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Research Article

Relative Survival of Vibrio parahaemolyticus Strains from Environment and Food Source in Water and Sediment of Cochin Estuary

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Abstract

The present study aimed to evaluate the relative survival of pathogenic *Vibrio parahaemolyticus* strains isolated from two different sources, in a tropical estuary along the southwest coast of India. Microcosms studies were conducted under controlled laboratory conditions. Each microcosm was inoculated with ~107 CFU/ml of the bacterial culture. Two multi-drug resistant *V. parahaemolyticus* strains carrying the virulence genes (*tdh* and *trh*) were used. The survival pattern of the strains was compared by analysing the role of various biological factors on the removal of these pathogens in the estuarine environment and the effect of temperature (25˚C, 30˚C, 35˚C) on their survival. Results highlighted that sediments of estuary provides a favourable environment for extended survival of the pathogen. The strain from food source had a selective advantage over the environmental source to survive better in the estuarine environments. The study revealed a significant difference in the survival pattern of the both strains at all temperatures. However, both exhibited extended survival at lower temperatures. Our results also disclosed that no universal pattern can be predicted for the survival of *V. parahaemolyticus* strains in the natural environments. This report highlights the existence of strain- wise variation in the survival of this pathogenic species in the natural environments.

Keywords: *Relative Survival; Multi-Drug Resistant; V. parahaemolyticus; Tropical Estuary*

Introduction

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium, naturally found from shallow coastal waters to the deepest parts of the ocean, and abundant in aquatic environments, including estuaries, marine coastal waters and sediments and aquaculture settings worldwide [1]. Among the infections associated with the genus *Vibrio*, the majority are caused by *V. parahaemolyticus* [2]. It is the leading causative agent of human acute gastroenteritis following the consumption of raw, undercooked, or mishandled marine products. Since its discovery, *V. parahaemolyticus* has been responsible for 20 - 30% of seafood borne diseases in many Asian countries [3]. While the incidence of most food- borne pathogens like *Listeria, Campylobacter* and *E. coli* O:157 have decreased from 1998 to 2008, incidence of *V. parahaemolyticus* has increased 47% within this time span [4].

Vibrio parahaemolyticus is autochthonous to estuarine environments, where it is exposed to frequent environmental variations in temperature, salinity, pH, nutrient levels, and presence of pollutants. Once introduced into the estuary, the survival of the organism is influenced by all these factors. Among the physical parameters, temperature is the major factor influencing its survival and virulence, whereas protozoan and bacteriophage predators constitute the predominant biological factors. In our previous study, we have observed high prevalence of multi- drug resistant *V. parahaemolyticus* in Cochin estuary [5]. High genetic heterogeneity was also observed among the strains [6]. Evaluation of the survival capabilities of this pathogen in the estuarine environment is significant as this estuary is one of

the most famous tourist hot spots, and shrimps grown here are exported worldwide. Moreover, a number of seafood industries depend on the estuary and it is a means of livelihood for the local fisher folk. However, the studies on removal kinetics of this pathogen in this estuarine environment are scarce. Hence, it is essential to study the level of survival and explore the environmental factors, which enhance the elimination of these pathogenic organisms in estuarine environments. To our knowledge, there are limited published data on survival of *Vibrio* in Cochin estuary. The present study aims to evaluate and compare the survival rates of genotypically different strains of *V. parahaemolyticus* from two sources, in this tropical estuary under controlled laboratory conditions. The study is very relevant as it may throw light on the possible potential health hazards posed by the extended survival of the pathogen, which may adversely affect the intended beneficial uses of the estuary.

