MICROBIAL FLORA AND LEVEL OF *VIBRIO PARAHAEMOLYTICUS* OF OYSTERS *(CRASSOSTREA VIRGINICA)*, WATER AND SEDIMENT FROM GALVESTON BAY[a]

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ABSTRACT

Aerobic plate counts at 25 C of freshly harvested oysters ranged from 2.3×10^4 to 3.0×10^7 and those of sediment samples from $<10^2$ to 3.0×10^6/g. Counts of water samples were nearly always $<10^2$/ml. *Vibrio, Aeromonas,* and *Moraxella* species predominated in the fresh oysters. *Vibrio parahaemolyticus* was isolated from 39 of 66 oyster samples and from 9 of 30 sediment and water samples. Isolation was most effective with prior enrichment of samples in trypticase soy broth with 7% NaCl and subsequent plating on thiosulfate citrate bile salts sucrose agar. *V. parahaemolyticus* was detected in only 1 of 8 refrigerated retail oyster samples. Aerobic plate counts at 25 C of refrigerated retail oysters were not much different from those of similar lots shucked under aseptic conditions in the laboratory (before shucking and washing in the plants). *Aeromonas* and *Moraxella* species were predominant in oysters at the retail level.

For many years isolations of *Vibrio parahaemolyticus* seemed to be limited to Japan where it is a major cause of gastroenteritis associated with consumption of seafoods particularly during summer months *(19)*. In recent years this organism has been isolated from shellfish and marine environments in the United States and many other countries *(2-4, 7-10, 16, 22, 32, 33)*. Although *V. parahaemolyticus* was the probable cause of several unconfirmed outbreaks of foodborne illness in the United States in 1969 *(25)*, the first confirmed outbreaks occurred in 1971 which involved consumption of crab *(14, 26)*. Other seafood-associated outbreaks of gastroenteritis caused by *V. parahaemolyticus* have been reported in 1972 *(27)*. Isolations of *V. parahaemolyticus* from oysters have been reported from widely different marine environments in the United States *(3, 4, 10, 22, 30)*. A seasonal incidence of this organism with a peak during the summer months is reported for oysters and/or water from Puget Sound *(3)*, Great Bay and Little Bay areas of New Hampshire *(4)*, and Chesapeake Bay *(8)*. A similar distribution was reported from Japan *(13)* and the Baltic Sea *(16)*.

Foodborne illness caused by *V. parahaemolyticus* in Japan usually is associated with consumption of raw seafoods. In the United States most seafoods are subjected to some heat treatment before consumption. Under these conditions outbreaks were caused by gross mishandling of the food. The oyster is the only mollusc that is generally eaten raw. Landings in the United States in 1971 were 54.6 million pounds valued at $30 million *(24)*. To evaluate the potential of oysters in foodborne illness, information is needed concerning the distribution of *V. parahaemolyticus* in oysters from sub-tropical waters such as the Northern Gulf of Mexico. Information about the effect of commercial handling practices on survival and/or growth of *V. parahaemolyticus* in oysters is scarce. With respect to the microbial flora of oysters, reports by Colwell and Liston *(5)*, Lovelace et al. *(11)*, and Murchelano and Brown *(15)* indicate that *Pseudomonas, Vibrio, Achromobacter, Alcaligenes,* and *Flavobacterium* species were predominant. The range for gram-positive forms was 15 to 20%. This report provides information about the level and type of microbial flora of oysters and the distribution of *V. parahaemolyticus* in oysters, water, and sediment.

MATERIALS AND METHODS

Samples

All samples were taken from various approved areas in Galveston Bay by personnel of the Texas State Department of Health at LaMarque or of the Marine Laboratory, Texas A&M University at Galveston, Tex. Oysters were collected in sterile plastic bags and transported to the laboratory in an insulated container at 5-10 C. Samples usually were examined within 6 to 12 hr after collection. Commercial oyster samples in the market surveys were obtained from two processing plants in the Texas Gulf Coast area. Preparation of oysters for microbiological examination was as described in *Recommended Procedures for the Examination of Sea Water and Shellfish (1)*.

[a]Published with the approval of the Director of the Texas Agricultural Experiment Station, College Station.

