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ENTEROVIRUS AND BACTERIAL EVALUATION OF MISSISSIPPI OYSTERS

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ABSTRACT The numbers of enteric viruses and fecal coliform bacteria in oysters and water samples collected along the Mississippi Gulf coast during 1979 were determined. Ten viral isolates, representing members of the poliovirus group, were identified from an approved oyster harvesting site. The number of virus isolations increased to 51 when oysters were collected from a prohibited harvesting location. The majority of isolates were identified as poliovirus type 1 or 2, coxsackievirus B3 and B4, and echovirus type 24. Fecal coliforms in water samples collected at approved and prohibited locations confirmed the classification assigned to each area by the Mississippi State Board of Health. The numbers of fecal coliforms in oyster samples collected at the identical sites did not reflect the levels observed in water samples. There was no positive correlation between indicator bacteria in the water column and the number of viruses in the shellfish examined. These results imply that viral analyses of shellfish may be needed as an adjunct to bacteriological analyses so that shellfish safety is verified.

INTRODUCTION

Since the early 1900s, bacterial indices (as exemplified by the coliform count) have been used to demonstrate the degree of fecal pollution of water, including marine waters (Hunt 1977). As pollution levels have increased, the number of approved shellfish reefs has declined. The shellfish industry has lost ground steadily and today is becoming more dependent on relaying and depuration practices for survival.

Viral contamination of polluted shellfish does occur (Bendenelli and Rucchi 1969, Metcalf and Stiles 1968, Fugate et al. 1975, Denis 1973, Goyal et al. 1979, Gerba and Goyal 1978, Gerba et al. 1979, Portnoy et al. 1975), and usually includes those groups with direct or indirect association with the alimentary tract of man or animals. The characteristics of particular viral groups also allow for survival and transmission via feces. The entero-, reo-, rota- and adenoviruses, and hepatitis A virus all are transmitted through sewage and have the potential to contaminate shellfish growing waters.

Enteric viruses do not reproduce in oysters but are concentrated from the surrounding water when the animal feeds (Chang et al. 1971). The digestive gland of the oyster harbors the greatest viral concentration (Liu et al. 1966) but other tissues will adsorb virus particles. Since polluted oysters usually contain low-level viral contamination, sample extraction and concentration are necessary. Recent investigations (Fugate et al. 1975; Sobsey et al. 1975, 1978; Tierney et al. 1980; Metcalf et al. 1980; Landry et al. 1980; Goyal et al. 1979; Herrman and Cliver 1968a, 1968b; Kostenbader and Cliver 1972; Vaughn et al. 1980; Ellender et al. 1980) have evaluated the extent of viral contamination of shellfish by various methods of extraction.

Many researchers now recognize that fecal coliform counts in surface waters do not reflect the level of viral

contamination of shellfish. Recent investigations (Gerba et al. 1979, Portnoy et al. 1975, Mackowiak et al. 1976) have shown that (1) enteric viruses can be present in acceptable levels, (2) viruses remain viable in the estuarine environment, (3) shellfish concentrate viruses from the surrounding water and prolong virus survival, (4) particulate matter in estuaries decreases virus inactivation, and (5) viruses adsorbed to particulates can be introduced into the water column by changes in the chemical and physical environment.

The purpose of this study was to evaluate fecal coliform and enterovirus levels in oysters taken from both approved and prohibited growing waters along the Mississippi Gulf coast. Data also were collected on the levels of fecal coliforms in surface and bottom water samples. A summary of results of the first year (1978) has been published (Ellender et al. 1980). This report presents the results of the 1979 collections and discusses the fluctuations of fecal coliform and enterovirus levels observed during the 2-year period, 1978–1979.

