Int.J.Curr.Microbiol.App.Sci (2017) *6*(9): 3808-3815

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 9 (2017) pp. 3808-3815**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.609.470

Growth Kinetics of *V. parahaemolyticus* in Post-Harvest Shellstock Clams (*Meretrix meritrx*) Spiked with Pathogenic Strains of *V. parahaemolyticus*

V. Alamelu[1*], B. K. Krishna[2], Dechamma Sony[2], M. N. Venugopal[2] and S. Malathi[2]

[1]Fisheries College & Research Institute, TNFU, Thoothukudi – 628 008, Tamil Nadu, India
[2]Department of Fisheries Microbiology, College of Fisheries, KVAFSU, Mangalore–575 002, Karnataka, India

**Corresponding author*

Keywords

V. parahaemolyticus, storage temperature, growth kinetics, environmental factor, strain variability

Article Info

Accepted:
30 August 2017
Available Online:
10 September 2017

A B S T R A C T

Pathogenic strains of *V. parahaemolyticus* exhibits growth variability at different storage temperatures and it plays an vital role in seafood safety risk assessment. The present study investigated the effects of storage temperatures on growth kinetics of total *V. parahaemolyticus* in artificially spiked post-harvest shell stock clams (*Meretrix meretrix*). Clams are consumed raw or undercooked. Vibrios accumulates in clams and that may exceed levels that cause human illness when post harvest temperatures are not properly controlled. Growth kinetics were produced by injecting clams with three different strains of *V. parahaemolyticus* and measuring viability rates at temperatures (10, 20, 30 and 37°C) at selected time intervals. At selected time intervals, separated samples of five clams, each were homogenated and analyzed for total *V. parahaemolyticus* count. The results revealed that the growth variability among strains increased as the growth conditions became more stressful in terms of temperature (20°C and 10°C). This study confirms that the storage temperatures as well as strain variability of *V. parahaemolyticus* have impacts on growth kinetics of total *V. parahaemolyticus* and which can be helpful for incorporating strain variability in microbial risk assessment.

Introduction

Vibrio parahaemolyticus is a gram negative, halophilic bacterium, naturally associated with marine environment (Zhang and Orth, 2013). Among *Vibrios, V. parahaemolyticus, V. vulnificus* and *V. cholerae* are the three primary human pathogens responsible for the majority of disease related to seafood-borne bacterial infections (Regunath, 2014). *Vibrios* spp. can be isolated from near-shore marine waters and estuarine environments, and are typically found in the water column during late spring or early summer and in the sediment during winter. The bacteria are associated with marine organisms including zooplankton and farmed aquatic animals (Kaneko *et al.*, 1977; Thompson *et al.*, 2004). Human disease usually occurs as medium to severe gastroenteritis, septicemia and wound infection. Clinical illness mostly associated with the pathogenic strains of *Vibrios* spp. Clams are filter feeders and may accumulate pathogenic microorganisms from contaminated waters. *Vibrios* spp. existing naturally in clam and oyster growing

environments (Cook *et al.*, 2002) and it can be concentrated in oysters through filtering of contaminated water. The densities of Vibrio spp. in both environment and clams are affected by several factors, particularly water temperature. In order to limit the growth of *Vibrio parahaemolyticus* in post-harvest shellstock clams, there is a need to establish time – temperature regulations that restrict maximum exposure time of clams to ambient temperatures. Earlier studies indicated that temperature abuse of clams by improper storage induces higher rates of *Vibrio parahaemolyticus* Generally, *Vibrios* spp. reported to multiply rapidly with a short generation time in seafood between 18 to 40^{0}C (). Other research findings suggest that refrigerated storage should be considered a critical control point (CCP) in hazard analysis and critical control points (HACCP) plans for raw clams. Higher storage temperature may lead to pathogen growth as well as spoilage. Further, growth behavior of total *Vibrios* inside bivalves has not been compared. Effective post-harvest treatments to eliminate pathogenic *Vibrio parahaemolyticus* are an important step to reduce the risk of infection associated with seafood consumption. No information is available on the growth of total *Vibrio parahaemolyticus* in clam, *Meretrix meretrix* stored at different temperatures. Since, the consumption of clams is more in the south west coast of India, this study was carried out with the purpose to prevent the occurrence of severe gastrointestinal and fatal human diseases due to the consumption of undercooked clams.

