

Evaluation of Two Direct Plating Methods Using Nonradioactive Probes for Enumeration of *Vibrio parahaemolyticus* in Oysters

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Oysters (*Crassostrea virginica*) were collected monthly from May 1998 to April 1999 from Mobile Bay, Ala., and analyzed to determine *Vibrio parahaemolyticus* densities at zero time and after 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 then analyzed 14 to 17 days later. The *V. parahaemolyticus* numbers were determined by the most-probable-number procedure using alkaline phosphatase-labeled DNA probe VPAP, which targets the species-specific thermolabile hemolysin gene (*tlh*), to identify suspect isolates (MPN-VPAP procedure). Two direct plating methods, one using a VPAP probe (Direct-VPAP) and one using a digoxigenin-labeled probe (Direct-VPDig) to identify suspect colonies, were compared to the MPN-VPAP procedure. The results of the Direct-VPAP and Direct-VPDig techniques were highly correlated ($r = 0.91$), as were the results of the Direct-VPAP and MPN-VPAP procedures ($r = 0.91$). The correlation between the Direct-VPDig and MPN-VPAP results was 0.85. The two direct plating methods in which nonradioactive DNA probes were used were equivalent to the MPN-VPAP procedure for identification of total *V. parahaemolyticus*, and they were more rapid and less labor-intensive.

Vibrio parahaemolyticus is an enteric pathogen found in estuaries and various types of seafood throughout the world (1, 2, 13–15). *V. parahaemolyticus* infections can cause gastroenteritis in humans and are most frequently associated with consumption of raw or undercooked seafood and seafood recontaminated with the bacterium after cooking (19). Consumption of raw shellfish, primarily oysters, has been linked to four multistate *V. parahaemolyticus* illness outbreaks involving 650 reported cases in the United States since 1997 (Washington in 1997 and 1998; New York and Texas in 1998) (4–6). All patient isolates obtained from the 296 reported *V. parahaemolyticus* infections in Texas were serotype O3:K6, which commonly causes outbreaks in Asia but had not been identified previously in the United States (6). These outbreaks increased concern about *V. parahaemolyticus* densities in oysters and focused attention on the development of more efficient methods for environmental monitoring of this pathogen.

The Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) most-probable-number (MPN) method (11) is most frequently used to enumerate *V. parahaemolyticus* in foods. The BAM-MPN method uses biochemical techniques to identify isolates and is time-consuming and labor-intensive. As an alternative, researchers recently described the use of nonradioactive DNA probes for identification of *V. parahaemolyticus* (17).

In the present study we compared two direct plating methods using nonradioactive DNA probes (Direct-VPAP and

Direct-VPDig) with a modification of the BAM-MPN method in which confirmation of the identities of *V. parahaemolyticus* isolates was accomplished with a DNA probe (MPN-VPAP) targeting the species-specific thermolabile hemolysin gene (*tlh*) (21). In the Direct-VPAP method we used a *tlh*-alkaline phosphatase (*tlh*-AP)-labeled DNA probe, and in the Direct-VPDig method we used a *tlh*-digoxigenin-labeled DNA probe for identification of *V. parahaemolyticus*. Pathogenic *V. parahaemolyticus* strains contain additional hemolysin genes, designated the thermostable direct hemolysin (*tdh*) gene and the thermostable direct related hemolysin (*trh*) gene (18, 20). Since *V. parahaemolyticus* densities in oysters can vary with the season, salinity, temperature, and storage parameters (8), the methods were tested under a variety of conditions over a 1-year period.

MATERIALS AND METHODS

Oyster collection and handling. Adult oysters (diameter, >7.82 cm; *Crassostrea virginica*) were harvested monthly from May 1998 through April 1999 by tonging in Mobile Bay, Ala. 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). At each sampling time, 12 oysters were chilled on ice and 60 to 80 oysters were held without icing at the ambient air temperature on the boat. The oysters were transported to the FDA Gulf Coast Seafood Laboratory on Dauphin Island, Ala., within 1 h of collection. The chilled oysters were analyzed within 2 h to obtain harvest (zero-time) levels of *V. parahaemolyticus*, and the remaining nonchilled oysters were placed in an incubator adjusted to 26°C.