Microbiological procedures

The shucked oysters were blended for 2 min in a sterile Waring blendor. A 50-g portion then was blended for 1.5 min with 450 ml of sterile 0.1% trypticase peptone (BBL) with 3% NaCl. Bacterial counts were determined with the spread plate method by placing 0.1 ml of appropriate dilutions on trypticase soy agar (TSA, BBL) plates with 3% NaCl. Plates were incubated at 25 C for 2 days. To determine microbial types, approximately 40 colonies were picked at random from countable plates. Diagnostic procedures and schemes for identification of the microbial flora were presented in a previous report (29).

Isolation of *V. parahaemolyticus* was accomplished by placing appropriate quantities of oyster-trypticase-peptone homogenate (equivalent to 1 to 10^{-4} g of oyster) in trypticase soy broth (TSB, BBL) with 7% NaCl. After 24 hr at 42 C, the tubes were streaked with a wire loop on thiosulfate citrate bile salts sucrose agar (TCBS, BBL) plates and also on a starch medium originally proposed by Twedt et al. (23) and later modified by Vanderzant and Nickelson (30). The latter medium, designated MT, consisted of 2% trypticase peptone, 0.2% yeast extract, 1% corn starch, 7% NaCl, and 1.5% agar (pH 8.0). Isolation was also made without enrichment by spreading 0.1 ml of oyster-trypticase homogenate and appropriate dilutions directly on the surface of MT plates. Plates were incubated aerobically at 42 C for 24 to 48 hr. Blue-green colonies from TCBS medium and white to creamy, circular, smooth, amylase-positive colonies from MT medium were picked as suspect *V. parahaemolyticus*. The tests applied to these isolates and typical reactions for *V. parahaemolyticus* are as follows: Gram reaction (neg.), morphology (rods, exhibiting pleomorphism), cytochrome oxidase (+), catalase (+), starch hydrolysis (+), triple sugar iron agar (alkaline/acid, H_2S-, gas-), lysine decarboxylase (+), ornithine decarboxylase (+), lysine deaminase (−), NH_3 from arginine (−), growth in 1% trypticase broth with 0, 3, 7, 8, and 10% NaCl (−+++−), indole production (+), methyl red (+), Voges-Proskauer (−), motility (+), nitrate reduction (+), citrate utilization (+), urease (−), gelatin liquefaction (+), sensitive to pteridine 0/129 (+) and novobiocin (+), acid from sucrose (−), lactose (−), mannitol (+), arabinose (+), and cellobiose (∓). Hemolytic activity against fresh human erythrocytes (Kanagawa test) and serological identification were also employed. Methods are described in detail in the *Bacteriological Analytical Manual for Foods* (28) and by Vanderzant and Nickelson (30).

RESULTS

Bacterial counts of freshly harvested oysters ranged from 2.3×10^4 to 3.0×10^7/g with an average (geometric) count of 4.0×10^6/g (Fig. 1). Bacterial counts of sediment samples ranged from $<10^2$ to 3.0×10^6/g, those of water samples were, except for one, always $<10^2$ per ml. The temperature of the waters from which the oysters were taken ranged from 12 C in November to 30 C in June, July, and September (Fig. 2). The salinity of the water ranged from 7 ppt in May to 26 ppt in April (Fig. 3).

In general, species of *Vibrio, Aeromonas,* and *Moraxella* were predominant in freshly harvested oysters (Table 1). These species constituted 20% or more of the microbial flora in 5 to 8 of the 12 monthly

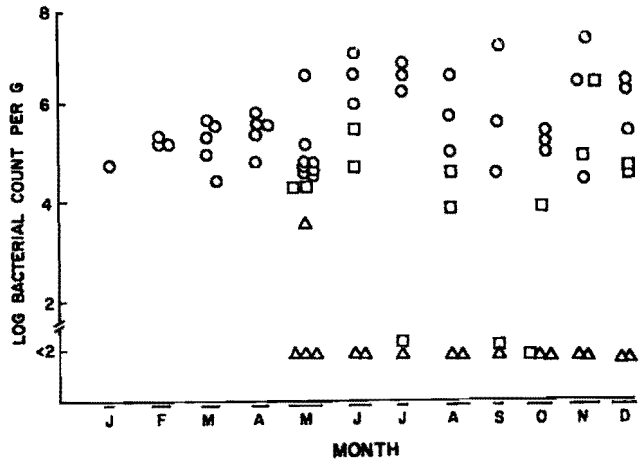

Figure 1. Aerobic plate count of freshly harvested oysters (open circles), water (open triangles), and sediment (open squares) samples.

