloidal material could envelop or adsorb the virus and protect it against the hostile environment (8, 14, 15, 65). Other factors that probably affect the survival of enteric viruses in the estuarine environment include the chemical composition of seawater, pH, salinity and the presence of various chemicals and heavy metal ions (1, 47, 68, 85).

The clumping of viruses and electrostatic adsorption of viruses onto clay and suspended solids followed by sedimentation leads to their accumulation in marine sediments (1, 14, 35). Although viruses are inactivated more readily in seawater than in freshwater (61), they can survive much longer if adsorbed to sediment (83).

Besides physical-chemical factors, biological parameters such as microbial antagonism and predation (16, 29, 82) or enzymatic degradation (16) could also influence virus survival in the environment. Non-sterile seawater is generally more virucidal than autoclaved or filtered sterilized seawater.

Inactivation of viruses in seawater may also vary from one virus type to another (16, 57). Lo et al. reported that with respect to survival in natural estuarine waters, viruses behaved as follows: coxsackieviruses > echoviruses > polioviruses (57).

E. Enteric Viruses in Estuarine Sediments

Large numbers of enteric viruses have been shown to be discharged into estuarine waters by off-shore sewage outfalls (20, 23, 33). These enteric viruses become readily associated with sediment in the estuarine environment and settle to the bottom where they accumulate (40, 53). One study demonstrated the potential for 10,000 times more virus in estuarine sediments than overlaying waters (32).

The accumulation of viruses in marine and estuarine sediments may play a major role in hydrotransportation, distribution and survival of these viruses (53). A study of virus survival in a sandy marine sediment has shown that the inactivation of poliovirus type 1 was 4.5-fold faster in seawater than in the sand (20). Other studies have indicated the sediments are able to protect viruses from

environmental inactivation (11, 12, 83). This protective effect explains why enteric viruses survive longer in systems containing sediment than when suspended in seawater (35, 79). The sediment protection could be physical, such as trapping of the virus in a surface opening or by stabilization of the virion by electrostatic forces (83).

Liew et al. reported that adsorption of enterovirus to estuarine sediments may play a significant role in protecting them against thermoinactivation (56). Sediment particles could also act as a buffer, adsorbing chemical inactivating agents present in the seawater (68).

It has been found that soil-bound viruses can be desorbed after a period of heavy rainful (55, 91), indicating that reversible binding does occur between virus and sediments. Sediment-associated viruses are thought to be readily released into the water column by physical and/or chemical changes in the environment such as storm, wave or boat action or by changes in the pH of the water brought about by industrial/agricultural runoff. Sediments can therefore act as a reservoir of virus in nature and play an important role in the persistence of virus in estuarine waters and shellfish. Schaub and Sagik demonstrated that the animal viruses adsorbed to particles retained their infectivity for mice and tissue culture (79). Therefore, sediment-associated viruses are significant from a public health standpoint.

F. Detection of Enteric Viruses in Estuarine Sediments

Reports of field studies suggested that sediment bound viruses did exist under natural conditions (20, 33, 34). The development of reliable, quantitative methods to evaluate virus levels in sediments is a first step toward an understanding of the level of virus contamination.

There have been many investigations designed to detect viruses in sewage sludge (38, 45, 58, 69, 90). However, few studies have concentrated on the detection of enteric viruses in sediments. The earlier investigations did not determine the efficiency of their methods and it is possible that past studies greatly underestimated the level of natural sediment associated viruses.

A critical defect in the study of virus-sediment association seems to be the inefficiency of the initial eluting step (34). The extraction methods used by past researchers relied on an alkaline buffered eluent (34). The method of Gerba (34) was choosen as the basis for development of an improved elution method because sample processing time was short and did not require expensive chemicals. However, the high pH of the eluents used in these experiments appeared to be virucidal (34, 84). Gerba et al. reported that the recoveries of virus in estuarine sediment from the Upper Texas Coast were very low using glycine buffer adjusted to pH 10 and 11.5 (ranged from $<0.1\%$ to 16%), whereas the addition of

supplements, such as EDTA or fetal calf serum, could improve the virus recovery (ranged from 12% to 108%).

Other eluents using slightly alkaline proteinaceous solutions were designed and tested. Organic eluents seem to have a better ability to detach virus from sediment than inorganic buffer solutions (54). Bitton reported that a 0.5% skim milk solution in Tris buffer at pH 9 could yield 94.8±11.3% poliovirus 1 recovery from soil (Eustis fine sand) (10). Landry stated that a 3% beef extract solution at pH 9 yielded a mean efficiency of 85% virus recovery from a variety of concentrating filters (54). A solution of 4% nutrient broth at pH 9 has also been used by Hill et al. to elute virus from membrane cartridge filters (42). Eluents that have been previously used for the elution of virus from other materials were applied to the elution of virus from sediment. Comparison of these eluents (sometimes with modifications) were made to determine their effectiveness.

An earlier investigation of virus extraction from Mississippi estuarine sediment with Gerba's procedure did not yield viruses (80). This indicated that the sediment quality of Mississippi estuarine could be different from those of Upper Texas Gulf Coast.

In this study, various eluent systems were employed and modified to develop and evaluate an efficient and reliable method to extract viruses from the estuarine sediments collected at Graveline Bayou, Mississippi.

CHAPTER III

MATERIALS AND METHODS

A. Laboratory Practice and Equipment

Aseptic techniques were used for all experiments performed.

All media and reagents were made with double distilled deionized water. Tissue culture media were filter sterilized (Millipore Co., 90 mm filter holder, 0.45 um pore size filter). Reagents were either filter sterilized or autoclaved at 15 psi, 121 ^{o}C for 15 minutes. Sterile 125 ml flasks (Kimax), beakers (Kimax) and centrifuge tubes (DuPont) were used for extraction procedures.

An incubator-shaker (New Brunswick Scientific Co., Inc.) was used for sediment-eluent mixing. Vertical-flow laminar-air hoods (Biogard Hood, the Baker Co., Inc.) were used for cell cultivation, filter sterilization, viral assay and experimental procedures requiring aseptic techniques. Cells were incubated in a 37 ^{o}C incubator (Hotpack Co.). Virus pools and final eluents were stored at -70 ^{o}C in a Bio-Freezer (Forma Scientific) until assay.

Virus containing glassware was placed in 3% Lysol (Steriling Drug, Inc.) and autoclaved before disposal. Glassware was soaked in a solution of 7x detergent (Linbro,

Inc.) overnight, rinsed with tap water and rerinsed with distilled water. The glassware was allowed to dry and then wrapped in aluminum foil and placed in the hot oven at 350 ^{o}F for two hours.

B. Cell Cultures

Viral assays were conducted using the Buffalo green monkey kidney (BGM) cell line which has been demonstrated to be more sensitive than primary rhesus or African green monkey kidney cells to enteric viruses (18).

BGM cells were grown in MEM-L15 medium (Appendix A) in 150cm^2 disposable Falcon tissue culture flasks (BBL). The cells were incubated at 37 ^{o}C until confluency. For subcultivation, monolayers were washed with trypsin-versine (ATV, Appendix A) 3 times, leaving an ATV residues (1 ml) after the third wash. The flasks were incubated for 15 minutes or until the cells detached from the flask surface and were microscopically observed to be free of clumps. Cells were resuspended in growth media and dispersed into sterile 150cm^2 culture flasks. For plaque assay, the same procedures were performed and the cells were dispersed into twenty 25cm^2 flasks. Cell passages between 120 and 200 were used for all viral assays.

