

Full Length Research Paper

## Occurrence of *Vibrio parahaemolyticus* in oysters (*Crassostrea gigas*) and mussels (*Perna perna*) of the seacoast of Santa Catarina, Brazil

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This research aimed to identify and quantify *Vibrio parahaemolyticus* in fresh oysters (*Crassostrea gigas*), mussels (*Perna perna*) and seawater from different regions of cultivation of bivalve shellfishes in the seacoast of Santa Catarina, Brazil. Samples were collected between October 2012 and December 2013 and 130 oysters samples (*Crassostrea gigas*), 215 mussels samples (*Perna perna*) and 222 seawater were collected. The occurrence of *V. parahaemolyticus* in oysters and mussels was low, 10.76 and 11.62% of the samples tested. Higher incidences of *V. parahaemolyticus* were observed in seawater (18%). The density of *V. parahaemolyticus* in summer (December to March) was significantly greater than those in the other 3 seasons ( $P < 0.01$ ). The occurrence of pathogenic *V. parahaemolyticus* in oyster, mussels and seawater was very low (<10%). It is recommend that control measures should be considered, including the establishment of an intensive and continuous monitoring of potentially pathogenic *V. parahaemolyticus* from all oyster-growing areas, the environmental parameters, and the assessment of the region-specific human health risk due to consumption of oyster.

**Key words:** Oyster, *Crassostrea gigas*, *Vibrio parahaemolyticus*, bivalve molluscs.

### INTRODUCTION

Seafood is recognized as a nutritious food choice, and is liked by increasing numbers of consumers worldwide (Hellberg et al., 2012). For the last two decades, there has been a fourfold growth in commercial aquaculture worldwide (Cabello, 2006). In Brazil, the production of bivalve shellfishes occurs mainly in the state of Santa

Catarina, in the southern region of Brazil, due to the excellent geographical conditions of this area for the cultivation of marine organisms, such as the presence of a large number of bays, which facilitates the establishment of marine farms (Coelho et al., 2003; Corrêa et al., 2007).

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Despite the increase, the main obstacles in the consumption of seafood are its high perishability and risk to health due to contamination by pathogens (Reyhana and Kutty, 2014). In addition to the indicators of faecal contamination, which are used to assess the microbiological quality of bivalve molluscs in Brazil, different species of the *Vibrio* genus occur naturally in marine, coastal and estuary environments, where some species such as *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* are potentially pathogenic for men, and may be present in fishes and raw shellfishes or partially subjected to cooking (Thompson et al., 2004). The possibility of seafood consumers to be infected by pathogenic vibrios by eating oysters depends on the microbiological quality of the marine habitat, as well as on the practices of handling and processing of these shellfish (Vieira et al., 2011). The occurrence of these bacteria is not related to the counts of *Escherichia coli* or thermotolerant coliforms, therefore the specific constant monitoring is required (Pereira, 2002; Oliver, 2006; Suffredini et al., 2014).

Infections caused by *Vibrio parahaemolyticus* have been reported in several countries in Asia (Chiou et al., 2000; Chowdhury et al., 2013; Kubota et al., 2008; Ma et al., 2014; Okuda et al., 1997; Tuyet et al., 2002; Vuddhakul et al., 2006), United States (Haendiges et al., 2014; Sims et al., 2011), in Europe only a few outbreaks or sporadic cases were reported in the last decade as a consequence of the consumption of local or imported seafood (Martinez-Urtaza et al., 2005; Ottaviani et al., 2008, 2010b, 2012; Quilici et al., 2005; Sala et al., 2009), and some South American countries like Chile (Fuenzalida et al., 2006; Cabello et al., 2007; Harth et al., 2009), Peru (Gil et al., 2007; Martinez-Urtaza et al., 2008) and Brazil (Leal et al., 2008) have also reported outbreaks.

Pathogenic strains of *Vibrio parahaemolyticus* can be differentiated from non-pathogenic strains with its ability to produce thermostable hemolysin (TDH), whose production is called the Kanagawa phenomenon. The pathogenicity of *Vibrio parahaemolyticus* is associated with the presence of the *tdh* and *trh* gene in oysters (Nishibuchi and Kaper, 1995).