Materials and Methodology

Description of the bacterial strains used

For the present study two strains of *V. parahaemolyticus*, PMIS2 (Genbank accession no: KM406325) and V10M1 (Genbank accession no: KT163390) were used. PM1S2 was previously isolated from sediment of Cochin estuary (environmental source) and V10M1 from flesh of shellfish *Villorita cyprinoides* (food source) which was collected from shellfish harvesting area in Cochin. Preliminary identification was done using the dichotomous key by Noguerela and Blanch [7] followed by molecular confirmation of species- specific *tlh* and *toxR* genes. The strains were subjected to RAPD-PCR and both belonged to different genotypes [6]. In addition, the pathogenic potential of the strains was confirmed by detection of the virulence genes *tdh* and *trh*. Another important fact to be noted was that both the strains were multi-drug resistant, with the following resistance patterns; PM1S2 (Amoxycillin, Ampicillin, Carbenicillin, Cephalothin, Colistin, Erythromycin, Enrofloxacin, Furazolidone Nitrofuratoin) and V10M1 (Ampicillin, Amikacin, Cefpodoxime, Gentamicin, Nitrofurantoin, Streptomycin). The isolation, characterisation and antibiotic resistance determination of the strains are described in detail in our previous study [5].

Preparation of bacterial inoculum

The strains were inoculated into Tryptone Soy Broth and incubated overnight at 37°C. After incubation the cells were concentrated by centrifugation at 13,000 rpm for 15 minutes. The pellet was washed thrice and re-suspended in 10 ml physiological saline. One ml (approximately 10^{7-8} CFU/ml) of the culture was inoculated into 250 ml Erlenmeyer flasks with 100 ml test solutions to give an initial inoculum density of 10^{6-7} CFU/ml.

Setting up of the microcosm

Water and sediments samples were freshly collected from the Cochin estuary and brought to the laboratory for setting up the microcosms. Water microcosms were prepared by adding 100 ml water and sediment microcosms prepared with 50 gm sediment and 50 ml overlaying estuarine water. Raw estuarine water (RW) and sediments (RS) were used as test solutions to study the effect of the self-contained biological factors such as protozoa and bacteriophages on the test organisms. The effect of protozoan predation on the test organisms was studied by addition of protozoan inhibitor cycloheximide (500 mg/l) into the raw water and sediments. Protozoa was identified by microscopy. Bacteriophages in the water sample were detected by double layer agar method and formation of plaques was noted. Autoclaved estuarine water (AW), devoid of all biological factors was used as test solution to study the effect of temperature (25°C, 30°C and 35°C) on the survival of the test organisms.

Enumeration techniques

Survival assay was conducted for a period of one month. Time zero (inoculation time) and subsequent samples were taken for plate counts. Samples were taken and enumerated after 1st, 3rd, 5th, 7th, 14th, 21st and 28th day by the drop plate method [8] using selective media TCBS agar. The plates were incubated at 37°C; the number of colonies was counted after 24h. All the plating were done in triplicates and the mean value and standard deviation were used to plot the survival curves. Any significant difference in survival of both the strains were analysed by analysis of variance (ANOVA) using the statistical package in Microsoft excel 2007.

Results and Discussion

In raw water microcosm (RW), V10M1 and PM1S2 showed a continuous steep reduction in the cell count and were not recoverable after 5 days. In raw sediments (RS) there was statistically significant difference in the survival of both strains ($p < 0.01$), PM1S2 demonstrated a steep reduction throughout the exposure time whereas V10MI showed a slight growth initially and then declined, and from 14th day onwards a steady growth was observed until 28th day. This shows that V10M1 could withstand the effect of biological factors in RS better than PM1S2 (Figure 1a and 1b). In autoclaved sediments (AS) both followed the same survival pattern (Figure 1b). However, in autoclaved water microcosm (AW) there was significant difference in the survival of the strains ($p < 0.01$). Prolonged survival was observed for V10MI compared to PM1S2. PM1S2 declined to zero by the end of 21st day whereas V10MI survived until 28th day, revealing the adaptive strategy of V10MI to withstand starvation effect (Figure 1a). However, there was variation in the survival kinetics of the strains, both exhibiting higher mortality in raw water and sediment microcosms compared to autoclaved water and sediment which was devoid of any biological factors (Figure 1a and 1b). This suggests the role played by various biological factors on the mortality of the test organisms. According to Abhirosh and Hatha [9], there are various physico-chemical and biological factors involved in the disappearance of pathogenic microorganisms in the aquatic environment.