Twelve of the oysters stored at 26°C were sampled and analyzed to determine *V. parahaemolyticus* densities at 5, 10, and 24 h after harvest. The current protocol for handling oysters from the Gulf of Mexico calls for refrigeration within 10 to 36 h, depending on the ambient water temperature at the time of harvest (10). A 24-h holding period at 26°C was chosen for this study in order to analyze oysters before, up to, and after the usual Gulf of Mexico oyster industry harvest times and refrigeration times. After 24 h, the remaining oysters were transferred to a refrigerator (3°C) and then analyzed 14 to 17 days later to simulate possible retail handling practices. The oysters were scrubbed, shucked,

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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] per liter, 0.21 g of KH_2PO_4 [Sigma] per liter; pH 7.4) (11), and the mixture was blended for 90 s with a sterile Waring blender in preparation for analysis (9).

Enumeration by the MPN method. The MPN method described in the FDA BAM (11) was used to estimate *V. parahaemolyticus* densities, except that a species-specific DNA probe targeting the *tlh* gene was used for identification (MPN-VPAP) (17) instead of biochemical utilization assays. This oligonucleotide probe conjugated with alkaline phosphatase (*tlh*-AP) was purchased from DNA Technology A/S (Aarhus, Denmark). Briefly, oyster homogenate was serially diluted, inoculated into a series of MPN tubes containing alkaline peptone water (11) (three tubes/dilution), and incubated for 16 to 18 h at 35°C, and then a loopful from each MPN tube showing growth was streaked onto a thiosulfate-citrate-bile salts (TCBS) agar plate. After 18 to 24 h of incubation at 35°C, suspect colonies from the TCBS agar (Difco) streak plates were transferred with sterile toothpicks into alkaline peptone water in individual wells of 96-well plates and incubated for 16 to 18 h at 35°C. Cells from the 96-well plates were transferred to *Vibrio vulnificus* agar (30 g of NaCl [Sigma] per liter, 10 g of cellobiose [Sigma] per liter, 20 g of proteose peptone [Difco] per liter, 0.06 g of bromthymol blue [Sigma] per liter, 25 g of agar [Difco] per liter) plates by using a 48-prong replicator (9). Colony lifts were prepared and tested for hybridization with the *tlh*-AP probe as described by McCarthy et al. (17) for confirmation of species identity. *V. parahaemolyticus* TX 2103 (a human stool sample isolate) and *V. vulnificus* MO6-24 (a human primary septicemia blood isolate) were used as positive and negative controls, respectively.

Direct-VPAP enumeration. Aliquots of oyster homogenate (0.2 g of a 1:1 [wt/wt] preparation in PBS [equivalent to 0.1 g], taken directly from a blender, or 0.1-ml portions from subsequent 10-fold dilutions in PBS) were spread plated onto T_1N_3 (1% tryptone [Difco], 3% NaCl, 2% agar; pH 7.2) plates. After overnight incubation at 35°C, colony lift, hybridization, and colorimetric detection analyses were done as described previously for the *tlh*-AP probe (17). The nucleotide base sequence of the alkaline phosphatase-labeled DNA probe was the sequence from bases 904 to 927 of the species-specific *V. parahaemolyticus* *tlh* gene (accession number M36437) (3, 17, 21). After color development, colonies that hybridized with the *tlh*-AP probe were determined visually.

Direct-VPDg enumeration. The *V. parahaemolyticus* species-specific *tlh* DNA fragments were synthesized with primers by PCR as described by Brasher et al. (3). Digoxigenin-labeled nucleotides were used to label the probe by the procedures of Boehringer Mannheim (*The Genius System User's Guide for Filter Hybridization*, version 2.9-92; Boehringer Mannheim Corp., Indianapolis, Ind.) and Weagant et al. (22). Nylon membranes (MagnaGraph; Osmonics-MSI, Minnetonka, Minn.) were placed onto tryptic soy agar (Difco) containing MgSO_4 (TSAMS agar) (40 g of tryptic soy agar per liter, 20 g of NaCl per liter, 1.5 g of MgSO_4 [Sigma] per liter) plates and spread plated with the dilutions of oyster homogenate described above. The plates were incubated for 3 h at 35°C to repair sublethally injured cells, and the membranes were then transferred (with the inoculated side of each membrane up) to TCBS plates and incubated overnight at 40°C. Probe and membrane preparation, hybridization, and colorimetric detection were performed as described in *The Genius System User's Guide for Filter Hybridization* (Boehringer Mannheim Corp.), as outlined by McCarthy et al. (17) and Weagant et al. (22).

