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Natural Enterovirus and Fecal Coliform Contamination of Gulf Coast Oysters

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ABSTRACT

The numbers of fecal coliforms and enteroviruses present in oysters and/or their growing waters of two Mississippi reefs were determined over a 12-month period. Bacterial and viral levels reflected the classification of the waters at each location as set by the Mississippi State Board of Health in compliance with the National Shellfish Sanitation Program, but statistically significant correlations between these levels were not observed. Twelve viral isolates were found at an approved oyster harvesting location, eight of which were identified as poliovirus type 1. At the prohibited site, 146 viruses were isolated including poliovirus types 1 and 2, echovirus type 24 and several isolates which remain to be identified. The number of virus isolates from samples from each location represented approximately 35% of the number of plaques observed; however, no consistent ratio of plaque to confirmed virus was demonstrated. The results suggest that the fecal coliform levels in oyster growing waters do not reflect the level of virus contaminaton in either approved or prohibited waters.

The extent to which shellfish growing waters have been polluted by fecal material has been clearly documented in this century by the frequent closure of productive reefs. Since the early 1900's, coliform bacteria have been used to gauge the degree of fecal pollution of water, including marine waters (*25*). Methods for detecting pathogenic bacteria in shellfish or their growing waters are available although the time and expense required may preclude their use in favor of indicator bacteria. In the last 15 years, shellfish viral contaminants have attracted more and more interest as evidenced by the increased number of technical papers dealing with this problem. Compared to bacteria, viruses are not as easily detected nor are the consequences of their presence always understood.

The presence of viruses in shellfish has been documented (*2,8,9,10,17,32,35,36,43*) and usually includes those groups with direct or indirect association with the alimentary tract of man or other homiothermic animals, and whose characteristics permit survival and transmission by feces. The enteroviruses, reoviruses, adenoviruses and hepatitis A virus are considered prime candidates for shellfish contamination. A more complete list and a consideration of the ecological and epidemiological significance of other possible viral contaminants was provided by Carrick and Sobsey (*3*).

Viral epidemics attributed to shellfish ingestion most frequently involve a hepatitis virus (*27,31,33,37,38, 40-42*), usually type A. Some evidence for hepatitis B virus transmission by feces or infected shellfish has been reported (*5,15,32*). At present there is no standard technique for isolating and quantifying hepatitis viruses in feces or shellfish, although several proposed methods of fecal detection are under investigation (*14,16,24,32*). The enteric viruses are more easily isolated by routine virological procedures and could perhaps serve as indicators of viral contamination of shellfish. They are important in that they can produce either acute or chronic disease, but most human infections probably remain subclinical. In certain instances, such syndromes as aseptic meningitis, paralysis, herpangina, pleurodynia, myocarditis, skin rash and coryza may occur. In view of the multitude of problems associated with enteric viral infection, it is surprising that so little information exists which supports or negates the importance of polluted shellfish in the transmission of enteric viral diseases.

Methods for detection of viruses in shellfish usually involve the assay of entire shellfish rather than dissection procedures which are designed to isolate infectious particles associated with the feeding, digestive and excretory systems. Viruses that enter oysters from the surrounding water do not reproduce and are often found in the digestive gland (*4,11*). It is possible for viruses to adhere to shellfish due to the charge differences between virus particles and mucous surfaces (*12*). The effect of bioaccumulation by these mechanisms permits viruses to be concentrated from the growing water at least by a factor of 60 (*36*).

Methods for recovery of viruses from shellfish may or may not employ concentration steps. Procedures that do not involve concentration steps are discussed in references 2, 11, 34 and 35, but are not applicable to the analysis of large quantities of shellfish tissue or are unlikely to demonstrate low level contamination. Recent investigations (*17,22,23,28,-30,45-47*) provided data that may reflect the level of contamination in shellfish tissue. Of the methods reported in those investigations, the Sobsey method (*47*) and subsequent revisions (*45,46*) are most often used and have provided the most consistent results.

In Mississippi, coastal estuaries receive the effluents of sewage disposal facilities. Those effluents have been

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extremely detrimental to the once viable shellfish industry. This report details the results of a study designed to isolate, enumerate and identify viruses from oysters collected from both approved and prohibited shellfish growing areas. Comparative fecal coliform analyses were also performed.

