Perkinsus marinus in the pleasure oyster Crassostrea corteziensis cultivated on the southeast coast of the Gulf of California, Mexico

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ABSTRACT. The protozoan Perkinsus marinus has been associated with high mortality episodes of the eastern oyster Crassostrea virginica in the USA. The presence of P. marinus on the pleasure oyster Crassostrea corteziensis cultivated in two estuaries on the southeast coast of the Gulf of California was evaluated. Oysters were collected monthly (September 2016 to September 2017) and analyzed using Ray’s fluid thioglycollate medium (RFTM) and polymerase chain reaction (PCR). Water parameters and oyster biometrics were also recorded. Pathogen prevalence increased over time from 0 to 100% in oysters from La Pitahaya, and from 0 to 83.33% in those from Bacorehuis. At both oyster farms, infection intensity was light (<1×10⁴ parasites g⁻¹ wet tissue), pathogen prevalence and infection intensity were correlated with oyster size and weight, and there was a strong correlation between P. marinus prevalence and intensity (La Pitahaya r = 0.91; Bacorehuis r = 0.82). The oysters that resulted positive for P. marinus by RFTM also were assayed using PCR. P. marinus presence was confirmed in 98.27% (114/116) of the oysters from La Pitahaya, and 95.83% (46/48) of those from Bacorehuis. The detection of P. marinus confirms that this pathogen is well established in the area with high expression during the warmer season. Despite the light infection intensity of this parasite at both sites, health surveillance of this bivalve in the region is highly advisable.

Keywords: Perkinsus marinus; Crassostrea corteziensis; pathogen; diseases; prevalence; infection intensity; Gulf of California

INTRODUCTION

Diseases of farmed mollusks cost billions of dollars each year, and the movement of exotic pathogens to new culture areas is one of the most important factors for disease dispersion (Lafferty et al., 2015). As in many countries (Soletechnick et al., 2002; Langdon et al., 2003; Taris et al., 2007), oyster production in northwestern Mexico is based on the Japanese oyster (Crassostrea gigas) (Chávez-Villalba, 2014). This bivalve was first introduced in the 1970s on the Pacific coast of the Baja California Peninsula (Isla Olivas, 1975), and later in the Gulf of California. Unusual episodes of high mortality in C. gigas associated with environmental factors (Cáceres-Martínez et al., 2018), poor culture management (Cáceres-Martínez & Vásquez-Yeomans, 2013), and pathogens (Cáceres-Martínez, 2000) have been reported since 1997 (Vásquez-Yeomans et al., 2004a). Therefore, the culture of the pleasure oyster Crassostrea corteziensis,
a native species from the Gulf of California, was suggested as an alternative to compensate for *C. gigas* losses (Chávez-Villalba et al., 2005). However, concerns about *C. gigas* mortality related, for example, to the presence of parasites have recently extended to other bivalve mollusk species in the region, including *C. corteziensis* (Cáceres-Martínez et al., 2016).

Different oyster species are affected by a variety of symbionts and pathogens that may cause some external and internal signs, including shell perforations (Gallo-García et al., 2004) and damage to internal tissues (Vásquez-Yeomans, 2006; Aguirre-Macedo et al., 2007; Cáceres-Martínez et al., 2010). In other cases, high mortality at different oyster ages has been detected (Chávez-Romero et al., 2011). Among oyster parasites reported in Mexico, Vásquez-Yeomans et al. (2004b) isolated bacteria in gill tissues from oysters collected during a mortality event in Baja California; Cáceres-Martínez & Vásquez-Yeomans (2013) reported the presence of a herpesvirus in *C. gigas* in Baja California, and Grijalva-Chon et al. (2013) detected a new type of ostreid herpes virus in *C. gigas* from the Gulf of California. Cáceres-Martínez et al. (2010) revealed the presence of Rickettsiales-like prokaryotes, turbellarians, encysted crustaceans, and ciliated protozoans in different tissues from *C. corteziensis* on the north-central coast of the Mexican Pacific. They concluded that the protozoan *Perkinsus marinus* represented the most significant risk to oyster health. The parasite presence in the Mexican Pacific derives from *C. virginica* mobilizations from the USA and the Gulf of Mexico. Cáceres-Martínez & Vásquez-Yeomans (2013) documented three records of *P. marinus* introductions in the Pacific coast: one from the Washington coast to the estuary Punta Banda, Baja California, in the 1970s; another from the transfer of oysters for commercial purposes from the Tamáhua Lagoon, Veracruz to Nayarit one decade later; and the last one by mobilization of the American oyster from Louisiana, USA, to San Jorge Bay, Sonora, to protect the oyster stock and production of the impact of Katrina hurricane, in 2005 (Cáceres-Martínez & Vásquez-Yeomans, 2013).

