

Seasonal Abundance of Total and Pathogenic *Vibrio parahaemolyticus* in Alabama Oysters

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Recent *Vibrio parahaemolyticus* outbreaks associated with consumption of raw shellfish in the United States focused attention on the occurrence of this organism in shellfish. From March 1999 through September 2000, paired oyster samples were collected biweekly from two shellfish-growing areas in Mobile Bay, Ala. The presence and densities of *V. parahaemolyticus* were determined by using DNA probes targeting the thermolabile hemolysin (*tlh*) and thermostable direct hemolysin (*tdh*) genes for confirmation of total and pathogenic *V. parahaemolyticus*, respectively. *V. parahaemolyticus* was detected in all samples with densities ranging from <10 to 12,000 g⁻¹. Higher *V. parahaemolyticus* densities were associated with higher water temperatures. Pathogenic strains were detected in 34 (21.8%) of 156 samples by direct plating or enrichment. Forty-six of 6,018 and 31 of 6,992 *V. parahaemolyticus* isolates from enrichments and direct plates, respectively, hybridized with the *tdh* probe. There was an apparent inverse relationship between water temperature and the prevalence of pathogenic strains. Pathogenic strains were of diverse serotypes, and 97% produced urease and possessed a *tdh*-related hemolysin (*trh*) gene. The O3:K6 serotype associated with pandemic spread and recent outbreaks in the United States was not detected. The efficient screening of numerous isolates by colony lift and DNA probe procedures may account for the higher prevalence of samples with *tdh*⁺ *V. parahaemolyticus* than previously reported.

Vibrio parahaemolyticus is a gram-negative, halophilic bacterium that occurs naturally in estuarine environments worldwide (21). Its densities in the environment and seafoods vary greatly by season, location, sample type, fecal pollution, and analytical methodology (5, 8–10, 14, 21, 22, 36). Pathogenic *V. parahaemolyticus* generally produces a thermostable direct hemolysin (TDH), the product of the *tdh* gene (20). More than 90% of clinical *V. parahaemolyticus* isolates but fewer than 1% of food or environmental strains produce TDH or possess *tdh* (9, 10, 14, 22, 26, 31, 35). The frequency of TDH or *tdh* detection in environmental samples and seafoods ranges from 0 to 6% (5, 14, 22, 26, 31, 34). Quantitative data for *tdh*⁺ *V. parahaemolyticus* have been reported in only one study and are based on one or two isolates from each of four oyster samples (10).

A series of oyster-associated outbreaks of *V. parahaemolyticus* (3, 4, 6, 7) in 1997 (Washington State) and 1998 (Texas, New York and Connecticut, and Washington State) prompted the development of nonradioactive DNA probes (28, 29) and direct-plating methods for rapid and efficient determination of the abundance of total and pathogenic *V. parahaemolyticus* in oysters. The Food and Drug Administration (FDA) initiated a surveillance program using these new methods in Alabama in March 1999. The objective of this study was to correlate quantitative data on the abundance of total and pathogenic *V. parahaemolyticus* with each other and with environmental pa-

rameters such as water temperature and salinity. Direct-plating methods were used to collect quantitative data for total and *tdh*⁺ *V. parahaemolyticus*. Additionally, the prevalence of total and *tdh*⁺ *V. parahaemolyticus* was determined as described in the FDA *Bacteriological Analytical Manual* (15), especially when levels were below the detection sensitivity of direct-plating methods. Pathogenic strains were serotyped to determine the prevalence of specific strains and the potential presence of outbreak strains.

MATERIALS AND METHODS

Sample collection. Oysters were collected biweekly with hand tongs from a small boat at two sites near Mobile Bay (Fig. 1) from March 1999 through September 2000. One site was on Cedar Point Reef (lower salinity) in the Mississippi Sound, and the other was in Dauphin Island Bay (higher salinity). Both sites are in National Shellfish Sanitation Program conditionally approved shellfish-growing areas with commercial harvesting. Oysters (25 from each site) were placed on top of bagged ice in insulated chests, transported to the FDA Gulf Coast Seafood Laboratory, and analyzed within 3 h of collection. Bottom (1 to 2 m) water temperature and salinity were determined with a model 85 salinometer (YSI, Yellow Springs, Ohio).