MATERIALS AND METHODS

Sampling procedures

Water and oyster (*Crassostrea virginica*) samples were collected from approved (Pass Christian reef) and prohibited (Graveline Bayou) shellfish growing areas (Fig. 1) from January through December, 1978. Table 1 outlines the types and numbers of samples taken. Samples were collected monthly, with the exception of oysters from Graveline Bayou for virological analysis, which were collected twice monthly. Surface (upper 0.25 m) water samples were collected in sterile wide-mouth jars while bottom samples were collected in a sterile bottle with the aid of a J-Z sampler. Water samples were collected at each of three locations in Graveline Bayou for 3 days before sampling the oysters and on the actual day of oyster sampling. Water samples were taken at three locations on the Pass Christian reef only on the day of oyster sampling. Oysters (3-5 inches long) were harvested with a hand dredge, culled and placed in an insulated box for shipment. All samples were kept at 4 C until processed.

Surface water temperatures were measured in situ with a mercury-in-glass thermometer. Salinity measurements were made on a portion of the water samples collected for bacteriological analysis using an AO Goldberg refractometer (No. 10402). Temperature and salinity data are expressed as averages of three replicate measurements.

Samples analysis

Fecal coliform analyses of water and oyster samples were conducted by methods previously described (*39*). Analyses were normally begun within 3 h after collection.

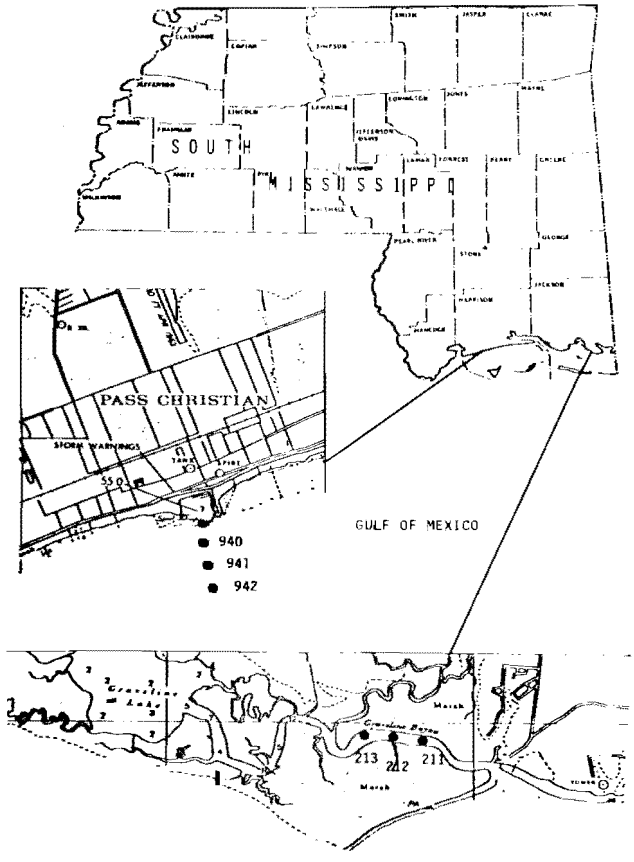

Figure 1. *Sample collection sites.*

TABLE 1. *Nature of samples.*

Location	Type sample	Purpose	Number of samples	Quantity
Pass Christian Reef	Surface water	FC[a]	3	100 ml
	Bottom water	FC	3	100 ml
	Oysters	FC	2	200 ml
	Oysters	V[b]	4	150 ml
Graveline Bayou	Surface water	FC	12	100 ml
	Bottom water	FC	12	100 ml
	Oysters	FC	2	200 g
	Oysters	V	6-7	150 g

[a]Fecal coliform analysis.
[b]Virological analysis.

Oysters (150-g lots) were extracted to determine virus concentration using a modification of the Sobsey procedure (*46*) as shown in Fig. 2.

Tissue culture assay

The Buffalo green monkey kidney cell line (BGM) (*1,6*) was used to analyze all oyster concentrates. Virus samples and/or dilutions (0.2 to 0.5 ml per 25 cm^2 plastic flask) were inoculated onto BGM monolayers (passages 100 to 120) which were incubated for 1 h at 37 C using a rocking apparatus (Bellco) at five rotations per minute. Growth medium for BGM cells consisted of MEM:L15 (1:1), 10% fetal calf serum and 1% L-glutamine (all purchased from Grand Island Biological Company).

Samples were quantitatively assayed by a modification of the plaque method reported by Dahling et al. (*6*). Plaque counts were made on a daily basis for 5 days or until no new plaques appeared for two consecutive days.