C. Viruses

Poliovirus type 1 (American Tissue Culture Collection (ATCC), VR-192, type 1, Sabin) and coxsackievirus B 3 (ATCC, VR-30, Nancy) were used in all experiments. Initial virus pools were prepared by inoculation of a known dilution

of test virus onto culture flasks containing confluent BGM monolayers. The flasks were placed on a rocker platform (Bellco Biological Glassware) at 37 ^{o}C for one hour to allow the virus to adsorb to the cells. Following adsorption, cell monolayers were incubated with 50ml fresh culture medium. These flasks were incubated at 37 ^{o}C until virtually all the cells (more than 98%) were infected. The flasks were stored at -70 ^{o}C in a Forma Freezer. Infected cells were lysed by rapid thawing at 37 ^{o}C.

The lysate was centrifuged at 1,000 xg (International Equipment Company) for 10 minutes to remove cell debris. The virus-containing supernatant was collected and dispersed in 1ml aliquots into 2ml sterile plastic ampules (Vanquard Inter.) and stored at -70 ^{o}C in a Forma Freezer. Virus pools were titered by plaque assay. Ampules used in seeded experiments were thawed rapidly at 37 ^{o}C in a water bath. Appropriate dilutions were prepared in MEM-L15 media.

D. Plaque Assay

The virus titer of each sample was determined by plaque assay using a double agar overlay technique. Plastic tissue culture flasks (25cm^2) containing confluent monolayers of BGM cells were used. Aliquots of diluted virus pool or eluent were diluted in MEM-L15 medium.

For inoculations, growth medium was removed from the flasks and 0.2ml of sample was placed into 3-4 replicate flasks. All flasks were placed on a rocker platform for one

hour at 37 °C to allow adequate virus adsorption and to redistribute the inoculum over the cell layer. The fluid residue was removed by aspiration prior to the addition of of 5ml first agar overlay medium (Appendix A).

The flasks were inverted after the overlay media solidified and incubated for three days at 37 °C. On day four, 5ml of a second agar overlay medium was allowed to solidify and the flasks then inverted and incubated at 37 °C. Plaques were counted on a daily basis for 6 days or until no new plaques appeared after 2 consecutive days.

E. Sediment Collection

Figure 1 is a map of the study area with the location of the sampling stations marked. Bayou Graveline near Pascagoula, Mississippi, is polluted with sewage and has been classified as a prohibited area since 1975 (67). Sediments used for this study were collected at station 2 and station 3 for the following reasons:

(a) for comparison with other data being accumulated on virus levels of Graveline oysters.

(b) to observe the recovery of virus from sediment of different compositions.

Sediment samples were collected by an individual of the Gulf Coast Research Laboratory using a hand-held Ekman standard dredge (Wildco, Cat. No. 196). The upper 2 to 3 cm of sediments (Figure 2) were removed from the dredge with a clean spoon and placed in a sterile plastic container.

Figure 1: The Map of Graveline Bayou

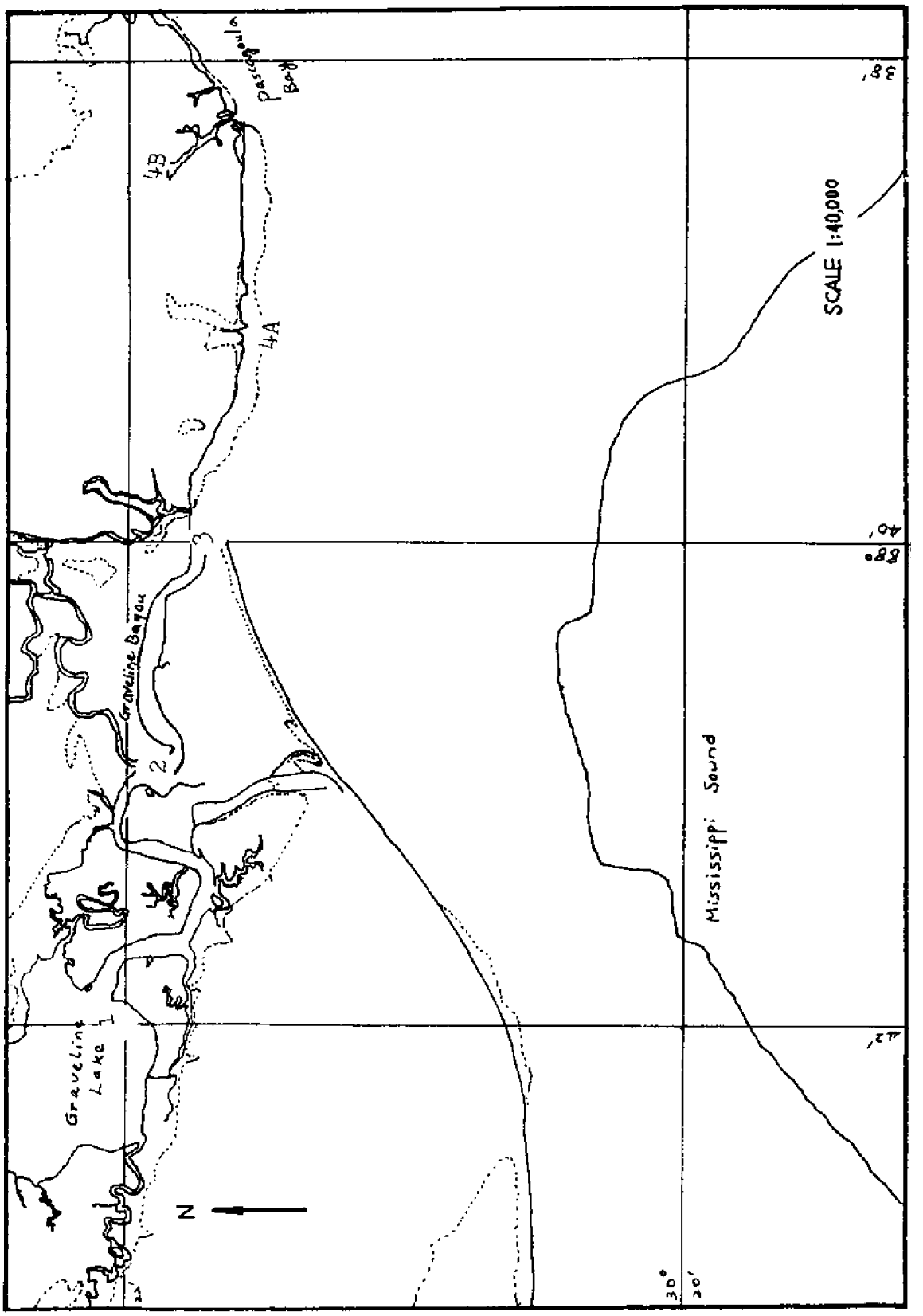
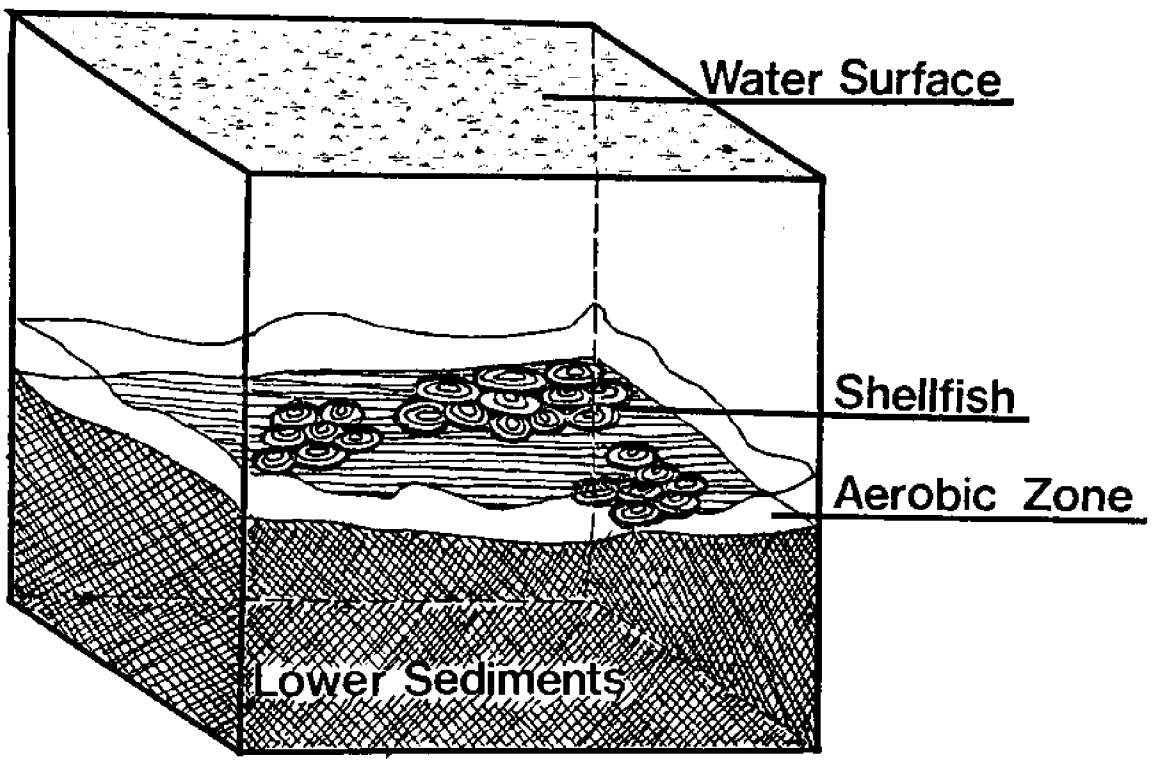