The concentration of *V. parahaemolyticus*, in oysters and mussels is directly related to water temperature, with a higher concentration being present when the bivalve molluscs are in warm water. Because of this, these microorganisms are rarely isolated when the water temperature is below 15°C (Pruzzo et al., 2005; Su and Liu, 2007). In Brazil, the temperature of sea waters is above 20°C in most of the year, favouring the occurrence of these microorganisms in the different stations.

This research aimed to identify and quantify *V. parahaemolyticus* in fresh oysters (*Crassostrea gigas*) and mussels from different regions of cultivation of bivalve shellfishes in the seacoast of Santa Catarina, Brazil.

## MATERIALS AND METHODS

### Collection and preparation of the samples

Between October, 2012 and December, 2013, 130 oysters samples (*Crassostrea gigas*), 215 mussels samples (*Perna perna*) and 222 seawater samples were collected directly from three geographical regions in Santa Catarina where there is shellfish farming in Brazil (Figure 1).

Each oysters and mussels sample consisted of 12 units. The oysters and mussels were transported to the laboratory in an isothermal box with packaged potable ice, and analyzed within 6 h of sampling.

The oysters and mussels were scrubbed under tap water to remove debris, allowed to dry, disinfected with 70% ethanol, and opened aseptically using a sterilized knife. The flesh and intervalve liquid were aseptically transferred to sterile bags and were homogenized for 1 min, forming the pool of 12 units.

### Isolation and enumeration of *Vibrio parahaemolyticus* in oyster and mussels samples

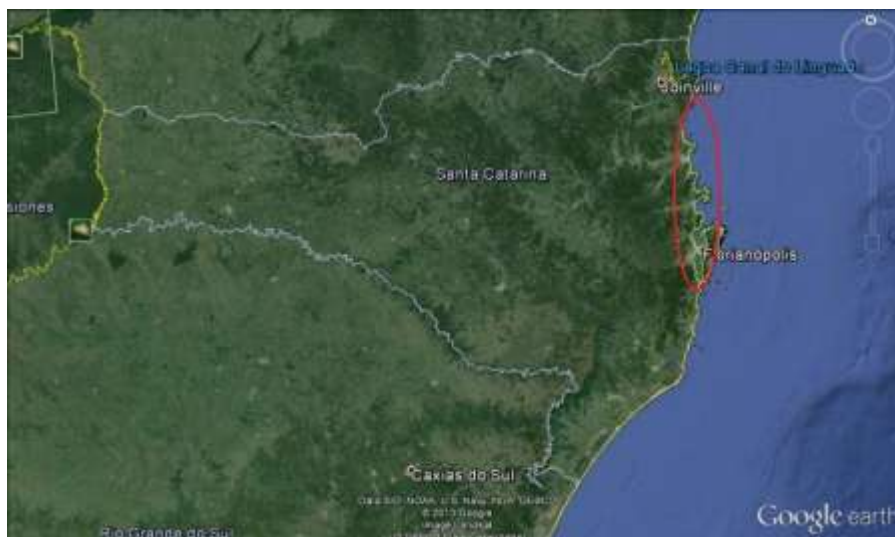
Enumeration of *V. parahaemolyticus* was performed using most probable number (MPN) technique (Kaysner and DePaola, 2004). Approximately 25 g of the homogenate was added to 225 ml of phosphate buffered saline (PBS). Serial 10-fold dilutions were prepared up to 1:106 and three aliquots of each dilution were inoculated into alkaline peptone water tubes and incubated overnight at 37°C. After incubation, a loopful from the top 1 cm, approximately, of each broth tube with turbid growth was streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates (Oxoid, UK) and incubated at 37°C for 24 h. Five to ten typical colonies from each plate were selected and isolated for identification. Sucrose-negative (blue-green on TCBS agar) colonies were submitted to confirmation as Gram-negative and oxidase-positive. Further biochemical differentiation for identification and confirmation of isolated items were performed using the API 20E system (bioMérieux, France). Total populations of *V. parahaemolyticus* in oysters and mussels were determined by converting numbers of APW tubes that were positive for *V. parahaemolyticus* to MPN g<sup>-1</sup> using the MPN table. All strains of *V. parahaemolyticus* were confirmed genotypically through the detection of the *tdh* gene by multiplex qPCR.