MATERIAL AND METHODS

Collection of Samples

Composite oyster samples (five 150-gram [gr] lots) (*Crassostrea virginica*) were collected from Pass Christian Reef (approved area) and from Graveline Bayou (prohibited area), and assayed for bacterial and viral levels. Pass Christian Reef was sampled monthly; Graveline Bayou was sampled biweekly. This sampling schedule was based on the premise that the majority of virus isolations would occur in samples of prohibited oysters. These samples were kept cool in an ice chest during transit and shucked upon arrival at the laboratory. Samples were either processed immediately or stored in sterile plastic bags at -75°C. Water samples (surface and bottom) at each location were collected using a J–Z sampler. At the Graveline Bayou location, water samples

were collected at three stations for three days prior to oyster sampling and on the days of oyster sampling. Three stations at the Pass Christian location were sampled on the day of oyster collection.

Temperature and salinity records were collected as described by Ellender et al. (1980).

Analysis of Fecal Coliforms and Viruses

Fecal coliform bacteria were determined in oyster and water samples according to standard methods (American Public Health Association 1970). Virus contamination of oyster samples was determined according to procedures described by Ellender et al. (1980). Briefly, samples were extracted by a modification of the adsorption-elution procedure of Sobsey et al. (1975). Sample concentrates were assayed according to the plaque method in the Buffalo green monkey (BGM) kidney cell line. Plaques were confirmed as viruses by three passages in BGM cells and were identified serologically.

RESULTS

Fecal coliform counts in oyster and water samples taken from Pass Christian and Graveline Bayou stations are summarized in Table 1. The three sampling stations at Pass Christian were located in the same general area and usually contained similar numbers of fecal coliform bacteria in surface and bottom water samples. On occasion, however, differences did exist between samples collected at the two levels, surface and bottom. Fecal coliform counts in the water generally were lower during the summer and fall, but increased during the winter and spring months. The most probable number (MPN) median fecal coliform for all surface and bottom water samples during 1979 was < 2 per 100 ml. Only those samples collected in February and December 1979, contained counts greater than 43 per 100 ml. Fecal coliforms in oysters sampled at Pass Christian ranged from < 20 to 11,000 per 100 gr. The median fecal coliform count of all samples collected during the 12-month period (January through December 1979) was 88 per 100 gr. Except for samples taken in December, there appeared to be no correlation between fecal coliform counts in water and oyster samples collected at the same time in that area.

As with the Pass Christian samples, data collected at all three Graveline Bayou stations on the same day were similar. However, data collected on each of the four consecutive sampling days could vary significantly depending on local meteorological and hydrographical conditions. Of the 24 surface and bottom water median values recorded (Table 1), only one did not exceed a value of 14 per 100 ml. In addition, 66% of the MPN values exceeded 43 per 100 ml, indicating that the "prohibited" classification of Graveline Bayou is warranted. Fecal coliform levels in oysters collected at Graveline ranged from < 20 to 1,700 per 100 gr. The median MPN value for all samples was 140 per 100 gr. This figure was higher than the median value recorded for all oyster samples examined from Pass Christian.

Virus isolations from oysters collected at the approved site are presented in Table 2. From 32 samples representing 343 oysters, 53 plaque-like isolates (PLI) were examined. Of these, ten (19%) were confirmed as viruses; all were classified as poliovirus type 1 or type 2. The largest number of viruses isolated in a single month was eight. These were found in February samples, but this finding was not consistent with virus isolations during other winter months. Two virus isolations were made during June and July. Overall, the low frequency of virus isolation at Pass Christian sampling sites was consistent with the general trend observed in the number of indicator bacteria collected at the same location. This finding is supported by the fact that only 4 of the 32 samples analyzed (13%) contained confirmed virus isolates.

TABLE 1.

Fecal coliform levels in water and oyster samples taken during 1979.

Month	Pass Christian Reef			Graveline Bayou		
	Water Median MPN/100 ml		Oyster	Water Median MPN/100 ml		Oyster
	Surface	Bottom	Mean MPN/100 gr	Surface	Bottom	Mean MPN/100 gr
January	23	20	90	38	25	70
February	47	70	50	650	730	120
March	< 2	3	130	68	75	30
April	27	26	130	420	340	49
May	8	< 2	7,800	48	56	750
June	3	< 2	250	87	60	320
July	< 2	< 2	400	18	13	120
August	< 2	< 2	20	33	34	94
September	< 2	< 2	80	37	36	190
October	< 2	3	40	97	83	140
November	10	3	360	880	700	1,200
December	60	120	3,300	230	260	1,100

TABLE 2.

