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Perkinsus marinus IN *Crassostrea gigas* AND *Chione fluctifraga* FROM KINO BAY, SONORA, MEXICO

Perkinsus marinus EN *Crassostrea gigas* Y *Chione fluctifraga* DE BAHÍA DE KINO, SONORA, MÉXICO

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ABSTRACT

At present, the Pacific oyster *Crassostrea gigas* is the most cultivated shellfish in northwest Mexico. Nonetheless, other mollusks species such as the black clam *Chione fluctifraga*, which can be found along with cultured *C. gigas* in Sonora, represents a profitable emerging fishery resource. Since 1997, the oyster industry along the Gulf of California has been affected by severe mortality episodes disturbing almost all the farming areas. In order to evaluate the prevalence of *Perkinsus marinus*, a monthly sampling of 30 oysters and 30 clams was carried out during a one-year period. A total of 360 oysters and 360 clams were analyzed by Ray's fluid thyoglicolate medium (RFTM) and PCR. The RFTM assay showed prevalence ranging from 3.3 to 60% for both oysters and clams through the study period, with infection levels from light to moderate, according to the Mackin scale. The PCR analysis was positive to *P. marinus* in the only one *C. gigas* with moderate infection in the RFTM analysis. The resulting sequence revealed 100% identity with the ITS region of *P. marinus*. To our knowledge, this is the first indication of *Perkinsus* sp. in the *C. fluctifraga* clam.

Keywords: *Perkinsus marinus*, *Crassostrea gigas*, *Chione fluctifraga*, mollusk diseases, aquaculture.

RESUMEN

El ostión japonés *Crassostrea gigas* es el molusco más cultivado en el noroeste de México, y la almeja negra *Chione fluctifraga* representa una pesquería comercial emergente, la cual cohabita con *C. gigas* cultivado en Sonora. Desde 1997, el cultivo de ostión se ha visto afectado por mortalidades en casi todos los sitios de cultivo a lo largo del Golfo de California. Con el fin de evaluar la prevalencia de *Perkinsus marinus*, un muestreo mensual de 30 ostiones y 30 almejas se llevó a cabo durante un año. Un total de 360 ostiones y 360 almejas fueron analizados por medio de cultivo de tejido en Tioglicolato (RFTM) y por PCR. La prueba de RFTM mostró prevalencias que van desde 3.3 a 60% tanto para ostiones como almejas en todos los meses muestreados y con niveles de infección muy ligero, a moderado, de acuerdo a la escala de Mackin. El análisis de PCR resultó positivo a *P. marinus* sólo en el ostión que presentó infección moderada en el análisis de RFTM. La sequencia resultante reveló 100% de identidad con la region ITS de *P. marinus*. Hasta donde sabemos, este es el primer indicio de *Perkinsus* sp. en la almeja *C. fluctifraga*.

Palabras clave: *Perkinsus marunus*, *Crassostrea gigas*, *Chione fluctifraga*, enfermedades de moluscos, acuacultura.

INTRODUCTION

Due to its fast growing rate, environmental adaptability, pathogen tolerance and national demand or consumption, the Pacific oyster *Crassostrea gigas* was introduced into some coastal lagoons and estuaries of northwestern Mexico in the 70's, especially in the states of Baja California, Sonora, Sinaloa and Nayarit, (Martínez-Córdova and Robles, 1990). Nowadays, this species is the most cultivated mollusk in the region (CONAPESCA, 2012). During 2012, the annual production of the Pacific oyster in northwestern Mexico reached 2,128 t (valuated at 3 million dollars) (CONAPESCA, 2012). On the other hand, a commercial emergent shellfish resource, the black clam *Chione fluctifraga,* which coexists with cultured *C. gigas* in Sonora, has been locally exploited for human consumption (Cáceres-Martínez *et al*., 1999). The geographical distribution of this clam comprises from Southern California to the Gulf of California

Since 1997, the oyster industry has been severely affected by episodes of major mortality disturbing almost all farmed oyster sites along the Gulf of California. Several hypotheses, as water contamination, red tides, fluctuations in food abundance and presence of pathogens, have been proposed to explain these events. Recently, during a mass mortality event (>90%) of cultured specimens of the Pacific oyster *C. gigas* in Sonora, the protozoan parasite *Perkinsus marinus* was detected for the first time (Enríquez-Espinoza *et al*., 2010). Nowadays, abnormal mortalities are not recorded; however, the status of *Perkinsus* prevalence in these apparently healthy cultures remains unknown. The aim of this work was to assess the yearly prevalence and the infection level of *P. marinus* in cultured *C. gigas* and in wild *C. fluctifraga* in the coast of Sonora.