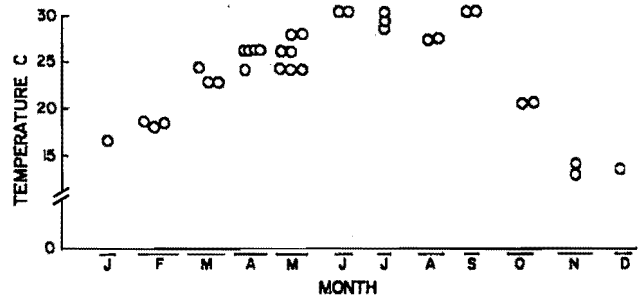

Figure 2. Temperature of waters from which oysters were taken.

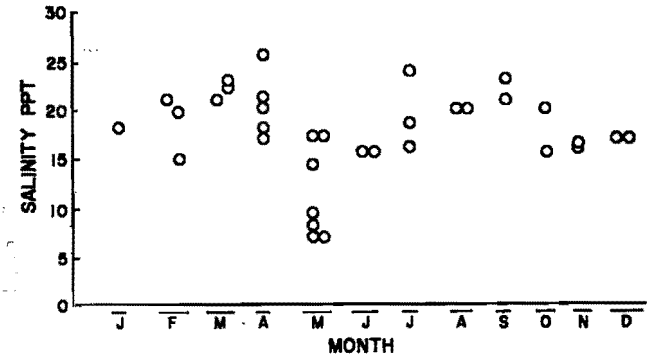

Figure 3. Salinity of waters from which oysters were taken.

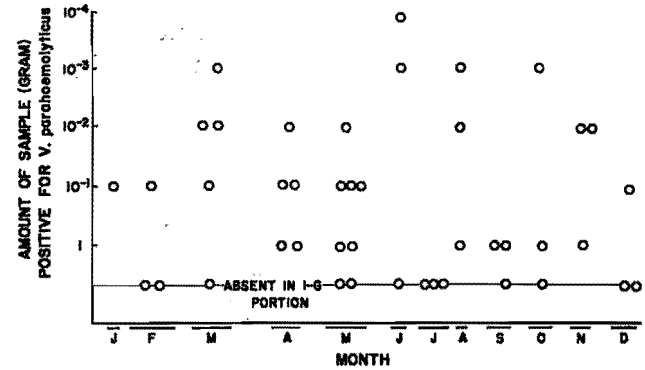

Figure 4. Level of *V. parahaemolyticus* in oysters from January through December 1972.

TABLE 1. DISTRIBUTION OF MICROBIAL FLORA OF 31 OYSTER SAMPLES

Type	% of total flora		Type present in no. of samples
	Range	Average	
Vibrio	0-80.8	22.3	23
Aeromonas	0-71.4	21.2	25
Moraxella	0-65.0	18.9	24
Pseudomonas	0-79.3	14.9	25
Acinetobacter	0-57.5	8.7	20
Coryneforms	0-63.3	7.1	13
Flavobacterium	0-30.0	1.5	5
Achromobacter	0-14.7	1.5	8
Bacillus	0-7.5	<1	3
Micrococcus	0-2.5	<1	1
Staphylococcus	0-2.5	<1	1
Enterobacteriaceae	0-32.5	3.2	8

TABLE 2. NUMBER OF SUSPECT AND CONFIRMED *V. parahaemolyticus* ISOLATES FROM OYSTERS, WATER, AND SEDIMENT SAMPLES[a]

Isolates	Oysters	Water & sediment
Suspect *V. parahaemolyticus*	278	41
No. and % of *V. parahaemolyticus*	80(29)	20(50)
No. and % of Kanagawa-pos. *V. parahaemolyticus*	16(20)	1(5)

[a]Includes isolates from MT medium with direct plating and from MT and TCBS media with enrichment in TSB (7% NaCl).

