EFFECT OF TIME LENGTH OF FROZEN STORAGE AND VACUUM PACKAGING ON SURVIVAL OF *Vibrio vulnificus*

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SUMMARY: Freshly harvested, shucked oysters were inoculated with approximately $1x10^6$ CFU/g of *Vibrio vulnificus*. Samples were then either packaged under normal atmospheric conditions or packaged under vacuum. Oysters were then frozen and stored at -20°C for 7, 14, 30, and 70 days.

Significant decreases ($P<.05$) in total aerobic bacteria and *V. vulnificus* were seen over the 70 day period. Greater numbers of *V. vulnificus* were found to survive in the inoculated samples than in the control samples ($P<.05$). Finally, vacuum packaged samples showed significantly lower mean concentrations of *V. vulnificus* over the 70 days than the normally sealed samples ($P<.05$), although this was less the case for concentrations of aerobic bacteria ($P=.08$).

INTRODUCTION: As seafood consumption increases in the United States, so does concern over its safety. One problem now facing shellfish consumers is *Vibrio vulnificus*. Although only first recognized in 1975 (HOLLIS et al,1976), *V. vulnificus* is quickly gaining notoriety. This exceptionally virulent and invasive gram-negative bacteria is often found in shellfish and waters of the Gulf of Mexico from April to October (MILLER,1988). It can cause infection in healthy persons and often death in compromised individuals. *Vibrio vulnificus* infection manifests itself in 3 clinical forms: primary septicemia, wound infection, and gastrointestinal illness. Since no programs exist to limit harvesting of shellfish to areas free of *V. vulnificus* (*FDA,1988*), public health education is of utmost importance. Shellfish consumers, especially those with liver or other chronic underlying illnesses, need to be aware of the dangers of eating raw or undercooked seafood.

Although *V. vulnificus* has been shown to grow at refrigerated temperatures (FDA,1988; MORGAN and GUTHRIE,1991), it is rapidly inactivated at commercial freezing temperatures (BOUTIN et al,1985). This was the first experiment, however, to study the fate of *V. vulnificus* in whole oysters frozen at -20°C.

Vacuum packaging has become a popular trend in the seafood industry. Not only is it visually pleasing to the consumer, it is also effective in the inhibition of bacterial growth at lower temperatures (OGRYDZIAK and BROWN, 1982).

The first objective of this study was to determine the viability of *V. vulnificus* inoculated into whole oysters when frozen and stored at -20°C for intervals of 0, 7, 14, 30, and 70 days. The second objective was to compare the effects of the normal heat seal with the effects of the vacuum seal on reducing bacterial levels in the oysters.

MATERIALS AND METHODS:

Inoculum Preparation

V. vulnificus ATCC 27562 (the species type strain) was held at room temperature on heart infusion agar (HIA) (Difco). Twelve tubes, each containing 3 ml of heart infusion broth (HIB) (Difco), were inoculated with a loopful of the *V. vulnificus* stock culture and incubated 16 hours at 30°C and 240 rpm. After gram-staining, pure cultures were pooled and washed twice with sterile phosphate buffered saline (PBS) (pH 7.5). After appropriate dilution with sterile PBS, the inoculum was plated onto HIA plates and incubated for 10 hours. Plates yielded approximately $1x10^8$ colony forming units (CFU)/ml of *V. vulnificus*. Since each oyster weighed about 10 g and the inoculum size was approximately 0.1 ml, the resulting *V. vulnificus* concentration was at least $1x10^6$CFU/g of oyster.

