Seasonal Abundance of \textit{Vibrio vulnificus} in Raw Oysters (\textit{Crassostrea virginica}) Harvested from Mandinga Lagoon System: A Food Safety Risk

Pardo-Sedas, V. T.*1, López-Hernandez, K. M.1, Martinez-Herrera, D. I.1, Flores-Primo, A.1, and Uzcanga-Serrano, R.1

*1Facultad de Medicina Veterinaria y Zootecnia, Universidad Veracruzana, Avenida Miguel Ángel de Quevedo s/n esq. Yáñez, Colonia Unidad Veracruzana, Veracruz, Veracruz, México CP 91710. Email: vpardos@yahoo.com.mx, vpardos@uv.mx

ABSTRACT
The seasonal abundance of \textit{Vibrio vulnificus} strains in raw American oysters (\textit{Crassostrea virginica}) was evaluated monthly during a one-year period (January to December) and the risk of exposure was assessed. A total of 80 medium-sized (7–8 cm long) live oysters were harvested monthly by divers at oyster beds in two harvesting sites of the Mandinga Lagoon System. After being cleansed, live oyster were analysed within 2 h of collection. The detection of \textit{V. vulnificus} species-specific hemolysin gene (\textit{vvhA}) and genotype gene targets for groups E (Environmental) and C (Clinical) densities was accomplished by MPN–PCR methodology. MPN values counts calculated with 3-tube MPN tables were normalized for appropriate analysis and the significant variations in the seasonal distribution were evaluated by analysis of variance (\(P < 0.05\)) and Tukey’s test. The FDA/FAO/WHO v.2005 software in combination with Microsoft Excel was used to run the simulations. Although no significant differences in density levels among seasons were detected, the highest mean \textit{V. vulnificus} (\textit{vvhA}+) densities were observed during summer and fall (1,100 MPN/g) seasons. The highest mean pathogenic \textit{V. vulnificus} (\textit{vvhA}+) type C density was found (360 MPN/g) during summer season as well; however, no \textit{V. vulnificus} (\textit{vvhA}+) type E densities were detected during the sampling period. Considering 10 h of storage out of refrigeration and an intake of 12 oysters (100 g) the model predicted that the higher mean risk per serving associated with the consumption of raw oysters contaminated with \textit{V. vulnificus} (\textit{vvhA}+) was \(4.0 \times 10^{-4}\) for both summer and fall seasons. Similarly, the model predicted for \textit{V. vulnificus} (\textit{vvhA}+) type C a mean risk per serving of \(4.3 \times 10^{-5}\) and \(3.0 \times 10^{-5}\) during summer and fall seasons, respectively. Although these results suggest that \textit{V. vulnificus} poses a low health risk level, these strains raise important health issues as \textit{V. vulnificus} (\textit{vvhA}+) type C are potentially virulent and may imply a risk of infection to the consumers of raw oysters. Hence, the monitoring of these pathogenic strains is crucial to seafood safety.

Keywords: \textit{Vibrio vulnificus}, raw oyster, health risk, seafood safety

INTRODUCTION
\textit{Vibrio vulnificus}, an etiologic agent of seafood-associated fatalities worldwide, is a Gram-negative, halophilic bacterium that has the ability to cause lethal infections including primary septicemia, wound infection and gastroenteritis (16). \textit{V. vulnificus} is more frequently associated with wound infections, with a case fatality rate as high as 30% (5), particularly in individuals with predisposing conditions, including patients with chronic liver disease, immunodeficiency, iron storage disorders, end-stage renal disease, and diabetes mellitus (5). \textit{V. vulnificus} exhibits a great deal of genotypic and...
phenotypic variation. The species is divided into three biotypes, all of which are able to cause human infection. However, biotype 1 is of greatest import to oyster producers and consumers. Biotype 1 strains of *V. vulnificus* have been further divided into two genotypes, C and E. The gene *vbg* (virulence correlated gene) has two alleles, *vbg*C and *vbg*E, representing clinical and environmental strains, respectively (4). This pathogen is commonly reported in many Asian countries, including China, Japan and Taiwan, and in USA as well (1).