Plaque identification

Individual plaques were picked when they were ≥ 1 mm in diameter. A Pasteur pipette, with a bent tip, moistened with 0.05 ml of growth medium was used to transfer an agar plug (area of plaque) to a holding medium (1 ml MEM per tube). Samples were passaged three times in BGM cells with a minimum of one filtration step (0.45 μm). Two blind passages were made of all samples not producing observable cytopathic effect. Plaques identified as viruses were titered and identified serologically (*21*).

Statistics

Bacterial and viral counts in water and oyster samples were subjected to a square root transformation before calculation of linear correlation coefficients (*48*).

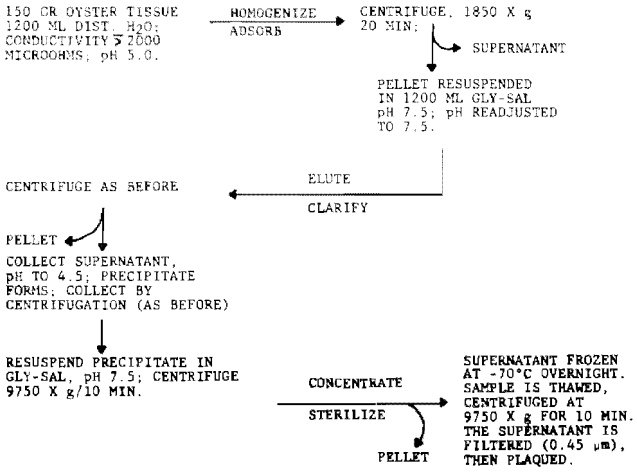

Figure 2. *Oyster extraction procedure for virus isolation.*

RESULTS

This investigation was an extension of a 1976-1977 study designed to compare the viral isolation efficiency of two oyster extraction methods (*13*). The Sobsey

procedure (*47*) was not satisfactory for examination of natural oyster samples and was modified by elimination of the intermediate filtration step and by substitution of the diluent for suspension of the final precipitate. The procedure described in Fig. 2 was tested with seeded oyster samples and yielded recoveries ranging from 61 to 73% (63% average). Oyster concentrates were clear and easy to filter-sterilize before assay. Bacterial and fungal contamination was minimal and individual flasks contained no particulate matter which interfered with visual analysis.

Table 2 presents the results of fecal coliform analyses of water and oyster samples performed during 1978. It is evident that the two areas differ significantly in bacteriological quality. The median fecal coliform MPN for all 71 water samples from the Pass Christian reef was less than 2 per 100 ml with only 8.45% of the samples exceedin an MPN of 43. The Graveline Bayou samples had a median fecal coliform MPN of 23 per 100 ml with 39.9% of the 276 samples exceeding of MPN of 43. These results confirm the approved and prohibited status of Pass Christian reef and Graveline Bayou, respectively.

TABLE 2. *Fecal coliform analysis of water and oyster samples.*

	Pass Christian Reef		Graveline Bayou	
	Water	Oyster	Water	Oyster
Month	Median fecal coliform (MPN/100 ml)	Mean fecal coliform (MPN/100 g)	Median fecal coliform (MPN/100 g)	Mean fecal coliform (MPN/100 g)
January	1400.0	73	1200.0	310.0
February	< 2.0	45	17.0	104.0
March	< 2.0	125	7.8	3,250.0
April	< 2.0	< 20	11.0	765.0
May	< 2.0	< 20	13.0	170.0
June	2.0	61	11.0	44.0
July	3.3	400	130.0	815.0
August	2.0	360	25.0	17,650.0
September	3.3	12,450	28.0	745.0
October	< 2.0	< 20	49.0	230.0
November	17.0	330	170.0	945.0
December	< 2.0	204	22.5	715.0

The fecal coliform counts from oysters (Table 2) also reflected the difference in water quality in those two areas. The median values for all samples taken from Graveline Bayou and Pass Christian were 410 and 78, respectively. There appears to be no apparent relationship between the coliform counts in the water and those in the oysters collected at the same time.

Viral isolates from approved (Table 3) and prohibited (Table 4) oyster samples also reflect the degree of fecal pollution at the two locations examined. Thirty-eight plaque-like isolates from Pass Christian were purified during the 12-month period. Of the 12 plaques confirmed as viruses, eight were identified as poliovirus type 1; four could not be typed. Most of the isolates were picked from April samples but these data could not be correlated with fecal coliform counts of that month.