Enrichment and isolation of *V. parahaemolyticus*. The formulation of the bacteriological media used, unless listed, was that in the FDA *Bacteriological Analytical Manual* (11), and the source was Difco Laboratories, Sparks, Md., unless stated otherwise. Paired samples of oysters (10 to 12 oysters/sample) from each site were scrubbed, shucked, diluted 1:1 with alkaline peptone water (APW), and blended for 90 s in a Waring blender (Waring, Hartford, Conn.). A 50-g portion of the APW-oyster homogenate was placed into a 1-liter Erlenmeyer flask containing 200 ml of APW (1:10) and incubated overnight at 35°C. Four thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates were streaked from each flask of APW-oyster enrichment for *V. parahaemolyticus* isolation (one 1.0- μ l loopful was taken from the surface pellicle, one was taken from the bottom, and two were taken after the flask was swirled). TCBS plates were incubated overnight at 35°C; up to 48 typical green (sucrose-negative) colonies were transferred with sterile toothpicks to either the first six or the last six

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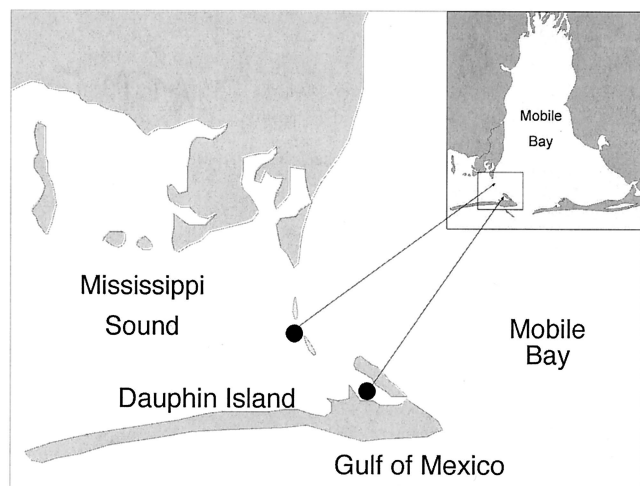


FIG. 1. Harvest sites near Mobile Bay.

columns of a 96-well plate preloaded with 100 μ l of APW. The 96-well plates were incubated at 35°C on a rocker for 4 to 6 h. Growth was transferred to duplicate *Vibrio vulnificus* agar (VVA) plates (37) with a 48-prong replicator (Bokel Industries, Feasterville, Pa.). The VVA plates were incubated overnight at 35°C.

Direct plating for *V. parahaemolyticus*. T₁N₃ agar (1% Tryptone, 3% NaCl, 2% agar) plates were spread plated in triplicate with 0.1-g (0.2 g of 1:1 APW-oyster homogenate) and 0.01-g (100 μ l of 1:10 APW-oyster homogenate from the flask described above) portions of homogenized oyster tissue. T₁N₃ spread plates were incubated overnight at 35°C; colony lifts for DNA probes were prepared as described below.

Colony hybridization for total and pathogenic *V. parahaemolyticus*. Colony lifts were prepared by placing Whatman 541 filters (8.5-cm diameter) onto appropriate VVA (enrichment) and T₁N₃ (direct plating) plates and applying gentle pressure with a plastic spreading rod. Colonies were tested for the presence of the thermolabile hemolysin (*tlh*) gene and the thermostable direct hemolysin (*tdh*) gene to verify the identity of the *V. parahaemolyticus* species and its pathogenicity, respectively (28, 29). Positive and negative controls spotted onto a separate strip of Whatman 541 filter paper were placed into each bag of five filters and included *V. parahaemolyticus* strains TX-2103, SPRC-10290, and GCSL-DI-B9 (*tlh*⁺ *tdh*⁺); Dal-1094 (*tlh*⁺ *tdh*); and *V. vulnificus* MO6-24 and *V. hollisae* 8393 (*tlh* *tdh*). Alkaline phosphatase-labeled DNA probes for the *tlh* and *tdh* genes were obtained from DNA Technology (Aarhus, Denmark); the probe sequences and hybridization conditions were as previously described (28, 29).

