The Effects of Freezing Temperatures and Times on the Inactivation of Human Pathogenic *Vibrio parahaemolyticus* in Gulf Coast Oysters (*Crassostrea virginica*)

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Introduction

Vibrio parahaemolyticus is a ubiquitous inhabitant of coastal marine water and is part of the natural flora of bivalve shellfish (Yeung and Boor, 2004). Molluscan shellfish extract, filter, and concentrate this organism from their environment. *V. parahaemolyticus* is the leading bacterial cause of seafood-associated gastroenteritis in the United States. Few studies have been performed on the sensitivity of clinical *V. parahaemolyticus* to freezing (both in solution and in *Cassostrea virginica* oysters). The goal of this research was to generate practical, applicable, and definitive data on freezing temperatures and storage times necessary for the reduction that oyster processors could employ to improve the safety of their oysters.

In the first phase of the study,ten clinical *V. parahaemolyticus.* strains known to have caused foodborne illness outbreaks were obtained from the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA). These strains were exposed to various storage time and temperature parameters to determine the necessary conditions to completely inactivate *Vibrio parahaemolyticus* in both mid-log and stationary phase After growth to mid-log and stationary phase (7 $\log_{10}$) in T_1N_1 broth (Trypticase 1%, NaCl 1%) , 4 ml of each strain was tested using all combinations of four temperatures (4, -10, -18, and -25°C) and 7 storage times (7, 14, 30, 45, 60, 90, and 120 days). The mid-log phase proved to be less virulent under freezing conditions than the stationary phase, as all ten strains stored at the mid-log phase were inactivated by day 90 (Table 1). From this work, strains BAC 983547, DIE 12-052499 and SPRC 10290 in the stationary phase were determined to be the most virulent under freezing conditions (Table 1) and were selected for phase two of the study. In phase two, these strains were propagated for assimilation in oysters and the storage study was repeated. Day 120 was added to the oyster storage study.

	Complete Inactivation (Day)							
	Stationary Phase				Mid-log Phase			
	4°C	-10°C	-18°C	-25°C	4°C	-10°C	-18°C	-25°C
AQ 4037 03:K6	90	30	60	90	45	7	15	30
ATCC 17802 non-03:K6	45	7	45	45	45	15	15	7
ATCC 17803 non-03:K6	30	7	30	30	15	7	7	15
BAC 98-3547 04:K55	**Viable day 90**	**Viable day 90**	**Viable day 90**	**Viable day 90**	**Viable day 90**	**45**	**60**	**Viable day 90**
CPA7-08-1699 04:K8	90	15	90	90	90	15	15	90
DIE 12-052499 01:KU(T)K	**Viable day 90**	**15**	**60**	**Viable day 90**	**90**	**15**	**60**	**90**
SPRC 10290 04:K12	**Viable Day 90**	**45**	**60**	**Viable day 90**	**Viable day 90**	**30**	**45**	**60**
SPRC 10293 01:K56	45	15	45	45	45	15	30	45
TX 2103 03:K6	Viable day 90	15	90	Viable day 90	90	30	30	90
TX 2062 03:K6	45	30	30	45	90	45	45	60

Table 1. Strains of clinical *Vibrio parahaemolyticus bulb data for inactivation in Stationary and Mid-log Phases*

stationary phase (7 $\log_{10}$) in T_1N_1 broth (Trypticase 1%, NaCl 1%) , pelleted cells were

reconstituted in phosphate buffered saline (PBS) and 4 ml of each strain was sealed in disposable transfer pipets (SAMCO, San Fernando, CA) and stored at temperatures of 4, -10, -18 and -25°C for 0, 7, 15, 30, 45, 60, and 90 days (See Table 1). Live oysters (*Crassostrea virginica*) were obtained from Cowart Seafood Corp, Lottsburg, VA and shipped overnight to the Virginia Tech Food Science Seafood Laboratory. The oysters were washed to remove any mud and placed in aerated artificial seawater tanks (20 ppt salinity in 30 liter tanks maintained at 18°C) for 4 hours before inoculation.