Statistical analyses. Bacterial densities were converted to base 10 logarithms before being analyzed by Microsoft Excel and SAS. Twelve-month geometric means were determined for each analytical method. Samples with nondetectable colonies were assigned the minimum detectable density on the basis of the volume examined. The statistical methods used included linear regression analysis to compare correlations between the analytical methods and analysis of variance to compare differences between treatments (Direct-VPAP, Direct-VPDg, and MPN-VPAP). An alpha level ($P < 0.05$) was considered a minimum level of significance for each statistical method. Within-treatment comparisons will be described elsewhere.

RESULTS AND DISCUSSION

Regression analyses. Figure 1 shows that there was close agreement between methods for enumerating *V. parahaemolyticus* in oysters under a variety of seasonal and storage conditions. It shows the regression lines and a line of identity for the two direct plating methods versus the MPN-VPAP method. The line of identity shows how the two direct plating methods compare with the MPN-VPAP procedure. The slopes

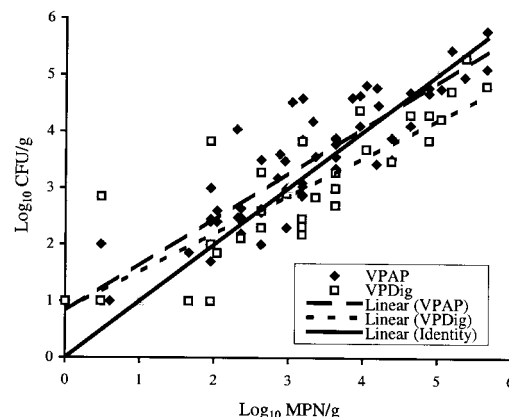


FIG. 1. *V. parahaemolyticus* densities (\log_{10} CFU/g or \log_{10} MPN/g) in oysters analyzed by the Direct-VPAP, Direct-VPDg, and MPN-VPAP methods. The line of identity shows the points for which the results of all three methods would be identical.

of the two direct plating regression lines are almost identical (the slope of the Direct-VPAP regression line is 0.91, and the slope of the Direct-VPDg regression line is 0.94). The *V. parahaemolyticus* estimates obtained with the methods were highly correlated for either the Direct-VPAP and Direct-VPDg procedures or the Direct-VPAP and MPN-VPAP procedures ($r = 0.91$); the correlation between the Direct-VPDg and MPN-VPAP procedures was 0.85. Data from studies or monitoring programs obtained with any of these methods could be compared (i.e., for risk assessment). The differences between the direct plating and MPN methods appeared to be greatest at lower *V. parahaemolyticus* densities and may be attributed to different detection sensitivities. Because the MPN-VPAP method is more sensitive (3 MPN/g for a 0.1-g sample and 0.3 MPN/g for a 1-g sample) than the direct plating methods (10 CFU/g for a 0.1-g sample), use of this method is recommended when low *V. parahaemolyticus* densities are suspected (e.g., during the winter when water temperatures are lower). Under warm conditions, either direct plating method offers an alternative that is more rapid, economical, and less labor-intensive than the BAM-MPN procedure. Similar direct plating methods used for *V. vulnificus* have shown that direct plating methods are more precise than MPN analyses (9).

In a previous study in which methods were compared, DePaola et al. (7) used resource-intensive biochemical tests to confirm the identities of suspect *V. parahaemolyticus* colonies and based direct plating estimates on only five suspect colonies per sample. The colony lift format used in this study eliminated the need to identify individual colonies and provided an efficient way to test all colonies on a plate.