Strain characterization. Isolates hybridizing with both the *tlh* and *tdh* DNA probes were recovered and purified on T₁N₃ agar. Isolates were identified as *V. parahaemolyticus* with API 20E diagnostic strips (bioMérieux, Hazelwood, N.J.). In addition to API 20E testing, urease production was also determined with Christensen's urea agar (17). Both DNA probes (28, 29) and a PCR method (2) were used to test for the presence of *tdh*, while the presence of *trh* and the thermolabile direct hemolysin gene (*tlh*), which is species specific, was determined by multiplex PCR (2). Test strains were grown, and their O:K serovars were determined by using specific antisera (Denka, Seiken Corp., Tokyo, Japan) as previously described (15).

Statistical analysis. Total *V. parahaemolyticus* counts were analyzed by regression of the mean log₁₀ density of replicate samples against environmental factors (water temperature, salinity, and harvest site). The effect of temperature was further summarized by rank correlation and the use of a smoothing technique (moving average) in which *V. parahaemolyticus* counts corresponding to temperatures within a range of 5°C were pooled to estimate geometric mean counts at the midpoint of successive intervals. Nondetectable counts (<10 CFU/g) of total *V. parahaemolyticus* were replaced by half of the limit of detection (5 CFU/g). Environmental effects on *tdh*⁺ *V. parahaemolyticus* counts were investigated by generalized linear regression. Counts of *tdh*⁺ *V. parahaemolyticus* were not log₁₀ transformed because of the high prevalence of nondetectable samples; they were modeled as either Poisson or binomially distributed, with *P* values adjusted for overdispersion by the method of scaling predicted variance by the deviance (30). Differences between the frequencies of detection of *tdh*⁺ *V. parahaemolyticus* by

direct plating versus enrichment were evaluated by McNemar's test, and differences in the frequency of detection of pathogenic *V. parahaemolyticus* at different sites were evaluated by Fisher's exact test. All statistical analyses were performed with the Statistical Analysis System (SAS Institute, Cary, N.C.).

RESULTS

Similar seasonal trends in total *V. parahaemolyticus* abundance at the two harvest sites are demonstrated in Fig. 2A (Cedar Point) and B (Dauphin Island Bay). In 1999, *V. parahaemolyticus* densities in oysters usually ranged from 100 to 1,000 CFU g⁻¹ from April through November at Cedar Point and April to mid-September at Dauphin Island Bay. From December through March, *V. parahaemolyticus* densities were generally <100 CFU g⁻¹. In 2000, only samples collected in May and June consistently exceeded 100 CFU g⁻¹ at either location. Samples collected in March 1999 and January 2000 were below the level of detection (10 CFU g⁻¹) by the direct-plating procedure, but *V. parahaemolyticus* was detected in all samples with the 25-g enrichments. Only one sample, collected from Cedar Point on 28 September 1999, exceeded 10,000 CFU g⁻¹. Except for three occasions (12 April 1999, 14 December 1999, and 20 September 2000), duplicate samples from the same site did not vary by more than 1 log.

Water temperatures from May through September in 1999 and 2000 were similar and exceeded 25°C. Mean salinities at Cedar Point and Dauphin Island Bay from May through September were approximately 6 ppt higher in 2000 (23.6 and 27.5, respectively) than in 1999 (17.6 and 20.7, respectively). In March 2000, temperatures were 1 to 5°C higher than in 1999 and *V. parahaemolyticus* levels increased earlier in 2000 than in 1999.