### Multiplex PCR for the detection of *roxB*, *tdh* and *trh* genes

The extraction of bacterial DNA was made in QiaCube equipment (Qiagen) using the DNeasy Blood and Tissue kit (Qiagen) with specific protocol for the equipment. Real time multiplex PCR was performed using the kit *V. parahaemolyticus* multiplex kit (Bioteccon). The target genes were the *Rox* to confirm the species, and *tdh* and *trh* genes of pathogenicity. The protocol used was indicated in the kit manual.

### Statistical analysis

Results of microbiological tests were transformed into log values and were assumed to be normally distributed; statistical analyses were performed in the Statistica 7.0® software (Stat-Soft, Inc., USA). To facilitate statistical analyses of quantitative data obtained by most probable number for counts *V. parahaemolyticus* when levels were below the limit of detection, there was substitution for 2 MPN g<sup>-1</sup> and test of significance of the observed differences in *V.*



**Figure 1.** The coastal region which is the cultivation area of bivalve molluscs in the Santa Catarina.

**Table 1.** Occurrence of *Vibrio parahaemolyticus* in oyster, mussels and seawater samples.

Sample	Season	Number of samples tested	Number of positive samples (%)	Level of <i>V. parahaemolyticus</i> (MPN/g)		
				< 100	10 <sup>2</sup> -10 <sup>3</sup>	10 <sup>3</sup> -10 <sup>4</sup>
Oyster	Summer	50	10 (20%)	-	6	4
Oyster	Autumn	25	1 (4%)	1	-	-
Oyster	Winter	25	-	-	-	-
Oyster	Spring	30	3 (10%)	2	1	-
Mussel	Summer	120	21 (17.5%)	4	8	9
Mussel	Autumn	25	1(4%)	1	-	-
Mussel	Winter	25	-	-	-	-
Mussel	Spring	45	3 (6.6%)	2	1	-
Seawater	Summer	120	37 (30.8%)	10	18	9
Seawater	Autumn	30	9(30%)	9	-	-
Seawater	Winter	30	2(6.6%)	2	-	-
Seawater	Spring	42	12 (28.5)	10	2	-

*parahaemolyticus* levels, environmental parameters in oysters and mussels across the 22 samplings sites was conducted using a one-way analysis of variance (ANOVA), an alpha level of 0.05 was considered using the minimum level for statistical significance.

## RESULTS AND DISCUSSION

The occurrence of *V. parahaemolyticus* in oysters and mussels was low, 10.76% and 11.62% of the samples tested (Table 1). Higher incidences of *V. parahaemolyticus* were observed in seawater (18%).

The densities of *V. parahaemolyticus* in oyster, mussels and seawater samples are listed in Table 1. They were higher in the summer months, especially in February and

March. The density of *V. parahaemolyticus* in summer (December to March) was significantly greater than those in the other 3 seasons ( $P < 0.01$ ). The occurrence of pathogenic *V. parahaemolyticus* in oyster, mussels and seawater was very low (<10%). Only 4 of 130 oysters, 5 of 215 and 5 of 220 seawater samples contained detectable levels of pathogenic strains. These results indicated that most *V. parahaemolyticus* in the environment were nonpathogenic to humans. Although, the levels of *V. parahaemolyticus* in oysters reported in this study were much lower, postharvest processing conditions and storage temperatures could allow contaminated *V. parahaemolyticus* to multiply to a higher level in market oysters. Studies have shown that the

populations of *V. parahaemolyticus* in unrefrigerated oysters could increase rapidly to reach 50-fold to 790-fold its original level within 24 h after harvest if oysters were exposed to an elevated temperature (Gooch et al., 2002).