Figure 2: Diagram of the Arobic Zone



These samples were placed into an insulated chest and returned to the laboratory.

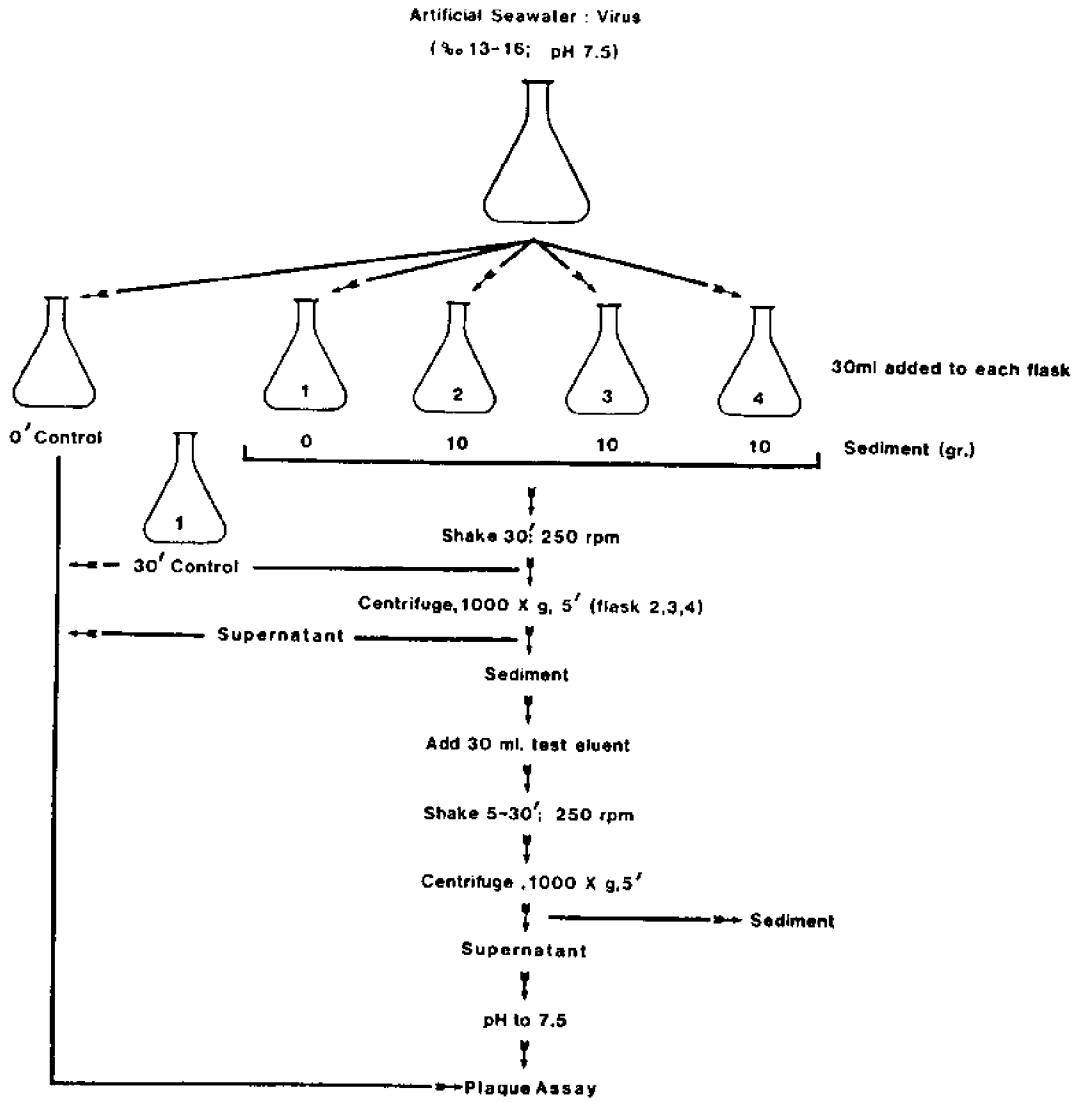
F. Artificial Seawater

Artificial seawater was prepared using Instant Ocean (R) (Eastlake, Ohio) artificial sea salts. The salinity, conductivity and pH of the seawater was measured with a model YSI 33 S-C-T meter. Only seawater with an average salinity of 15‰, a conductivity of 20,000 uMHO and a pH of 7.5 was used in these experiments.

G. Experimental Procedures

Figure 3 shows the procedure of all seeded-control experiments. When dry sediment was prepared, the sediment was washed with 2 equal volumes of deionized water, placed overnight in a drying oven at 60 ^{o}C, then brought to room temperature and pulverized. When wet sediment was employed, it was autoclaved at 15 psi, 121 ^{o}C for 15 minutes. A small aliquot was weighed and dried and the water content of the wet sediment was determined. Ten gram amounts of sediment were placed in 125 ml flasks followed by 30 ml artificial seawater containing 10^{5}-10^{6} PFU/ml poliovirus type 1 or coxsackievirus type B 3. A flask containing no sediment was used as input control. These flasks were shaken for 30 minutes at 250 rpm, then centrifuged at 1,000 xg for five minutes. The supernatants were collected to determine the percentage of virus adsorption. Sediments were resuspended in

Figure 3: Sediment Extraction Procedure



30 ml test eluent and again subjected to shaking and centrifugation. The pH of the supernatants was adjusted to pH 7.5 and frozen at -70 ^{o}C until virus assay.