Natural viral analysis of approved oysters, Pass Christian Reef, 1979.

Month	Number of Oysters/ Number of Samples	Number of Samples Containing Confirmed Virus Isolates	Number of Plaque-like Isolates Total: per 100 gr	Number of Plaques Identified as Viruses Total: per 100 gr
January	30/ 3	0	20.0: 4.4	0.0:0.0
February	31/ 4	2	17.0: 2.8	8.0:1.3
March	29/ 3	0	1.0: 0.2	0.0:0.0
April	23/ 3	0	3.0: 0.7	0.0:0.0
May	31/ 3	0	0.0: 0.0	0.0:0.0
June	28/ 3	1	3.0: 0.7	1.0:0.2
July	36/ 3	1	1.0: 0.2	1.0:0.2
August	29/ 2	0	6.0: 2.0	0.0:0.0
September	36/ 2	0	0.0: 0.0	0.0:0.0
October	23/ 2	0	0.0: 0.0	0.0:0.0
November	28/ 2	0	1.0: 0.3	0.0:0.0
December	18/ 2	0	1.0: 0.3	0.0:0.0
Total	343/32	4	53	10

Of 94 oyster samples (representing 1,043 oysters) collected at Graveline Bayou during 1979, 18 (19%) contained virus particles (Table 3). In prohibited oysters, 673 plaque-like isolates were found; 51 viruses were confirmed. The March samples accounted for 74% of the PLI, and 71% of the confirmed viruses. Viruses also were isolated during the months of April (3), June (1), July (2), August (3), September (5), and December (1). Of all virus isolations at the Graveline Bayou location, 83% were poliovirus types 1 and 2; 7% were represented by coxsackievirus types B3 and B4; 1% by echovirus type 24, and the remaining 9% could not be identified by the serological methods employed.

Table 4 summarizes the physical data collected during 1979. Surface- and bottom-water salinities did not vary significantly on a month-to-month basis at the Pass Christian site. The averages of the salinities recorded during 1979 were 11.5 and 14.7 parts per thousand (ppt) for surface and bottom waters, respectively. In general, the higher values were recorded during the winter and fall months. Salinity extremes were greater in Graveline Bayou than on Pass Christian Reef. Average surface and bottom salinities in Graveline Bayou were 7.8 and 9.2 ppt, respectively. As expected, temperatures were highest from May to September at both sampling locations.

It is clear from a summary of project data (Table 5) that the percentage of samples containing virus is not consistent

TABLE 3.

Natural viral analysis of prohibited oysters, Graveline Bayou, 1979.

Month	Number of Oysters/ Number of Samples	Number of Samples Containing Confirmed Virus Isolates	Number of Plaque-like Isolates Total: per 100 gr	Number of Plaques Identified as Viruses Total: per 100 gr
January	39/ 3	0	17.0: 3.8	0.0:0.0
February	91/ 9	0	42.0: 3.1	0.0:0.0
March	75/10	3	503.0:33.5	*36.0:2.4
April	89/10	2	70.0: 4.7	3.0:0.2
May	88/10	0	0.0: 0.0	0.0:0.0
June	104/10	1	2.0: 0.1	1.0:0.1
July	125/10	2	19.0: 1.3	2.0:0.1
August	117/ 9	3	5.0: 0.4	3.0:0.2
September	114/ 8	3	6.0: 0.5	5.0:0.4
October	88/ 7	0	0.0: 0.0	0.0:0.0
November	74/ 6	0	1.0: 0.1	0.0:0.0
December	21/ 2	1	4.0: 1.3	1.0:0.3
Total	1043/94	18	673	51

*Forty plaques purified and typed.