Materials and Methods

Bacterial cultures

Three pathogenic strains of *V. parahaemolyticus,* each possessing VP5 (*tdh*$^+$*trh*$^-$), VP22 (*tdh*$^-$*trh*$^+$) and VP12 (*tdh*$^+$*trh*$^+$) virulence genes were used in this study. These strains obtained from Dept. of Fisheries Microbiology, UNESCO-MIRCEN Centre for Biotechnology, Mangalore, India. Each culture was individually enriched in 10 ml of sterile alkaline peptone water (APW) supplemented with 1.5% NaCl at 37^{0}C overnight. The enriched cultures were pooled into a sterile centrifuge tube and centrifuged at 5^{0}C (600g rpm). The pelleted cells were collected and resuspended in sterile phosphate buffered solution to prepare 10^5 CFU/ml for inoculation.

Clam preparation

Live clams were collected from Mangalore coast in India and shipped to laboratory in a cooler with gel packs. The clams were briefly rinsed with seawater to remove mud from the shell and kept in glass tanks for depuration. The live clams were used for this study.

Challenging clams with *V. parahaemolyticus*

The artificially challenged live clams were transferred to plastic tray covered with sterile polythene cover to keep the clams active. The live clams were separated into three lots. Each were injected with different strains of *V. parahaemolyticus* at the level of 10^5 CFU/ml using the syringe. Clams were inoculated with *V. parahaemolyticus* artificially (10^5 CFU/ml) at room temperature. Inoculated clams were placed in plastic tray with sterile plastic cover and stored at 10, 20 and 37^{0}C.

Effects of different storage temperature on growth kinetics of Total *V. parahaemolyticus* (TVC)

The growth kinetics of *V. parahaemolyticus* at different storage temperatures were determined at 8 h time interval for up to 25 h, 72 h and 136 h at 37, 20 and 10^{0}C respectively. At each time interval, 5 clams were taken out from the pack for analyses.

Changes in growth kinetics for all the three strains of *V. parahaemolyticus* which was spiked artificially in clams were determined using spread plating (FDA, 2005). The optimum storage temperature of *V. parahaemolyticus* is 37^0C, hence the clams stored at 37^0C was used as reference in this study.

Microbiological analysis

For *V. parahaemolyticus* analysis, about 2 g of shucked meat of 5 clams were placed in mixer grinder and blended at high speed for 1 min. Buffered saline solution can be used as a diluents in Vibrio sp. Assays (Mahmoud *et al.*, 2009). The use of PBS recommended in the Australian standard methods (SAA, 1977). The blended samples were diluted in 10 fold serial dilutions with PSS and 100 µl was plated in duplicate on TCBS agar. The TCBS plates were incubated at 37^0C for 18 to 24 h. Plated dilutions yielding 30 to 300 CFU/plate were counted manually, and the number of CFU/g of homogenate was calculated. The study was continued until the clams were visibly gapped.

Statistical analysis

Differences between the growth of total *V. parahaemolyticus* at storage temperatures for selected time interval were analyzed with Two way ANOVA followed by Duncan post hoc test using SPSS 21. Significant differences between means of treatments were established at level of $P<0.05$. Prior to analysis, quantitative variables were log transformed to improve homoscedasticity and linearity.