CHAPTER IV

RESULTS AND DISCUSSION

A. Introduction

Only a few methods have been published which examine the elution of viruses from sediment (4, 10, 32, 34). The use of dried sediments by Gerba and his co-workers was not a suitable procedure for testing virus elution from Mississippi sediments. Instead, this study discovered that wet, autoclaved sediments were more easily handled and paralleled natural sediments in color and consistency. Drying of sediment was found to alter the physical and chemical properties of samples; autoclaving did not visibly change the nature of a sediment (Lytle, T. and Lytle, J., Gulf Coast Laboratory, Ocean Springs, Mississippi, personal communication).

B. Adsorption Studies

Table 1 summarizes experiments studying the adsorption of viruses to dry or wet estuarine sediments. The ability of sediment to adsorb virus was not influenced by either the location of sampling or the condition of the sediment prior to analysis. For all experiments, virus adsorption ranged from 99.6 to 99.9%. A pattern of adsorption was also observed when different viruses were

Table 1

Adsorption of Viruses to Estuarine Sediments in Artificial Seawater

STATION	SEDIMENT TYPE	TYPE OF VIRUSES	NUMBER OF TRIALS	AVERAGE PFU/ml ADDED TO SEAWATER	AVERAGE PFU/ml AFTER REMOVAL OF SEDIMENTS	AVERAGE % VIRUSES ADSORPTION
3	Dry	Poliovirus 1	19	1.2×10^6	1.4×10^3	99.9
3	Wet	Poliovirus 1	86	1.4×10^6	5.4×10^2	99.9
2	Wet	Poliovirus 1	18	1.1×10^6	3.0×10^3	99.7
3	Wet	Coxsackievirus B 3	12	4.9×10^5	2.0×10^3	99.6
2	Wet	Coxsackievirus B 3	6	3.6×10^5	5.5×10^2	99.8

used; poliovirus and coxsackievirus removal from 30 ml seawater by ten gram amounts of sediments was consistent and averaged a three log decrease in virus titer. These data indicate that virus which is introduced into the estuarine environment rapidly binds to suspended sediment particles and can become part of the aerobic zone. The average viral titers of the 0 minute controls (without shaking) and the 30 minute controls are shown in Table 2. Although the titer dropped to some extent during the 30 minute interval, there was not a significant difference between virus levels. This titer drop may be due to the inactivation or aggregation of virus. This observation is consistent with earlier published reports (37) which stated that virus aggregation is increased at low pH levels. In these experiments, a constant seawater pH (7.5) was used and could account for the small decrease in titer observed. Temperature could have influenced the virus titer since all trials were conducted at ambient temperatures which fluctuated in the laboratory during the year. For all subsequent experiments, the 30 minute control was adopted as the input virus titer.

C. Extraction of Sediment Bound Viruses

1. The Glycine Elution Method

Sediments collected at station 3 were used during the initial part of this study. The results of Table 3 show that the virus recoveries using the Glycine elution

Table 2

The Effect of Shaking on Virus Titer of Seawater

TYPE OF VIRUSES	NUMBER OF TRIALS	AVERAGE PFU/ml OF 0 MINUTE CONTROLS	AVERAGE PFU/ml OF 30 MINUTE CONTROLS
Poliovirus 1	19	1.4×10^6	1.2×10^6
Poliovirus 1	86	1.6×10^6	1.4×10^6
Poliovirus 1	18	1.9×10^6	1.1×10^6
Coxsackievirus B 3	12	5.0×10^5	4.9×10^5
Coxsackievirus B 3	6	3.6×10^5	3.6×10^5

Table 3

Elution of Poliovirus 1 from Sediments[a]

by Glycine Elution Method

ELUENT	ELUTING pH	POLIOVIRUS INPUT (PFU/ml)	RECOVERY OF VIRUS FROM SEDIMENT (PFU/ml : %)
0.25M Glycine[b]	10.0	1.8×10^6	8.0×10^3 : 0.4
	10.5	1.8×10^6	1.0×10^4 : 0.9
	11.0	5.6×10^5	5.3×10^3 : 1.0
	11.5	1.8×10^6	9.6×10^3 : 0.5
0.05M EDTA[c] in 0.25M Glycine	10.0	5.6×10^5	1.1×10^4 : 1.4
	10.5	2.0×10^6	2.1×10^4 : 1.0
	11.0	5.6×10^5	1.3×10^4 : 1.7
	11.0	2.0×10^6	2.3×10^4 : 1.1
	11.0	5.6×10^5	1.8×10^4 : 0.9
10% FCS[d] in 0.25M Glycine	11.0	8.7×10^5	3.3×10^4 : 4.3
0.05M EDTA + 10% FCS in 0.25M Glycine	11.0	8.7×10^5	2.0×10^4 : 2.6
	11.5	8.7×10^5	1.8×10^4 : 2.4

a. Sediments collected at station 3

b. All experiments done in triplicate, figures are average of 3 trials at each level

c. EDTA: ethylenediaminetetraacetate

d. FCS: fetal calf serum

method were inadequate but consistent (less than 5%). The glycine buffer without supplement gave poor recovery (less than 1%) although Wallis et al. reported that glycine buffer adjusted to pH values above 10.0 could successfully elute enterovirus from membrane filters (88). Gerba et al. used glycine buffer to elute virus from dry sediments collected along the upper Texas Gulf Coast and found that only when the glycine buffer was adjusted to pH 11.0 did satisfactory virus extraction (16%) occur. When the pH value of the eluent was 11.5, less than 0.1% virus was eluted. When the pH value of the eluent was lowered to 10.5 or 10.0, the percentage of virus recovery dropped significantly (1.5% and 0.5% respectively) (34). In these experiments, significant differences in virus recoveries using 0.25M glycine was not observed. The eluent, 0.25M glycine, was modified to include 0.05M EDTA (ethylene-diaminetetraacetate) or 10% fetal calf serum or both. Earlier reports stated that metal cations in sediment enhanced the adsorption of viruses to solids (34, 79, 87). However, the addition of a chelating agent (EDTA) to sediment eluent did not markedly enhance the recovery of virus from estuarine sediment samples (recovery percentage varied from 0.9% to 1.7%). The average virus recovery by adding EDTA at pH 11.0 (1.4%) is slightly higher than the virus recovery using glycine buffer alone at pH 11.0 (1.0%).

Calf serum has been reported to be capable of

eluting virus adsorbed to various surfaces (35, 88). The addition of calf serum alone or in combination with EDTA allowed virus recoveries of 4.3% and 2.5%, respectively. The serum possibly afforded a viral protective effect, resulting in the preservation and protection of viruses against inactivation by high pH. However, this modification did not give suitable virus recovery.

The results of the preliminary experiments using dry sediments and the glycine elution method were extended to include autoclaved sediments (Table 4). All trials used pH 11.0 because this alkaline condition had produced the best recovery from dry sediments. Glycine alone recovered only 5.2% of seeded virus. The addition of 0.05M EDTA as a supplement increased this average percentage only to 7.5. Other modifications were likewise not effective including variation in the concentration of EDTA (2.3% recovery) or the selection of a second chelating agent, sodium oxalate (4% recovery). The highest recovery (10.9%) was achieved when 10% newborn calf serum was added.

