Densities of total and pathogenic *Vibrio parahaemolyticus* in Oysters (*Crassostrea virginica*) from the Pueblo Viejo Lagoon, Veracruz, Mexico.

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Introduction

The oyster is an important fishery resource in the State of Veracruz. However its commercialization has a low cost due to its low innocuous nature. Several studies have demonstrated that human activity is responsible for the ecological imbalance of the coastal lagoon ecosystems, which potentiate the incidence of pathogenic agents that pose a risk to human health. *Vibrio parahaemolyticus* is considered as one the main etiological agents causing gastroenteritis as result of the consumption of raw and partially raw mollusks. A rapid detection of pandemic strains of *V. parahaemolyticus* will help to improve seafood safety and mitigate the economic and social impact in the area. In response to this problematic, the Veracruz State Government, the Instituto Nacional de Pesca (INAPESCA) and the Instituto Tecnológico de Boca del Río (ITBOCA) have decided to develop a management plan for the lagoon systems. In order to conduct a risk assessment of this potential pathogenic bacterium in Veracruz lagoons, the ITBOCA, in conjunction with the Comisión Federal para la Protección contra Riesgos Sanitarios (COFEPRIS) are currently developing an integrated vigilance program of total and pathogenic *V. parahaemolyticus* in oysters harvested in Veracruz lagoons. In this regard, the main objective of this study was to determine the densities of total and pathogenic *V. parahaemolyticus* in oysters in Pueblo Viejo Lagoon, Veracruz.

Materials and methods

Sample collection

Oysters (90 of each site) were collected at five sites in the lagoon, from February through August 2009. Seasons were defined as winter (February), dry (May) and rain (August). Sites included the most important commercially bank along the lagoon. Oysters were placed on top of bagged ice in insulated coolers at 4 °C (Elliot *et al*., 1995; Tomlinson, 1992), transported to the laboratory and analyzed within 24 h of collection. Once in the laboratory, oysters were cleaned of debris and attached algae, and shucked under strict sterile conditions.

Densities of V. parahaemolyticus by MPN-PCR method

Oysters (100 g) were blended with 250 mL of alkaline peptone water (APW) at pH=8.0, containing 3% sodium chloride (NaCl), until obtaining a homogenate. Serial dilutions (1:10, 1:100 and 1:1000) were developed by resuspending an aliquot (1 mL) of the homogenized in 9 mL of APW (3% NaCl). An enumeration technique by most probable number (MPN) was performed as previously described (Lee *et al*., 2008; Miwa *et al*., 2006). Tubes were incubated at 35 °C for 18-24 h. The levels of *V. parahaemolyticus* tlh^+, tdh^+ and trh^+ were determined from three-tube series by MPN-PCR. Aliquots of 600 µL were obtained of each tube, and the commercial Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, USA) was used for DNA extraction. PCR reactions were carried out using primers and conditions previously reported (Luan *et al*., 2006). A positive result was considered when fragments of 450 bp for *V. parahaemolyticus* (tlh^+), 270 bp for *V. parahaemolyticus* (tdh^+)

and 500 bp for *V. parahaemolyticus* (*trh*$^+$) were visualized. Numbers of positive and negative tubes for PCR amplifications were scored and densities of total and pathogenic *V. parahaemolyticus* were calculated from the MPN table (Blodgett, 2000).

Results

Densities of total (*tlh*$^+$) and pathogenic (*tdh*$^+$ and/or trh$^+$) *V. parahaemolyticus* in oysters collected from the five sites in the lagoon at different seasons are shown in Table 1. Densities of *V. parahaemolyticus* (*tlh*$^+$) ranged from 26.66 MPN (rain) to > 16000 MPN/g (winter). The highest density of total *V. parahaemolyticus* was found in sites 4 and 5. *V. parahaemolyticus* total (*tlh*$^+$) could be detected in all samples analyzed during the three seasons. *V. parahaemolyticus* showed a high prevalence (61.47%) in oyster samples during the winter season (Fig. 2). Samples positives for pathogenic *V. parahaemolyticus* (*tdh* or *trh*) showed the higher densities during the dry (209.40 MPN/g) and the winter seasons (1461.33 MPN/g), respectively. As shown in Table 1, oysters collected during the dry season at sites 1-5 harbored *tdh*-positive *V. parahaemolyticus* (10.16 to 209.4 MPN); but during the winter *V. parahaemolyticus* (*tdh*$^+$) was detected only at site 4 (37.66 MPN). *V. parahaemolyticus* positive for the *tdh* or *trh* genes was detected in 23.69% and 24.44% of samples, respectively (Fig. 2). The presence of pathogenic *V. parahaemolyticus* (*tdh*$^+$ and *trh*$^+$) in oysters was low at the three seasons. Samples collected in winter (sites 3 and 4) and rainy (sites 3, 4 and 5) period were positive for *tdh* and *trh* genes. The highest density of *tdh*- and *trh*-positive *V. parahaemolyticus* (104.3 MPN/g) was found in site 4 during winter season (8.14%) (Tab. 1 and Fig. 2).