Different *Perkinsus*-like parasites have been reported in wild and cultivated bivalve species in and around the Gulf of California using different techniques given their presence, detection, confirmation, and infection degree. For instance, *P. marinus* was found in natural populations of *C. corteziensis* on the central coast of the Mexican Pacific (Cáceres-Martínez et al., 2008) and the mangrove oyster *Saccostrea palmula* along the Sinaloa coast (Cáceres-Martínez et al., 2012), as well as in the wild population of the black clam *Chione fluctifraga* in Kino Bay, Sonora (Enríquez-Espinoza et al., 2015). *Perkinsus* sp. has been diagnosed in a natural bank of the pen shell *Atrina maura* in Sinaloa (Góngora-Gómez et al., 2016) and cultivated Japanese oysters along the coast of Sonora in the Gulf of California (Enríquez-Espinoza et al., 2010) and on the north-central coast of Sinaloa (Villanueva-Fonseca & Escobedo-Bonilla, 2013). Cáceres-Martínez et al. (2016) determined the prevalence and intensity of *P. marinus* in two cultured populations of *C. corteziensis* in the state of Nayarit, Mexico, from 2007 to 2014 and pointed out that no unusual mortalities were related with the presence of the parasite.

The presence of *P. marinus* in pleasure oysters farmed off the north coast of Sinaloa, Mexico, remains unknown. Assessing the possible impact of this pathogen on *C. corteziensis* cultivated in this state is of paramount importance. Thus, this study’s objective was to evaluate *P. marinus* in *C. corteziensis* at two oyster farms on the southeast coast of the Gulf of California.

**MATERIALS AND METHODS**

**Oysters**

*Crassostrea corteziensis* specimens were sampled at two oyster farms located in the Bacorehuis (26°06'-26°32'N, 109°01'-109°20'W) and La Pitahaya (25°21'-25°24'N, 108°30'-108°45'W) estuaries in northern Sinaloa, Mexico. According to the farmers’ information, the oyster seed was produced at the Centro de Reproducción de Especies Marinas del Estado de Sonora (CREMES) of the Instituto de Acuacultura del Estado de Sonora, O.P.D., which were certified as parasites-free. The small oysters (4.5 mm size range) were transported to the cultural sites and acclimated following standard procedures (Gallo-García et al., 2001). As traditionally performed in the region, oysters were cultured in a suspended long-line system, according to Villanueva-Fonseca et al. (2017). Thirty oysters were sampled monthly in both sites throughout a single culture cycle from September 2016 to September 2017 (13 months). The mean initial shell height (SH, major axis) and total weight (TW) were 4.35 ± 0.78 mm and 0.01 ± 0.004 g. The specimens were randomly collected from several oyster trays each month and transported live in 20 L plastic tanks for further analysis at the Centro Interdisciplinario de Investigaciones para el Desarrollo Integral Regional (IPN-CIIDIR) Unidad Sinaloa, Guasave, Sinaloa, Mexico. At each sampling, the oyster biometrics (SH and TW) and water parameters (temperature, salinity, dissolved oxygen, pH, transparency, depth, chlorophyll-*a* concentration, total suspended solids, and particulate organic and inorganic matters) were recorded in order to assess any possible correlation with parasite preva-
Perkinsus marinus diagnosis