MATERIALS AND METHODS

Sampling

A monthly sampling was carried out during one year in a culture site at Estero La Cruz, Sonora, Mexico (28° 47' 34"

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N, 111° 53′ 27″ W). Each sampling consisted of 30 adult *C. gigas* (10.35 ± 1.82 cm length) and 30 *C. fluctifraga* (4.58 ± 1.39 cm length) per month during the period from December 2009 to November 2010. Oysters and clams were transported alive to the laboratory facilities at the University of Sonora for further analysis.

RFTM assay

To induce hypnospore formation, sections of gills, mantle, rectum and digestive gland were incubated in Ray´s fluid thyoglicolate medium (RFTM) with added antibiotics (500 U penicillin G, 500 mg dihydrostreptomycin and 200 U nystatin per mL of medium). Tissues were incubated for 4-7 days at 22-24°C in the dark. Tissue fragments were macerated with a scalpel on a slide and stained with Lugol´s solution. Then, they were covered with a cover slip and incubated at room temperature for 10 min. Slides were observed under a light microscope for hypnospore presence. The intensity of infection was measured according to the Mackin scale (Ray, 1954) modified by Craig *et al*. (1989), which is based on the number of hypnospores per slide. This method assigns a numerical value ranging from 0 to 5 where 0 = negative (not infected), 0.5 = very light (less than 10 parasites per preparation), 1 = light (11-100 parasites per preparation), 2 = light/moderate (over 100 parasites per preparation, some areas without parasites and others with 25-50 cells localized), 3 = moderate (over 100 parasites with localized concentrations of more than 50 cells and some areas without parasites), 4 = moderately heavy (over 100 cells but less than a half of the tissue is covered) and 5 = heavy infection (most of the tissue is covered by a large number of parasites). The prevalence was calculated through Thrusfield (1995) formula, as the percentage of organisms showing hypnospores from the total number of each sample.

PCR amplification

Tissues from gills, mantle and digestive gland of each mollusk were preserved in 95% ethanol for PCR analysis; no PCR analysis was done for RFTM cultures. DNA extraction was carried out using the QIAamp DNA Mini Kit according to the manufacturer´s instructions (QIAGEN, Hilden, Germany). PCR reactions of all 360 oysters and 360 clams were done with Ready-to-Go PCR beads (GE Healthcare, Little Chalfont, UK) using 30 ng of DNA and 10 pmol of each primer in a total volume of 25 μL. PCR reactions were performed using the primers Perk ITS-85: 5′-CCG-CTT-TGT-TTG-GAT-CCC-3′ (Casas *et al*., 2002) and PmarITS600R: 5′-CGA-GTT-TGC-GAG-TAC-CTC-KAG-AG-3′ (Audemard *et al*., 2004) to amplify a 475 bp region of the internal transcribed spacer (ITS) of the small subunit ribosomal RNA gene (SSU rRNA). PCR conditions were as those described previously (Enríquez-Espinoza *et al*., 2010), an initial denaturalization at 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final step of 72°C for 7 min. The obtained PCR products were electrophoresed in 2% agarose gels, dyed with ethidium bromide and visualized under a UV transilluminator.

DNA sequencing

The PCR amplicons were excised and purified using the Rapid Gel-Extraction System (Marligen Bioscience). The cycle sequencing was done in a Gene Amp PCR System 9700 (Applied Biosystems) using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). The sequences in both senses were obtained by capillary electrophoresis in an ABI Prism 3130 Genetic Analyzer. The obtained sequences were manually edited with ChromasPro v1.41. The identity of the obtained sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) at The National Center for Biotechnology Information (NCBI), and a multiple sequence alignment was performed with ClustalX (Thompson *et al*., 1997) against the *P. marinus* sequences available in GenBank.