Table 1. Densities of total and pathogenic *V. parahaemolyticus* in oysters by MPN-PCR (MPN/g).

Season	Site	*tlh*$^+$	*tdh*$^+$	*trh*$^+$	*tdh*$^+$/*trh*$^+$
Winter	1	79.53	0.00	0.00	0.00
	2	94.23	0.00	0.00	0.00
	3	166.66	0.00	112.00	62.80
	4	> 16000	37.66	1461.33	104.03
	5	> 16000	0.00	0.00	0.00
Dry	1	178.10	77.30	0.00	0.00
	2	41.13	41.13	0.00	0.00
	3	40.56	10.16	0.00	0.00
	4	238.06	132.56	0.00	0.00
	5	282.47	209.40	0.00	0.00
Rain	1	125.03	0.00	0.00	0.00
	2	26.66	0.00	0.00	0.00
	3	42.83	0.00	0.00	34.50
	4	122.66	44.33	20.36	10.01
	5	319.50	159.96	0.00	22.13

Discussion

Aquaculture is a high-impact economic activity and high growth potential whose main goal is to generate products and sub-products for human consumption with high quality ranges. For it, products must be considered as safe and pathogen-free. Risk assessment of *V. parahaemolyticus* in seafood requires quantitative information of the microorganism, particularly about haemolysins genes. In this study, the highest densities of *V. parahaemolyticus* (*tlh*$^+$) in oysters, occurred during the winter season (> 16 000 MPN / g), which coincides with studies that suggest that vibrio populations migrate in winter to the sediments, so it is more likely to be available and in association with benthic organisms. The abundance of total *V. parahaemolyticus* in oysters was in agreement with other studies (Cabanillas-Beltrán *et al*., 2006; DePaola *et al*., 2003). In Mexico, there is no regulation of the permissible limits for *V. parahaemolyticus* in oysters. Nevertheless, in this study the densities

of total *V. parahaemolyticus* were higher than the limits set by international organizations as NSSP USA (5000 MPN/g), FAO/WHO and FDA (10000 MPN/g).

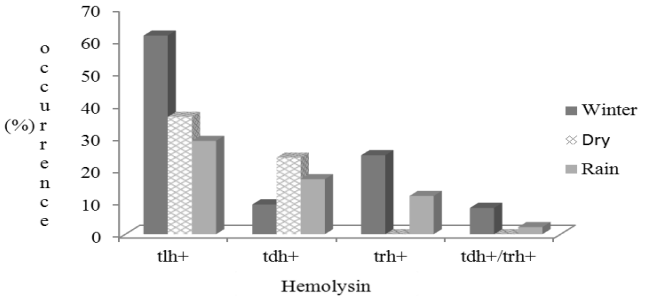

Fig. 1. Ocurrence of *tlh*, *tdh* and *trh* genes in oysters from the Pueblo Viejo Lagoon.

An interesting fact was observed with regard to pathogenic *V. parahaemolyticus*. In a previous study, *V. parahaemolyticus* (*trh*$^+$) was no detected in any of the strains isolated from oyster in the lagoon (Cabrera-Garcia *et al*., 2004). In this report, *trh* gene was detected during the winter and the rainy seasons. The detection and quantification of bacteria in seafood and the environment is extremely challenging so it is necessary to perform continuous monitoring of potential pathogens. Otherwise, pathogenic *V. parahaemolytiucs* densities were affected by the sampling site, as an increase in *V. parahaemolyticus* (*tdh*$^+$ and *trh*$^+$) was observed in oysters taken from areas near to polluted sources with anthropogenic impact.

Conclusions

The increase of potentially pathogenic *V. parahaemolyticus* (*tdh*$^+$ and *trh*$^+$) in areas that present high anthropogenic contamination shows the need to clean up the lagoon and indicates that a rapid detection of pandemic *Vibrio parahaemolyticus* strains will help to improve seafood safety and reduce the risk of infection caused by this microorganism, helping with the mitigation of economic, social and environmental impact in the region.

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