Results and Discussion

The growth kinetics of *V. parahaemolyticus* VP5 (*tdh*$^+$*trh*$^-$) was observed to increase from 4.37 to 6.59 $\log_{10}$ CFU/g on 10 h and further decreased to a level of 3.82 $\log_{10}$ CFU/g on 20 h of storage at 37^0C (Table 1). Similarly, the total *V. parahaemolyticus* count of *V. parahaemolyticus* VP22 (*tdh*$^-$*trh*$^-$) and VP12 (*tdh*$^+$*trh*$^+$) strain was increased from 4.25 to 4.52 $\log_{10}$ CFU/g on 15 h and from 4.18 to 4.81 $\log_{10}$ CFU/g on 15 h and later with increased storage time, the growth and survival of total *V. parahaemolyticus* was reduced significantly to a level of 3.41 $\log_{10}$ CFU/g and 3.91 $\log_{10}$ CFU/g on 20 h as shown in Fig.I. It was observed that the significantly higher growth rate was observed in VP5 (*tdh*$^+$*trh*$^-$) strain ($P<0.05$) when compared to VP22 (*tdh*$^-$*trh*$^+$) and VP12 (*tdh*$^+$*trh*$^+$) strains at 37^0C (Fig.5). Duncan test indicated that the total *V. parahaemolyticus* was statistically significant for all the three strains of *V. parahaemolyticus* at 37^0C. The results were similar to the study of Cook and Ruple (1989) found a significant rise in Vibrio levels in post-harvest shellstock oysters stored at 22 and 30^0C. Total *V. parahaemolyticus* count for VP5 (*tdh*$^+$*trh*$^-$) strain increased over time at 20^0C (Fig. 2). At 20^0C, the *V. parahaemolyticus* count was observed at 32 h of storage at 20^0C with about 3.62 to 4.03 $\log_{10}$ CFU/g from the 0 h (Table 2). The same trend was observed in the remaining two strains of *V. parahaemolyticus*. total *V. parahaemolyticus* count of *V. parahaemolyticus* VP22 (*trh*$^+$*tdh*$^-$) and VP12 (*tdh*$^+$*trh*$^+$) were increased from 3.69 to 4.50 $\log_{10}$ CFU/g and 3.29 to 4.22 $\log_{10}$ CFU/g on 40 h and 48 h respectively (Figure. II). Piquer *et al.*, (2011) reported that the there was an approximate increase of 2.5 and 3 $\log_{10}$ CFU/g in Total *V. parahaemolyticus* for pacific oysters stored at 20^0C for 3 days and 6.2^0C for 10 days, respectively. Total *V. parahaemolyticus* have also been measured in American oysters, where an increase of approximately 3 log units was observed after storage at 7^0C and 21^0C for 10 days (Lorca *et al.*, 2001).

Fig.1 Total *V. parahaemolyticus* Count (TVC) of VP5 (*tdh*$^+$*trh*$^-$), VP22 (*trh*$^+$*tdh*$^-$) and VP12 (*tdh*$^+$*trh*$^+$) at 37^{0}C

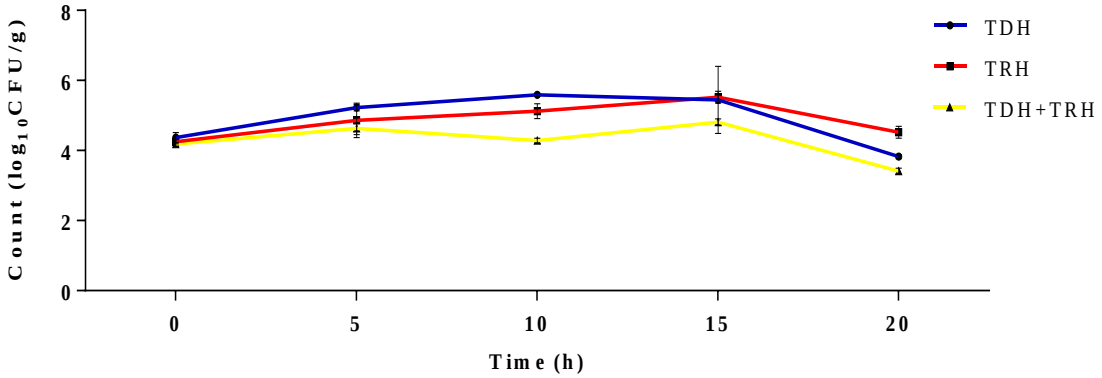

Fig.2 Total *V. parahaemolyticus* Count (TVC) of VP5 (*tdh*$^+$*trh*$^-$), VP22 (*trh*$^+$*tdh*$^-$) and VP12 (*tdh*$^+$*trh*$^+$) at 30^{0}C

