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NOTE

Perkinsus marinus in *Crassostrea gigas* in the Gulf of California

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ABSTRACT: To determine the agent responsible for the massive mortalities of the Pacific oyster *Crassostrea gigas* in northwest Mexico, 30 oysters were sampled after a severe mortality event in 2006 along the Sonoran coast. Histological analyses revealed the presence of a protozoan and Ray's fluid thioglycollate medium (RFTM) assays showed the presence of *Perkinsus* sp., identified as *P. marinus* from the DNA sequence of the internal transcribed spacer (ITS) of the ribosomal RNA (rRNA) gene complex. PCR analyses for *Marteilia refringens*, *M. sydneyi*, and *Haplosporidium costale* were negative. *P. marinus* presence in the Pacific oyster may be responsible for massive mortalities of the oyster, along with other environmental factors in the Gulf of California.

KEY WORDS: *Perkinsus marinus* · Pacific oyster · *Crassostrea gigas* · Aquaculture · Gulf of California · Ray's fluid thioglycollate medium · Ribosomal DNA

INTRODUCTION

The Pacific oyster *Crassostrea gigas* is a species widely cultured around the world, but sometimes mass mortalities jeopardize the farming activity; there are several pathogen species that threaten the organisms' health and that may be partially responsible for summer mortalities, together with environmental and reproductive factors (Samain et al. 2007).

In Mexico the culturing of *Crassostrea gigas* using several culture methods began in the 1970s along the Baja California Peninsula coast, with further success along the Mexican Pacific coast, including the Gulf of California (Gallo-García et al. 2001, Vásquez-Yeomans et al. 2004). The cultures were successful for >10 yr, but by the end of the 1990s the cultures started to decline because of severe mortality episodes affecting all size-classes, with Sonora and Baja California Sur being the more affected states (Cáceres-Martínez & Vásquez-Yeomans 2003). At present oyster culture activity has stagnated due to poor investment, lack of incentives, and unknown causes of mortality. To explain the mortalities, multiple hypotheses have been expressed by farmers: El Niño, contamination, red tides, fluctuations in food abundance, poor genetic variability, and unknown pathogens.

In 2006 multidisciplinary research began to investigate the mortality in *Crassostrea gigas* cultures along the Sonoran coast in the Gulf of California. A pathological research approach was used to explore the possible presence of protozoan parasites because several species have been associated with massive mortalities in mollusc cultures around the world (Berthe et al. 2004, Burreson & Ford 2004, Villalba et al. 2004, Audemard et al. 2008). The present study confirms the presence of *Perkinsus marinus* in *C. gigas* as a probable contributor to the massive mortalities in the farmed oysters of the Gulf of California.

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MATERIALS AND METHODS

From July to August 2006 severe mortality devastated the oyster cultures along the Sonoran coast. In September 2006, 30 live oysters were taken from 3 culture sites (10 oysters each) along the Sonoran coast at Estero Santa Barbara, Estero La Cruz, and Estero Morua with shell lengths between 7 and 10 cm (Table 1). The oysters were transported to our laboratory for histopathological analyses using standard hematoxilin-eosin staining to look for lesions in the intestine, gonad, gills, and mantle according to OIE (2009), Bondad-Reantaso et al. (2001), and Bower et al. (1994). At the same time, Ray's fluid thioglycollate medium (RFTM) assay (OIE 2009) was done on all oysters using small tissue sections of the abductor muscle, intestine, and digestive gland, which were stained with Lugol's iodine on a glass slide and observed microscopically. This test is considered presumptive for *Perkinsus* spp., although some dinoflagellate species and a *Perkinsus* spp.-like organism can give positive results with RFTM (Novoa et al. 2002). Remaining tissues were frozen at –70°C.