Mean densities by method. The 12-month geometric mean *V. parahaemolyticus* densities for the three methods for samples obtained at zero time and 5, 10, and 24 h and 14 to 17 days after harvest are shown in Table 1. These methods were tested by using oysters that were growing at wide ranges of temperature and salinity and there were no significant differences between method means at any time ($P > 0.12$) after either storage under warm conditions or long-term refrigeration. The counts ranged from <10 to 800 CFU/g or 0.9 to 900 MPN/g at

TABLE 1. Twelve-month mean *V. parahaemolyticus* values for the three methods for samples obtained at zero time and after 5, 10, and 24 h and 14 to 17 days

Method	Log ₁₀ CFU/g or MPN/g				
	Zero time	5 h	10 h	24 h	14–17 days
Direct-VPAP	1.94 ± 0.18 (12) ^a	2.58 ± 0.24 (12)	3.35 ± 0.29 (12)	4.72 ± 0.22 (12)	3.94 ± 0.28 (11)
Direct-VPDig	1.85 ± 0.25 (10)	2.45 ± 0.32 (10)	ND ^b	4.23 ± 0.50 (5)	3.54 ± 0.25 (10)
MPN-VPAP	1.57 ± 0.34 (10)	2.27 ± 0.33 (12)	3.25 ± 0.35 (12)	4.39 ± 0.31 (12)	3.55 ± 0.39 (12)

^a Mean ± standard error. The numbers in parentheses are the numbers of observations. The method-hour interaction was not significant ($P > 0.12$).

^b ND, not determined.

harvest, depending upon the season. The levels of *V. parahaemolyticus* recovery in this study (i.e., Direct-VPAP 12-month geometric mean of 86 CFU/g) agree closely with those previously reported for Gulf Coast oysters (8). The mean *V. parahaemolyticus* density was 110 CFU/g in a seasonal survey when the hydrophobic grid membrane filter method was used, and the highest densities occurred in spring and summer (8).

The *V. parahaemolyticus* densities increased during storage at 26°C by 1.4 and 3 logs (12-month means) after 10 and 24 h, respectively. After 14 to 17 days of refrigeration at 3°C, the mean count decreased by only 0.9 log from the 24-h level, suggesting that long-term refrigeration may not substantially reduce the numbers of bacteria present in raw oysters. Johnson and Liston (12) observed similar decreases in *V. parahaemolyticus* densities in naturally contaminated oysters stored at 11°C (0.8-log reduction after 8 days) and 5°C (1.6-log reduction after 14 days).

The Direct-VPAP mean was slightly higher than the other two means at each time point. The Direct-VPDig method included a repair step on magnesium-supplemented TSAMS agar (16), but the levels of recovery were comparable to those obtained with the Direct-VPAP method. The repair step was performed to account for any cellular damage due to temperature or salinity either before harvest or during storage and to compare the bacterial counts obtained by this method with those obtained by the Direct-VPAP procedure. This repair step may be insufficient to overcome the subsequent inhibition on the selective TCBS medium. The selective components of TCBS medium include oxgall, sodium citrate, and an alkaline pH (pH 8.6). The T₁N₃ agar used with the Direct-VPAP method was not selective, but its high salt concentration may inhibit some competing microflora. While optimization studies were not conducted with T₁N₃ agar, this medium was simple and economical to prepare, required no repair step, limited colony spreading, and gave good levels of *V. parahaemolyticus* recovery under all experimental conditions. The Direct-VPAP method can be completed in 1 day, compared with 2 days for the Direct-VPDig method and 3 to 4 days for the BAM-MPN method.

In conclusion, recent *V. parahaemolyticus* illness outbreaks emphasized the need for rapid, quantitative methods for environmental monitoring of *V. parahaemolyticus* levels in the environment. Two direct plating methods (the Direct-VPAP and Direct-VPDig methods) using nonradioactive DNA probes were equivalent to the MPN-VPAP procedure and provided a faster alternative for *V. parahaemolyticus* enumeration and confirmation in oyster samples.

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