In comparison, plaque-like isolates from oysters collected from Graveline Bayou totalled 416. Of this number, 146 or 35%, were identified as viruses. Of 55 random isolates identified, 50 were polio type 1, one was polio type 2, one was echovirus type 24 and three were unidentifiable by the procedures employed. Plaque-like isolates were not evenly distributed among the samples for a given month. The percent of positive 150-g samples (Table 4) ranged from 10 to 55%. In July, one of 10 samples contained virus as compared to five of nine samples in October.

TABLE 3. *Viral analysis of approved oysters, Pass Christian Reef.*

Month	# Oysters/ # samples	*	# Plaque-like isolates Total/100 g	# Plaques identified as viruses Total/100 g
Jan.	24/2	0	2/0.6	0/0
Feb.	32/2	0	4/1.3	0/0
Mar.	24/2	0	6/2.0	0/0
Apr.	30/2	1	19/6.3	11/3.6
May	21/1	0	0/0	0/0
June	38/2	0	0/0	0/0
July	32/2	0	0/0	0/0
Aug.	36/2	0	2/0.6	0/0
Sept.	33/2	0	0/0	0/0
Oct.	26/2	1	2/0.6	1/0.3
Nov.	21/1	0	0/0	0/0
Dec.	33/2	0	3/1.0	0/0
TOTAL	350/22		38	12

*Number of samples containing confirmed virus isolates.

TABLE 4. *Viral analysis of prohibited oysters, Graveline Bayou.*

Month	# Oysters/ # Samples	*	# Plaque-like isolates Total/100 g	# Plaques identified as viruses Total/100 g
Jan.	139/10	5	88/5.8	31/2.2
Feb.	65/5	1	38/5.0	5/0.7
Mar.	53/3	1	18/3.0	3/0.5
Apr.	56/3	1	5/1.1	3/0.7
May	102/6	3	51/5.6	18/2.0
June	200/13	3	38/1.9	12/0.6
July	180/10	1	44/2.9	23/1.5
Aug.	89/7	3	39/5.0	11/1.4
Sept.	191/10	5	46/2.7	26/1.5
Oct.	129/9	5	15/1.1	10/0.7
Nov.	88/8	1	9/0.8	3/0.3
Dec.	35/3	1	25/5.5	1/0.2
TOTAL	1327/87		416	146

*Number of samples containing confirmed virus isolates.

Generally, in both areas bottom water salinities were higher than surface salinities (Tables 5 and 6). The salinities of the waters over the Pass Christian reef remained fairly constant during the year except for two periods following heavy rainfall when the salinities were significantly reduced. The water salinities in Graveline Bayou ranged from a low of 2.0 to a high of 28 ppt. On one occasion, the salinity fluctuated as much as 15 ppt over the 4-day sampling period.

Temperatures of surface waters at the approved reef ranged from 8 C in January to 31 C in June. The same general trend in temperature fluctuation was observed at Graveline Bayou.

Correlation coefficients which compared fecal coliforms in surface and bottom waters, fecal coliforms in oyster tissue and plaque-like and actual virus isolates from oyster samples are presented in Table 7. A significant ($P \leq 0.1$) positive correlation was found between fecal coliforms in bottom and surface waters but not between those and fecal coliforms in oyster tissue.

TABLE 5. *Physical data on water samples from Pass Christian reef.*

Date	Surface Temperature[a] (C)	Surface Salinity[a] (ppt)	Bottom Salinity[a] (ppt)[b]
1/27/78	8.0	2.0	5.5
2/28/78	14.0	16.0	19.6
3/30/78	19.0	14.6	15.6
4/27/78	21.0	14.6	15.0
5/31/78	30.0	7.0	8.3
6/28/78	31.0	13.8	14.6
7/3/78	28.0	18.0	18.0
8/30/78	29.0	14.6	18.6
9/28/78	27.0	17.3	17.3
11/2/78	23.0	18.0	18.3
11/30/78	17.0	17.6	18.3
12/14/78	11.0	15.3	20.0

[a]An average of three measurements.
[b]Parts per thousand.

Significant correlations between fecal coliforms in waters or oysters and plaque-like or virus isolates in oysters were not observed. Although the number of confirmed viral isolates began as plaque-like isolates, no correlation could be found to indicate a relationship on a month-to-month basis.