TABLE 3. NUMBER OF SAMPLES OF OYSTERS, WATER, AND SEDIMENT WITH SUSPECT AND CONFIRMED *V. parahaemolyticus* AS DETERMINED ON MT AND TCBS MEDIA

No. and type of sample	Method used		
	MT[a]	MT(ER)[b]	TCBS(ER)[c]
Oysters, suspect[d] 66	2	52	59
Oysters, confirmed 39	2	16	34
Water and sediment, suspect 16	1	11	13
Water and sediment, confirmed 9	0	5	7
Total suspect, 82	3	63	72
Total confirmed, 48	2	21	41

[a]MT = Sample plated directly on MT medium.

[b]MT(ER) = Sample plated on MT medium following enrichment in TSB (7% NaCl).

[c]TCBS(ER) = Sample plated on TCBS agar following enrichment in TSB (7% NaCl).

[d]Includes 43 samples of freshly harvested oysters shucked in the laboratory and 23 samples of commercially shucked oysters.

averages. Large variations between samples were noted for *Vibrio, Aeromonas, Moraxella, Pseudomonas, Acinetobacter,* coryneform bacteria, and *Flavobacterium. Bacillus Micrococcus, Staphylococcus,* and *Enterobacteriaceae* were not often detected and only in low numbers.

V. parahaemolyticus was present (in 1 to 10^{-4}-g portions) in 30 of the 43 freshly harvested oysters. In only a few of the samples was *V. parahaemolyticus* present in 0.001-g portion (Fig. 4). *V. parahaemolyticus* was detected in 9 of the 30 water and sediment samples and only in 1- or 0.1-g portions.

A comparison of the frequency of isolation of *V. parahaemolyticus* with three different procedures showed that by direct plating on MT medium *V. parahaemolyticus* was confirmed in only 2 of the 43 oyster samples. With prior enrichment in TSB (7% NaCl) and subsequent plating on MT medium, *V. parahaemolyticus* was detected in 13 samples, with a similar enrichment but plated on TCBS agar in 25 samples. It should be recognized that by direct plating on MT medium, 0.1 ml of a 1:10 dilution of the sample was spread on the plates, which will not detect very low levels of *V. parahaemolyticus.* A comparison of the levels of *V. parahaemolyticus* in oysters as determined by plating enrichments (TSB, 7% NaCl) on MT and TCBS agar media showed higher levels on TCBS agar with 18 samples, higher levels on MT medium with 6 samples, and a similar level with 6 samples.

Many of the typical colonies, both on MT and TCBS agar media, which were picked for further identification were "false-positive." With enrichment in TSB, the percentage of false-positive isolates for TCBS agar (33%) was much smaller than on MT medium (72%). Many of those on MT medium were *V. alginolyticus.* The number of suspect *V. parahaemolyticus* isolates and the percentage confirmed are presented in Table 2. Only 29% of the suspected isolates could be confirmed by further testing. Twenty percent of the confirmed *V. parahaemolyticus* showed beta hemolysis with fresh human erythrocytes (Kanagawa reaction).

The number of samples with suspect and confirmed *V. parahaemolyticus* is given in Table 3. *V. parahaemolyticus* was confirmed in 39 of 66 oyster samples and in 9 of 30 water and sediment samples. Percentage of confirmation was greater (57 versus 33%) when isolates were picked from TCBS plates as compared with MT(ER) plates.

Eight market surveys were conducted between May to December 1972 in which oysters were sampled at various stages during processing and marketing (Table 4). Aerobic plate counts of oysters in the shell (shucked in the laboratory) ranged from 1.5×10^5 to 3.0×10^7/g with an average (geometric) count of 3.9×10^6/g. Bacterial counts of identical lots at the retail level were similar or slightly lower than before processing. *V. parahaemolyticus* was present at low levels (0.01- to 1-g portion) in 5 of the 8 freshly harvested samples but none of these samples contained *V. parahaemolyticus* (in 1-g portion) at

TABLE 4. AEROBIC PLATE COUNT AND LEVEL OF *V. parahaemolyticus* IN OYSTERS AT VARIOUS STAGES DURING PROCESSING AND MARKETING IN 8 MARKET SURVEYS