Generally speaking, the glycine elution method did not appear to be applicable for virus extraction from either dry or wet type sediments collected at Graveline Bayou, station 3. Wallis reported that highly alkaline glycine buffer appeared to be necessary for efficient virus elution (87). At these high pH levels, the repulsive

Table 4

Elution of Poliovirus 1 from Wet Sediments[a]

by Glycine Elution Method

ELUENT	ELUTING pH	POLIOVIRUS INPUT (PFU/ml)	RECOVERY OF VIRUS FROM SEDIMENT (PFU/ml : %)
0.25M Glycine[b]	11.0	3.8×10^6	2.0×10^5 : 5.2
0.05M EDTA in 0.25M Glycine	11.0	3.8×10^6	2.6×10^5 : 7.0
0.10M EDTA in 0.25M Glycine	11.0	1.2×10^6	2.8×10^4 : 2.3
		1.2×10^6	7.5×10^4 : 6.0
10% NCS[c] in 0.25M Glycine	11.0	8.0×10^5	8.8×10^4 :10.9
0.05M Sodium oxalate in 0.25M Glycine	11.0	8.0×10^5	3.2×10^4 : 4.0

a. Sediment samples were collected at station 3

b. Experiments were done in triplicate

c. NCS: newborn calf serum

forces between the viral protein surface and the sediment particles would be increased. However, the variation of alkaline pH levels used in this study did not improve the virus recovery by the glycine elution method. It is probable that the high pH used in this eluent system may have caused considerable inactivation of virus (34). Jacubowski has suggested that a lowering of the pH may reduce the risk of viral inactivation (46).

Although glycine has been recommended as a standard eluent for the recovery of enteric viruses from water, wasterwater (2) and sediments (34), it appears incapable of extracting virus from Mississippi estuarine sediment samples. Recently, a number of alkaline proteinaceous substances have been used for virus extraction. However, most work was performed using sewage sludge. In order to determine whether these methods would improve virus elution from sediments, further experimentation was conducted.

2. The Skim Milk Elution Method

The elution method (10) using skim milk was evaluated in experiments summarized in Tables 5 and 6. In this study, most trials were done using 0.5% skim milk dissolved in pH 9.0 Tris (hydroxymethyl aminomethane) buffer. The skim milk alone at pH 9.0 gave low poliovirus recovery in experiments using dry type sediment (0.1%-0.5% recovery) (Table 5) and wet type sediment (0.2% recovery) (Table 6).

Table 5

Elution of Poliovirus 1 from Dry Sediments[a]

by the Skim Milk Elution Method

ELUENT[b]	ELUTING pH	POLIOVIRUS INPUT (PFU/ml)	RECOVERY OF VIRUS FROM SEDIMENT (PFU/ml : %)
0.5% skim milk	9.0[c]	8.7×10^5	2.5×10^3 : 0.3
	9.0	8.9×10^5	4.2×10^3 : 0.5
	10.0	8.9×10^5	3.0×10^3 : 0.5
	11.0	8.9×10^5	1.0×10^3 : 0.1
0.5% skim milk +10% FCS	9.0[c]	8.7×10^5	3.8×10^4 : 4.3

a. Sediments were collected at station 3

b. Skim milk was prepared in Tris buffer

c. Experiments were done in triplicate

Table 6

Elution of Poliovirus 1 from Wet Sediment[a]

by the Skim Milk Elution Method

ELUENT[b]	POLIOVIRUS INPUT (PFU/ml)	RECOVERY OF VIRUS FROM SEDIMENT (PFU/ml : %)
0.5% skim milk[c]	8.7×10^5	2.0×10^3 : 0.2
0.5% skim milk + 10% NCS[d,e]	1.9×10^6	2.1×10^5 :11.0
0.5% skim milk + 10% NCS + 0.05M EDTA[d,f]	2.0×10^6	1.0×10^5 : 5.0
0.5% skim milk + 0.25% SDS[g]	2.5×10^6	5.4×10^4 : 2.2
0.5% skim milk + 10% NCS + 0.25% SDS	2.5×10^6	8.2×10^4 : 3.3

a. Sediments were collected at station 3

b. Eluents were prepared in Tris buffer at pH 9.0

c. Experiment was done in triplicate

d. Results are average of 3 seperate experiments

e. NCS: newborn calf serum

f. EDTA: ethylenediaminetetraacetate

g. SDS: sodium dodecyl sulfate

The skim milk method was modified to attempt to increase virus recoveries from dry type sediment. Increasing the alkaline nature of the eluent (pH 9.0 to 11.0) did not increase the release of sediment bound virus (0.1%-0.3%) but possibly inactivated additional virus. Only the addition of calf serum increased the virus recovery from dry sediment (4.3% virus recovery) and wet sediment (average 11.0% virus recovery). The addition of both calf serum and EDTA yielded an average of 5.0% virus recovery.

Another modification was to add 0.25% SDS (sodium dodecyl sulfate) to the test eluent. SDS as a detergent was added as a supplement to skim milk in an attempt to increase sediment particle repulsion. However, the addition of SDS appears not to enhance viral desorption (2.2% - 3.3% recovery) and was often found to be detrimental to cell cultures even in the presence of calf serum.

The addition of calf serum or EDTA did improve the eluting capacity of the skim milk solution; however, virus recovery by these modified eluents was still not satisfactory. The overall results using the skim milk elution method indicate that the technique is not an ideal choice for elution of virus from Mississippi sediment samples.

3. The Beef Extract Elution Method (Extractions used only wet sediments)

Two types of beef extract were used in this study:

paste (Difco) and powdered (Inolex). Table 7 summarizes the results of elution experiments using 3% Difco beef extract at pH 9.0 and wet autoclaved sediments. The eluents prepared from paste type beef extract did not release a large amount of sediment-bound poliovirus (2.5%-6.6% recovery). This was also true when the time of sample agitation and the pH of the eluent were altered (3.2%-3.7% virus recovery at pH 10.0 and 2.0%-2.2% recovery at pH 11.0). Another attempt to modify this method by increasing the concentration of protein and/or by adding supplements also failed to increase the extent of virus elution (Table 8). Ten percent Difco beef extract solution at pH 9.0 eluted 2.8% of adsorbed virus; however, the addition of calf serum or SDS resulted in virus recoveries of only 3.0-3.2%. A 10% solution of Difco beef extract in McIlvaine buffer (4) containing sufficient Na_2HPO_4 to bring the molarity of the salt to 0.05 and sufficient citric acid to maintain the pH at approximately 7 eluted more virions (11.8%) than a 10% solution of Difco beef extract as eluent protein (Table 9). Three percent Inolex beef extract at pH 9.0 produced an average of 46.6% virus recovery. The same eluent at pH 10.0 eluted 37-65% virus. Ten to twelve percent recovery was obtained by Inolex beef extract solution at pH 11.0. Perhaps, as discussed before, the high pH is harmful to the virus (34).

The results of Table 10 demonstrate the extraction

Table 7

Effect of pH on Elution of Poliovirus 1 from Wet Sediments[a] by 3% Difco Beef Extract[b]

AGITATION TIME (MINUTE)	ELUTING pH	POLIOVIRUS INPUT (PFU/ml)	RECOVERY OF VIRUS FROM SEDIMENT (PFU/ml : %)
30	9[c]	1.0×10^6	3.4×10^4 : 3.4
	9	1.2×10^6	8.0×10^4 : 6.6
	9	1.0×10^6	2.5×10^4 : 2.5
15	10[c]	1.0×10^6	3.2×10^4 : 3.2
	10	1.0×10^6	3.7×10^4 : 3.7
10	11[c]	1.0×10^6	2.2×10^4 : 2.2
	11	1.0×10^6	2.0×10^4 : 2.0

a. Sediments were collected at station 3

b. Difco beef extract was prepared in Tris buffer

c. Experiments were done in triplicate

Table 8

Elution of Poliovirus 1 from Wet Sediment[a]

by Difco Beef Extract

ELUENT	ELUTING pH	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
10% beef extract[b,c]	9	2.5×10^6	7.0×10^4 : 2.8
10% beef extract[b] + 10% NCS	9	2.5×10^6	7.5×10^4 : 3.0
10% beef extract[b] + 10% NCS + 0.25% SDS	9	2.5×10^6	8.0×10^4 : 3.2
10% beef extract in 0.05M Na_2HPO_4 + 1.2g/l citric acid	7	8.0×10^5	9.5×10^4 :11.8