When the results of fecal coliform and virus analyses of Graveline Bayou oysters are graphically compared (Fig. 3), they reflect the lack of statistical correlation. The variations observed in three particular months are of interest. In March, the number of fecal coliforms rose while the number of plaque-like and confirmed virus isolates decreased or remained constant. In May, the number of plaque-like and confirmed virus isolates increased, but did not correlate with the decreasing number of fecal coliforms. The numbers of both fecal coliforms and plaque-like isolates increased in August, but the number of confirmed virus isolates remained at approximately the level of the previous month.

DISCUSSION

The two locations chosen for this study were selected because previous observations had shown that each was ecologically, topographically and bacteriologically distinct. The Pass Christian reef lies in open waters of the Mississippi Sound and is not readily influenced by rapid environmental changes. Conversely, Graveline Bayou is greatly influenced by local rainfall and tidal flushing and may change rapidly within the short time period. The maximum sampling effort was expended at this location primarily to increase the probability of virus recoveries. Oyster harvesting has not been permitted in Graveline Bayou since 1975. The bayou begins at the Mississippi Sound and runs 4389 m to Lake Graveline (95 hectares). Bayou depth varies from 0.6-3.7 m. The average sill depth at the bayou's mouth is 15 cm at mean low tide. Several sources of sewage pollution contribute to the closure of Graveline Bayou to shellfishing harvesting: Del Flore treatment plant, 1097 m east of the bayou's mouth and the Gautier Point treatment facility, 3474 m east of Graveline. Septic tanks near the lake and new housing development near the bayou's mouth also

TABLE 7. *Correlation coefficients, Graveline Bayou isolates.*

	FCWS	FCWB	FCO	PLI	VI
FCWS	1.000				
FCWB	0.748**	1.000			
FCO	0.052	0.414	1.000		
PLI	0.079	0.069	0.191	1.000	
VI	0.405	0.446	0.290	0.455	1.000

**$P < 0.1$ level of significance
FCWS, FCWB, FCO, PLI, VI represent fecal coliform water surface, MPN 100 ml; fecal coliform water bottom, MPN 100 ml; fecal coliform oyster, MPN 100 gr^{-1}; plaque-like isolates, 100 gr^{-1}; viruses identified 100 gr^{-1}.

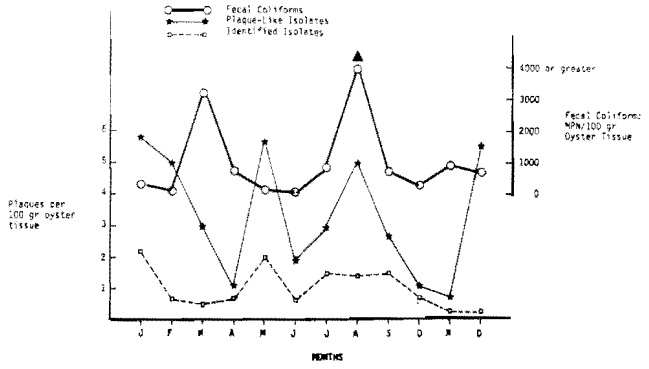

Figure 3. *Fecal coliform, plaque-like isolates and confirmed virus levels per 100 g of oyster tissue. Samples collected in Graveline Bayou, 1978.*

TABLE 6. *Physical data on Graveline Bayou water samples.*

Dates[a]	Surface Temperature[b] (C)	Surface Salinity[b] (ppt)[c]	Bottom Salinity[b] (ppt)[c]
1/24/78 to 1/27/78	9.0 to 12.5	2.0 to 7.0	2.0 to 10.3
2/25/78 to 2/28/78	11.0 to 14.5	6.0 to 12.0	10.3 to 17.6
3/28/78 to 3/31/78	17.5 to 19.5	11.6 to 21.3	17.0 to 22.0
4/25/78 to 4/28/78	19.0 to 23.5	14.6 to 19.6	15.0 to 19.6
5/28/78 to 5/31/78	27.0 to 30.0	4.0 to 5.8	4.8 to 6.0
6/25/78 to 6/28/78	29.0 to 32.0	8.5 to 17.3	10.0 to 17.3
7/31/78 to 8/3/78	28.0 to 32.0	6.2 to 18.6	7.5 to 22.0
8/27/78 to 8/30/78	27.0 to 30.0	14.0 to 16.0	14.0 to 16.0
9/25/78 to 9/28/78	27.0 to 29.0	16.0 to 16.6	16.0 to 16.6
10/30/78 to 11/2/78	21.0 to 23.0	24.0 to 25.6	24.0 to 25.6
11/27/78 to 11/30/78	17.0 to 20.0	21.0 to 22.0	21.0 to 22.0
12/11/78 to 12/14/78	8.0 to 12.0	20.0 to 28.0	22.0 to 28.0