Sample Preparation

Freshly harvested, shucked Gulf Coast Oysters (*Crassostrea virginica*) were obtained from a local processor in Dickinson, Texas and remained on ice overnight until processing was begun the next morning. Aseptic techniques were employed throughout the preparation of the samples. Oysters were weighed out into 125 g $\pm$ 5 g samples using only those oysters ranging in approximate size from 6.0-14.0 g. Sixty samples were formed, placed in quart-size zippered storage bags, and replaced in the ice. The 60 sample bags were then randomly split into 2 treatment groups: 30 to be used as controls and 30 to be inoculated with *V. vulnificus*. Using a 1cc syringe fitted with a 3/8 inch, 26 gauge needle, individual oyster were then inoculated with approximately 0.1 ml of the *V. vulnificus* suspension into the gut region,

returned to the bags and replaced on ice. The inoculum size was based on the estimated size of each oyster (0.07 ml per 7.0 g oyster, 0.1 ml per 10.0 g oyster, etc...). Control samples were not injected. Each group of 30 sample bags was then randomly separated into 2 groups: 15 to be heat sealed under normal atmospheric conditions and 15 to be heat sealed under vacuum. Bags were then folded back on themselves to remain open, and individually placed into a low oxygen permeable plastic bag. An absorbent paper towel was placed over the opening of the zippered bag to prevent the oyster liquor from interfering with the heat seal site on the packaging bag, which must remain dry. Bags to be normally sealed were then heat sealed and replaced in the ice. Using the same procedures, samples to be vacuum sealed were, after total evacuation of air, also heat sealed and replaced in the ice. All bags, except for Day 0, were then placed in a -20°C freezer. Samples were frozen for intervals of 7, 14, 30, and 70 days. Three samples from each group (control-normal sealed, control-vacuum sealed, inoculated-normal sealed, and inoculated-vacuum sealed), were processed at each interval.

Sample Processing

Immediately after the sorting, sealing, and freezing of all oysters, the 12 samples of Day 0 oysters were analyzed. Day 0 oysters were processed after packaging but before freezing. Using sterile instruments, each bag was opened and a 50 g $\pm$ 0.5 g sample was measured out. The sample was placed in a sterile stainless steel blender and 450 ml of chilled PBS (pH 7.5) were added (FDA,1988). The sample was blended on high speed for 90 seconds. Decimal dilutions of homogenate were then performed through 10^{-7} in 9 ml PBS. Seven ml of alkaline peptone water (APW) (pH 8.5) was then inoculated with 1 ml of each dilution in a 3-tube per dilution most probable number (MPN) series (FDA,1984; FDA,1988). Blenders were washed, autoclaved, and cooled between the processing of each sample. All inoculated APW tubes were vortexed and incubated at 35-37°C for 12-16 hours (FDA,1988). After such time, tubes showing turbidity were reported and streaked onto 2 Y-plates (Baxter), one containing cellobiose-polymyxin B-colistin (CPC) agar (MASSAD and OLIVER,1987) and the other containing Modified CPC agar (TAMPLIN et al,1991). After incubation at 40°C for 18-24 hours (FDA,1988), plates were examined for the presence of presumptive *V. vulnificus* growth. Typical *V. vulnificus* colonies on CPC and Modified CPC appeared as flat, yellow colonies of approximately 2 mm in diameter (FDA,1988). Both opaque and translucent colonies were present. *Vibrio vulnificus* colonies were easily distinguished from non-cellobiose fermenters, which appear greenish-brown to purple (FDA,1988). Colonies exhibiting the above traits, were presumed to be *V. vulnificus* and recorded.

The MPN estimate of *V. vulnificus* per gram of oyster was then calculated based on the number of turbid APW tubes later exhibiting characteristics of *V. vulnificus* on the selective agars. The FDA-BAM 3-tube most probable number determination tables were used in these calculations (FDA,1984).

At each of the previously mentioned intervals, 12 samples (3 from each group) were removed from -20°C storage and thawed rapidly under cool running tap water. Processing was carried out as detailed previously.

Statistical Analysis

General Linear Models Procedure was performed on the $\log_{10}$ of the estimated CFU/g of oyster (SAS,1988). Significant mean differences for each variable were determined using the Student-Newman-Keuls test. Finally, CPC and Modified CPC agars were compared for sensitivity using a t-test. Day 0 results were included as a point of reference, but not included in the analysis, since they were not subject to freezing temperatures, as were all other samples.