*V. vulnificus* is a naturally occurring opportunistic pathogen commonly found in estuarine waters of tropical climates (15). The consumption of raw oysters, particularly those harvested from the Gulf of México in warmer months, has been strongly correlated with serious foodborne illness caused by *V. vulnificus* (8). In México, Quitones *et al.* (11) isolated *V. vulnificus* from oyster samples collected from Pueblo Viejo Lagoon, Veracruz during the warmer months. This may imply increased risk of infection to the consumers of raw oysters. Nevertheless, there are few reports of risk assessments for *V. vulnificus* associated with raw oyster consumption. Mandinga Lagoon System (MLS) is one of the most important shellfish-production estuarine lagoon systems on the Mexican Gulf coast, which supplies fishery resources (oysters, shrimps, fishes, crabs) to nearby local restaurants, mostly from Veracruz – Boca del Río and México cities, where oysters are marketed and consumed raw by the local and tourist population (López-Hernández *et al.*, 2015). As there is limited information about the presence and abundance of pathogenic *V. vulnificus* in oysters from this important lagoon system, the aim of this study was to determine the seasonal abundance of *Vibrio vulnificus* in raw oysters harvested from MLS for a better assessment of the public health risk associated with oyster consumption.

**MATERIALS AND METHODS.** Sample collection and processing. American oyster (*Crassostrea virginica*) samples were collected monthly during a one-year period (January to December). A total of 80 medium legal-sized (7–8 cm long) (13) live oysters were harvested by divers at oyster beds in two harvesting sites of the MLS (Figure 1) and immediately transported to the laboratory. Dead animals were discarded and the remaining oysters were scrubbed and rinsed under cold running tap water to remove debris and attached algae, and analyzed within 2 hours of collection. The detection of *V. vulnificus* species-specific hemolysin gene (*vwhA*) densities was accomplished by MPN–PCR methodology (López-Hernández *et al.*, 2015) for *V. vulnificus*. Briefly: The quantification of densities of *V. vulnificus* species-specific hemolysin gene (*vwhA*) and genotype gene *vbg* targets for groups *vbg*E and *vbg*C strains was expressed by the Most Probably Number (MPN) method with the 3-tube test series MPN chart and the results expressed as *V. vulnificus* MPN/g of oysters (USDA, 2008).
Figure 1. Location of the study region and map of the MLS. The MLS is in the state of Veracruz, Mexico and flows parallel to the northwestern coastline of the Gulf of Mexico, between 19°02′ N and 96°06′ W in Alvarado, Veracruz. The MLS is constituted by Larga, Chica and Granada lagoons and exits into the Gulf of Mexico by the Boca del Río, close to Veracruz City. Sites of oyster samples collection monitored for one-year period: bank A (Canal de Mundinga located close to the human settlement Mundinga) and bank B (Las Garzas located close to mangrove islands).

Presumptive strains that were confirmed *V. vulnificus* *vwhar*+, type E, and C in the direct PCR were scored positive for the respective gene and stored in slants of Trypticase Soja Agar (TSA) (BIOXON Becton Dickinson de México S.A de C.V., México) at −20°C for preservation until identification. Detection of *V. vulnificus* specific-species genes, and genotypes (E and C) were determined by conventional PCR according to the procedures described by Brauns et al. (2) and Rosche et al. (12), respectively. PCR assays were performed using specific primers (Sigma-Aldrich QUIMICA S.A. de C.V., Mexico) for species and identification of genotype. DNA of strain CAIM 610 (2) was used as positive control for the (*vwhar*) gene, and CAIM 1859 and CAIM 1860 were used as positive controls for genotypes E and C (12).