Water temperature and salinity were significantly associated (*P* < 0.05) with log₁₀ total *V. parahaemolyticus* counts. The abundance of total *V. parahaemolyticus* in Alabama oysters was seasonal and correlated well (*r* = 0.51, *P* < 0.0001) with the water temperature. The effect of the harvest site on total *V. parahaemolyticus* densities was not significant. The best estimate of the temperature effect was a 1.2-fold increase in the number of *V. parahaemolyticus* CFU per gram per degree Celsius. The effect of salinity was quadratic, with a predicted optimum of 17 ppt. The linear regression model that best fits the data is log₁₀(*V. parahaemolyticus*/g) = -1.53 + 0.082 × temperature + 0.20 × salinity - 0.006 × salinity².

The model has an *R*² of 0.42, indicating that 42% of the observed variation in log₁₀ total *V. parahaemolyticus* density was attributable to differences in water temperature and salinity. The relationship of *V. parahaemolyticus* densities in oysters versus water temperature from Cedar Point Reef and Dauphin Island Bay Reef, Ala., is shown in Fig. 3. The mean density rose rapidly as the temperature increased above 20°C, reaching an apparent plateau at temperatures above 25°C.

Pathogenic strains (i.e., *tdh*⁺) were detected at both sites by the direct-plating and enrichment methods. The frequencies of isolation of pathogenic *V. parahaemolyticus* by direct plating and enrichment are shown in Table 1. Paired samples from the same collection were both positive for *tdh*⁺ on 2 (2.6%) of 78 occasions by direct plating and on only 1 (1.3%) of 78 occasions by enrichment. The rates of detection from individual samples were higher, with 20 (12.8%) of 156 positive by direct