RESULTS AND DISCUSSION: Variables that were analyzed for each data set included: day (days of frozen storage), treatment (whether or not oysters had been inoculated with *V. vulnificus*), the interaction between day and treatment, type of seal (normal or vacuum), the interaction between day and seal, the interaction between treatment and seal, and finally, the interaction between day, treatment, and seal. Before analysis, all data underwent log transformation.

Survival of aerobic bacteria in oysters frozen at -20°C : (Figure 1)

Total bacteria, as estimated by MPN determination, decreased between 2.5 and 3.4 log units over the 70 days of storage. Of all the variables, only the length of frozen storage, was statistically significant in reducing the

Figure 1: Survival of Aerobic Bacteria in Frozen Oysters.

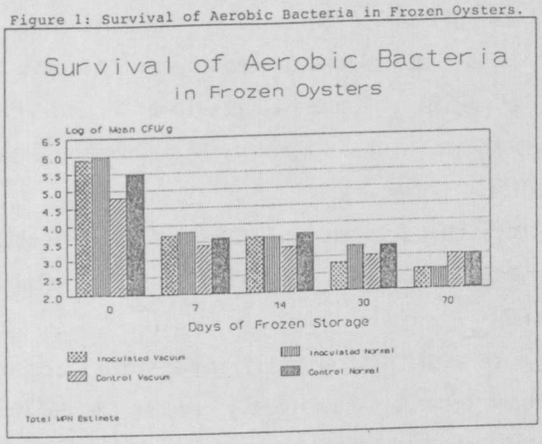


Figure 2: Survival of V.vulnificus in Frozen Oysters (CPC agar).

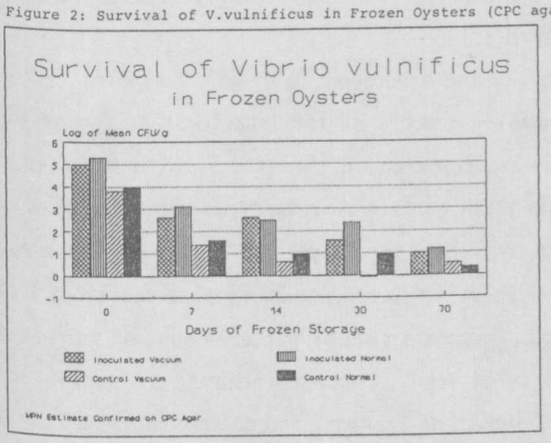
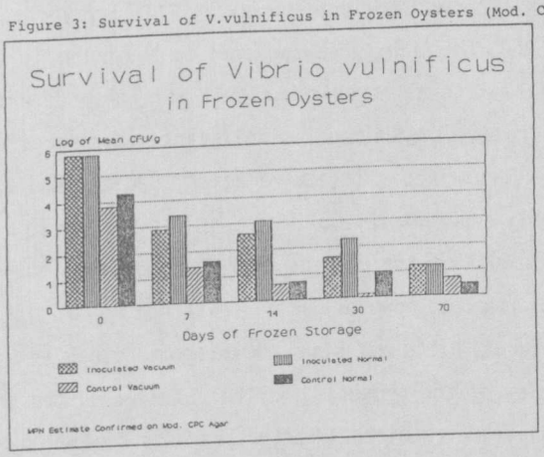


Figure 3: Survival of V.vulnificus in Frozen Oysters (Mod. CPC agar).



numbers of total aerobic bacteria (P<.05). Whether or not the type of seal helped reduce total bacterial numbers is questionable (P=.08). Mean bacterial values for Days 7 and 14 were not significantly different (P<.05). Both Days 30 and 70, however, had significantly different means of bacterial levels (P<.05). The type of seal and treatment group had no significant effect (P>.05) on the reduction of aerobic bacterial counts over the 70 day storage period.