a. Sediments were collected at station 3

b. Protein and supplements prepared in Tris buffer (0.05M)

c. Experiment was done in triplicate

Table 9

Elution of Poliovirus 1 from Wet Sediments[a]

by Inolex Beef Extract[b] Elution Method

ELUTING pH	ELUTING TIME	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
9[c]	30	1.0×10^6	5.0×10^5 :50.0
9	30	1.2×10^6	3.0×10^5 :25.0
9	30	1.0×10^6	5.2×10^5 :52.0
9	30	1.8×10^6	8.0×10^5 :44.4
10[c]	15	1.0×10^6	3.7×10^5 :37.0
10	15	1.0×10^6	6.5×10^5 :65.0
11[c]	5	1.0×10^6	1.0×10^5 :10.0
11	5	1.0×10^6	1.2×10^5 :12.0

a. Sediments were collected at station 3

b. Three percent beef extract (lot number 5485-1A) was prepared in Tris buffer

c. Experiments were done in triplicate

Table 10

Elution of Poliovirus 1 from Wet Sediment[a] by Inolex Beef Extract[b] in Different pH and/or Buffer Solution

BEEF EXTRACT CONCENTRATION[c]	ELUTING pH	BUFFER	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
3%	7	Tris	4.8×10^4	1.2×10^4 :25.0
3%	7	Glycine	4.8×10^4	1.2×10^4 :25.0
10%	7	McIlvaine	8.0×10^5	2.5×10^5 :31.2
3%	8	Tris	4.8×10^4	1.5×10^4 :31.0
3%	9	Tris	4.8×10^4	2.0×10^4 :42.0
3%	9	Glycine	4.8×10^4	1.6×10^4 :33.0

a. Sediments were collected at station 3

b. Inolex beef extract lot number 5485-1A

c. Time of elution: 30 minutes

efficiency of Inolex beef extract at different pH conditions and/or different buffer solutions. Beef extract at pH 9.0 gave the best recovery. A solution of 3% Inolex beef extract in Tris buffer at pH 9.0 yielded higher results (42 % recovery) than that in glycine buffer (33.0% recovery). At pH 7.0, no difference was observed between Tris and glycine buffers (both 25% recovery). A 31.2% recovery was achieved when 10% Inolex beef extract was dissolved in McIlvaine buffer.

Many past investigations have utilized 3% beef extract but no specific concentration is characterized as a standard amount. Landry et al. reported that powdered beef extract concentrations of less than 3% appeared to be as effective as 3% beef extract for virus reconcentration from wastewater effluent samples (54). Berg et al. demonstrated that a 10% solution of powdered beef extract consistently eluted more of the poliovirus adsorbed to river water solids than a 3% solution of beef extract (4). To determine if different concentration of Inolex beef extract had different capacities to elute virus from Mississippi estuarine sediment, experiments were conducted using 1%, 2%, 3%, 5%, 8%, 10%, 15%, 20%, 30% and 40% beef extract. As shown on Table 11, virus recovery increased until approximately 15% was reached. This implies that some components in powdered beef extract could affect the release of virus from sediment samples and thus the higher

Table 11

Effect of Concentration of Inolex Beef Extract[a] at pH 9 on the Elution of Poliovirus 1 from Wet Sediments[b]

CONCENTRATION OF BEEF EXTRACT (%)	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
1	1.8×10^6	3.5×10^5 :19.4
2	1.8×10^6	5.0×10^5 :27.8
3[c]	1.3×10^6	5.7×10^5 :43.8
5	4.8×10^4	2.0×10^4 :42.0
8	4.6×10^4	2.1×10^4 :46.0
10	4.6×10^4	2.2×10^4 :48.0
15[d]	9.2×10^5	4.4×10^4 :47.8
20[d]	9.2×10^5	1.4×10^5 :15.2
25	1.8×10^6	3.0×10^5 :16.6
30[e]	1.2×10^6	1.5×10^5 :12.5
40	4.6×10^4	1.0×10^4 :22.0

a. Beef extract lot number 5485-1A

b. Sediments were collected at station 3

c. Results are the average of four experiments

d. Results are the average of two experiments

e. Results are the average of three experiments

concentration of beef extract could release more viruses. Concentrations greater than 15% gave variable results. The soupy consistency of high concentrations of powdered beef extract might either interfere with the desorption of virus from sediment or affect the attachment of virus to cells used in this assay. A similar pattern of recovery was observed by Berg et al. (4) when the concentration of beef extract reached the 10-20% or higher range.

Table 12 shows the virus recovery at different elution times. Hurst (45) reported that a 30 second or 1 minute mixing time could yield similar or higher results than longer periods, for example, 15 minutes when conducting experiments of activated sludge. In this study, 3% Inolex beef extract recovered the greatest amount of virus following the 30 minute elution (average 43.8%). When the time of elution was increased to 45 minutes, the percentage of virus recovery decreased (34.2%).

The Inolex beef extract method of elution was also modified by the addition of supplements and pH variation. The results are shown in Table 13 and indicate that neither calf serum nor (0.025%-0.25%) SDS increased the recovery of virus. It is possible that these supplements adversely affected the desorption capacity of beef extract. In only two instances (3% Inolex beef extract + 0.025% SDS and 3% Inolex beef extract + 0.025% SDS + 10% calf serum) did a supplement (s) allow satisfactory virus recovery.

Table 12

Effect of Elution Time on the Virus Recovery

ELUTION TIME[a] (MINUTE)	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
5	1.9×10^6	6.0×10^5:31.5
15	1.9×10^6	6.5×10^5:34.2
30[b]	1.3×10^6	5.7×10^5:43.8
45	1.9×10^6	6.5×10^5:34.2

a. Experiments were conducted using 3% Inolex beef extract (lot number 5485-1A) at pH 9 and wet type sediment collected at station 3

b. Results are the average of four experiments

Table 13

Elution of Poliovirus 1 Using Wet Sediments[a] and Inolex Beef Extract Elution Method

ELUENT	ELUTING pH	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
3% IBE[b] + 10% NCS[c]	9	8.0×10^5	1.0×10^5 :12.5
	9	9.0×10^5	2.0×10^5 :22.0
	10	8.0×10^5	2.3×10^5 :28.7
	11	8.0×10^5	2.0×10^5 :25.0
3% IBE + 0.25% SDS[d]	9	2.5×10^6	3.3×10^5 :13.3
3% IBE + 0.025% SDS	9	9.0×10^5	3.5×10^5 :38.0
10% IBE + 0.25% SDS	9	2.5×10^5	3.8×10^5 :15.0
3% IBE + 0.025% SDS	9	8.0×10^5	1.5×10^5 :18.7
+ 10% NCS	9	9.0×10^5	3.0×10^5 :33.0

a. Sediments were collected at station 3

b. IBE: Inolex beef extract

c. NCS: newborn calf serum

d. SDS: sodium dodecyl sulfate

Berg et al. (6) reported that different lots of beef extract could have different eluting capacities. Six different lots of beef extract were compared and the results are recorded in Table 14. In general, the six lots produced approximately the same recoveries of poliovirus (average of 32%).

The effect of the method of sediment-eluent agitation was also determined (Table 15). After stirring for 15 minutes, 18.3%-20.5% of adsorbed virus was released. From the sediment, 23.3%-24.4% virus was recovered after stirring for 30 minutes. The average virus recovery using this procedure appeared to be lower than the recoveries of preliminary experiments. This indicates that when the powdered beef extract system was used, agitation on a magnetic stirrer resulted in a lower efficiency than shaking at 250 rpm. A longer elution time (30 minutes) produced a slightly higher virus recovery than a 15 minute stirring.