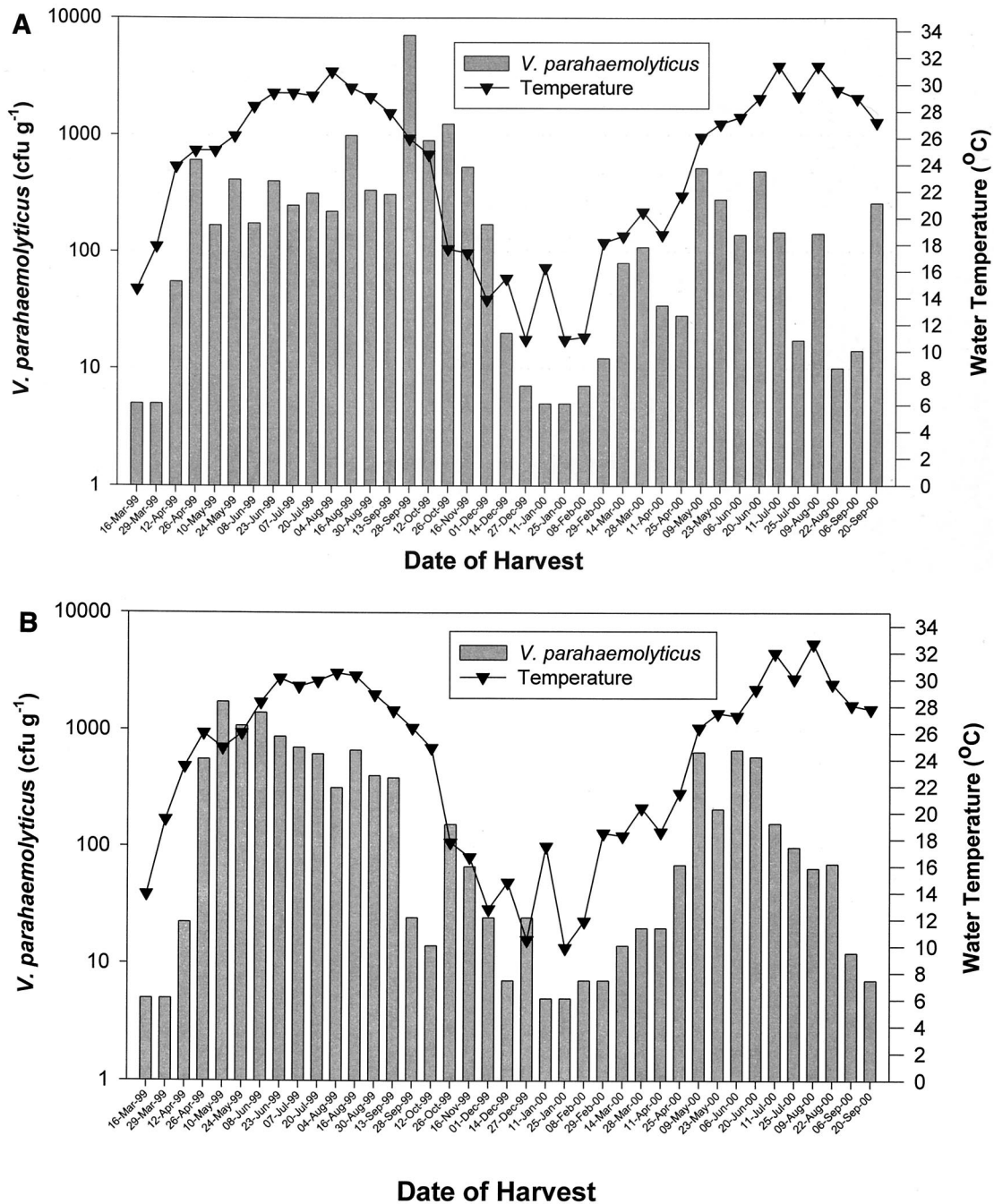


FIG. 2. Density of *V. parahaemolyticus* in oysters from Cedar Point Reef (A) and Dauphin Island Bay Reef (B), Ala. Each bar represents the geometric mean of two samples. Bars at 5 CFU g⁻¹ indicate that samples were taken but *V. parahaemolyticus* was not detected in either sample (count, <10 CFU g⁻¹).

plating and 15 (9.6%) of 156 positive after enrichment. Overall, the observed difference in the frequency of detection of the *tdh* gene by direct plating versus the enrichment method was not significant. Combining results from replicate samples and detection methods, pathogenic strains were detected on 20 (51.3%) of 39 sampling occasions at Dauphin Island Bay compared to 10 (25.6%) of 39 sampling occasions at Cedar Point ($P < 0.05$) and in 34 (21.8%) of 156 samples overall.

The number of *tdh*⁺ *V. parahaemolyticus* colonies was low

and variable across samples collected under similar conditions; however, a higher isolation frequency was observed when water temperatures and total *V. parahaemolyticus* densities were low (Table 1). The results of regression analyses to estimate the magnitude of this effect were found to be sensitive to the specification of link function or model structure. The most consistent effect identified by regression analysis was sampling site. The ratio of pathogenic to total *V. parahaemolyticus* isolates after APW enrichment was significantly higher at Dau-

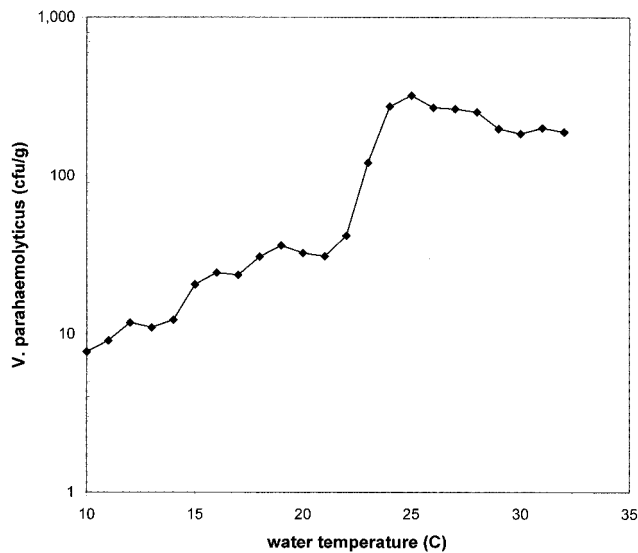


FIG. 3. Relationship between water temperature and *V. parahaemolyticus* density in oysters between Cedar Point Reef and Dauphin Island Bay Reef, Ala.

phin Island Bay (1.35%) than at Cedar Point (0.17%). The average *tdh*⁺ *V. parahaemolyticus* density determined by direct plating was 2.7 CFU g⁻¹ at Dauphin Island Bay compared to 1.3 CFU g⁻¹ at Cedar Point ($P < 0.05$).

