Study on the Effect of Different NaCl Concentrations on the Growth Kinetics of Total Vibrio Parahaemolyticus Count in Post Harvest Shellstock Clams (Meretrix Meretrix)

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Abstract

Growth variability of pathogenic strains of V. parahaemolyticus plays an important role on food safety risk assessment. V. parahaemolyticus is ubiquitous in marine, brackish and estuarine waters throughout the world, where fluctuations in salinity pose a major challenge to the osmotic stress of the organism. V. parahaemolyticus is a moderately halophilic and having an absolute requirement for NaCl for their growth and survival. This study investigated the effects of different NaCl concentrations on the growth kinetics of total V. parahaemolyticus count (TVC) in post harvest shellstock clams which was spiked artificially with three virulent strains of V. parahaemolyticus at 1, 2 and 3.5% NaCl concentrations for selected time intervals stored at 370C. The results revealed that strain growth variability increased as the growth conditions became more stressful in terms of temperature. Moreover, the strains with VP5 (tdh+trh-) and VP22 (trh+tdh-) exhibited higher growth variability than VP12 (tdh+trh+), revealing that gene heterogeneity might have possible relations with the growth variability. This study illustrates that the growth environments as well as genotypes have impacts on strain growth variability of V. parahaemolyticus that can be helpful for incorporating strain variability in microbial risk assessment.

Keywords: *V. parahaemolyticus, TVC, growth kinetics, environmental factor, salinity, strain variability*

Introduction

V. parahaemolyticus is a gram negative, moderately halophilic bacterium that occurs in worldwide marine and estuarine environments raises concerns on food safety as well as human health due to their potential to cause disease and its entirely depending on the environment (Ceccarelli et al., 2013; Su and Liu, 2007; Zhang and Orth, 2013; Nelapati et al., 2012). *V. parahaemolyticus* is the leading cause of seafood borne bacterial gastroenteritis in the world and it is often associated with the consumption of raw or undercooked seafood, especially mollusacn shellfish that include oysters followed by clams and mussels. However, not all the strains of *V. parahaemolyticus* are pathogenic. *V. parahaemolyticus* strains have a number of different virulent factors including adhesions such as thermostable direct hemolysin (TDH) followed by thermostable direct hemolysin related hemolysin (TRH) and Type three

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system (TTSS) respectively (Makino et al., 2003). In addition, some *V. parahaemolyticus* strains gain a T3SS2, *tdh* and *trh* genes which lead to a number of strains with different degrees of pathogenicity.

The prevalence and distribution of *V. parahaemolyticus* is known to be influenced by several environmental factors including the water temperature, salt concentration (Cabrera – Garcia et al., 2004). Despite the advances in hygiene practices and food processing, seafood borne pathogen *V. parahaemolyticus* still represents a significant threat to human health worldwide. As a marine enteric pathogen, *V. parahaemolyticus* encounters a variety of salinity and temperature conditions in the marine environment. During harvesting of the seafood it colonizes, and during postharvesting processing (Huang et al., 2012; Johnson et al., 2010; Mahoney et al., 2012) *V. parahaemolyticus* requires a minimum of 0.5% NaCl for growth. It can grow in media containing up to 10.5% NaCl, but optimal growth occurs at 3% NaCl (Naughton et al., 2009; Ongagna-Yhombi et al., 2013). Generally, *V. parahaemolyticus* is faced with NaCl concentrations of 3.5% of salinity (35 ppt) whereas in estuarine systems and within oysters it must adapt to changes in salinity. Climate anomalies pose a serious threat on the humans due to the increased growth and survival of pathogenic strains of *V. parahaemolyticus* i.e., fluctuations in NaCl concentrations. Therefore, this study was carried out to measure the effects of different NaCl concentrations as well as the influence virulent genes on the growth kinetics of total *V. parahaemolyticus* count in clams that are challenged artificially with three different strains of *V. parahaemolyticus* viz. VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+). This study will throws the light on the data gaps about the effect of different NaCl concentrations on the growth kinetics of total *V. parahaemolyticus* count in clams.