4. The Nutrient Broth Elution Method (Extractions used only wet sediments)

Studies to evaluate the elution efficiency of nutrient broth as elutent are available but are not associated with sediment virology as compared to beef extract systems. However, as shown on Table 16, a 4% nutrient broth solution at pH 7.5 is very efficient for eluting virus from Mississippi sediments. The time of

Table 14

Comparison of the Eluting Capacity of Different Lots of Inolex Beef Extract[a,b]

LOT NUMBER	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
5485	8.3×10^5	3.3×10^5 :39.1
	1.7×10^5	5.5×10^4 :33.3
5667	8.3×10^5	3.3×10^5 :39.1
	1.6×10^5	4.3×10^4 :25.7
3924	1.6×10^5	4.0×10^4 :24.2
011961	8.3×10^5	2.3×10^5 :27.1
	8.3×10^5	3.0×10^5 :36.1
	1.7×10^5	5.3×10^4 :31.8
014507	8.3×10^5	3.8×10^4 :22.7
012036	8.3×10^5	5.0×10^4 :30.3

a. Three percent solution of different lots Inolex beef extract at pH 9 were used. The eluting time is 30 minutes

b. Supplied by Inolex Corp. in 100 gram quantities

Table 15

Mechanical Effect on Virus Elution[a]

ELUTING TIME (MINUTE)	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
15	1.8×10^6	3.3×10^5 :18.3
15	1.8×10^6	3.7×10^5 :20.5
30	1.8×10^6	4.2×10^5 :23.3
30	1.8×10^6	4.4×10^5 :24.4

a. Experiments were conducted using wet sediment collected at station 3 and 3% beef extract in Tris buffer at pH 9. Sediment-eluent agitation performed using a magnetic stirrer in place of the shaker apparatus.

Table 16

Elution of Poliovirus 1 Using 4%

Nutrient Broth[a] and Wet Sediment

ELUTING TIME (MINUTE)	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
10	1.9×10^6	1.0×10^6 :52.6
15	1.9×10^6	1.2×10^6 :63.2
30[b,c]	9.0×10^5	2.3×10^5 :25.0
30[d]	1.9×10^6	1.1×10^6 :57.9

a. Eluent was adjusted to pH 7.5

b. Experiment was done in triplicate

c. Sediment (station 3) was collected during summer, 1980

d. Sediment (station 3) was collected during winter, 1980

elution was not considered critical but the time of sample collection did infuence the virus recovery. Overall, this method offers the advantages of higher virus recoveries, economy and elution at neutral pH (7.5).

5. Survey of Factors Influencing Virus Extraction

It is obvious that one of the major problems in working with sedimentary materials is the uncertainty of the recovery of virus from sedimentary particles. In this study, fluctuation of the results was observed regardless of the elution method employed. There are many factors that can result in the fluctuation including the plaque assay system, eluting procedures and sediment quality. The plaque assay system has been widely used as a standard assay method in many virological studies. It is possible that multiple viruses aggregate and are assayed as one plaque forming unit (30). However, using 30 minute controls and triplicate replicates should minimize the effect. Another possible reason is that viruses might be inactivated in the process of adsorption or desorption or both. It is also possible that sediment composition plays a distinct role in virus recovery. To generate information to aid in the understanding of the extent of viral entrapment by sediments, sand, silt and clay values of Graveline sediments (station 2 and 3) were determined during each quarter of 1980. As shown on Table 17, fluctuation in the composition of sand, silt and

Table 17

Composition of Sediment Samples

STATION	DATE COLLECTED	SEDIMENT (%) SAND	SILT	CLAY	SIZE TERM
2	3/14/80	10.8	39.7	49.5	Very fine, sandy mud
	6/16/80	5.5	63.7	30.8	Silt
	9/15/80	18.0	50.3	31.7	Very fine, sandy mud
	12/15/80	6.2	39.0	54.8	Mud
3	3/14/80	60.2	26.3	13.5	Muddy, fine sand
	6/16/80	92.9	5.1	2.0	Fine sand
	9/15/80	89.4	3.0	7.6	Clayey, fine sand
	12/15/80	74.2	12.6	13.2	Muddy, fine sand

clay did occur. These variations implied that a universal eluent may be required to evaluate virus recovery from sediments of different composition.

6. Virus Extraction from Station 2 Sediment

The four elution methods and modifications were utilized to determine their eluting capacity for a sediment with different composition. Table 18 demonstrates the virus recovery from sediments collected at station 2. As expected, fluctuation still existed in experiments conducted using station 2 sediment samples. The results of virus recovery appeared to correspond to the results of station 3 sediment analysis. The glycine elution method yielded 0.7% virus recovery. As before, the addition of 0.05M EDTA or 10% calf serum or both only increased the virus recovery slightly (0.8% to 4.2%). The elution method of Bitton also gave poor, unreliable virus recovery (0.3% to 0.5%) and was not improved by the addition of 10% calf serum (0.5% to 0.8%).

As observed in experiments conducted using sediments from location 3, 3% Difco beef extract gave poor recovery of virus from station 2 samples. Powdered beef extract containing eluents produced satisfactory virus recoveries. Experiments conducted using 3% Inolex beef extract and 10% Inolex beef extract yielded 25.0% and 34.6% recovery, respectively. Desirable increases in virus elution were not realized by the addition of 10% calf serum (30%

Table 18

Elution of Poliovirus 1 from Wet Sediments[a]

ELUENT	ELUTING pH	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
0.25M Glycine[b]	11	1.0×10^6	6.5×10^3 :0.1
0.05M EDTA in 0.25M Glycine	11	1.0×10^6	1.5×10^4 :1.5
10% NCS in	11	1.0×10^6	8.0×10^3 :0.8
0.25M Glycine	11	1.3×10^6	5.5×10^4 :4.2
0.05M EDTA + 10% NCS in 0.25M Glycine	11	1.0×10^6	2.5×10^4 :2.5
0.5% skim milk	9[b]	1.0×10^6	4.5×10^3 :0.5
	9	1.3×10^6	3.5×10^3 :0.3
0.5% skim milk	9	1.0×10^6	7.5×10^3 :0.8
+ 10% NCS	9	1.3×10^6	6.5×10^3 :0.5
0.5% skim milk	9	1.0×10^6	7.5×10^3 :0.8
+ 0.025% SDS	9	1.3×10^6	8.0×10^3 :0.6
0.5% skim milk + 10% NCS + 0.025% SDS	9	1.3×10^6	1.5×10^3 :0.1
3% Difco[b] beef extract	9	1.0×10^6	7.5×10^3 :0.8
3% Difco beef extract + 10% NCS	9	1.0×10^6	2.5×10^5 :2.5

Table 18: continued

3% Inolex beef extract[b] lot # 5485-1A	9	1.0×10^6	2.5×10^5	:25.0
3% Inolex beef extract lot # 5485	9	8.3×10^5	2.5×10^5	:30.1
3% Inolex beef extract lot # 5667	9	8.3×10^5	3.5×10^5	:42.1
3% Inolex beef extract lot # 011961	9	8.3×10^5	7.2×10^5	:27.1
3% Inolex beef extract+10% NCS lot # 5485-1A	9	1.0×10^6	3.0×10^5	:30.0
10% Inolex beef extract lot # 5485-1A	9	1.3×10^6	4.5×10^5	:34.6
4% Nutrient broth	7.5	1.0×10^6	4.0×10^5	:40.0

a. Sediments were collected at station 2

b. Experiments were done in triplicate

recovery). Three different lots of powdered beef extract were tested and produced 27.1% to 42.1% recovery. Four percent nutrient broth (pH 7.5) also allowed a high percentage of virus recovery (40%).

