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Growth and Survival of *Vibrio parahaemolyticus* in Postharvest American Oysters

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ABSTRACT

Oysters at the retail stage of distribution generally contain greater densities of *Vibrio parahaemolyticus* than do oysters at harvest. The objective of this study was to determine the effects of postharvest storage at 26 and 3°C on the growth and survival of naturally occurring *V. parahaemolyticus* in shellstock American oysters (*Crassostrea virginica*). Oysters were collected monthly from May 1998 through April 1999 from Mobile Bay, Alabama, and their *V. parahaemolyticus* densities were determined after 0, 5, 10, and 24 h of postharvest storage at 26°C. After 24 h of storage at 26°C, oysters were transferred to a refrigerator at 3°C and analyzed 14 to 17 days later. *V. parahaemolyticus* numbers were determined by a direct plating method involving an alkaline-phosphatase-labeled DNA probe that targets the species-specific thermolabile hemolysin gene (*tlh*-AP) to identify suspect isolates. From April to December, when water temperatures at harvest were >20°C, the geometric mean harvest density of *V. parahaemolyticus* was 130 CFU/g. When water temperatures were <20°C, the geometric mean harvest density was 15 CFU/g. After harvest, *V. parahaemolyticus* multiplied rapidly in live oysters held at 26°C, showing a 50-fold increase (1.7 log CFU/g) at 10 h and a 790-fold increase (2.9 log CFU/g) at 24 h (April through December). Average *V. parahaemolyticus* numbers showed a sixfold decrease (0.8 log CFU/g) after approximately 14 days of refrigeration. These results indicate that *V. parahaemolyticus* can grow rapidly in unrefrigerated oysters.

Vibrio parahaemolyticus gastroenteritis is one of the leading bacterial infections associated with seafood consumption in the United States and is usually linked to the consumption of raw or undercooked seafood and seafood contaminated after cooking *(17, 29).* Consumption of raw shellfish, primarily oysters, was linked to four multistate *V. parahaemolyticus* illness outbreaks in the United States in 1997 and 1998 *(1, 4, 5, 9).*

Kaneko and Colwell *(20, 21)* were the first to report a seasonal cycle for *V. parahaemolyticus* in the United States. In the Pacific Northwest, Kelly and Stroh *(23)* found *V. parahaemolyticus* in the environment only during the summer months, when water temperatures were ≥17°C and salinities were ≤13 ppt. Also in the Pacific Northwest, Kaysner et al. *(22)* isolated *V. parahaemolyticus* from the water column when temperatures ranged from 15 to 22°C. The seasonal and geographical distributions of *V. parahaemolyticus* in U.S. coastal waters and oysters were investigated by DePaola et al. *(10). V. parahaemolyticus* levels in both water and oyster samples were correlated with water temperature but not with salinity and were 233 times as high in oysters as in the overlying waters. The highest concentrations of *V. parahaemolyticus* (10^2 to 10^3 CFU/g) were found in Gulf Coast oysters collected in late spring and summer.

The densities of *V. parahaemolyticus* found in shell oysters at the retail stage of distribution *(14)* are greater than those observed in oysters at harvest *(10).* Under approved shellfish harvesting and handling guidelines, shellfish may remain unrefrigerated for as long as 10 h after harvest, even when temperatures exceed 27°C *(32).*

Vibrio vulnificus may increase 10- to 100-fold in 10 h when oysters are stored at ambient temperatures after harvest *(6).* Cook and Ruple *(8)* studied changes in the levels of *Vibrionaceae* in postharvest shellstock oysters during commercial transport and storage at various temperatures (at 10°C, at 22°C, in ambient air, and at 30°C); these investigators observed temperature-dependent increases of 1 to 4 orders of magnitude in *Vibrionaceae* as temperature increased. Johnson et al. *(19)* determined that *V. parahaemolyticus* multiplied within shellstock oysters stored at 35°C for 2 to 3 days and survived storage at 4°C for at least 3 weeks with no apparent decrease in numbers. Goatcher et al. *(15)* observed that large numbers of *V. parahaemolyticus* inoculated on the surfaces of processed oysters were reduced by several log cycles within 24 h at 5°C.

Limitations associated with methods for isolation and identification have curtailed precise quantitative studies on the growth and survival of *V. parahaemolyticus* in oyster shellstock. Recent studies have indicated that a direct plating method involving a DNA probe (direct-VPAP [*Vibrio parahaemolyticus* alkaline phosphatase]) for the identification of *V. parahaemolyticus* in oysters is rapid, efficient, reliable, and precise *(11, 14, 16)* and produced counts com-

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parable to the Food and Drug Administration's *Bacteriological Analytical Manual* most-probable-number (MPN) procedure *(13)* for enumeration of *V. parahaemolyticus.*

The objectives of this study were (i) to determine *V. parahaemolyticus* levels in oysters at harvest, (ii) to examine *V. parahaemolyticus* growth in postharvest oysters stored at 26°C, and (iii) to study the effect of long-term refrigeration at 3°C on *V. parahaemolyticus* survival in postharvest oysters.