Materials and Methods

Clam Samples

Live clams were collected from south west coast of Karnataka, Mangalore in India and shipped to laboratory in a cooler with gel packs and transported within 2 h. The clams were briefly rinsed with tap water to remove excess mud from the shell as indicated by American public health association for the bacteriological examination of shellfish (IOS, 2007) and kept in glass tanks for depuration. The live clams were used for the following experiments.

Bacterial Cultures

Three pathogenic strains of *V. parahaemolyticus,* each possessing VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) virulence genes were used in this study. The 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 370C overnight. The enriched cultures were pooled into a sterile centrifuge tube and centrifuged at 50C (6000g rpm). The

pelleted cells were collected and resuspended in sterile phosphate buffered solution to prepare 105 CFU/ml for inoculation.

Challenging clams with *V. parahaemolyticus*

The adductor muscles of clams were directly injected with 0.1 ml of the inoculam using 1 ml syringe equipped with 23 gauge needle (Treumo), similar to the method previously reported (Garnier et al., 2007). Then, the injected live clams were transferred to an glass tanks containing 50 L of seawater maintained at a desired NaCl concentration (1, 2 and 3.5%). Air was pumped into the tank to keep the clams active. Growth kinetics of total *V. parahaemolyticus* in clams were determined at 0 to h for 1% NaCl followed by 0 to h for 2% NaCl and 0 to h for 3.5% NaCl concentrations respectively using the spread plating method according to the FDA`s Bacteriological Analytical Manual (FDA, 2009).

Effects of different NaCl concentrations on growth kinetics of total *V. parahaemolyticus* count (TVC)

V. parahaemolyticus (105 CFU/ml) were inoculated artificially into Clams at room temperature. Inoculated clams were placed in glass tanks of 1%, 2% and 3.5% NaCl concentrations. The growth kinetics of total *V. parahaemolyticus* at different NaCl concentrations were determined in every 8 h for up to 112 h at 3.5%, 88 h at 2% and 64 h at 1% respectively. At each time interval, 5 clams were taken out from the tanks for analyses. Changes in growth kinetics of TVC for all the three strains of *V. parahaemolyticus* which was spiked artificially in clams were determined using spread plating as previously described.

Microbiological Analysis

For TVC analysis, about 2 g of shucked meat of 5 clams were placed in mixer grinder and blended at high speed for 1 min. Phosphate buffered solution can be used as 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 PBS and 100 µl was plated in duplicate on TCBS agar. The TCBS plates were incubated at 370C for 16 to 18 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 different NaCl concentrations 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.

Results and Discussion

According to Lopez-Hernandez *et al*., (2015), salinity was the primary factor governing the temporal distribution of *V. parahaemolyticus*, whereas seawater temperature had a secondary effect and only modulated the abundance in periods of reduced salinities. In view of the above

the study was carried out on the growth of total *V. parahaemolyticus* count (TVC) at different NaCl concentrations of 1%, 2% and 3.5% for selected time intervals. The analyses continued until the clam shells visibly gaped. The optimal growth condition at 3.5% was used as reference in this study.

This study shows that the total *V. parahaemolyticus* count is increased over time at 3.5%. The growth of all the three strains of *V. parahaemolyticus* viz. VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) was studied. At 3.5%, the growth of *V. parahaemolyticus* viz. VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) strains showed a similar trend of growth rate. Initially, the growth was increased from 3.65 to 4.61 log10 CFU/g and from 3.18 to 4.53 log10 CFU/g by 64 h of storage. Subsequently, the growth and survival of the two strains were decreased to a level of 3.92 log10 CFU/g and 3.15 log10 CFU/g at 112 h whereas the growth of *V. parahaemolyticus* VP5 (*tdh*+*trh*-) strain was increased from 3.25 to 3.68 log10 CFU/g at 40 h of storage and further the growth and survival was decreased to 2.75 log10 CFU/g at 112 h of storage. Among all the strains *V. parahaemolyticus* VP5 (*tdh*+*trh*-) strain had a lower population density when compared with other two strains (Table 1). Duncan post hoc test indicated that there was a significant difference between the strains as well as in time intervals pertaining to the growth of pathogenic *V. parahaemolyticus*, VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) at 3.5% (P□0.05) at 24 h followed by 40 h, 64, 88, 96 and 112 h (Fig. 1) Further, Phuvasate and Su (2012) observed reductions of greater than 3.0 log (MPN/g) *V. parahaemolyticus* in laboratory contaminated Pacific oysters depurated in 30 ppt seawater at temperatures ranging from 7 to 150C for five days with no mortality. Thus, he concluded that it is clear that salinity per se is not necessarily detrimental to *V. parahaemolyticus* but may favor growth and survival if the water is rich in organic matter.