Table 2 lists the sources and serotypes of the pathogenic *V. parahaemolyticus* isolates tested. All of the strains that hybridized with both the *tlh* and *tdh* DNA probes were confirmed as *V. parahaemolyticus* by the API 20E system. Except for a single O4:K8 isolate from an oyster sample collected at Cedar Point reef on 16 August 1999, all 39 pathogenic isolates were *trh*⁺ and *urease*⁺ by both the API 20E system and Christensen's urea agar (data not shown). Pathogenic *V. parahaemolyticus* isolates were serologically heterogeneous. In fact, five different serotypes of *tdh*⁺ *V. parahaemolyticus* were isolated from a single sample from Dauphin Island Bay on 16 March 1999. The O3:K6 serotype was not detected.

TABLE 2. Sources and serotypes of *tdh*⁺ *V. parahaemolyticus* isolates

Harvest date	Site/sample ^a	No. of <i>tdh</i> ⁺ isolates ^b	Serotype(s)
16-Mar-99	DI/A	6	O4:K9
16-Mar-99	DI/B	7	O1:K22, O1:K56, O4:K9, O1:Kuk, ^c O11:K40
24-May-99	DI/B	2	O1:Kuk
08-Jun-99	DI/B	1	O1:K56
07-Jul-99	DI/B	1	O1:K56
16-Aug-99	CP/B	1	O4:K8
27-Dec-99	DI/A	4	O11:Kuk
11-Jan-00	DI/A	1	O11:Kuk
11-Jan-00	DI/B	11	O11:Kuk
25-Jan-00	DI/B	1	O1:K30
14-Mar-00	DI/A	1	O1:K55
23-May-00	DI/B	1	O8:K70
06-Jun-00	DI/B	1	O4:K9
20-Jun-00	DI/B	1	O1:K54

^a DI: Dauphin Island Bay; CP: Cedar Point. A and B are subsamples.

^b A total of 39 isolates were tested.

^c uk, capsular antigen untypeable with available antisera.

DISCUSSION

The abundance of total *V. parahaemolyticus* in Alabama oysters at both sites was seasonal and was in agreement with previous studies (9, 21, 24). The abundance of *V. parahaemolyticus* was affected more by temperature than by salinity; however, *V. parahaemolyticus* levels were lower in the summer of 2000 than in that of 1999 but the temperatures were similar. Salinities were closer to the optimum of 17 ppt during the summer of 1999 than in that of 2000, and this may account for the difference. On some occasions, *V. parahaemolyticus* levels appeared to fluctuate independently of temperature and salinity. *V. parahaemolyticus* levels were more than 2 logs higher in a sample collected at Cedar Point on 20 September 2000 than in one collected on the same day at Dauphin Island Bay or in samples collected 2 weeks earlier at both locations, when the temperatures and salinities were similar. The oyster, as a living host, may have contributed to the variation in *V. parahaemolyticus* numbers independently of temperature and salinity be-

TABLE 1. Number of pathogenic (*tdh*⁺) *V. parahaemolyticus* isolates versus temperature and the total number of *V. parahaemolyticus* isolates