Epidemiological data from CDC on association with *V. parahaemolyticus* gastroenteritis with tdh-carrying strains in the period 2001–2004 and US risk assessment studies on oysters (FDA, 2005), support the assumption that *V. parahaemolyticus* risk is proportional to exposure to different levels of pathogenic *V. parahaemolyticus* (WHO, 2011). According to some studies, pathogenic *V. parahaemolyticus* levels may be reliably estimated from total *V. parahaemolyticus* levels (Miwa et al., 2003; Nordstrom et al., 2007). On the other side, other studies showed that the ratio between total and pathogenic *V. parahaemolyticus* in the environment may be quite variable over time, as in the case of the monitoring performed in Alaskan waters, where percentage of potentially pathogenic strains in two consecutive summers (2004 and 2005) changed from 74 to 30% (WHO, 2011). Such variability, together with the limited number of quantitative data on *V. parahaemolyticus* levels in the environment and in shellfish harvested in regions as Europe (Cantet et al., 2013), Asia (Deepanjali et al., 2005), South America (Garcia et al., 2009), which are occasionally involved in outbreaks, underline the need for analytical assays which allow the enumeration of both total and potentially pathogenic (tdh and/or trh positive) *V. parahaemolyticus* strains. Trouble variables for the presence of *V. parahaemolyticus* in seafood have been shown in studies by many researchers around the world, using conventional bacteriological methods. The results found in this study are in agreement with the results reported by Nordstrom et al. (2007), a study conducted in Alaska (USA), Cabello et al. (2007) in Chile, Gil et al. (2006) in Peru and Quintoil et al. (2007), India.

Higher incidence of *V. parahaemolyticus*, however, was found using conventional methods of wild mullet in Italy (Serracca et al., 2011), cockles in Indonesia (Zulkifli et al., 2009), various seafood in India (Chakraborty et al., 2008) and in the USA mussels (Lu et al., 2006). Furthermore, the lower incidence of 8% (Hassan et al., 2012), were reported in the Netherlands seafood. Ramos et al. (2014) found an incidence of *V. parahaemolyticus*, 30.0% in samples of oysters and 33.3% in water samples from cultivation sites in Bahia Sul in Florianopolis, in the study region of this work.

Several factors are involved in the distribution and survival of microorganisms in estuarine ecosystems such as biotic and abiotic parameters of water, such as temperature, salinity, pH and turbidity (Ristori et al., 2007; Strom and Paranjpye, 2000). The concentration of *V. parahaemolyticus* in seawater increases with increasing temperature and is correlated with the seasonal increase in the occurrence of sporadic cases of infections in months with higher temperature (Hlady and Klontz, 1996).

The presence of *V. parahaemolyticus* seems to be constant where the sea water temperature is  $>10^{\circ}\text{C}$ , unlike what occurs in Europe, where isolation of this pathogen decreases during the winter months (Baker-Austin et al., 2013). Hence, *V. parahaemolyticus* can be considered ubiquitous in the marine environment. The World Health Organization (WHO, 2011) listed the optimum temperature for *V. parahaemolyticus* growth as  $37^{\circ}\text{C}$ , with a wide growth range of  $5\text{--}43^{\circ}\text{C}$ . Several studies have shown a positive correlation between contamination of raw shellfish by *V. parahaemolyticus* and water temperature with higher frequencies being detected during warmer months in spring and summer seasons than in winter (DePaola et al., 2003; Parveen et al., 2008; Johnson et al., 2012; Ceccarelli et al., 2013).

The data provided in this study on contamination levels of total and potentially pathogenic *V. parahaemolyticus* and seasonal distribution, will help in defining appropriate monitoring programs and post-harvest policies for this hazard. The acquisition of further quantitative information on *V. parahaemolyticus* distribution in production areas and marketed products (exposure assessment), together with studies on the effectiveness of post-harvest treatments, will help in the definition of codes of practice for vibrios in shellfish and improve the safety of products.

## Conclusion

In conclusion, these results demonstrate greater seasonal variations in total and pathogenic *V. parahaemolyticus* densities in oysters. Hence, there may be more uncertainty in the use of densities of total *V. parahaemolyticus* organisms as alternative for risk predictions as was previously recognized. These findings can provide a reference for the comprehensive management and control of the harvesting areas. Therefore, it is recommended that control measures should be considered, including the establishment of an intensive and continuous monitoring of potentially pathogenic *V. parahaemolyticus* from all oyster-growing areas, the environmental parameters, and the assessment of the region-specific human health risk due to consumption of oyster. Thus, more research is needed to assess differences in virulence among various toxigenic strains and to assess and manage the risk of illness due to human exposure to oysters harvested in contaminated environments under the light of the climate change.

## Conflict of interests

Authors have not declared any conflict of interests.

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