Table 1: Total *V. parahaemolyticus* counts for pathogenic strains grown in clams at 3.5% NaCl

Time interval	Pathogenic *V. parahaemolyticus*		
	VP5 (*tdh*+*trh*-) (log10 CFU/g)	VP22 (*tdh*-*trh*+) (log10 CFU/g)	VP12 (*tdh*+*trh*+) (log10 CFU/g)
0	3.25	3.65	3.18
8	3.36	4.01	3.34
16	3.50	4.23	3.51
24	3.67	3.88	3.75
32	3.77	4.29	4.21
40	3.68	4.41	4.25
48	3.67	4.48	4.36
56	3.59	4.51	4.44
64	3.32	4.61	4.53
72	3.16	4.47	4.25
80	3.23	4.32	4.22
88	3.12	4.20	3.82
96	2.94	4.14	3.36
104	2.85	3.85	3.33
112	2.75	3.92	3.15
12	3.25	3.65	3.18

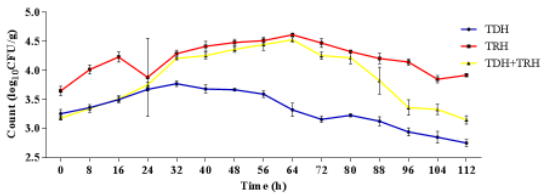

Fig. 1: Total *V. parahaemolyticus* Count (TVC) of VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) at 3.5% NaCl

At 2% NaCl concentration, the growth of all the three strains of *V. parahaemolyticus* viz. VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) were exhibited similar growth trend such as 3.29 to 3.96 log10 CFU/g, 3.19 to 3.85 log10 CFU/g and 3.23 to 2.85 log10 CFU/g at 56 h, 48 h and 40 h respectively. Later, with increased time period, the growth and survival of all the three strains of *V. parahaemolyticus* found to be decreased to a level of 2.37 log10 CFU/g, 2.52 log10 CFU/g and 2.45 log10 CFU/g by 88 h of storage at 2% NaCl concentration respectively (Table 2). Duncan test showed that there was a significant difference in growth of total *V. parahaemolyticus* between the time interval (P□0.05) and between the strains of VP12 (*tdh*+*trh*+) and *trh*+*tdh*- followed by VP5 (*tdh*+*trh*-) and VP22 (*trh*+*tdh*-) respectively (P□0.05) at 48, h 56 h followed by 64 h, 80 h and 88 h respectively (Fig. 2).

Table 2: Total *V. parahaemolyticus* counts for pathogenic strains grown in clams at 2%

Time interval	Pathogenic *V. parahaemolyticus*		
	VP5 (*tdh*+*trh*-) (log10 CFU/g)	VP22 (*tdh*-*trh*+) (log10 CFU/g)	VP12 (*tdh*+*trh*+) (log10 CFU/g)
0	3.29	3.19	3.23
8	3.33	3.41	3.25
16	3.52	3.61	3.47
24	3.60	3.80	3.34
32	3.58	3.64	3.64
40	3.72	3.95	3.65
48	3.87	3.85	4.68
56	3.96	3.71	3.86
64	3.62	3.72	3.70
72	3.23	3.39	3.17
80	2.62	2.85	2.85
88	2.37	3.19	2.45