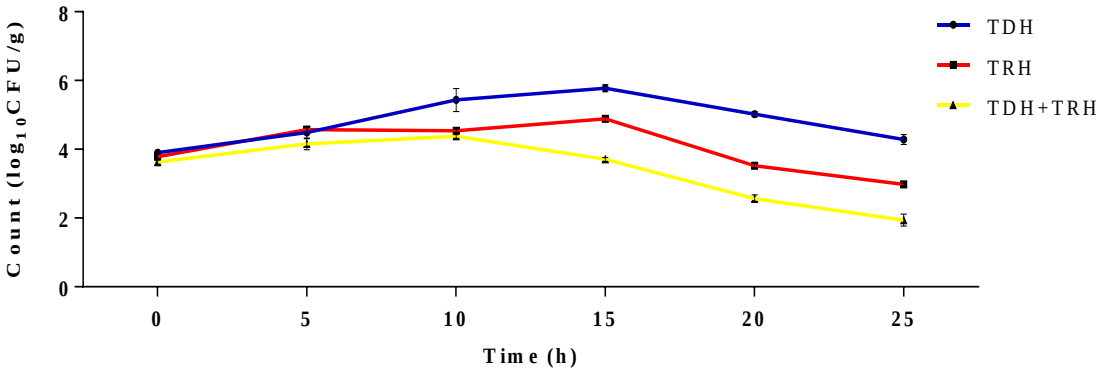

Fig.3 Total *V. parahaemolyticus* Count (TVC) of VP5 (*tdh*$^+$*trh*$^-$), VP22 (*trh*$^+$*tdh*$^-$) and VP12 (*tdh*$^+$*trh*$^+$) at 20^{0}C

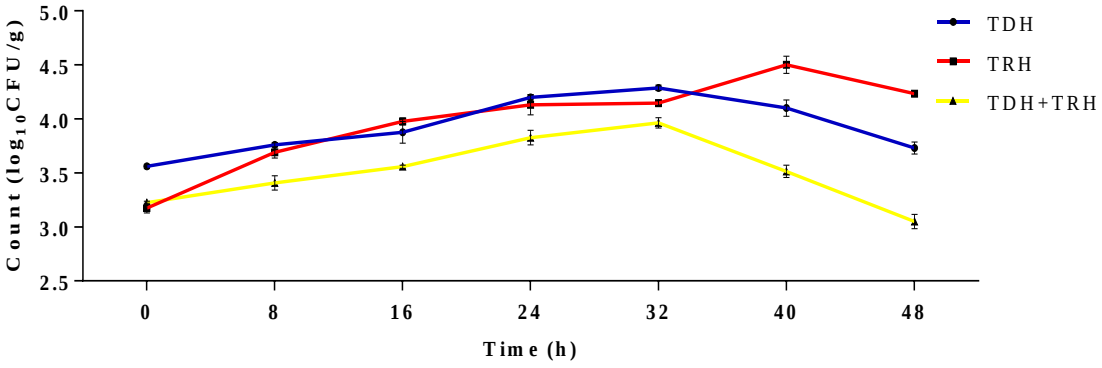

Fig.4 Total *V. parahaemolyticus* Count (TVC) of VP5 (*tdh*$^+$*trh*$^-$), VP22 (*trh*$^+$*tdh*$^-$) and VP12 (*tdh*$^+$*trh*$^+$) at 10^{0}C