TABLE 4.

Salinity (ppt) and temperature (°C) measurements, 1979

| | Pass Christian | | | Graveline Bayou | | |
| | Salinity | | | Salinity | | |
Month	Surface	Bottom	Temp.	Surface	Bottom	Temp.
Jan	13	14	6	16	19	7
Feb	7	10	15	2	3	11
Mar	5	5	19	3	3	15
Apr	2	2	20	1	1	19
May	4	8	22	4	3	21
Jun	14	14	29	12	12	26
Jul	8	19	32	4	7	32
Aug	19	19	31	10	11	30
Sep	17	18	29	9	10	30
Oct	19	19	17	15	16	21
Nov	18	18	11	10	13	15
Dec	12	12	9	8	12	7

TABLE 5.

Project summary: 1978–1979.

| | 1978 | | 1979 | | Total | |
	PC[1]	GB[2]	PC	GB	PC	GB
Number of samples examined:	22	87	32	94	54	181
Number of samples containing virus:	2	30	4	18	6	48
Percent of samples containing virus:	9	35	13	19	11	27
Number of months in which viruses were isolated:	2	12	3	7	5	19
Percent of months virus isolated:	17	100	25	58	21	79

[1] Pass Christian (approved) Reef
[2] Graveline Bayou (prohibited) Reef

on a year-to-year basis. Only 9% of 1978 Pass Christian Reef samples contained virus as compared to 13% in the following year. The reverse occurred at the prohibited sampling location (1978, 35%; 1979, 19%). These trends again are observed in the data of the number of months in which viruses were isolated. During the 12 months of 1978, only 2 months (17%) yielded samples containing viruses. This percentage increased to 25% during 1979. All 12 months of 1978, Graveline Bayou samples were positive for virus. This figure, however, dropped to 7 months (58%) during the subsequent 12-month period. Although the number of samples collected at the Graveline Bayou site during the 2-year period was higher than the number of approved-site samples collected, statistical testing did demonstrate that the two locations were significantly different in relation to the number of virus-containing samples that were examined (probability [P] ≤ 0.01).

Tables 6 and 7 indicate the degree of correlation for all measured parameters during the 24 months of sampling. They indicate the inability of fecal coliform water analyses to predict the contamination of shellfish meats by virus particles. At the approved collection site, strong correlations were observed between surface- and bottom-water salinities, temperatures and salinities in the water column, and fecal coliform counts in surface and bottom waters. Plaque-like isolates did correlate with confirmed virus levels. Certain moderate relationships between different parameters also were present in Graveline Bayou samples. Moderate correlations did exist between (1) fecal coliforms in oyster tissue and the number of PLI observed, and (2) confirmed virus isolations and the month of the year. A weak positive correlation was observed between the numbers of plaque-like isolates and the numbers of confirmed viruses.

DISCUSSION

The results of this study stress the need to better understand the complex relationships of shellfish virology. Virus isolations during the 2-year period were variable and often clustered in small groups of samples. This suggests that intermittent contamination, rather than chronic contamination, of shellfish-growing waters plays a significant role in the level of contamination observed and may require frequent sampling by health agencies responsible for classification of shellfish-growing waters. Graphic representations of fecal coliform analysis of water and oyster samples followed cyclic patterns which generally were higher during the winter and summer months at each of the locations tested. However, such patterns were not related to the individual month of sampling and they underscore the need to analyze samples frequently from a shellfish-harvesting area for reef classification.

Those data suggest that virus levels in shellfish are not always consistent with the levels of indicator bacteria used to classify estuarine growing areas. This failure of the bacterial-indicator concept lends credence to the search for a virus which could signify a potentially dangerous situation. Often polioviruses are suggested as a representative group since they are commonly found in human feces as a result of widespread vaccination. In this study, the majority of oyster-associated viruses were poliovirus type 1. Type 2 was isolated infrequently. Since there are three serotypes in the vaccine in approximately equal proportions, the frequent isolation of a single type from oyster samples could mean that the extraction method is more selective for that serotype, that the viruses are not shed in equal numbers, or that the estuarine environment or the shellfish population favors the viability on only certain strains. Laboratory experiments do not suggest that extraction procedures are selective, but other work (R. D. Ellender and D. W. Cook, unpublished) has implied that oysters do not take

TABLE 6.