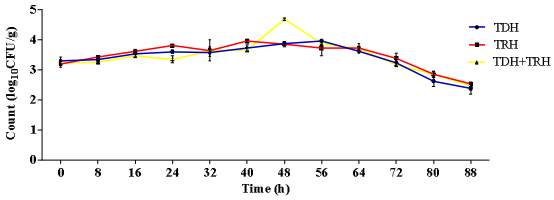

Fig. 2: Total *V. parahaemolyticus* Count (TVC) of VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) at 2% NaCl

Effect of 1% NaCl concentration on the growth of total *V. parahaemolyticus* count (TVC) was found to be decreased from 3.37 to 1.99 log10 CFU/g and 3.21 to 1.88 log10 CFU/g respectively, at 64 h of storage in VP5 (*tdh*+*trh*-) and VP12 (*tdh*+*trh*+). In contrast, the growth of VP22 (*trh*+*tdh*-) was found be decreased to a level of 3.27 from 2.30 log10 CFU/g as shown in Table 3. The ANOVA and Duncan post hoc test showed that there was a significant difference between the strains in terms of growth at 1% NaCl (P□0.05) at 16 h followed by 32 h, 48 h, 56 h and 64 h respectively (Fig.3).

Table 3: Total *V. parahaemolyticus* counts for pathogenic strains grown in clams at 1% NaCl

Time interval	Pathogenic *V. parahaemolyticus*		
	VP5 (*tdh*+*trh*) (log10 CFU/g)	VP22 (*tdh*-*trh*+) (log10 CFU/g)	VP12 (*tdh*+*trh*+) (log10 CFU/g)
0	3.37	3.29	3.21
8	3.41	3.45	3.24
16	3.51	3.55	3.37
24	3.49	3.54	3.47
32	3.30	3.46	3.30
40	2.95	3.18	2.85
48	2.92	2.97	2.52
56	2.24	2.61	2.16
64	1.99	2.30	1.88

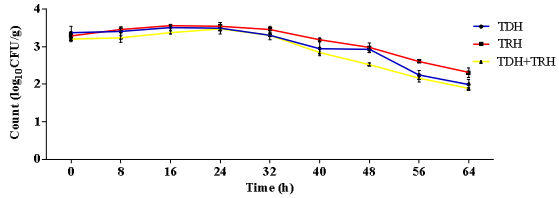

Fig. 3: Total *V. parahaemolyticus* Count (TVC) of VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) at 1% NaCl

The growth and survival of TVC at different NaCl concentrations of 1%, 2% and 3.5% for selected time intervals revealed that there was a significant difference between the growth and survival of all the three strains of *V. parahaemolyticus*, VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) at 1%, 2% and 3.5% (P□0.05). However, vibrios tolerate a wide range of salinity (Farmer et al., 2005), with high levels detected at moderate salinities (Martinez-Urtaza et al., 2008).

Among all, *V. parahaemolyticus* VP5 (*tdh*+*trh*-) had the lower growth rate while other two had slightly higher growth rate at 3.5%. Overall, at 3.5%, 2% and 1% NaCl concentration, there was significant difference between the growth of total *V. parahaemolyticus* count of all the three strains of *V. parahaemolyticus*, VP5 (*tdh*+*trh*-), VP22 (*trh*+*tdh*-) and VP12 (*tdh*+*trh*+) (P□0.05) as well as between the different NaCl concentrations of 3.5%, 2% and 1% respectively. Studies on the effect of NaCl on the growth of total *V. parahaemolyticus* (TVC) is scarce in India. In this study, it was confirmed that the growth of total *V. parahaemolyticus* showed a largest variation in non optimal condition like 1% and 2% NaCl for all the three strains of *V. parahaemolyticus* as noted by Mudoh *et al*., (2001). Overall, the present study confirmed that growth in 1% NaCl is a stress condition for the organism and reduces its ability to tolerate other stresses. The optimum salinity for *V. parahaemolyticus* in oysters is 23 per cent although salinities ranging from 10 to 36 per cent were reported to support relatively high numbers in bivalve shellfish (U.S. FDA,2005; Martinez-Urtaza *et al*., 2010). Liu *et al.*, (2016) also confirmed that the *V. parahaemolyticus* strains reached the largest maximum growth rate in such an optimal growth condition at 3%.

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