MATERIALS AND METHODS

Oyster collection and handling. American oysters (*Crassostrea virginica*) were collected monthly from May 1998 through April 1999 in Mobile Bay, Alabama. The salinity and temperature of the surface water in the harvest area were measured with a model 85 dissolved oxygen conductivity meter (Yellow Springs Instrument Co., Yellow Springs, Ohio). Twelve oysters were chilled on ice, and 60 to 80 oysters were held at ambient temperature on the boat. Oysters were transported to the Food and Drug Administration's Gulf Coast Seafood Lab on Dauphin Island, Alabama, within 1 h of collection. The chilled oysters were analyzed within 2 h to obtain harvest levels (0 h) of *V. parahaemolyticus*; the remaining nonchilled oysters were placed in an incubator at 26°C.

Twelve oysters were randomly chosen from those stored at 26°C and analyzed at each time point (5, 10, and 24 h after harvest). After 24 h, the remaining oysters were refrigerated at 3°C and analyzed 14 to 17 days later to simulate possible retail handling practices. The oysters were scrubbed and shucked; the meats and liquor from the 12 oysters were pooled and mixed with an equal weight (1:1) of sterile phosphate-buffered saline (PBS) (7.65 g of NaCl per liter, 0.724 g of anhydrous Na_2HPO_4 [Sigma, St. Louis, Mo.] per liter, 0.21 g of KH_2PO_4 [Sigma] per liter [pH 7.4]) *(13),* and the mixture was blended for 90 s with a sterile Waring blender in preparation for the analysis *(12).*

Enumeration by direct-VPAP. Aliquots of oyster homogenate (0.2 g of 1:1 [wt/wt] in PBS [equivalent to 0.1 g] taken directly from a blender or 0.1-ml portions taken from tenfold dilutions in PBS) were spread plated onto T_1N_3 (1% tryptone, 3% salt, 2% agar per liter [pH 7.2]) plates. After overnight incubation at 35°C, colony lifts and hybridization with the *tlh*-AP DNA probe for species confirmation were performed as described by McCarthy et al. *(24).* The alkaline phosphatase–conjugated oligonucleotide probe was prepared with DNA Technology A/S (DK-8000 Aarhus C., Denmark) by a proprietary process. Filter preparation, hybridization, and colorimetric detection were performed as described by Wright et al. *(33)* for *V. vulnificus,* except that the hybridization temperature was 54°C. Probe-positive (purple-brown) colonies were counted at each spread-plated dilution.

Statistical analyses. Bacterial numbers were converted to base 10 logarithms for statistical analysis *(30).* When *V. parahaemolyticus* was not detected, a value of 5 CFU/g was used (half the limit of detection for a 0.1-g sample) in the calculations. The significance of differences observed between *V. parahaemolyticus* densities obtained at different sampling times up to 24 h were determined by the paired-comparisons *t* test. The maximum growth rate at 26°C was estimated by regression analysis. Data obtained when the harvest water temperature was <20°C (January through March) were excluded from the regression analysis to estimate growth because the growth of *V. parahaemolyticus* in oysters while they acclimate to 26°C is slower and because ambient air temperatures are typically well below 26°C during this period. The signed-rank test was used to determine the significance of the effect of storage at 3°C for 14 to 17 days. Calculations were performed by using Statistical Analysis Systems (SAS Institute Inc., Cary, N.C.) and Microsoft Excel. An alpha level of 0.05 was considered the minimum level for significance.

TABLE 1. *Harvest densities (0 h) of* V. parahaemolyticus *in oysters collected monthly over the course of a year*[a]

Month	Direct-VPAP value (CFU/g)	Water temp (°C)	Salinity (ppt)
January	70	10.0	12.0
February	10	18.0	4.0
March	<10	18.0	8.5
April	10	24.0	8.7
May	<100	28.0	12.0
June	300	32.5	20.0
July	50	32.5	25.0
August	370	32.5	15.0
September	200	30.5	22.0
October	300	27.0	18.0
November	280	22.0	20.0
December	250	20.0	18.0

[a] Positive bacterial colonies were counted at each time point and adjusted for the dilution to yield the direct-VPAP value.