RESULTS

The RFTM assay showed the presence of enlarged circular blue-black hypnospores characteristic of *Perkinsus* spp., along the sampled months, both in oysters as well as in clams (Fig. 1), with prevalence ranging from 3.3 to 60% (Fig. 2). Positive organisms to the RFTM assay showed a light infection according to the Mackin scale, with an exception of one oyster from the sampling on September 2010, which showed a moderate infection level. From the 360 oysters analyzed by PCR, only one oyster sampled in September, with a moderate infection level, showed an amplicon of the expected size (475 bp) (Fig. 3) confirming the presence of *P. marinus* in C. *gigas*. From the 360 clams, none was positive in the PCR analysis. The resulting sequence revealed 100% identity with the ITS region sequences of *P. marinus* previously reported in the Gulf of California (GenBank accession no. GQ861511 and JQ266240).

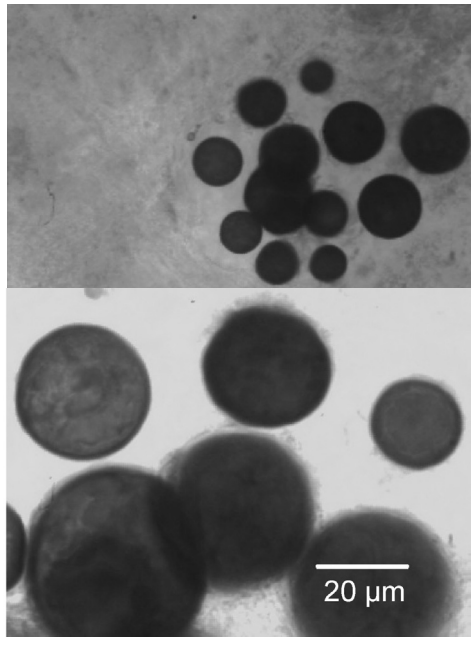

Figure 1. Hypnospores present in *Crassostrea gigas* tissues incubated in RFTM.

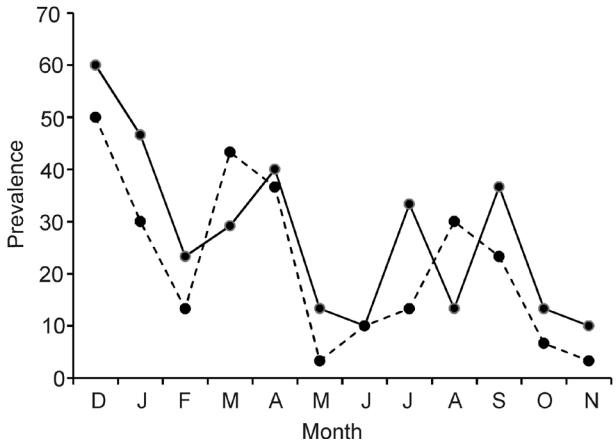

Figure 2. Prevalence percentage of hypnospores obtained from the RFTM assay during December 2009 to November 2010. Continuous line, *Crassostrea gigas*; interrupted line, *Chione fluctifraga*.

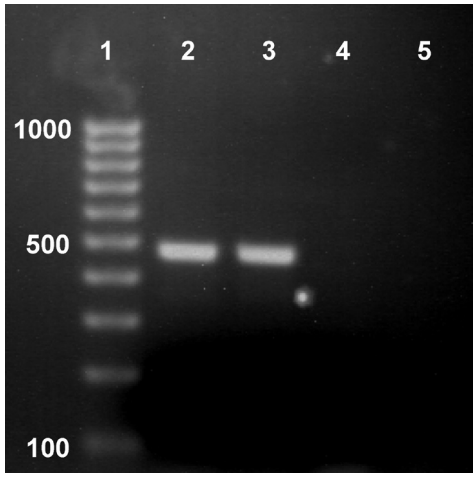

Figure 3. Agarose gel for PCR amplifications with PerkITS-85 and PmarITS-600R primers. Lane 1, 100 bp molecular standard; lane 2, amplicon obtained from alcohol-fixed tissue from oyster with moderate infection level according to the RFTM assay; lane 3, amplicon obtained from tissue incubated in RFTM; lane 4, oyster negative to RFTM assay; lane 5, negative control without DNA.