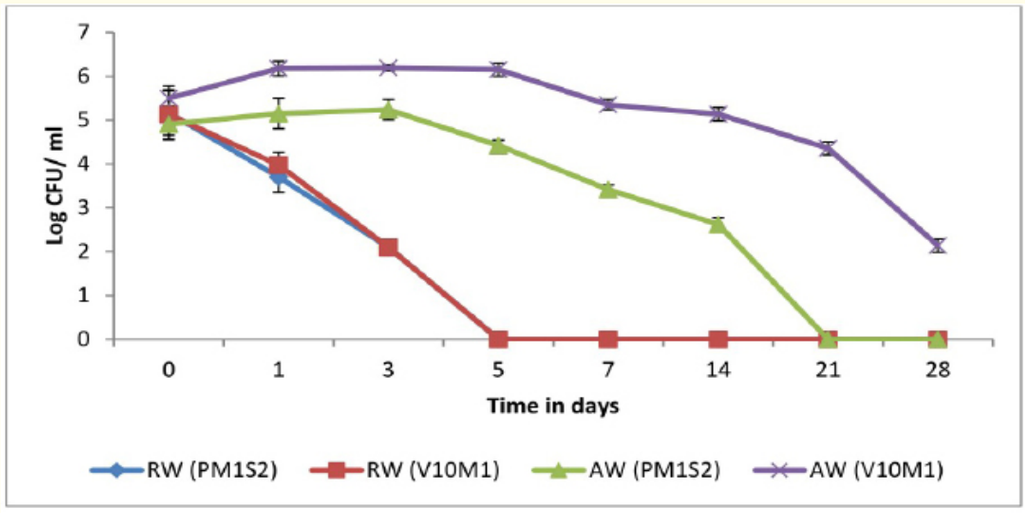

Figure 1a: *Effect of biotic factors on survival of V. parahaemolyticus strains PM1S2 and V10M1 in water of Cochin estuary. RW raw water microcosm, AW autoclaved water microcosm.*

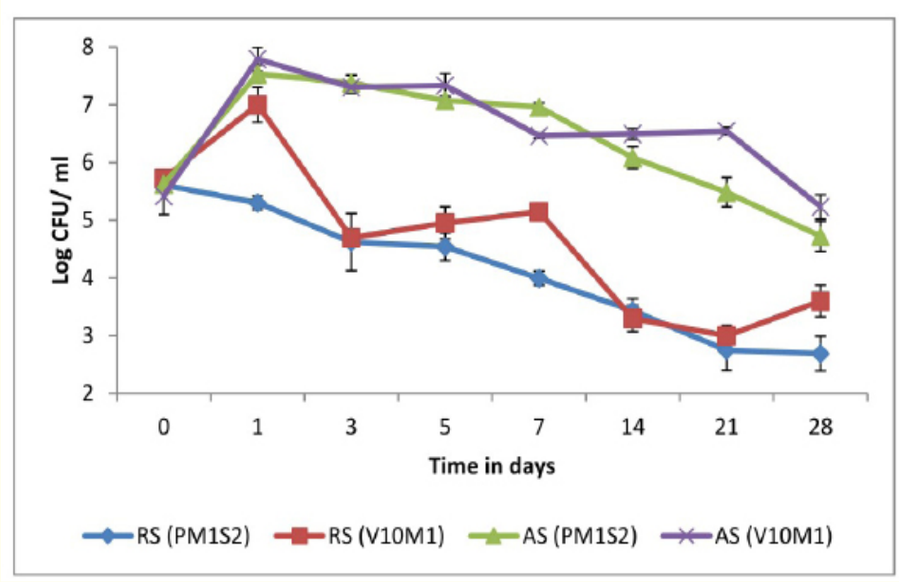

Figure 1b: *Effect of biotic factors on survival of V. parahaemolyticus strains PM1S2 and V10M1 in sediment of Cochin estuary. RS raw sediment microcosm, AS autoclaved sediment microcosm.*

In cycloheximide treated microcosms that is devoid of protozoans, the strains survived better compared to the non-treated microcosms. There was significant difference in the survival of both strains in treated sediments compared to non- treated sediments ($p < 0.01$) (Figures 2a and 2b). This highlights the role played by protozoan predators in the mortality of test organisms in raw water and sediments. The protozoans identified in the present study include *Vorticella, Tintinnids, Euglena, Phacus, Strombidium, Favella, Tintinnopsis, Centropyxis, Difflugia, Stylonichia*. Previous study by Hahn and Hofle [10] have already documented that protozoan graz¬ing was the significant factor responsible for the removal of bacterial population in aquatic envi¬ronments.