Using Cook's (2003), protocol for obtaining cell suspensions, 20 mls of the resulting bacterial concentration of 10^8 CFU/ml was used to inoculate tanks containing oysters, resulting in a final concentration of $\pm 10^5$ cells/ml seawater. The temperature in the room was raised to 22°C (71.6°F). After 18 hours, the oysters were removed and randomly placed into Styrofoam® containers. The oysters were then stored at 4°C, -10°C, -18°C, or -25°C for 0, 7, 15, 30, 45, 60, 90, and 120 days. At each sampling day, nine oysters were removed from each storage container and divided into three replicate samples. The oysters were shucked, and the FDA's Bacteriological Analytical Manual (BAM) preferred laboratory procedures for microbiological analyses of seafoods were followed for *Vibrio* isolation and identification. Equal parts oyster and PBS were homogenized for 2 min using a Seward Stomacher 400 Circulator (Tekmar Co., Cincinnati, OH). From this, serial dilutions were made in PBS and Most Probable Numbers (MPN) of *V. parahaemolyticus* were determined by inoculating 3 x 1 ml portions of the 1:10, 1:100, 1:1000, and 1:10,000 dilutions into 10 ml of Alkaline Peptone Saline (APS). The tubes were incubated overnight at 35°C. From the top 1 cm of the APS tubes containing the three highest dilutions of sample showing growth, a 3-mm loopful was streaked onto TCBS (Thiosulfate Citrate Bile Salts Sucrose) for *V. parahaemolyticus.* The TCBS plates were incubated at 35°C overnight. Typical blue-green colonies on the TCBS were counted and recorded. Representative colonies were picked for confirmation using API 20E® diagnostic strips. The number of MPN tubes containing confirmed *V. parahaemolyticus* colonies was compared with the 3-tube-MPN chart and the results expressed as *V. parahaemolyticus* MPN/g of oysters.

Results

Figures 1, 2, and 3 illustrate the MPN for each of the three strains under each set of storage parameters. For DIE 12-052499, storage at -10°C for both 90 and 120 days resulted in greater than a 4.4 log reduction. For SPRC 10290, storage at -10°C for 60 and 120 days resulted in a 3.8 log reduction, storage at -18°C for 120 days resulted in 3.8 log reduction. The third strain, BAC 98-3547 also exhibited log reductions of 3.9 and 3.8 at -10°C for 90 and 120 days respectively, and a 3.7 log reduction at -18°C for 120 days storage.

Conclusion

The recommended storage parameters for inactivation of clinical strains of *V parahaemolyticus* include -10°C for 90 days, or -18°C for 120 days. The level of oyster uptake of the *Vibrio* inoculum was 10^5; however, we feel that the storage parameters listed above could easily inactivate clinical *Vibrio parahaemolyticus.* concentrations greater than 5.0 log in oysters.

References

Cook, D.W. 2003. Sensitivity of *Vibrio* species in phosphate-buffered saline and in oysters to High-pressure processing. *Journal of Food Protection.* 66(12): 2276-2282

Yeung, P.S.M. and K.J. Boor. 2004. Epidemiology, Pathogens, and Prevention of Foodborne *Vibrio parahaemolyticus* infections. *Foodborne Pathogens and Disease.* 1(2): 74-88.

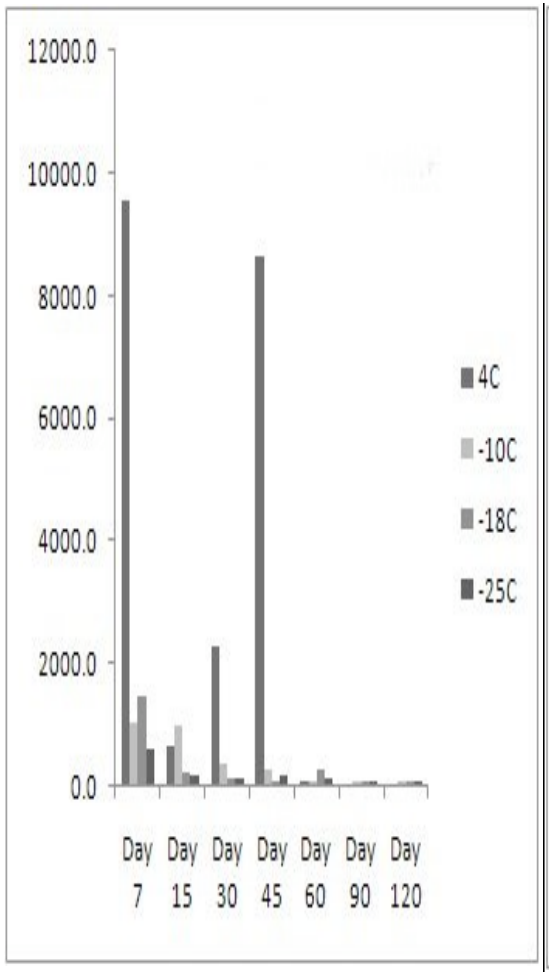

Figure 1. MPN for *V. parahaemolyticus* DIE 12-052499 Temperature and Storage Data