Sample	APC per g[a]	Portion of sample (g) positive for *V. parahaemolyticus* [TCBS(ER)][b]							
In shell (shucked in lab.)	1.5×10^5-3.0×10^7 (Avg. 3.9×10^6)	1	10^{-2}	–[c]	10^{-2}	–	–	1	10^{-1}
After shucking	3.0×10^5-6.5×10^6 (1.3×10^6)	–	–	1	–	–	–	10^{-1}	1
After shucking and washing	6.7×10^4-3.0×10^5 (5.0×10^5)	–	1	10^{-3}	1	–	1	–	1
At retail level	8.2×10^4-8.7×10^6 (6.0×10^5)	–	–	10^{-3}	NT[d]	–	–	–	–

[a]APC = Aerobic plate count
[b]TCBS(ER) = Level of *V. parahaemolyticus* by plating on TCBS medium following enrichment in TSB with 7% NaCl.
[c]1-g portion negative for *V. parahaemolyticus*.
[d]NT = Not tested.

TABLE 5. DISTRIBUTION OF MICROBIAL FLORA OF OYSTERS BEFORE SHUCKING AND AT RETAIL LEVEL IN 8 MARKET SURVEYS

Microbial Type	Before shucking[a]			At retail level		
	% of total flora		Type present in no. of samples[b]	% of total flora		Type present in no. of samples[c]
	Range	Average		Range	Average	
Vibrio	0-25.0	5.8	3	0-60.0	15.4	3
Aeromonas	0-71.4	37.3	5	0-44.4	18.9	6
Moraxella	3.6-41.4	26.5	6	2.5-37.5	19.8	7
Pseudomonas	0-46.7	11.7	4	0-12.5	6.1	5
Acinetobacter	0-13.3	5.1	3	0-60.0	12.1	4
Coryneforms	0-44.8	7.9	2	0-85.0	13.2	2
Flavobacterium	0-7.5	2.3	3	0-52.9	8.3	2
Achromobacter	0	0	0	0-7.5	1.6	2
Bacillus	0-6.9	1	1	0	0	0
Micrococcus	0-2.5	<1	1	0	0	0
Staphylococcus	0	0	0	0-2.5	<1	1
Enterobacteriaceae	0-5.0	2	2	0-18.5	4.5	3

[a]Shucked under aseptic conditions in laboratory.
[b]Based on 6 samples.
[c]Based on 7 samples.

the retail level. In one oyster sample *V. parahaemolyticus* was present in 0.001-g portion after shucking and washing and at the retail level, and not before shucking and washing. The cause of this is not known. A comparison of the microbial flora of oysters shucked in the laboratory (market survey samples) and shucked, washed oysters in cans at the retail level (Table 5) indicates that species of *Aeromonas* and *Moraxella* predominated.

DISCUSSION

In this study, *V. parahaemolyticus* was present in 70% of the freshly harvested oysters and in 30% of the water and sediment samples. Although the highest level of *V. parahaemolyticus* was recorded in June, no definite seasonal incidence was apparent. This is probably because of the relatively high water temperature in Galveston Bay as compared with Puget Sound, Chesapeake Bay, and estuaries of New Hampshire where numbers are greater in summer months and decrease sharply when water temperatures drop below 15 C *(3, 4, 8)*.

In general, *V. parahaemolyticus* was detected more frequently and at somewhat higher levels by plating on TCBS agar following enrichment of samples in TSB with 7% NaCl. In addition, fewer false-positive isolates were picked from TCBS than from MT medium. The percentage of oyster samples positive for *V. parahaemolyticus* and the level of this organism were somewhat lower than reported by Baross and Liston *(3)*. This probably resulted from a difference in classification of *V. parahaemolyticus*. In their study, hemolytic vibrios meeting the general classification of *V. parahaemolyticus* without regard to sucrose fermentation were reported as *V. parahaemolyticus*. In the present study only isolates with all basic characteristics of *V. parahaemolyticus* including lack of sucrose fermentation, identical to Sakazaki's biotype 1 *(20)*, were included as confirmed *V. parahaemolyticus*. If samples with sucrose-fermenting strains of "*V. parahaemolyticus*" had been included, 94% of the freshly harvested samples would have contained this organism. Japanse workers *(12, 21)* reported that 88-96% of *V. parahaemolyticus* cultures isolated from patients with gastroenteritis are beta-hemolytic against fresh human erythrocytes (Kanagawa-positive), but only 0.5-1% of the isolates from raw fish. In their opinion, human pathogenicity is closely related to this specific hemolytic activity. Administration of non-hemolytic strains to human volunteers confirmed their lack of pathogenicity. In our

study, 20% of fresh isolates of *V. parahaemolyticus* from oysters were Kanagawa-positive. Some loss of hemolytic activity occurred when these isolates were maintained on laboratory media for 6 to 12 months. Pathogenicity of these strains to humans has not yet been established. A majority (79%) of *V. parahaemolyticus* isolates from oysters, water, and sediment were serologically nontypable.