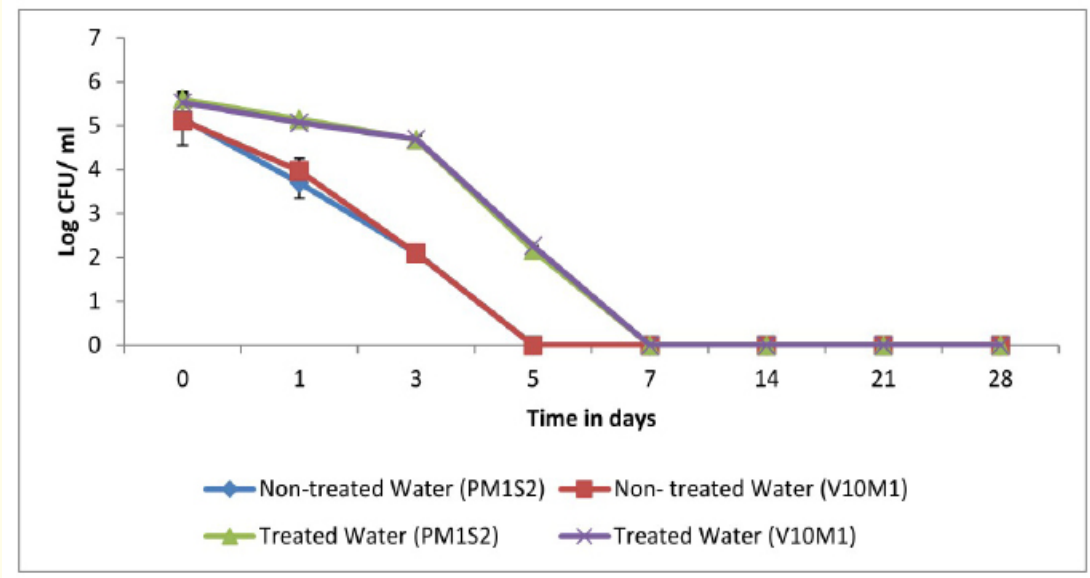

Figure 2a: *Effect of protozoan predation on survival of V. parahaemolyticus PM1S2 and V10M1 in water of Cochin estuary.*

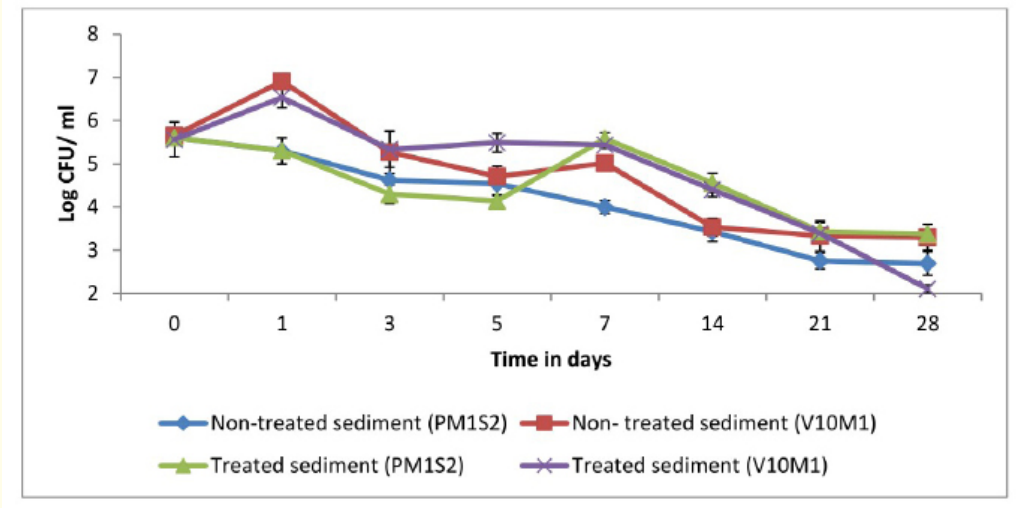

Figure 2b: *Effect of protozoan predation on survival of V. parahaemolyticus PM1S2 and V10M1 in sediment of Cochin estuary.*