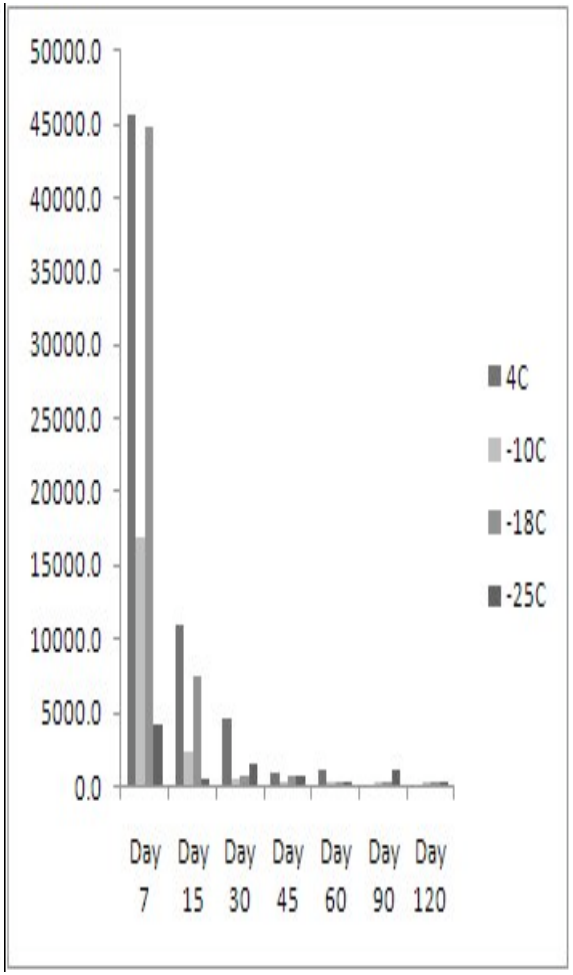

Figure 2. MPN for *V. parahaemolyticus* SPRC 10290 Temperature and Storage Data

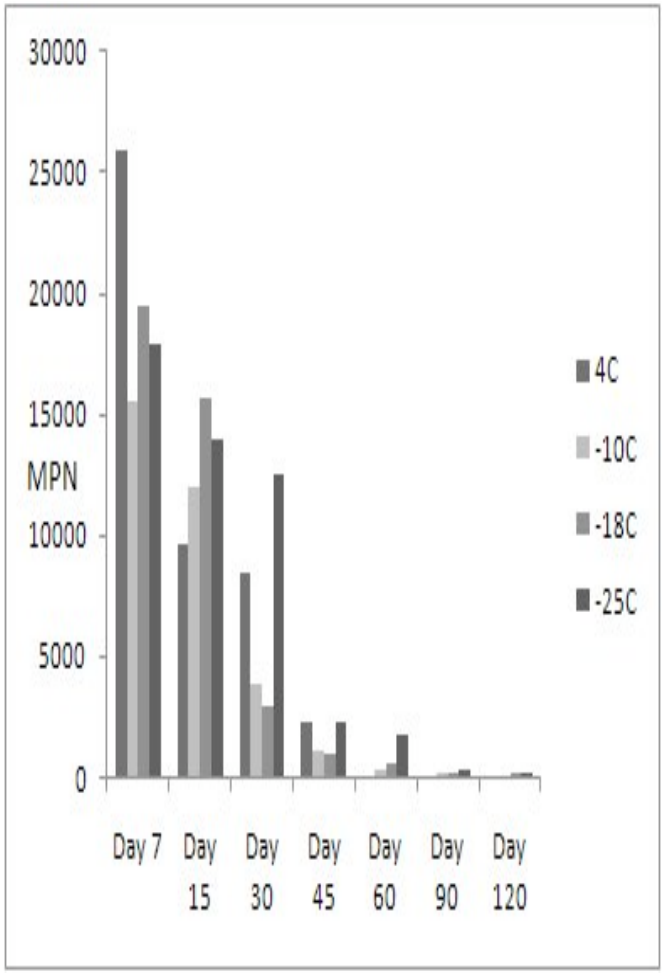

Figure 3. MPN for *V. parahaemolyticus* BAC 98-3547 Temperature and Storage Data