Temp (°C) or <i>V. parahaemolyticus</i> count (log ₁₀ g ⁻¹)	No. of samples	No. of <i>tdh</i> ⁺ samples (direct plates)	No. of <i>tdh</i> ⁺ isolates/ no. of <i>tlh</i> ⁺ isolates from direct plates (%)	No. of <i>tdh</i> ⁺ samples (enrichments)	No. of <i>tdh</i> ⁺ isolates/ no. of <i>tlh</i> ⁺ isolates from enrichments (%)
Temp					
<15	22	2	3/63 (4.8)	4	19/858 (2.2)
15–20	30	1	1/459 (0.22)	3	17/1,134 (1.5)
20–25	18	1	1/660 (0.15)	2	2/641 (0.31)
25–30	68	14	24/5,206 (0.46)	6	8/2,699 (0.30)
>30	18	2	2/604 (0.33)	0	0/686 (0.0)
Vibrio counts					
<1	28	5	8/0 ^a	4	30/1,077 (2.8)
1–2	46	2	2/170 (1.2)	4	7/1,555 (0.45)
2–3	65	11	19/2,504 (0.76)	4	5/2,646 (0.19)
>3	17	2	2/4,318 (0.05)	3	4/740 (0.54)
Overall	156	20	31/6,992 (0.44)	15	46/6,018 (0.76)

^a Ratio of *tdh* to *tlh* is indeterminate.

cause of fluctuations in physiology resulting from reproductive status, diet, and health.

V. parahaemolyticus levels in Alabama oysters during the summer of 1999 varied little (10^2 to 10^3 g^{-1}) and were consistent with observations in Galveston Bay after the 1998 outbreak (10). The erratic trend of total *V. parahaemolyticus* levels in Alabama oysters during the summer of 2000 was similar to that observed in Washington State oysters after an outbreak in 1997 (10). *V. parahaemolyticus* levels greater than 10^4 g^{-1} were observed on one occasion in this study; this was consistent with a growing body of evidence that this level seldom occurs in oysters prior to harvest (1, 9, 10, 22, 33). These levels stand in contrast to those reported in a recent study of U.S. market shellstock oysters (5) that frequently found *V. parahaemolyticus* levels of greater than 10^4 g^{-1} in Gulf Coast oysters and that suggested that these high levels are probably the result of postharvest growth.

The use of a direct-plating method for total *V. parahaemolyticus* probably accounts for the close agreement in *V. parahaemolyticus* levels between replicate samples. A strong correlation ($r = 0.78$) between the direct-plating and *Bacteriological Analytical Manual* most-probable number methods for determining total *V. parahaemolyticus* densities in market level oysters has been reported (16).

Pathogenic *V. parahaemolyticus* was detected in 20 (12.8%) of 156 samples by the quantitative direct-plating procedure. The rate of pathogenic *V. parahaemolyticus* detection by an equivalent direct-plating procedure in an earlier study in Galveston Bay was considerably lower, at 3 (2.8%) of 106 samples (10). Other researchers employed direct-plating methods (9, 23–25) and found much lower frequencies of samples yielding either TDH⁺ (1%) or *tdh*⁺ (4%) *V. parahaemolyticus* isolates. This may be because they tested relatively few selected typical colonies for pathogenicity instead of using the colony lift format as in the present study. The qualitative procedure (enrichment of 25-g portions of oyster homogenate, followed by isolation on TCBS) yielded a lower rate of pathogenic *V. parahaemolyticus* detection (9.6%) than the quantitative (0.1 g of oyster homogenate) spread plating procedure. Both methods detected a prevalence of pathogenic *V. parahaemolyticus* higher than the 0 to 6% reported in previous studies (5, 14, 22, 26, 31). The low frequency at which both paired samples were found to have the *tdh* gene by either method (1.3% by enrichment and 2.6% by direct plating) suggests that collection of multiple samples at each site would substantially increase the detection of pathogenic *V. parahaemolyticus*.

The average *tdh*⁺ *V. parahaemolyticus* density determined by direct plating was 2.7 CFU g^{-1} at Dauphin Island Bay and 1.3 CFU g^{-1} at Cedar Point. Similar densities of *tdh*⁺ *V. parahaemolyticus* were reported in a previous study of Pacific Coast oysters (10). Alabama oysters were not linked to any *V. parahaemolyticus* illnesses during the study.

