Effect of Frozen Storage and Vacuum-Packaging on Survival of Vibrio Vulnificus in Gulf Coast Oysters (Crassostrea virginica)

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ABSTRACT

Vibrio vulnificus contamination of raw oysters is a serious public health hazard, therefore, it is necessary to investigate the persistence of V. vulnificus in harvested and stored oysters. For this study, triplicate oyster samples were split into four treatment groups: control, normal-packaged; control, vacuum-packaged; inoculated, normal-packaged; and inoculated, vacuum-packaged. Oysters in the inoculated groups were individually injected with V. vulnificus to a level of approximately 1 × 10⁶ CFU/g. Control oysters were already naturally contaminated to a level of approximately 1 × 10⁵ CFU/g. Oysters were then packaged, frozen and stored at -20°C. On day 0 and days 7, 14, 30 and 70 post-freezing, concentrations of total aerobic bacteria and V. vulnificus were determined using a 3-tube most probable number (MPN) estimation from enrichment Alkaline Peptone Water tubes with subsequent presumptive V. vulnificus growth on modified Cellobiose-Polymyxin B-Colistin agar. Length of frozen storage had a significant effect on decreasing total aerobic bacteria (from approximately 10⁶ CFU/g to approximately 10⁵ CFU/g) and V. vulnificus (from approximately 10⁵ CFU/g to approximately 10⁴ CFU/g). Also, vacuum-packaged samples showed significantly lower concentrations of V. vulnificus over the length of the study than did the normal-sealed samples.

Key Words: Vibrio, Vibrio vulnificus, oysters, shellfish, frozen storage, vacuum-packaging

One concern facing the shellfish industry is V. vulnificus. This gram-negative bacterium can cause infections in previously healthy persons, but usually it causes severe clinical disease with high case fatality rates in affected individuals with chronic underlying illness (6-8,15,27). Disease often results following wound infection or consumption of contaminated raw shellfish, especially oysters (1,3,4,6,15,27,30). Vibrio vulnificus can be frequently found in Gulf Coast oysters and waters during the warm-water season (5). It has been estimated that in April through October, 40% or more of Gulf Coast oysters may be carrying V. vulnificus (17).

Numerous experiments with V. vulnificus have shown it to be susceptible to reduced temperatures (10-14,18,20,23,24). In some of these studies V. vulnificus levels declined in oyster homogenates (20), shucked whole oysters (11-13,20) and commercial shellstock (10-13), but usually some viable organisms remained in these products. Some studies have exposed V. vulnificus-contaminated products to freezing temperatures (9,11). Following an initial bacterial count decline during the freezing process, numbers remained constant at -80°C storage (9) while they continually declined at -20°C (9,11).

A new popular processing trend in the seafood industry is the use of vacuum-packaging. Not only does it provide good product presentation, at lower temperatures it is also effective in inhibiting bacterial growth at lower temperatures resulting in reduced spoilage and extended product shelf-life (2,19).

The objectives of this experiment were to determine the viability of V. vulnificus inoculated into whole oysters when frozen and stored at -20°C under vacuum-packaged or normal-packaged conditions.

MATERIALS AND METHODS

Vibrio vulnificus culture and inoculum preparation

Vibrio vulnificus ATCC 27562 (the species type strain) was cultured in heart infusion broth (HIB) (Difco Laboratories, Detroit, MI). Bacterial cells were collected and washed twice with cold 0.01 M phosphate buffered saline (PBS) (pH 7.5) by centrifugation (5,000 × g, 10 min, 4°C). The cell pellet was resuspended in cold PBS and concentration adjusted according to a previously determined A₅₆₀ concentration curve (data not shown). Actual inoculum concentration was tested by triplicate spread plating onto Heart Infusion Agar (HIA) (Difco) plates from serial decimal dilutions of the inoculum in PBS. The initial inoculum level was subsequently determined as approximately 1.26 × 10⁶ CFU/ml.

Sample preparation

Freshly harvested and shucked Gulf Coast oysters (Crassostrea virginica) were obtained from a Dickinson, TX, processor. Oysters were drained of their liquor and portioned into a total of 60 (for 5 time periods × 4 treatment groups × 3 replicates) samples each weighing 125 g ± 5 g, using only whole oysters ranging in size from 6.0 to 14.0 g. The samples were randomly split into four treatment groups: Control Normal-Sealed (CN); Inoculated Normal-Sealed (IN); Control Vacuum-Sealed (CV); and Inoculated Vacuum-Sealed (IV). Inoculated oysters were individually injected (26 ga. needle) with approximately 0.1 ml of the prepared inoculum into the gastrointestinal tract region, thus, an average 10 g oyster received an inoculated V. vulnificus concentration of...
approximately $1 \times 10^6$ CFU/g of oyster tissue. Control oysters were not inoculated. The oyster sample groups were then placed in $7 \times 12$ in. low oxygen permeable bags (2.3 L capacity; water vapor transmission: 0.5 to 0.6 g/24 h, 100 in.$^2$, 100°C, 100% RH; oxygen transmission: 3 to 6 cm$^3$/24 h, 1 m$^2$, 1 atm, 40°C, 0% RH; Cryovac No. B540; W. R. Grace and Co., Duncan, SC) before sealing by a heat sealer/vacuum packager (Multivac A300 Table Top Model, Sepp Haggenmueller, Germany). Samples to be vacuum-packaged were sealed after evacuation of air. No vacuum was applied to the normal-sealed samples and remaining headspace was approximately 5 to 10% compared to the product volume. After sealing, all bags were placed in a -20°C laboratory freezer, except Day 0 samples which were immediately processed for bacterial enumerations as initial reference points.