RESULTS AND DISCUSSION

Harvest densities. Densities of *V. parahaemolyticus* in 12 pooled oysters obtained at the time of harvest (0 h) each month are listed in Table 1. When water temperatures were >20°C (April through December), the geometric mean harvest density was 130 CFU/g. From January through March, when water temperatures were <20°C, the geometric mean harvest density was 15 CFU/g. Harvest densities were ≤10 CFU/g during February, March, and April.

The data demonstrate a seasonal trend of *V. parahaemolyticus* abundance in Gulf Coast oysters that is similar to that for *V. vulnificus (26).* This finding is consistent with those of previous studies identifying water temperature as a major factor in the seasonal and geographical distribution of *V. parahaemolyticus* in shellfish growing areas *(10, 22). V. parahaemolyticus* counts in April and May were lower than expected, possibly because of reduced salinities *(23)*; Ellison et al. *(14)* also reported relatively low *V. parahaemolyticus* densities in Florida retail oysters during April and May 1998. However, when initial levels were below the limit of detection, oyster incubation at 26°C for 24 h consistently yielded detectable numbers. This finding suggests that the bacterium was most likely present in numbers below the limit of detection, or perhaps in a viable but nonculturable state *(28).*

Growth during storage. *V. parahaemolyticus* multiplied rapidly in oysters stored at 26°C, increasing 50-fold (April-to-December mean) between harvest and 10 h (Fig. 1). The 10-h sampling time was selected as a reference point because it is the maximum allowable time that shellfish from states confirmed as original sources of product associated with two or more *V. vulnificus* illnesses may remain unrefrigerated after harvest *(32).* Increases of >10-

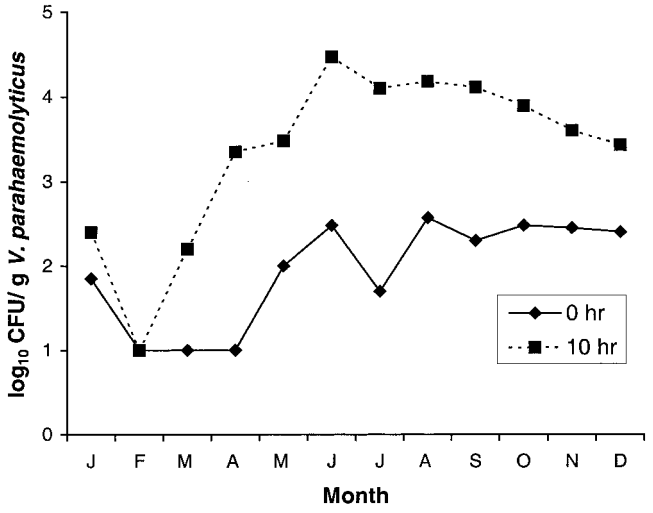

FIGURE 1. *Numbers of* V. parahaemolyticus *in shellstock oysters at harvest and after 10 h of storage at 26°C. For March, bacterial counts at harvest were below the level of detection.*

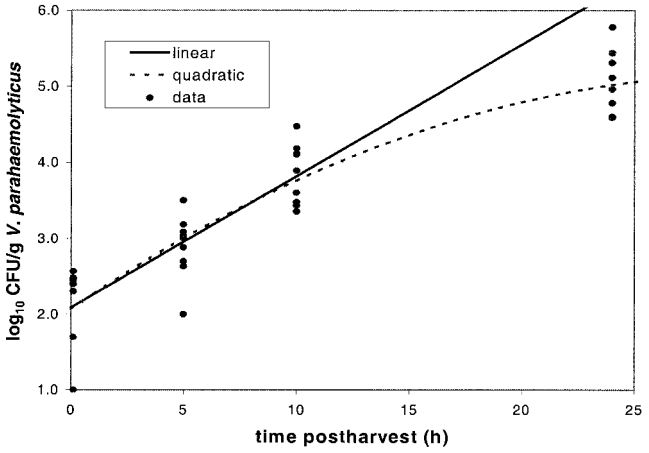

FIGURE 2. Vibrio parahaemolyticus *data for April through December at 0 to 24 h fit to linear and quadratic regression analyses.*

fold in *V. parahaemolyticus* populations were observed after 10 h at 26°C in all months except January and February. In January, the oysters were at 10°C at the time of harvest and warmed slowly in the 26°C incubator (requiring 7 h to reach 26°C; data not shown). *V. parahaemolyticus* numbers increased 500-fold (2.7 logs) after 24 h of storage at 26°C in January. The lowest salinity of the study was observed for the February samples (4 ppt), which may account for the reduced *V. parahaemolyticus* growth (*V. parahaemolyticus* numbers increased 79-fold [1.9 logs] after 24 h in February). Similar water temperature and *V. parahaemolyticus* concentrations at harvest were observed in March, but salinity was higher (8.5 ppt) and *V. parahaemolyticus* concentrations increased 790-fold (2.9 logs) after 24 h of storage.