[a]Represents the last 4 days of each month.
[b]An average of three measurements.
[c]Parts per thousand.

contribute sewage. From the sound, sewage enters Graveline by the westerly current drift in conjunction with tidal currents and prevailing southeast winds.

In our literature survey, we could find few previous studies which analyzed the virological content of oysters over an extended period. The lack of such studies is surprising when one considers the inability of the coliform standard to adequately predict increased viral contamination and the possible epidemiological consequences. Undoubtedly hepatitis outbreaks transmitted by shellfish are well documented (*18*), but similar occurrences of enterovirus disease transmission are by their very nature difficult to identify. The ingestion of raw or incompletely cooked oysters presents the potential for enterovirus transmission and the isolation of these viruses from shellfish has been observed on more than one occasion (*17,21,34,35*).

The modifications of the Sobsey extraction procedure, which have been made in our laboratory over the last 3 years, resulted from a desire to process naturally polluted oyster samples. During 1978, a wide seasonal variation of environmental parameters occurred, but no significant changes in the procedure were required. Most problems usually occurred when the final precipitate was suspended. Heavy contamination and/or inability to filter the concentrate before assay demanded the most attention. The more turbid the final concentrate, the more likely that the plaque assay would be adversely affected. When the concentrate was frozen, then thawed, centrifuged and filtered, less than 0.1% of the virus was lost to the precipitate and the filter.

Fecal coliform levels are used to verify the classification of shellfish-growing waters. The present classification system does, in general, protect the public from diseases transmitted by shellfish, but it is by no means considered infallible, especially in regard to the level of viral contamination (*17,20,21*). The problem is compounded by the lack of valid correlations between fecal coliform levels in waters and oyster samples taken simultaneously at the same site (Table 2). One factor which could account for that lack of correlation is salinity which on occasion varied as much as 15 ppt during the 4-day sampling period. Similar fluctuations were noted in the fecal coliform counts in water which in one instance changed by 2 logs in one 3-day period.

The expected rise and fall of surface water temperatures during the year did not correspond to the fluctuation of either fecal coliform or virus levels. At the Pass Christian reef, the highest recorded values for temperature, fecal coliform and virus counts occurred in the months of June, January and April, respectively. The same parameters recorded at Graveline Bayou corresponded to the months of August and January. Although our studies and those of other investigators (*21*) do not indicate correlations between salinity and temperature versus fecal coliforms in water and oysters and virus in oyster, fluctuation of those parameters would affect indicator ratios and could produce significant variation in the data used for sanitary surveys.

The plaque procedure used contributed to the problems of sample assay. For all samples examined, over 60% of all plaques were not of viral origin. This discrepancy could be due to artifacts in the flasks, limited chemical or biological contamination or failure of the isolate to replicate in the BGM cell line. This complication can be avoided using an all-or-none quantal assay in addition to the plaque assay. Studies of minimal viral contamination of oysters that compare all or none versus plaque assay methods would more clearly define the most appropriate method.

Most of the purified viral isolates (85%) was identified as poliovirus type 1. This observation is not unusual (*21*) and probably reflects the wide-spread distribution of oral polio vaccine. What is surprising is the very low numbers of other polio types observed. Perhaps environmental factors or certain aspects of the oyster extraction procedure contributed to the failure to detect viruses that are shed by the fecal route. Although Katzenelson and Kedmi (*26*) did not express this particular concern, they did suggest that additional research be done to develop a cell system with greater potential for multiple-virus assay.

These data again emphasize the need to re-evaluate the use of the coliform standard for verification of shellfish growing waters. As the fecal coliform standard is routinely used, consideration for keeping the total coliform standard should be given since Goyal et al. (*21*) have demonstrated a relationship between viruses in estuarine water and the total coliform counts in water and oysters. The relationships of viruses in shellfish to viruses in estuarine sediments should be defined since recent studies (*7,19,44,49*) confirmed that sediments can contribute large numbers of viruses to the water column and possibly to feeding shellfish.

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