Sample processing
Samples were processed for V. vulnificus concentration at Day 0 and Days 7, 14, 30 and 70 post-freezing. Triplicates from each treatment group (CN, CV, IN, IV) were processed at each interval. Each bag was aseptically opened and a 50 g ± 0.5 g sample was measured. The sample was placed in a sterile stainless steel Waring commercial blender with 450 ml cold PBS. The diluted sample was blended on high speed for 90 s followed by serial decimal dilutions in PBS. Alkaline Peptone Water (APW) (1% peptone, 1% sodium chloride [NaCl], pH 8.5), in 7-ml amounts, was inoculated with 1 ml of each dilution in a 3-tube MPN series (29). The APW tubes were incubated at 35 to 37°C for 12 to 16 h (26,30). Incubated broths showing turbidity were streaked (one loopful) onto Y-petri plates of Cellobiose-Polymyxin B-Colistin (CPC) agar (16,22,26) modified to contain 400,000 U/L of colistin methanesulfonate (28). The inoculated selective agar plates were incubated at 40°C for 18 to 24 h. Growth on modified CPC agar was examined for the presence of presumptive V. vulnificus colonies, typically flat, yellow colonies (due to the fermentation of the cellobiose) of approximately 2 mm in diameter (16,26,30). Presumptive V. vulnificus colonies were easily distinguished from non-cellobiose fermenters, which appeared green-brown to purple (16,30).

The MPN estimates of V. vulnificus per gram of oyster were calculated based upon the number of turbid APW tubes that provided presumptive V. vulnificus growth on the modified CPC agar plates. The MPN estimates of total aerobic bacteria in the samples were estimated solely from those tubes showing turbidity regardless of subsequent growth on the selective agars. The U.S. Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM) 3-tube MPN determination tables were used in calculations (29).

Statistical analysis
All data were analyzed using the Statistical Analysis System (SAS) (25) following $\log_{10}$ transformation of the raw data. Significant mean differences for each variable were determined using the non-parametric Student-Newman-Keuls test. The Day 0 results were included in the figures as a reference point, but not included in the statistical analysis because those samples had not been subjected to freezing treatment.

RESULTS
Survival of aerobic bacteria in oysters frozen at -20°C
The numbers of total aerobic bacteria, as estimated by MPN determination, decreased by 2 to 3 log units over the 70 days of storage (Fig. 1). Only the length of frozen storage was statistically significant in reducing the numbers of total aerobic bacteria (P <0.05). The packaging conditions did not significantly affect the total aerobic bacteria levels over the length of the study.

Survival of V. vulnificus in oysters frozen at -20°C
Several variables showed significant effects as V. vulnificus levels declined by 3 to 4 log units over the length of the study in all four treatment groups as determined with modified CPC agar (Fig. 2). Each successive time interval (7, 14, 30 and 70 days) showed a significant decrease in V. vulnificus numbers (P <0.05). Oysters that had been inoculated had significantly higher levels of V. vulnificus than did the controls (P <0.05). In addition, those samples that were vacuum-packaged showed a significantly greater decrease in V. vulnificus numbers than did the normally-sealed samples (P <0.05).

DISCUSSION
Due to the severity of V. vulnificus infections following the consumption of contaminated Gulf Coast oysters
the public health community has been investigating possible methods by which the concentrations of this pathogen may be reduced in oysters. This study has been successful to this end. Freezing oysters under vacuum-packaged conditions at -20°C was significantly effective in reducing loads of V. vulnificus. While much of the decline in V. vulnificus numbers occurs early post-freezing, as evidenced by day 7 results, levels do continue to decrease upon prolonged frozen storage. Day 30 samples, however, still contained up to 2 log units of V. vulnificus. Even at day 70, inoculated samples contained 1 log unit of the bacteria and control uninoculated samples still contained some low viable numbers of this pathogen.

Because the infectious dose is unknown, there may still be enough bacteria to cause an infection in some high-risk consumers (10,13). This reduction of bacteria by freezing and vacuum-packaging must be weighed against the economic aspects of the process. Requirements of long-term frozen storage, including such factors as storage space for freezing, time/length of storage and documentation of the process, may prove too costly to be widely employed in reducing V. vulnificus loads of shellfish species, Crassostrea virginica and Mercenaria campechiensis. Appl. Environ. Microbiol. 45:1221-1228.