DNA extraction was done of all the RFTM cultures and all frozen tissue using the QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN). PCR was done using the *Perkinsus* spp.-specific internal transcribed spacer (ITS) region primers PerkITS-85 (5′-CCG CTT TGT TTG GAT CCC-3′) and PerkITS-750 (5′-ACA TCA GGC CTT CTA ATG ATG-3′) (Casas et al. 2002, Audemard et al. 2004). These primers amplify DNA for all *Perkinsus* spp. with the exception of *P. qugwadi*. PCR reactions were done with Ready-to-Go PCR beads (GE Healthcare) with 30 ng DNA and 0.01 µg of each primer in a total volume of 25 µl. Because PCR conditions recommended by Casas et al. (2002) and Audemard et al. (2004) generated weak and inconsistent DNA bands, PCR assays were done by looking for the best PCR conditions, which were 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. Consistency was checked with 5 replicates of the PCR analysis.

PCR products of the PerkITS-85 and PerkITS-750 primers were visualized on 2% agarose gels stained with ethidium bromide. The DNA bands were purified using the Rapid Gel-Extraction System (Marligen Bioscience). To corroborate the species identity, the primer PmarITS-600R (5′-CGA GTT TGC GAG TAC CTC KAG AG-3′) (Audemard et al. 2004), specific to *Perkinsus marinus*, was used with primer PerkITS-85 in PCR reactions with 60 ng of purified amplicon generated by the primers PerkITS-85 and PerkITS-750. For this PCR reaction, Ready-to-Go PCR beads were used with the same PCR conditions detailed in the previous paragraph. All reactions included a negative control without DNA. PCR products from RFTM cultures were sequenced by Macrogen.

The sequences obtained were analyzed using the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI), USA. A multiple sequence alignment was also done using ClustalX (Thompson et al. 1997) with some *Perkinsus* spp. sequences reported in GenBank.

Additionally, nested PCR reactions were done using frozen tissue to look for *Marteilia refringens* using primers MT-1 (5′-GCC AAA GAC ACG CCT CTA C-3′) and MT-2 (5′-AGC CTT GAT CAC ACG CTT T-3′) for a first reaction, and MT-1B (5′-CGC CAC TAC GAC CGT AGC CT-3′) and MT-2B (5′-CGA TCG AGT AAG TGC ATG CA-3′) for the nested reaction, according to procedures described in López-Flores et al. (2004). *M. sydneyi* was screened with primers PRO2 (5′-TCA AGG GAC ATC CAA CGG TC-3′) and LEG1 (5′-CGA TCT GTG TAG TCG GAT TCC GA-3′) according to Kleeman & Adlard (2000). For *Haplosporidium costale*, the primers SSO-A (5′-CAC GAC TTT GGC AGT TAG TTT TG-3′) and SSO-B (5′-CGA ACA AGC GCT AGC AGT ACA T-3′) were used according to OIE (2003). All PCR products were visualized on 2% agarose gels.

RESULTS

The observation of histological sections showed alterations in the tissue structure in various organs, such as the intestinal epithelium, gills, digestive gland, and connective tissue. In the intestinal epithelia, the arrangement of the columnar cells showed severe changes, accompanied by an eosinophilic reaction, presumably indicating the presence of parasites in the tissue. Also, gill lesions showed severe vacuolization, necrosis, and the invasive presence of unidentified rosette-like parasites. In the digestive gland, tubules were also seen with the same types of parasites with alteration of the epithelium, probably as a result of this

Table 1. *Crassostrea gigas*. Sampling locations, number, and size (mean ±SD of *C. gigas* analyzed for the presence of *Perkinsus marinus*

Sampling location	Geographical coordinates	No. of oysters	Shell length (cm)
Estero Santa Barbara	26° 41′ 39″ N, 109° 39′ 80″ W	10	9.25 ± 0.92
Estero La Cruz	28° 47′ 34″ N, 111° 53′ 27″ W	10	7.55 ± 0.86
Estero Morua	31° 17′ 19″ N, 113° 26′ 64″ W	10	8.35 ± 0.58