Once at the lab, oysters (N = 30 ind month\(^{-1}\) site\(^{-1}\)) were immediately processed for *Perkinsus marinus* diagnosis. Fouling organisms and mud were removed from each oyster using a soft brush and a stream of freshwater. Next, specimens were opened, and the soft tissue divided into two subsamples. One subsample from each oyster was incubated in Ray’s Fluid Thioglycollate Medium, RFTM (Ray, 1966; OIE, 2012) with the addition of antibiotics (penicillin and streptomycin) and an antifungal (nystatin) and incubated in the dark for 7 d at room temperature (23-25°C) to induce hypnospore formation; the other subsample was placed at -70°C for confirmatory PCR analysis. Subsequently, the tissues were digested (NaOH 2M, 60°C for 2-6 h) and rinsed with deionized water; next, they were placed on a slide, stained with 3% Lugol’s solution, and observed under light microscopy (100x) for the presence of spherical, blue, or bluish-black *P. marinus* hypnospores. Samples with a high number of hypnospores were serially diluted to reduce the total cell number to a manageable number.

The number of hypnospores per gram of oyster tissue was calculated. The effect of *P. marinus* in the sampled oysters was evaluated based on the infection intensity and prevalence. The infection intensity was classified as negative (0 hypnospores), light (<1x10\(^4\) hypnospores g\(^{-1}\)), moderate (1x10\(^4\) to 5x10\(^5\) hypnospores g\(^{-1}\)), or heavy (>5x10\(^5\) hypnospores g\(^{-1}\)) (Bushek et al., 1994). The pathogen prevalence was defined as the number of infected oysters in the sample and expressed as a percentage of the total number of oysters sampled (Thrusfield, 1995).

Specimens with a positive RFTM result were subjected to PCR. Total deoxyribonucleic acid (DNA) extraction was achieved using a DNAzol\(^\oplus\) kit following the manufacturer’s protocol (Molecular Research Center, Inc., Cincinnati, OH). At the same time, PCR was performed using the primers PmarITS-70F (5’-CTT-TTG-YTW-GAG-WGT-TGC-GAG-ATG-3’) and PmarITS-600R (5’-CGA-GTT-TGC-GAG-TAC-CTC-KAG-AG-3’) that amplify a 509 of the ITS region of the *P. marinus* rRNA gene complex. The PCR mix was prepared as follows: 100 ng of DNA template; 1×PCR Buffer (10 mM Tris-HCl, pH 8.5, KCl 50 mM); MgCl\(_2\) 1.5 mM; 0.2 mM of a mixture of dATP, dCTP, dGTP, and dTTP; 0.01 mM of each primer; and 1.5 U of proofreading *Taq* DNA polymerase (Bioline, Boston, MA, USA) in a final volume of 25 µL. Amplification conditions were: an initial denaturation step of 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final elongation of 72°C for 5 min (Audemard et al., 2004). Also included in the reactions were a positive control (total genomic DNA of *P. marinus* infected tissue from *C. corteziensis*) and negative control in which sterile water was added instead of the template. All PCR analyses were performed in triplicate. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide (1 µg mL\(^{-1}\)), and visualized using ultraviolet light.

Statistical analysis

All data were tested for normality (Lilliefors test) and heteroscedasticity (Bartlett’s test) to select appropriate statistical analyses. Prevalence values were arcsine transformed before analysis. The differences in the number of hypnospores per gram and the parasite prevalence in the oysters were compared between the two sites every month using a Mann-Whitney U test and a paired-sample t-test, respectively. Spearman's rank correlation was used to relate the infection intensity and prevalence data with the oyster biometrics and environmental parameters at the two oyster farms. All statistical analyses were performed using Statistica (Statsoft Inc., Tulsa, OK, USA). The significance level was set to α = 0.05 for all tests.