Statistical analysis. Most probable number (MPN) 3-tube chart and formulas corresponding 95% confidence limits were used to identify MPN for each sample as previously described (17). Significant differences in the seasonal distributions of Log10 MPN/g *V. vulnificus* densities were analyzed by Analysis of Variance (*P* < 0.05) and Tukey's test using Minitab v.16.0 (Minitab, Inc., State College PA). *V. vulnificus* counts were Log10 transformed to appropriately normalize the data for ANOVA. Nondetectable values of *V. vulnificus* counts (<0.30 MPN/g) were replaced by half of the detection limit in oysters for statistical purposes. The FDA/FAO/WHO v.2005 software in combination with Microsoft Excel was used to run the risk simulations.

RESULTS AND DISCUSSION

Although no significant differences in density levels among seasons were detected, the highest mean *V. vulnificus* (*vwhar*+) densities were observed during summer (351.7 MPN/g) and the lowest in winter (4.2 MPN/g) seasons. Moreover, positive samples were found during all seasons of a one-year period. The highest mean pathogenic *V. vulnificus* (*vwhar*+) type C density was found (14.65 MPN/g) during
The summer season as well. No *V. vulnificus* (*vwha*) type E densities were detected during the sampling period. The highest mean *V. vulnificus* (*vwha*) densities were detected during summer and fall when the average water temperature in the MLS were 29.29 and 27.27°C, respectively. Meanwhile, mean *V. vulnificus* (*vwha*) densities decreased during winter when the average water temperature decreased to 25.62°C.

<table>
<thead>
<tr>
<th>Seasons</th>
<th>V. vulnificus vwha density (MPN/g mean and range)</th>
<th>Genotype E density (MPN/g mean and range)</th>
<th>Genotype C density (MPN/g mean and range)</th>
<th>Water Temperature (°C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter</td>
<td>4.2 ± 3.6a (-0.30 – 7.7)</td>
<td>&lt;0.30</td>
<td>1.65 ± 1.5a (-0.30 – 3.0)</td>
<td>25.62 ± 3.15a</td>
</tr>
<tr>
<td>Spring</td>
<td>184.1 ± 448.7a (-0.30 – 1,100.0)</td>
<td>&lt;0.30</td>
<td>1.283 ± 2.4a (-0.30 – 6.2)</td>
<td>28.79 ± 0.72a</td>
</tr>
<tr>
<td>Summer</td>
<td>551.7 ± 600.7a (-0.30 – 1,100.0)</td>
<td>&lt;0.30</td>
<td>14.65 ± 17.5a (-0.30 – 36.0)</td>
<td>29.29 ± 0.80a</td>
</tr>
<tr>
<td>Fall</td>
<td>366.9 ± 567.9a (-0.30 – 1,100.0)</td>
<td>&lt;0.30</td>
<td>2.75 ± 1.5a (-0.30 – 5.2)</td>
<td>27.27 ± 1.51a</td>
</tr>
</tbody>
</table>

Means with different literals are statistically different (*P < 0.05*) between seasons; *(9)*

Our results agree with those reported by Johnson *et al.* (7) who observed the highest *V. vulnificus* levels in oysters harvested from the U.S. Gulf of Mexico between May and October (median monthly concentration of 2300 MPN/g), with a reduction to fewer than 10 MPN/g from November to March. *V. vulnificus* proliferates in areas or during months where the water temperature exceeds 18°C, and culturable concentrations of *V. vulnificus* are generally lower when water temperatures are cooler. Thus, *V. vulnificus* colonization of oysters may be influenced by water parameters such as temperature or salinity (10). Quiñones *et al.* (11) isolated *V. vulnificus* from oyster samples collected from Pueblo Viejo Lagoon, Veracruz, and observed that overall 27% (39/143) of the oyster samples were (*vwha*).