As discussed above, our results show that for both station 2 and 3 sediment samples, the powdered beef extract and the nutrient broth elution systems appeared to be better than the glycine or the skim milk methods.

7. Elution of Sediment-bound Coxsackievirus

The conditions governing the adsorption-elution of the poliovirus have been shown to similar to other enteroviruses (87, 88). Although poliovirus 1 has been regarded as the model virus in this kind of investigation, experiments were conducted to determine whether the test eluents had similar eluting capacities for other enteroviruses. Coxsackievirus type B 3 was employed in separate series of experiments. Table 19 shows the elution of coxsackievirus B 3 from wet sediments collected at station 3. The lower recoveries of virus were associated with the glycine (3.8%-4.8%) or the skim milk procedures. Beef extract and nutrient broth elution methods produced higher recoveries (12.1%-15.8%) which were low in comparison to the elution of sediment bound poliovirus. The four eluent systems were also employed to elute coxsackievirus type B 3 from wet sediments collected at station 2. Again the lower virus recoveries were yielded

Table 19

Elution of Coxsackievirus B 3 from Wet Sediments[a]

ELUENT	ELUTING pH	ELUTING TIME (MINUTE)	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
0.25M Glycine[b]	11	10	6.5×10^5	2.5×10^4 : 3.8
0.25M Glycine + 0.05M EDTA	11	10	6.5×10^5	3.1×10^4 : 4.8
0.5% skim milk[b]	9	30	6.5×10^5	3.9×10^4 : 6.0
0.5% skim milk + 10% NCS	9	30	6.5×10^5	7.0×10^4 :10.7
3% Inolex beef extract	9[b]	30	3.3×10^5	4.0×10^4 :12.1
	9	30	6.5×10^5	9.8×10^4 :15.0
10% Inolex beef extract	9	30	3.3×10^5	4.3×10^4 :13.1
3% Inolex beef extract + 10% NCS	9	30	3.3×10^5	4.2×10^4 :12.7
4% Nutrient broth	9	30	3.3×10^5	3.5×10^4 :15.4

a. Sediments were collected at station 3

b. Experiments were done in triplicate

by the glycine elution method (4.2%-11.1%) and the skim milk elution method (6.7%-11.7%). Higher virus recoveries were associated with the beef extract elution method (18.8% -29.0%) and nutrient broth elution method (25.9%) (Table 20).

In conclusion, both poliovirus 1 and coxsackievirus B 3 can readily adsorb to both station 2 and station 3 sediments. However, the efficiency of elution of sediments-bound viruses varied with the virus types and sediment quality. Among the four elution systems examined in this study, the beef extract and nutrient broth elution methods are superior to the glycine and skim milk elution methods in extracting both poliovirus 1 and coxsackievirus B 3 from both station 2 and station 3 sediments.

Table 20

Elution of Coxsackievirus B 3 from Sediments[a]

ELUENT	ELUTING pH	ELUTING TIME MINUTE	VIRUS INPUT (PFU/ml)	VIRUS RECOVERY (PFU/ml : %)
0.25M Glycine[b]	11	10	3.6×10^5	1.5×10^4 : 4.2
0.25M Glycine + 0.05M EDTA	11	10	3.6×10^5	4.0×10^4 :11.1
0.5% skim milk[b]	9	30	3.0×10^5	2.0×10^4 : 6.7
0.5% skim milk + 10% NCS	9	30	3.0×10^5	3.5×10^4 :11.7
3% Inolex beef extract[b]	9	30	3.2×10^5	9.3×10^4 :29.0
10% Inolex beef extract	9	30	3.2×10^5	8.3×10^4 :25.9
3% Inolex beef extract + 10% NCS	9	30	3.2×10^5	6.0×10^4 :18.8
4% Nutrient broth[b]	7.5	30	3.2×10^5	8.3×10^4 :25.9

a. Wet sediments were collected at station 2

b. Experiments were done in triplicate

APPENDIX A

MEDIA AND REAGENTS

BGM Growth Medium

MEM-L15	500.0 ml
Newborn Calf Serum	50.0 ml
Antibiotic-Antimycotic Mixture (100X)	5.5 ml
L-Glutamine (29.2 mg/ml) Solution (100X)	5.5 ml
7.5% Sodium Bicarbonate Solution	5.0 ml

MEM-L15: Minimum Essential Medium-Leibovitz's L15 Medium. Powdered medium was dissolved in double distilled water and sterilized by membrane filtration.

ATV(10X) Versene Dispersant

NaCl	80.0 g
KCl	4.0 g
Dextrose	10.0 g
Trypsin (Difco 1:250)	5.0 g
Na_2HCO_3	5.8 g
EDTA	2.0 g
Distilled H_2O	QS to 1 liter

Overlay Medium

First Agar Overlay:

Modified Autoclavable MEM	100 ml
Newborn Calf Serum	4 ml
Antibiotic-Antimycotic Mixture (100X)	1 ml
L-Glutamin (29.2 mg/ml) Solution (100X)	1 ml
7.5% Sodium Bicarbonate Solution	3.5 ml
Agar Noble (Difco)	1.5 ml

Second Agar Overlay

Modified Autoclavable MEM	100 ml
Newborn Calf Serum	2 ml
Antibiotic-Antimycotic Mixture (100X)	1 ml
L-Glutamin (29.27 g/ml) Solution (100X)	1 ml
7.5% Sodium Bicarbonate Solution	3.5 ml
Neutral Red Solution (1:300)	2 ml
Agar Noble (Difco)	1.5 ml

Autoclavable MEM was dissolved in double distilled water and autoclaved for 15 minutes with agar noble in it. Other ingredients were added after cooling the MEM-agar solution to 45 ^{o}C.

REAGENTS

Antibiotic-Antimycotic Mixture

Penicillin, Streptomycin and Fungizone (PSF, 100X) purchased from Grand Island Biological Company, Cat. No. 600-5240

7.5% Sodium Bicarbonate

7.5 g of Sodium Bicarbonate (Na_2HCO_3) was dissolved in 100 ml of double distilled water and autoclaved.

Newborn Calf Serum

NCS (100X) purchased from Grand Island Biological Company, Cat. No. 230-6010

L-Glutamine

L-Glutamine (200 mM) purchased from Grand Island Biological Company, Cat. No. 320-5030.

Tris (hydroxymethyl) aminomethane Buffer

24.2 g Tris (hydroxymethyl) aminomethane was dissolved in 1000 ml double distilled water (0.2 M) and served as stock solution. 0.2 M HCl or 0.2 M NaOH were added to adjust to required pH.

APPENDIX B

EXPERIMENTAL MATERIALS

ITEM	MANUFACTURE
Centrifuge	International Eq. Co.
Filter holder	Millipore Co.
Forma bio-freezer	Forma Scientific
Incubator	Hotpack Co.
Incubator shaker	New Brunswick Scientific
Inverted microscope	Olympus
Magnetic stirrer	E. H. Sargent & Co.
Media filter (90 mm, 0.45 um)	Millipore Co.
Media holding tank for sterilization	Gibco Co.
Nalgene filter unit	Nalge Co.
pH meter	Sargent-Welch Scientific
Rocker platform	Bellco Co.
Salinity (S ‰), temperature (^{o}C), conductivity (uMHO) and pH meter	Simpson Electric Co.
Sterile plastic ampule	Vanguard International
Tissue culture flask	Curtin Matheson Sci.
Verticle-flow laminar air hood	Baker Co., Inc.

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