RESULTS

Growth and mortality of oysters and environmental data

After one year of culture, the SH and TW of *Crassostrea corteziensis* from the La Pitahaya and Bacorehuis oyster farms were 62.74 ± 3.74 and 68.82 ± 6.77 mm and 35.09 ± 4.94 and 38.75 ± 11.27 g, respectively. The daily length and weight growth rate were 0.14 mm d\(^{-1}\) and 0.08 g d\(^{-1}\) in La Pitahaya and 0.18 mm d\(^{-1}\) and 0.09 g d\(^{-1}\) in Bacorehuis. The mortality was recorded during the autumn and winter seasons, from October 2016 to February 2017 at La Pitahaya and from November 2016 to January 2017 at Bacorehuis. Final survival was 64.43 and 75.56% for La Pitahaya and Bacorehuis farms, respectively. The mean water parameters for the La Pitahaya and Bacorehuis estuaries are shown in Table 1.

Perkinsus marinus diagnosis

The RFTM test shows that out of 390 oysters analyzed 116 (29.74%) and 48 (12.31%) from the La Pitahaya and Bacorehuis estuaries, respectively, tested positive for presumptive *Perkinsus marinus* hypnospores by RFTM, which showed spherical cells (5-70 µm diameter) stained black or bluish-black with Lugol’s solution (Fig. 1).
Table 1. Mean water parameters in the La Pitahaya and Bacorehuis estuaries during 13 cultivation months (September 2016-September 2017).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>La Pitahaya</th>
<th>Bacorehuis</th>
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<tbody>
<tr>
<td>Temperature (°C)</td>
<td>27.64 ± 4.97</td>
<td>27.90 ± 4.18</td>
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<tr>
<td>Salinity</td>
<td>33.61 ± 2.18</td>
<td>32.38 ± 4.55</td>
</tr>
<tr>
<td>Dissolved oxygen (mg L(^{-1}))</td>
<td>5.84 ± 0.93</td>
<td>5.49 ± 1.98</td>
</tr>
<tr>
<td>pH</td>
<td>7.03 ± 0.84</td>
<td>7.10 ± 0.89</td>
</tr>
<tr>
<td>Transparency (m)</td>
<td>0.64 ± 0.29</td>
<td>0.53 ± 0.32</td>
</tr>
<tr>
<td>Depth (m)</td>
<td>1.45 ± 0.45</td>
<td>0.86 ± 0.30</td>
</tr>
<tr>
<td>Chlorophyll-a (mg m(^{-3}))</td>
<td>6.70 ± 4.63</td>
<td>4.41 ±3.35</td>
</tr>
<tr>
<td>Total suspended solids (mg L(^{-1}))</td>
<td>40.71 ± 12.68</td>
<td>37.15 ± 30.78</td>
</tr>
<tr>
<td>Particulate organic matter (mg L(^{-1}))</td>
<td>19.63 ± 2.38</td>
<td>19.68 ± 4.65</td>
</tr>
<tr>
<td>Particulate inorganic matter (mg L(^{-1}))</td>
<td>29.78 ± 10.55</td>
<td>27.46 ± 26.75</td>
</tr>
</tbody>
</table>

Figure 1. Detection of presumptive *Perkinsus marinus* hypnospores in *Crassostrea corteziensis* from a) La Pitahaya and b) Bacorehuis estuaries with Ray’s fluid thioglycolate medium (RFTM) (100x).

Both the prevalence and the infection intensity of *P. marinus* in *C. corteziensis* at both oyster farms increased over time (Fig. 2).