Plaque assay demonstrated plaque formation and this reveals the presence of bacteriophages specific to *V. parahaemolyticus* in the estuary. The mortality that was observed in cycloheximide treated microcosms may be considered to be induced by bacteriophage or by competing autochthonous bacteria. In the present study, besides protozoans, bacteriophages and competition with other autochthonous bacteria were also identified to be another major biological factors responsible for the mortality of the test organisms. This clearly indicated the role played by biological factors in the removal of these pathogens from the aquatic environments. This is in agreement with the previous study on survival of *V. parahaemolyticus* from Vembanad Lake [11].

Another observation was that, when compared to water microcosms, extended and better survival was observed in sediment microcosms, which may be attributed to the presence of high nutrients in the sediment. The sediments are known to protect the bacteria from the deleterious effect of UV radiation from the sun, predation, and even high salinity in marine environments [12]. This is in agreement with a previous report where *V. fluvialis* showed prolonged survival in sediment microcosms [13]. This highlights that sediments of Cochin estuary could act as permanent reservoirs or repository for these pathogenic bacteria as it showed extended survival in estuarine sediments, which in turn would pose serious public health concern. Similar results were also reported from Vembanad Lake where the lake sediments acted as repository for pathogens [14]. As the shipping channel in the Cochin estuary is dredged on a regular basis in order to facilitate movement of ships to Cochin port, the resuspension of sediment is real. In addition, the better growth of *V. parahaemolyticus* in autoclaved sediments may be possibly due to the absence of competing organisms and abundance of available nutrients. This is in agreement with previous report by Hood and Ness [15]. Autoclaving results in release of those nutrients from the sediments that are usually in bound form in the natural environments [16].

When survival of the strains was compared at different temperatures, we could again observe significant difference in their survival rates ($p < 0.01$, Figures 3a and 3b). The survival pattern of both *V. parahaemolyticus* varied. PM1S2 showed a slight growth until the 3rd day at 25˚C and 30˚C and then it started declining gradually. However, at 35˚C slight growth was demonstrated during 1st day and then it reduced steeply. The cell count declined to zero by the end of 14th day at 35˚C and 21st day at 30˚C, whereas at 25˚C the survival persisted until the end of the experiment. V10MI survived better at all the temperatures until the end of the experiment. It demonstrated a steady growth until 3rd day at 25˚C, afterwards increased logarithmically until 7th day, and later started declining until the end. At 30˚C there was slight growth up to 5th day and declined thereafter but never reached zero until 28th day. At 35˚C, a slight growth was observed initially, but later declined logarithmically until the end. The strain demonstrated prolonged survival compared to PM1S2. Even though the survival pattern varied, we observed a similar trend of extended survival of both strains of *V. parahaemolyticus* at 25˚C compared to 30˚C and 35˚C, indicating that they survived better at lower temperatures.