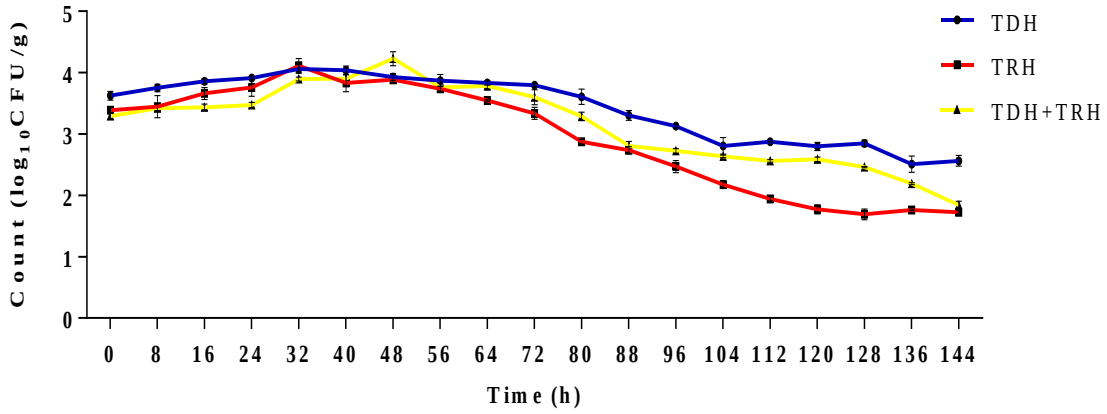

Table.1 Total *V. parahaemolyticus* counts for pathogenic strains grown in clams at 37^{0}C

Time interval	Pathogenic *V. parahaemolyticus*		
	VP5 (*tdh*$^+$*trh*$^-$) (log_{10} CFU/g)	VP22 (*tdh*$^-$*trh*$^+$) (log_{10} CFU/g)	VP12 (*tdh*$^+$*trh*$^+$) (log_{10} CFU/g)
0	4.37	4.25	4.18
5	5.22	4.86	4.63
10	6.59	5.12	4.28
15	5.44	4.52	4.81
20	3.82	4.52	3.41

Table.2 Total *V. parahaemolyticus* counts for pathogenic strains grown in clams at 30^{0}C

Time interval (h)	Pathogenic *V. parahaemolyticus*		
	VP5 (*tdh*$^+$*trh*$^-$) (log_{10} CFU/g)	VP22 (*tdh*$^-$*trh*$^+$) (log_{10} CFU/g)	VP12 (*tdh*$^+$*trh*$^+$) (log_{10} CFU/g)
0	3.90	3.7833	3.63
5	4.48	4.5733	4.15
10	5.43	4.5400	4.37
15	5.77	4.8833	3.70
20	5.02	3.5200	2.56
25	4.28	2.9800	1.94

Table.3 Total *V. parahaemolyticus* counts for pathogenic strains grown in clams at 20^0C

Time interval	Pathogenic *V. parahaemolyticus*		
	VP5 (*tdh*$^+$*trh*$^-$) (log_{10} CFU/g)	VP22 (*tdh*$^-$*trh*$^+$) (log_{10} CFU/g)	VP12 (*tdh*$^+$*trh*$^+$) (log_{10} CFU/g)
0	3.56	3.17	3.22
8	3.76	3.69	3.40
16	3.87	3.97	3.55
24	4.20	4.13	3.82
32	4.28	4.14	3.96
40	4.10	4.50	3.51
48	3.73	4.23	3.05
56	3.56	3.17	3.22
64	3.76	3.69	3.40
72	3.87	3.97	3.55

Table.4 Total *V. parahaemolyticus* counts for pathogenic strains grown in clams at 10^0C

Time interval	Pathogenic *V. parahaemolyticus*		
	VP5 (*tdh*$^+$*trh*$^-$) (log_{10} CFU/g)	VP22 (*tdh*$^-$*trh*$^+$) (log_{10} CFU/g)	VP12 (*tdh*$^+$*trh*$^+$) (log_{10} CFU/g)
0	3.62	3.38	3.29
8	3.75	3.44	3.41
16	3.85	3.66	3.43
24	3.91	3.75	3.47
32	4.06	4.11	3.89
40	4.03	3.83	3.90
48	3.93	3.88	4.22
56	3.87	3.73	3.75
64	3.83	3.54	3.78
72	3.79	3.33	3.60
80	3.60	2.87	3.28
88	3.30	2.73	2.80
96	3.13	2.47	2.72
104	2.80	2.17	2.63
112	2.87	1.91	2.55
120	2.79	1.77	2.58
128	2.84	1.69	2.46
136	2.50	1.76	2.19

There was significant difference ($P<0.05$) in the growth and survival of total vibrio among the three strains. The effect of time and storage temperature on total *V. parahaemolyticus* was statistically significant among the three strains of *V. parahaemolyticus* at 8 h, 24 h, 32 h and 48 h at 20^0C ($P<0.05$).