Survival of *Vibrio vulnificus* in oysters frozen at -20°C

Recovery on CPC Agar : (Figure 2)

Vibrio vulnificus counts, as estimated by MPN determination and growth on CPC agar, decreased between 3.3 and 4.1 log units over the 70 day study. Day, treatment, the interaction of day and treatment, and the type of seal all had statistically significant (P<.05) effects on concentrations of *V. vulnificus*. Each successive time interval showed a significant decrease (P<.05) in *V. vulnificus* numbers. Oysters that had been inoculated had significantly higher levels (P<.05) of *V. vulnificus* than did the controls. Also, vacuum packaged samples showed a significantly greater decrease (P<.05) in *V. vulnificus* numbers than did the normally sealed samples.

Recovery on Modified CPC Agar : (Figure 3)

Similar results were obtained on Modified CPC agar. *Vibrio vulnificus* levels, as estimated by MPN determination and growth on Modified CPC agar, decreased between 3.1 and 4.6 log units over the 70 day period. Day, treatment, the interaction of day and treatment, and seal all had significant effects (P<.05) on the final concentrations of *V. vulnificus* at Day 70. While Days 7, 14, and 30 showed significant decreases (P<.05) in *V. vulnificus* numbers, there was no further significant decline (P<.05) between Day 30 and Day 70. Inoculated oysters showed significantly greater numbers (P<.05) of *V. vulnificus* than did the control groups at Day 70. Finally, vacuum packaging again showed a significantly greater decrease (P<.05) in *V. vulnificus* concentrations than did the normally sealed samples.

CPC and Modified CPC agars were compared using a t-test. More *V. vulnificus* was detected using the Modified CPC agar (P=0.0376), indicating that it is more sensitive for *V. vulnificus*. This may be because the Modified CPC

is less inhibitory, having 3.5 times less colistin. Additionally, the antibiotic solution in Modified CPC is not filter sterilized, as it is in the CPC. This may allow more polymyxin B to pass into the final mixture, and thus screen out more of the other vibrios, allowing for increased growth of *V. vulnificus*. Finally, since the CPC is autoclaved (Modified CPC is not), some components of the agar may be altered or destroyed in this process. This may explain the greater recovery on the Modified CPC agar.

CONCLUSION: Due to the severity and increasing frequency of *V. vulnificus* infections following the consumption of Gulf Coast oysters, the public health community has been researching possible methods by which the concentrations of this pathogen may be reduced in shellfish.

This study has been successful to this end. After analyzing *V. vulnificus* concentrations in the oysters, it was first determined that length of frozen storage significantly reduces ($P<.05$) loads of *V. vulnificus*. Secondly, those oysters in the inoculated treatment group, showed greater levels ($P<.05$) of *V. vulnificus* at analysis than did those in the control treatment group. Finally, vacuum packaging resulted in significantly greater reductions of numbers of *V. vulnificus* when compared to the normally sealed samples.

Levels of total aerobic bacteria were also determined during the experiment. Of all variables tested, only length of frozen storage had a significant reducing effect ($P<.05$) on numbers of total bacteria in the oysters.

Two *V. vulnificus* selective agars, CPC and Modified CPC were compared in this study. The Modified CPC was significantly superior ($P<.05$) to CPC in its ability to select and differentiate *V. vulnificus* recovered from oysters.

While much of the decline in *V. vulnificus* numbers occurs by Day 7, levels do continue to decrease up to Day 70. Day 30 samples, however, still contained up to 2 log units of *V. vulnificus*. Day 70 samples contained 1 log unit of the bacteria. This may still be enough to cause an infection. This reduction of bacteria must be weighed against the economics of the process. Further studies on the practicality of long-term frozen storage of oysters in vacuum packaged bags are required. Factors such as freezer space, time length of storage, and documentation of the process may prove too costly to be widely employed in industry. Organoleptic quality of the oysters after lengthy storage must also be considered. Meanwhile, until effective *V. vulnificus* reducing storage procedures are widely implemented, people are advised to eat their shellfish thoroughly cooked, and for those individuals with compromising health conditions, to avoid consumption of shellfish altogether.

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