DISCUSSION

Despite there were no abnormal mortality events reported during the period of the present study, and all the bivalves analyzed did not present gross signs of illness, the oyster culture industry in Sonora had been facing a severe crisis by recurrent mortality outbreaks in the past years probably due to multiple factors (Enríquez- Espinoza *et al*., 2010).

The presence of round blue-blackish hypnospores, indicative of *Perkinsus* spp. by the RFTM assay, supports the presence of this protozoan in wild *C. fluctifraga* and in apparently healthy *C. gigas* cultured in Sonora in a low to moderate prevalence. In some cases, the RFTM assay resulted positive, but histology and PCR analysis yielded negative results, this may be due to a low intensity along with localized infections and low amount of pathogen DNA (Burreson, 2008; Sanil *et al*., 2012; Villanueva-Fonseca and Escobedo-Bonilla, 2013). For this reason, the OIE (World Organization for Animal Health) recommends the RFTM assay as the gold-standard since it is considered even more sensitive than histology (Audemard *et al*., 2008; OIE, 2003). These findings agree with our results, since only one oyster with moderate infection could be detected positive to *P. marinus* by PCR. Therefore, despite that the RFTM assay is presumptive, the use of PCR as confirmatory tests have demonstrated the presence of *Perkinsus marinus* in cultured oysters in Sonora.

PCR amplification of the ITS region and its DNA sequencing confirmed the identity of *Perkinsus*, having 100% identity with the previously reported sequence of *P. marinus* from *C. gigas* from the Gulf of California (GQ861511) by Enríquez-Espinoza *et al*. (2010) and the JQ266240 sequence obtained from *C. corteziensis* from Nayarit (Escobedo-Fregoso *et al*., 2015).

Despite the occurrence of *P. marinus* in the culture site, no abnormal mortalities or signs of illness were recorded. This can be due to several factors such as the low prevalence and the intensity levels. Besides, is has been demonstrated the low susceptibility of *C. gigas* to *P. marinus* (Calvo *et al*., 1999; La Peyre *et al*., 1995; Meyers *et al*., 1991). Moreover, virulence genotypes of *P. marinus* must be taken into account, as several genotypes of *P. marinus*, infecting *C. corteziensis* have been detected, which have high similarity to genotypes with low to moderate virulence (Escobedo-Fregoso *et al*., 2015). In the same oyster cultures of La Cruz, Grijalva-Chon *et al.* (2013) reported a new oyster herpesvirus type 1 (OsHV-1) genotype in healthy *C. gigas*, which emphazises the importance of surveillance programs to beware of outbreaks triggered by the complex relationship between the environment, the susceptibility of oysters and the presence of pathogens.

Although the presence of *P. marinus* has been well documented from the Eastern coast of the USA until the Gulf of Mexico (Villalba *et al*., 2004), in recent years, *P. marinus* has been detected in oysters in the Pacific coast of Mexico (*C. corteziensis)* (Cáceres-Martínez *et al*., 2008). Later, Enríquez-Espinoza *et al.* (2010) reported *P. marinus* in farmed *C. gigas* from the Gulf of California. Further, *P. marinus* was first detected in the oyster *Saccostrea palmula* also in the Pacific coast of Mexico (Cáceres-Martínez *et al*., 2012), and Villanueva-Fonseca and Escobedo-Bonilla (2013) recorded *Perkinsus* sp. in cultured *C. gigas* from Sinaloa, with a low prevalence. The spread of *P. marinus* on its distribution and host range is a major concern for the oyster farmers, since *P. marinus* could infect other populations of wild mollusks.

CONCLUSION

Although *P. marinus* is present in a low prevalence and light infection levels in healthy *C. gigas* in Sonora, a surveillance program is highly important to follow the sanitary situation in the culture sites, since environmental or physiological factors could trigger mortalities. Even when *C. gigas* has low susceptibility to *P. marinus*, and no apparent disease is registered at the moment, sanitary measures to control movement of bivalves from some culture sites to other should be enforced, since other mollusk species could

be seriously affected with the dispersion of this parasite.

To our knowledge, this is the first indication of *Perkisnsus* sp. in the clam *C. fluctifraga* by RFTM. Nevertheless, additional tests should be carried out to confirm the presence and/or an actual infection of this parasite on this species, such as histology, *in situ* hybridization or other molecular tools. It is important to know the role of this mollusk as host or reservoir in the spread of *Perkinsus* sp.

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