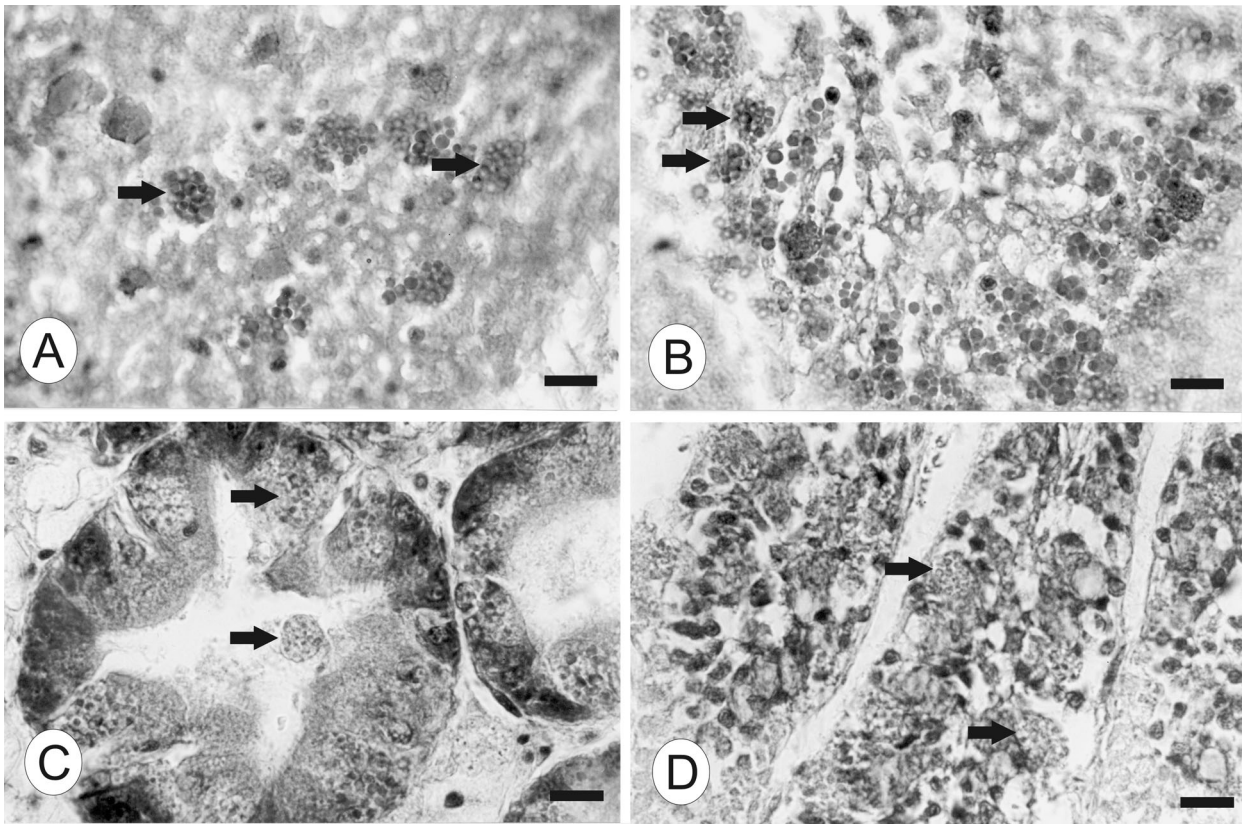

Fig. 1. *Crassostrea gigas*. Histological tissue sections depicting severe damage caused by the invasive presence of schizonts (black arrows) in the (A,B) gut, (C) digestive gland, and (D) gills. Scale bar = 15 µm

invasion (Fig. 1). These histological lesions coincided with the ones reported for *Perkinsus marinus* infection of *Crassostrea virginica* (Burreson et al. 1994), where rosettes were seen in the digestive epithelia, similar to those found in *C. gigas* of Sonora. The tissues analyzed by the RFTM assay showed dark blue spherical bodies, which were hypnospores characteristic for *Perkinsus* spp. (Fig. 2). There was 100% prevalence of infection for samples from the 3 locations, with infection intensities ranging from 3558 ± 3197 to 165 112 ± 155 404 hypnospores g^{-1} tissue (Table 2).