For La Pitahaya, *P. marinus* was not detected (September-November 2016 and February 2017) or occurred with a low prevalence (December 2016, March-May 2017). Prevalence increased from June to September 2017, reaching 100% in the last two months. For Bacorehuis, the parasite was not detected from October to December 2016 and February 2017 or occurred with a low prevalence (January, March-July 2017). Prevalence increased in August, reaching 83.33% in the last month. At the end of the culture cycle, the number of hypnospores per g of wet tissue (wt) of the oysters cultivated at the La Pitahaya farm reached 9,242 hypnospores g\(^{-1}\) wt, approximately three times that found in the oysters from the Bacorehuis farm (3,226 hypnospores g\(^{-1}\) wt). The infection intensity was light at both oyster farms (<1×10\(^4\) parasites g\(^{-1}\)). Between sites, the prevalence was similar (\( t = 1.37, P = 0.18 \)) but the parasite load was significantly different (\( W = 8.33, P = 0.0008 \)). Table 2 shows the correlations between the prevalence and intensity of infection with oyster measurements and physicochemical parameters. The infection prevalence was strongly correlated with the infection intensity (La Pitahaya \( r = 0.91 \); Bacorehuis \( r = 0.82 \)).

The specimens testing positive for *Perkinsus* by RFTM were subjected to *P. marinus* species identification by PCR analysis. At La Pitahaya and Bacorehuis, 114/116 (98.3%) and 46/48 (95.8%) cases were confirmed, respectively (Fig. 3).

**DISCUSSION**

Cáceres-Martínez *et al.* (2008, 2010, 2012) and Martínez-García *et al.* (2017) reported the occurrence of *Perkinsus marinus* in cultivated and wild bivalves along the north-central coast of the Mexican Pacific, suggesting a possible horizontal transmission of the pro-
Perkinsus marinus

Figure 2. Intensity (hypnosposes g⁻¹ wet tissue weight) and prevalence of the infection of Perkinsus marinus in C. corteziensis in a) La Pitahaya and b) Bacorehuis estuaries.

Figure 3. Agarose gel electrophoresis of PCR products. Lanes: 1) Perkinsus marinus (positive control), 2) sterile water (negative control), 3-4) fresh tissue (La Pitahaya), 5) fresh tissue (Bacorehuis), 6) molecular weight markers (1 Kb ladder).

Table 2. Spearman’s rank-order correlations between prevalence and intensity of Perkinsus marinus infection, with oyster measurements and environmental parameters for Crassostrea corteziensis cultivated in the La Pitahaya and Bacorehuis farms, Sinaloa, Mexico. r: correlation, P: p-value. SL: shell length, SH: shell height, SW: shell weight, BW: body weight, DO: dissolved oxygen. Only significant correlations (P < 0.05) are shown.

<table>
<thead>
<tr>
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<th>La Pitahaya</th>
<th>Bacorehuis</th>
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<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>Prevalence vs. SL</td>
<td>0.744</td>
<td>0.0099</td>
</tr>
<tr>
<td>Prevalence vs. SH</td>
<td>0.845</td>
<td>0.0034</td>
</tr>
<tr>
<td>Prevalence vs. SW</td>
<td>0.806</td>
<td>0.0052</td>
</tr>
<tr>
<td>Prevalence vs. BW</td>
<td>0.867</td>
<td>0.0027</td>
</tr>
<tr>
<td>Intensity vs. SL</td>
<td>0.810</td>
<td>0.0050</td>
</tr>
<tr>
<td>Intensity vs. SH</td>
<td>0.908</td>
<td>0.0017</td>
</tr>
<tr>
<td>Intensity vs. SW</td>
<td>0.885</td>
<td>0.0021</td>
</tr>
<tr>
<td>Intensity vs. BW</td>
<td>0.919</td>
<td>0.0014</td>
</tr>
<tr>
<td>Prevalence vs. DO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intensity vs. DO</td>
<td>0.638</td>
<td>0.0269</td>
</tr>
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</table>
marinus (Cáceres-Martínez & Vásquez-Yeomans, 2013; Cáceres-Martínez et al., 2016). According to Soniat (1996), the low levels of prevalence and intensity of *P. marinus* in young oysters can be explained because their cell division rate is similar to *Perkinsus*. While they are adults, their growth rate decreases, and the parasite can reach higher infection intensity, which is consistent with this work, because bigger oysters (>50 mm shell height) presented the highest prevalence and infection intensity of *P. marinus*. Contrary findings for other cultivated oyster species are reported by Enríquez-Espinoza et al. (2015) and da Silva et al. (2016), who found that infection rates were independent of oysters’ size. According to Paytnner et al. (2010) and Ehrich & Harris (2015), larger oysters have a larger gill area that consequently leads to a higher filtration rate favoring infection through the increased ingestion of parasite cells, which could explain our results. It is also possible that larger oysters may have been infected at earlier stages, and the parasite had more time to proliferate within the host, as well as the fact that the older the oysters are, their growth rate decrease favoring the parasite proliferation; questions that deserve further research. The mean prevalence of *P. marinus* infecting *C. corteziensis* ranged from moderate (29.74% La Pitahaya) to low (19.30% Bacorehuis) by RFTM; the infection prevalence exceeded 80% at the end of the culture cycle. Although the MFTR staining technique does not discriminate between species of the genus Perkinsus spp., it is cheap, simple (OIE, 2012), more sensitive when compared with histology (McLaughlin & Faisal, 1999) and the infection data collected may be counted (Auderman et al., 2008) and categorized on a scale (Mackin, 1962; Bushek et al., 1994). Also, RFTM is considered a reliable preliminary method for carrying out subsequent molecular assays (such as PCR) in processed tissues in helping to confirm parasite’s species and, as in this case, to explain better the prevalence of *P. marinus* infection in *C. corteziensis*.