Although positive samples were found during all seasons of a one-year period, a seasonal fluctuation was observed. Isolation rates from oysters were significantly higher in June than in the period from November to February (*p < 0.0002*), indicating that water surface temperatures (≥24°C) are more favorable for *V. vulnificus* during the summer months.

An important finding in our study is the isolation of *V. vulnificus* of the C genotype. This is the first study to report the presence of *V. vulnificus* C in oysters from the Mexican coastline of the Gulf of Mexico. A seasonal trend for C genotype was observed, as the mean *V. vulnificus* C densities were detected when water temperatures were ≥27°C; meanwhile, the mean densities decreased as temperature decreased during winter. However, *V. vulnificus* E was not detected over the one-year period. It is unclear if levels of the two genotypes are unique to certain environmental conditions. These results seem to indicate that C strains have evolved to cope with the stresses associated with changing environment. The fact that oysters have C genotype strains as the dominant strain type further suggests the possibility that those oysters harboring larger densities of this genotype would be likely to be more infective to humans as *V. vulnificus* C type is more infectious (18).
Table 2. Estimated risk for *Vibrio vulnificus* in raw oysters *Crassostrea virginica* harvested from MLS over a one-year period.

<table>
<thead>
<tr>
<th>Season</th>
<th><em>Vibrio vulnificus</em> mean density (Log$_{10}$ MPN/g)</th>
<th>Expected cases per 100,000 servings consumed by at risk population</th>
<th>Lower confidence limits expected cases per 100,000 servings consumed by at risk population</th>
<th>95% confidence limits</th>
<th>Upper 95% confidence limits</th>
<th>Lower 95% confidence limits</th>
<th>Upper 95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>vwhr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>0.328</td>
<td>2.5</td>
<td>1.7</td>
<td>3.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>0.249</td>
<td>2.9</td>
<td>3.1</td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>1.509</td>
<td>4.0</td>
<td>3.1</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fall</td>
<td>0.665</td>
<td>3.9</td>
<td>2.2</td>
<td>4.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>0.224</td>
<td>1.3</td>
<td>0.1</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>0.112</td>
<td>2.1</td>
<td>0.2</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>1.178</td>
<td>4.3</td>
<td>3.5</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fall</td>
<td>0.440</td>
<td>3.0</td>
<td>0.2</td>
<td>3.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As shown in Table 2, considering 10 h of storage out of refrigeration and an intake of 12 oysters (100 g), the model predicted a higher mean risk per serving associated with the consumption of raw oysters contaminated with *V. vulnificus* (vwhr+), 4.0 cases per 100,000 servings during summer and fall seasons. Similarly, the model predicted for *V. vulnificus* (vwhr+) type C a mean risk of 4.3 and 3.0 cases per 100,000 servings during summer and fall seasons, respectively. Moreover, the legal Mexican bacteriological limit (14) of no detection of *V. vulnificus* in 50 g of oyster flesh was not accomplished. FAO/WHO (3) reported a risk assessment for primary septicaemia cases associated with consumption of raw oysters from the Gulf Coast of USA with mean densities of 57,000 and 80 MPN/g during summer and winter harvest seasons, respectively. Given an average serving size of 196 g (14 oysters), these average numbers correspond to *V. vulnificus* ingested per serving of $1.1 \times 10^8$ and $1.6 \times 10^8$, respectively. However, variation in water and air temperatures and the characteristics of harvesting duration and storage time have the effect of increasing the variation of *V. vulnificus* numbers at each point along the harvest-to-consumption continuum.

Although these results suggest that *V. vulnificus* poses a low health risk level, the *V. vulnificus* (vwhr+) type C raise important health issues as these strains are potentially virulent and may imply a significant risk of infection associated with consumption of raw oysters and exposure to seawater. Thus, there is a need for tiered regulation that simultaneously reduces infection risk without ceasing to provide raw shellfish altogether. Hence, the monitoring of these pathogenic strains is crucial to seafood safety.

REFERENCES