At 10^0C, total *V. parahaemolyticus* count for VP5 (*tdh*$^+$*trh*$^-$) strain was initially showed an increasing trend from 3.62 to 4.06 $\log_{10}$ CFU/g in 0 to 32 h. After 32 h of storage, the significant reduction was noted in total *V. parahaemolyticus* to a level of 2.56 $\log_{10}$ CFU/g (Table 3). Total *V. parahaemolyticus* of *V. parahaemolyticus* VP22 (*trh*$^+$*tdh*$^-$) and VP12 (*tdh*$^+$*trh*$^+$) strains were also in similar manner viz. 3.38 to 4.11 $\log_{10}$ CFU/g in 32 h further decreased to a level of 1.72 $\log_{10}$ CFU/g and 1.84 $\log_{10}$ CFU/g in 144 h, respectively. In contrast, Total *V. parahaemolyticus* remained approximately 3.3 $\log_{10}$ CFU/g or below at 5^0C and 10^0C indicating that low temperatures can suppress *total vibrio* growth (Mudoh *et al.,* 2014). This study also corroborates the findings of Cook and Ruple (1989) as well as Lorca *et al.,* (2001). They had reported similar total vibrio results with post-harvest shell stock oysters stored at 7^0C, 13^0C, 21^0C, 22^0C and 30^0C for a 10 day period in oysters. Duncan post hoc test indicated the effect of time and temperature on total *V. parahaemolyticus* was statistically significant among the three strains of *V. parahaemolyticus* at 10^0 C at 120 h, 128 h, 136 h and 144 h ($P<0.05$) as shown in Fig.III. Results presented here showed that keeping cold at 10^0C was an effective method to limit the total *Vibrios* in clams. For reducing the total *V. parahaemolyticus* contamination in foods, the cold temperatures should be maintained from shipping, retail and home refrigeration.

The growth of total *V. parahaemolyticus* count in three different strains of *V. parahaemolyticus*, VP5 (tdh+trh-), VP22 (tdh-trh+) and VP12 (tdh+trh+) was highest of 6.59 log10 CFU/g on 10 h at storage temperature of 37^0C followed by 30^0C and 20^0C in VP5 (tdh+trh-) strain. Among these three strains, VP5 (tdh+trh-) strain had shown significantly highest growth rate than other two strains at all storage temperatures. Similar trend was noted by Piquer *et al.,* (2011) in oysters. He reported that the there was an approximate increase of 2.5 and 3 log10 CFU/g in Total *V. parahaemolyticusfor* pacific oysters stored at 20^0C for 3 days and 2^0C for 10 days, respectively. Total *V. parahaemolyticus* have also been measured in American oysters, where an increase of approximately 3 log units was observed after storage at 7^0C and 21^0C for 10 days (Lorca *et al.,* 2001) and an increase of approximately 1 log10 was observed after storage at 22^0C for days (Cook and Ruple, 1989). Differences among studies could be due to different oyster species and medium compositions (eg. NaCl concentration) as well as storage temperatures. When the total *V. parahaemolyticus* count was compared to the growth of *V. parahaemolyticus*, it shows higher growth rates than total *V. parahaemolyticus* count at temperatures above 20^0C, 30^0C and 37^0C.

V. parahaemolyticus level increased over time with storage temperatures. Lorca *et al.,* (2001) confirmed the similar results for *V. vulnificus* in shellstock oysters stored at 7^0C, 13^0C and 21^0C for a 10 day period. Further, Cook and Ruple (1989) also reported a similar rise in *Vibrios* levels in post-harvest shell stock oysters stored at 22^0C to 30^0C. Few years before, Parveen *et al.,* (2012) also observed that the *V. parahaemolyticus* multiplied rapidly when oysters were stored at 15 or above, with no growth at 5^0C and 10^0C. Based on the results, this study suggests that storage of shellstock clams at or below 10^0C is effective for preventing total Vibrio growth in shellstock clams.