Reduction in the level of *V. parahaemolyticus* after processing at the retail level was probably caused by a dilution effect of water during washing after shucking and the sensivity of this organism to refrigeration *(6, 31)*. In addition, washing may have reduced the salinity of the surface area of the oyster. *V. parahaemolyticus* is unstable without salt and is destroyed readily under these conditions *(6)*. No relation could be established between level of *V. parahaemolyticus* and aerobic plate count.

Oysters harvested from approved waters usually are of acceptable quality at time of harvesting with areobic plate counts (APC) at 35 C on Standard Methods agar (SMA) ranging from a few hundred to a few thousand per gram. High aerobic plate counts of shucked oysters at the wholesale level reflect microbial growth in the interval between harvesting and shucking, and improper handling or inadequate refrigeration during processing and shipping *(17, 18)*. To be acceptable at this point the APC at 35 C must not exceed 5×10^5/g with a fecal coliform density (MPN) of not more than 230/100 g. The relatively high bacterial counts of freshly harvested oysters in this study most likely reflect certain changes in the agar plate method such as *(a)* plate incubation at 25 C, *(b)* use of TSA as compared with SMA plating medium, and *(c)* inclusion of NaCl in the plating medium, which allowed a greater number of bacteria to develop. A comparison of bacterial counts of a limited number of samples on TSA (3% NaCl, at 25 C) and SMA (no added NaCl, at 35 C) showed counts on TSA 1.6 to 3.7 logs higher. The average (geometric) count on TSA was 4×10^5, that on SMA 10^3 per g. Higher counts on TSA also may have resulted from improved recovery of sublethally injured cells. Colwell and Liston *(5)* also reported higher counts with plate incubation at 25 than at 37 C.

Which of the bacterial counts, at 35 C on SMA without salt or at 25 C on TSA with salt provides more useful information is a controversial point. High viable counts at 35-37 C in seafoods usually indicate unsanitary handling. High viable counts at 20-25 C reflect increases in count of psychrotrophic and some mesophilic species initially present on the freshly harvested seafood or acquired by contact with contaminated equipment or surfaces in the plant. These species are frequently responsible for quality deterioration during refrigerated storage. For some of these species a temperature of 35-37 C may constitute the upper limit for growth on laboratory media. In addition, some species of the natural microbial flora of shellfish exhibit a partial or complete salt dependence as demonstrated by a growth stimulating effect of NaCl added to standard media *(5)*.

The bacterial counts of freshly harvested oysters from Galveston Bay were somewhat higher (geometric mean 4×10^5/g) than counts of oyster gill tissue (10^3-10^4/g) or mantle fluid (10^4/ml) from Chesapeake Bay oysters *(5)*. This difference in count may have been caused by differences in marine environment or enumeration techniques. Bacterial counts of oysters in excess of 10^6/g were recorded more often from May to September probably because of higher air and water temperatures. The slightly lower counts of oysters at the retail level as compared with the shellstock probably reflects the effacts of sanitary handling, washing, and adequate refrigeration.

The microbial flora of freshly harvested oysters from Galveston Bay was dominated by gram-negative rods (*Vibrio, Aeromonas, Moraxella, Pseudomonas, Acinetobacter*) and was similar to that of Pacific oysters (*Crassostrea gigas*) from Washington State *(5)* and oysters (*Crassostrea virginica*) from Long Island Sound and Chesapeake Bay *(11, 15)*. In addition to *V. parahaemolyticus, V. alginolyticus* and *V. anguillarum* were isolated from oysters. Among *Vibrio, V. alginolyticus* and *V. parahaemolyticus* usually were predominant throughout the year. *V. alginolyticus* predominated in August and September. Baross and Liston *(3)* reported that *V. alginolyticus* was abundant in the summer only which suggests that the temperature of the water is a critical factor.

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