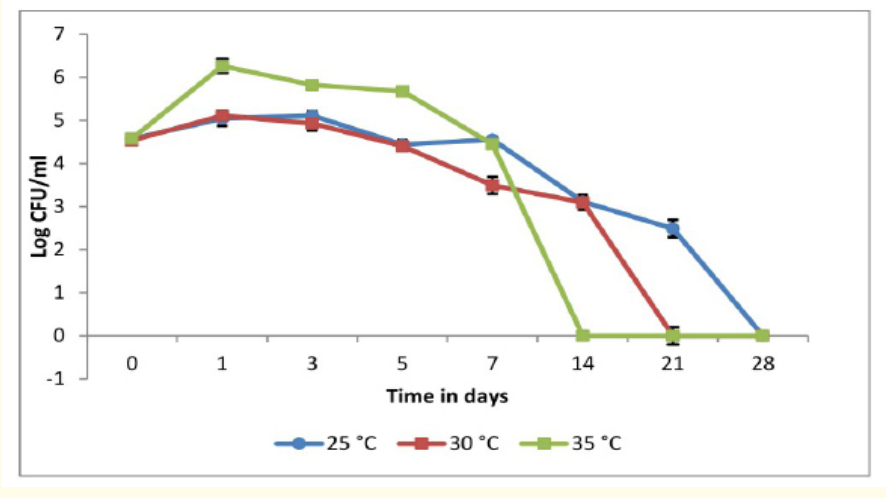

Figure 3a: *Survival curves of V. parahaemolyticus PM1S2 at different temperatures (25°C, 30°C and 35°C).*

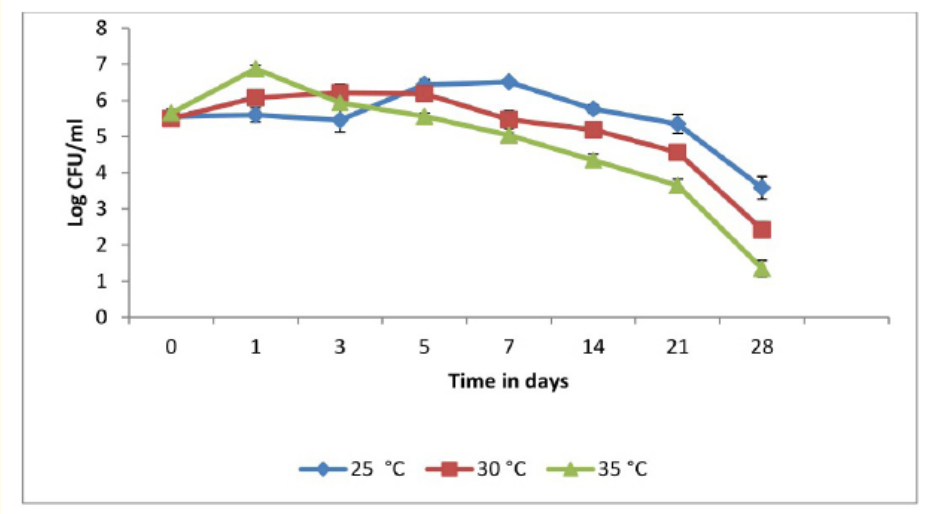

Figure 3b: *Survival curves of V. parahaemolyticus V10M1 at different temperatures (25°C, 30°C and 35°C).*

The survival rate of the strains in different microcosms varied with the genotype/isolation source. It reveals that no uniform pattern exists for the survival and spread of these pathogens in the natural environments. Previous studies by Warner and Oliver [17] on two genotypes of *V. vulnificus* reported genotype wise variation in the survival rates; the E-genotype was found at higher levels than genotype-C in oysters. Even though the exact mechanism is not known, our results suggest the selective advantage of *V. parahaemolyticus* from food source to survive better in Cochin estuarine environments than the strain from environmental source.

Conclusion

Cochin backwaters are responsible for the rich fishery potential of Kerala and are a major source of livelihood for the local fishermen community. The estuary is also a famous tourist hot spot for recreational activities. Estuary being a receptacle for various kinds of domestic and industrial effluents is subjected to considerable pollution and poses health hazard to people who use this natural water body for recreation and livelihood. Hence, the persistence of multi- drug resistant pathogenic *V. parahaemolyticus* strains in the estuary raises serious concern about the public health. Even though the study highlights the role of biological factors in the self- purification of the estuarine environments, introduction of high load of pathogens into the system through untreated sewage may disrupt this balance. Hence, the concerned authorities should take necessary control measures to check the pollution to ensure seafood safety and to prevent the potential spread of any outbreaks in the present study areas.

From the present study it is clearly evident that strain- wise variations exists in the survival kinetics of pathogenic *V. parahaemolyticus* strains. This makes the situation worse as no universal pattern could be predicted for survival and spread of these pathogens in the natural environments. In addition, it is also impossible to make conclusive statements on the survival of the species based on studies of individual strains. Further detailed studies are required to obtain a clear pattern of their survival kinetics.

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