From April through December, average *V. parahaemolyticus* densities increased consistently at all sampling times. Average monthly increases above harvest levels were 6-, 50-, and 790-fold (0.8, 1.7, and 2.9 log CFU/g) at 5, 10, and 24 h, respectively. All differences were highly significant ($P < 0.0001$). Miles et al. *(25)* developed a predictive mathematical model to ascertain the effect of temperature (8 to 45°C) and water activity (0.936 to 0.995) on the growth rate of *V. parahaemolyticus.* They found growth rates to be fourfold higher in broth than we observed for shellstock oysters at 26°C.

In a regression analysis of all data up to 24 h, the inclusion of a quadratic temperature effect provided a better fit to the data than did a linear-only model ($P < 0.05$) (Fig. 2). This finding suggests that the exponential phase of growth ends sometime prior to 24 h, and therefore data obtained up to 10 h postharvest (April through December) were used to estimate the maximum growth rate at 26°C. The standard deviations were 0.53, 0.42, 0.40, and 0.41 log CFU/g at 0, 5, 10, and 24 h, respectively. The estimate of the slope of the linear regression was 0.17 log/h ($P < 0.0001$). This growth rate corresponds to a doubling time of 1.8 h during the exponential phase of growth.

The use of a linear regression to estimate the growth rate assumes that there is no lag phase. This assumption is appropriate for *V. parahaemolyticus* growth in live oysters because there is no abrupt change in temperature, pH, water activity, or nutrient availability; substantial changes in these parameters would induce a lag phase *(2, 3).* From April through December, water temperatures at harvest were within 6°C of the storage temperature of 26°C. Similarly, small differences between air and water temperature would be typical in Gulf Coast harvest areas during warm-weather months (data not shown), and therefore the absence of a lag phase under conditions of commercial harvest would be expected. Additionally, the data did not indicate a lag phase, as the growth rate between 0 and 5 h of storage was similar to that between 5 and 10 h. The maximum observed *V. parahaemolyticus* density in the present study was 5.8 log CFU/g. This value is comparable to maximums of 6.3 log MPN/g *(7)* and 5.0 CFU/g *(14)* observed in recent surveys of retail oysters.

The winter of 1998 to 1999 was one of the warmest on record for the Gulf Coast because of the influence of El Niño *(9).* The average monthly temperatures in Mobile, Alabama, for November 1998 to February 1999 were approximately 2°C higher than normal (http://www.nwsmobile.noaa.gov); the water temperatures reflect this.

Survival after refrigeration. Mean *V. parahaemolyticus* counts for oysters decreased sixfold (0.8 log CFU/g) after storage at 3°C for 14 to 17 days. A similar trend was reported for *V. vulnificus* numbers at lower temperatures *(8).* Johnson and Liston *(18)* allowed depurated oysters to take up *V. parahaemolyticus* from filtered seawater and then refrigerated them at 5°C. After 14 days of storage, the bacterial counts had decreased 1.6 logs from the initial count. Johnson et al. *(19)* observed that the bacteria could survive in refrigerated (4°C) shellstock oysters for at least 3 weeks. Thomson and Thacker *(31)* inoculated sterile oyster homogenates with 10^4 *V. parahaemolyticus* cells and stored them at 0, 4, 8, 10, and 12°C. At 0 and 4°C, *V. parahaemolyticus* numbers decreased to almost zero after 1 week; at 8°C, the cells did not multiply but remained viable

for 3 weeks. Multiplication occurred at 10 and 12°C, with cells remaining viable for 4 weeks or longer.

In previous studies involving oysters, investigators often resorted to eliminating background microflora by autoclaving processed oysters or homogenates and then artificially contaminating them with the bacterium of interest. For instance, Thomson and Thacker *(31)* and Muntada-Garriga et al. *(27)* inoculated tubes of sterile oyster homogenate with *V. parahaemolyticus* to study survival at different temperatures. The latter researchers observed a 1,000-fold decrease in 1 week at 4°C.

Oyster pH values were measured during the March and April experiments; the mean pH dropped gradually from 6.4 at harvest to approximately 6.2 after 14 to 17 days of storage at 3°C. This pH decrease was probably too slight to affect *V. parahaemolyticus* survival.

The present study represents the first time *V. parahaemolyticus* growth and survival in live oysters has been determined by precise, direct plating methods and DNA probes for identification. These conditions should produce more reliable data on the behavior of *V. parahaemolyticus* in live shellstock oysters than have previous studies involving shucked oysters or oyster homogenates.

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