The results of the molecular analysis (PCR) for the positive RFTM specimens indicated parasite incidence exceeding 95%; this is comparable with the PCR results reported by da Silva et al. (2016) in three month old *Crassostrea gasar* in polyculture with shrimp but higher than that reported for cultivated *C. gigas* (Villanueva-Fonseca & Escobedo-Bonilla, 2013) and wild pen shell *Atrina maura* (Góngora-Gómez et al., 2016) from locations near the La Pitahaya Estuary. These differences may be due to several factors, such as the low prevalence by RFTM and the light intensity levels obtained in the present assay and the susceptibility of other species to *P. marinus* (Calvo et al., 1999). Moreover, the sample size used for parasite detection combined with the low infection intensities (Reree et al., 2008; Sabry et al., 2009; Dantas-Neto et al., 2015) may have affected the output results. Finally, the different environmental conditions at each place also contribute to determining the host-parasite interaction (Villalba et al., 2004).

Of particular interest is the low infection level obtained for this oyster species on the north-central coast of Sinaloa, which coincides with the conclusions obtained by Cáceres-Martínez et al. (2012), Villanueva-Fonseca & Escobedo-Bonilla (2013), and Góngora-Gómez et al. (2016), who reported low infection by the same protozoan in other bivalve species in the region. While the production of commercially important bivalve species has not been affected significantly by *Perkinsus* sp. due to their low susceptibility to this pathogen, it nonetheless represents a potential disease vector for other mollusk species in the region (Villalba et al., 2004). Therefore, sanitary measures to control the movement of bivalves from one culture site to another, and continuous monitoring of the protozoan in both cultivated and wild populations of bivalve mollusks should be implemented.

**CONCLUSIONS**

The infection intensity observed in this study was low. The presence of *Perkinsus marinus* in the north-central coast of Sinaloa confirms that this pathogen is well established in the area with high expression during the warmer season, as concluded by Cáceres-Martínez et al. (2016) for the same oyster species in natural populations of the state of Nayarit. Although no Perkinsus-related mortality has been reported for the north of the Sinaloa state, health surveillance of bivalve populations in the region is highly advisable, particularly for the native oyster *Crassostrea corteziensis*, which is an important component of the oyster industry.

**ACKNOWLEDGMENTS**

Authors thank the Instituto Politécnico Nacional for funding (SIP-IPN 20161188 and 20171303) and logistic support by COFFA and EDI. L.C. Villanueva-Fonseca is a recipient of a doctoral fellowship from Instituto Politécnico Nacional (BEIFI Grant) and CONACYT. Thanks to Kristin Sullivan for the english edition of the manuscript.

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Received: 11 November 2019; Accepted: 24 January 2020