Correlation Coefficients: Pass Christian Reef
January 1978 to December 1979

	Month	Water Salinity Surface	Water Salinity Bottom	Temperature °C	Fecal Coliforms Surface Water 100 ml	Fecal Coliforms Bottom Water 100 ml	Fecal Coliforms Oyster Tissue 100 gr	Plaque-like Isolates: 100 gr	Confirmed Virus Isolates: 100 gr
Month	1.00000								
Water salinity									
Surface	*0.41101	1.00000							
Bottom	0.32886	*0.92442	1.00000						
Temperature °C	0.36257	*0.67603	*0.88680	1.00000					
Fecal coliforms:									
Surface water:									
100 ml	0.28354	0.13814	0.15305	0.13364	1.00000				
Bottom water:									
100 ml	0.11745	0.15036	0.16546	0.15509	*0.75231	1.00000			
Oyster tissue:									
100 g	0.01410	0.03685	0.07147	0.08420	0.10549	0.00211	1.00000		
Plaque-like									
isolates: 100 gr	0.22307	0.15595	0.20168	0.23140	0.08989	0.07562	0.24259	1.00000	
Confirmed virus									
isolates: 100 gr	0.18918	0.09406	0.11528	0.11215	0.08654	0.08488	0.13768	*0.78575	1.00000

*Numbers significant at $P \leqslant 0.05$ level of significance.

TABLE 7.

Correlation Coefficients: Graveline Bayou
January 1978 to December 1979

	Month	Water Salinity Surface	Water Salinity Bottom	Temperature °C	Fecal Coliforms Surface Water 100 ml	Fecal Coliforms Bottom Water 100 ml	Fecal Coliforms Oyster Tissue 100 gr	Plaque-like Isolates: 100 gr	Confirmed Virus Isolates: 100 gr
Month	1.00000								
Water salinity									
Surface	0.18698	1.00000							
Bottom	0.21181	*0.96757	1.00000						
Temperature °C	0.02834	0.00204	0.07350	1.00000					
Fecal coliforms:									
Surface water:									
100 ml	0.14853	0.02910	0.08070	0.07254	1.00000				
Bottom water:									
100 ml	0.03053	0.21243	0.18425	0.40043	*0.48042	1.00000			
Oyster tissue:									
100 g	0.31583	0.22894	0.21780	0.03852	0.10075	0.15296	1.00000		
Plaque-like									
isolates: 100 gr	0.27614	0.10952	0.11902	0.21291	0.09917	0.12708	*0.55320	1.00000	
Confirmed virus									
isolates: 100 gr	*0.55797	0.19078	0.19876	0.10390	0.22032	0.07284	0.05285	*0.45160	1.00000

*Numbers significant at $P \leqslant 0.05$ level of significance.

up coxsackievirus A-9 or echovirus type 3 as efficiently as poliovirus type 1.

These studies also demonstrate that classification of oyster-growing waters eventually may require additional changes which relate to virus contamination. The approved location examined in this study did contain low-level virus concentrations. If this observation is true for other "approved" oyster beds, the use of the fecal coliform

indicator concept will lose credibility.

Recent studies (R. D. Ellender, unpublished) suggest that viruses which enter the marine ecosystem adsorb to estuarine sediments which preserve their infectivity. Sediment may act as virus reservoirs which, when influenced by chemical or physical action, release virus back into the water column. Shellfish contamination may thus occur by a mechanism which is independent of the numbers of indicator bacteria in the growing waters. If it can be shown that viruses or fecal coliforms in sediments do correlate with virus numbers in shellfish, an additional mechanism will be available to predict potential disease outbreaks from the consumption of shellfish products.

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