Similar numbers of colonies were examined for the *tdh* gene after enrichment and direct plating. The *tdh* gene was detected in a small proportion of *V. parahaemolyticus* isolates after enrichment (46 of 6,018) and in directly plated oyster homogenates (31 of 6,992). The *tdh*⁺ colonies recovered from enrichments and by direct plating were low in number and unevenly distributed. Few total or *tdh*⁺ *V. parahaemolyticus* colonies were found on direct plates during the winter, whereas a con-

stant number (47 isolates per sample) was found in enrichments throughout the year. In two samples collected at Dauphin Island Bay (16 March 1999 and 11 January 2000), 13 and 12 of 47 isolates, respectively, obtained after enrichment were *tdh*⁺; other samples yielded much fewer *tdh*⁺ isolates. The ratio of pathogenic to total *V. parahaemolyticus* was seasonal; pathogenic strains were more prevalent in the winter. The greater prevalence of pathogenic *V. parahaemolyticus* in the winter was not observed in previous surveys, and this may explain why *V. parahaemolyticus* illnesses are less seasonal on the Gulf Coast than in other regions (19). The greater rate of detection of pathogenic *V. parahaemolyticus* in the winter may be due to a decreased prevalence of total *V. parahaemolyticus* and not to a greater abundance of pathogenic strains. During warm weather, *V. parahaemolyticus* multiplies in oysters (18); it probably multiplies in many other marine environments that contain adequate nutrients. Growth slows or stops as winter approaches, and we are not aware of data suggesting that *tdh*⁺ strains are more cold tolerant than other *V. parahaemolyticus* strains.

Vibrios are associated with mammals and birds (11, 27), and animal passage can select for virulent strains (13). Feces from warm-blooded animals could be a more significant source of vibrios under cold conditions that are unfavorable for environmental growth. Pathogenic strains of *V. cholerae* were most prevalent during the winter in Apalachicola Bay, Fla. (12). A wildlife refuge borders Dauphin Island Bay, and diverse species of birds are abundant year round. During the winter of 2000, raccoons were routinely sighted feeding on oysters along the shoreline of Dauphin Island Bay. Raccoons have been shown to harbor high densities of *V. parahaemolyticus* in their intestines (11).

Nearly all (97.4%) of the *tdh*⁺ *V. parahaemolyticus* strains examined in this study produced urease and possessed the *trh* gene. These traits are normally associated with *V. parahaemolyticus* from the Pacific Northwest and have not been reported in seafood or environmental samples from the Gulf Coast (22). Some have hypothesized that these traits may increase virulence, but the recently emerged O3:K6 clone associated with pandemic spread and high attack rates did not possess these traits (32). Additionally, approximately 40% of recent (1997 to 2001) human *V. parahaemolyticus* isolates did not produce urease or possess *trh* (Cheryl Bopp, Centers for Disease Control and Prevention, personal communication, June 2002). While diverse serotypes were found in this study, no O3:K6 strains were observed.

This study provides novel information on the abundance, ecology, and characteristics of pathogenic *V. parahaemolyticus* in U.S. Gulf Coast oysters. The higher prevalence of pathogenic *V. parahaemolyticus* observed in the present study is probably due to the testing of many more colonies than in most previous investigations. Extensive quantification of pathogenic *V. parahaemolyticus* in any environmental matrix has not previously been reported. Our quantitative data suggest a higher ratio of pathogenic to total *V. parahaemolyticus* strains in oysters during cooler weather and may explain why illness along the Gulf Coast is more prevalent in winter months than expected on the basis of levels of total *V. parahaemolyticus*. U.S. isolates of *tdh*⁺ *V. parahaemolyticus* possessing the *trh* gene and producing urease were previously reported only along the

Pacific coast; our results indicate that the overwhelming majority of pathogenic strains from the Gulf Coast also possess these potential virulence traits. These data should be valuable for assessment of the human health risk due to consumption of raw oysters.

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