Total *V. parahaemolyticus* count of VP5 (tdh+trh-) strain was shown increased slightly and further decreased from 3.62 to 4.06 log10 CFU/g in 0 to 32 h at 100C. After 32 h of storage, the significant reduction was noted in Total *V. parahaemolyticus* to a level of 2.56 log10 CFU/g. Total *V. parahaemolyticus* of VP5 (trh+tdh-) and VP12 (tdh+trh+) strains

were also exhibited similar manner viz. 3.38 to 4.11 log10 CFU/g in 32 h further decreased to a level of 1.72 log10 CFU/g and 1.84 log10 CFU/g in 144 h, respectively. In contrast, total *V. parahaemolyticus* count remained approximately 3.3 log10 CFU/g or below at 50C and 10^0C indicating that low temperatures can suppress total Vibrio growth (Mudoh *et al.,* 2014). This study also corroborates the findings of Cook and Ruple (1989) as well as Lorca *et al.,* (2001). They had reported similar total Vibrio results with post-harvest shell stock oysters stored at 7^0C, 21^0C, 22^0C and 30^0C for a 10 day period in oysters. The growth of *V. parahaemolyticus* could be affected by many factors including temperature and salinity, in which temperature is the key controlling factor in seafood processing, preservation and distribution operations. Therefore, it is important to understand the growth or death responses of the *V. parahaemolyticus* to environmental temperature and it could help to correlate contamination level and storage temperature conditions. The findings of this study will serve as a basis for the strengthening of temperature management strategies and provide valuable safety information to producers and consumers.

Acknowledgements

The author thank the Dean, College of Fisheries, Mangalore for the facilities and support to carry out the research work.

References

Cook, D. W. and Ruple A. D., 1989. Indicator bacteria and Vibrionaceae multiplication in post-harvest shellstock oysters. *J. Food Prot*., 52:343–349

Cook, D.W., Bowers, J.C. and Depaola, A., 2002. Density of total and pathogenic (tdh+) *Vibrio parahaemolyticus* in Atlantic and Gulf coast molluscan shellfish at harvest. J. Food Prot., 65:1873–1880

FDA, 2005. Quantitative risk assessment on the public health impact of pathogenic Vibrioparahaemolyticus in raw oysters. US Food and Drug Administration, Washington, DC.

Kaneko, T. and Colwell, R. R., 1977. The annual cycle of *Vibrio parahaemolyticus* in Cheasapeake Bay. Microb. Ecol., 4: 135-142

Lorca, T. A., Pierson, M. D., Flick, G. J. and Hackney, C. R., 2001. Levels of Vibrio vulnificus and organoleptic quality of raw shellstock oysters (*Crassostrea virginica*) maintained at different storage temperatures. *J.Food Prot*., 64:1716–1721

Mahmud, Z.H., Neog, S.B., Kassu, A., Wada, T., Islam, M.S., Nair, G.B. and Ota, F., 2007. Seaweeds as a reservoir for diverse *Vibrio parahaemolyticus* populations in Japan. *Int. J. Food Microbiol*., 118: 92-96

Mudoh, M.F., Parveen, S., Schwarz, J., Rippen, T. and Anish Chaudhuri., 2014. The effects of storage temperature on the growth of *Vibrio parahaemolyticus* and organoleptic properties in oysters. Front. In Public Health, 6(45): 1- 7

Reghunath, P., 2014. Roles Of Thermostable Direct Hemolysin (Tdh) And TDH-related hemolysin (TRH) in Vibrio parahaemolyticus. Frontiers in Microbiology., 5:1-4

Thompson, F.L., Iida, T. and Swings, J., 2004. Biodiversity of *Vibrios*. Microbiol. Mol. Biol. Rev., 68: 403-431

Zhang, L. and Orth, K., 2013. Virulence determinants for Vibrio parahaemolyticus infection. Curr. Opin. Microbiol., 16:70–77

How to cite this article:

Alamelu V., B. K. Krishna, Dechamma Sony, M. N. Venugopal and Malathi S. 2017. Growth Kinetics of *V. parahaemolyticus* in Post-Harvest Shellstock Clams (*Meretrix meritrx*) Spiked with Pathogenic Strains of *V. parahaemolyticus*. *Int.J.Curr.Microbiol.App.Sci.* 6(09): 3808-3815. doi: https://doi.org/10.20546/ijcmas.2017.609.470