PCR analyses for *Marteilia refringens*, *M. sydneyi*, and *Haplosporidium costale* were negative. Only the samples from Estero La Cruz were positive with both combinations of PerkITS-85 plus PerkITS-750 and PerkITS-85 plus PmarITS600R primers, which consistently amplified bands of 666 and 475 bp (Fig. 3) from RFTM tissue cultures. By the time we had determined what the best PCR conditions were, the fresh frozen tissue samples were lost and no PCR analyses were possible. Two sequences were obtained; the one obtained with primers PerkITS-85 and PmarITS-600R confirmed the *Perkinsus marinus* identity and matched the sequence obtained with primers PerkITS-85 and PerkITS-750 (GenBank accession no. GQ861511). The entire sequence of 666 bp that included the primer recognition sequences was analyzed by BLAST in GenBank and showed 98 to 100% identity with the ITS region of 58 *P. marinus* entries, having 100% identity with 2 of them (GenBank accession nos. AY295199 and AY295198), 93 to 94% identity with 23 *P. olseni* entries,

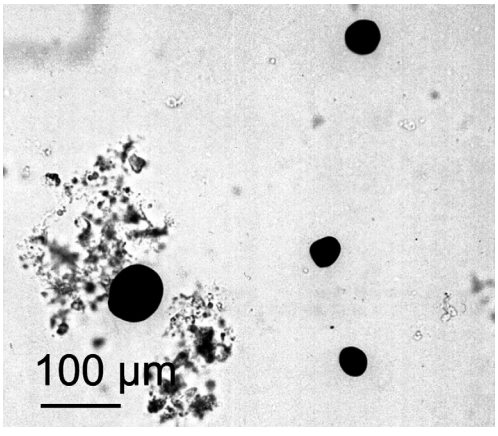

Fig. 2. *Perkinsus marinus* from *Crassostrea gigas*. *P. marinus* hypnospores from Ray's fluid thioglycollate medium culture and stained with Lugol's iodine, obtained from the gut of *C. gigas*

Table 2. *Perkinsus marinus* in *Crassostrea gigas*. Prevalence of infection, quantification, and PCR analysis. RFTM: Ray's fluid thioglycollate medium

Sampling location	Histology prevalence (%)	RFTM prevalence (%)	Hypnospores g^{-1} tissue (mean ± SD)	PCR from RFTM tissue
Estero Santa Barbara	100	100	3558 ± 3197	Negative
Estero La Cruz	100	100	165 112 ± 155 404	Positive
Estero Morua	100	100	6220 ± 4609	Negative

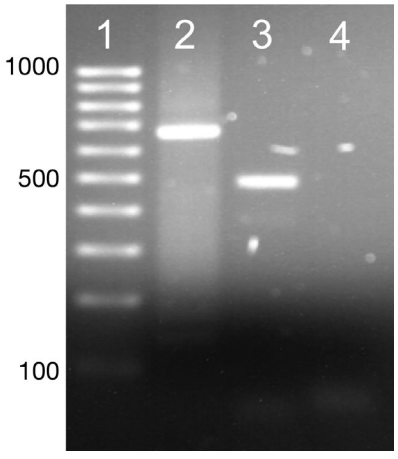

Fig. 3. Agarose gel of PCR amplicons obtained from Ray's fluid thioglycollate medium cultures. Lane 1: 100 bp molecular standard. Lane 2: amplicon with primers PerkITS-85 and PerkITS-750. Lane 3: amplicon with primers PerkITS-85 and PmarITS-600R. Lane 4: negative control

94% identity with 13 *P. atlanticus* entries, 92 to 93% identity with 3 *P. honshuensis* entries, and had coverage of 93 to 100% overall.

DISCUSSION

The massive mortality in *Crassostrea gigas* cultures along the Pacific coast of Mexico, mostly from October to November and March to May, has severely threatened the existence of >30 oyster farms. In our search for a pathogen involved in those mortalities, our histological analyses suggested the presence of a protozoan of an unknown species. The RFTM analysis, a routine and standard method to detect the presence of *Perkinsus* spp. in mollusc species (Burreson 2000, Coss et al. 2001, Reece & Dungan 2005), was positive in our samples. The discrepancy between positive RFTM assay and negative PCR results has been explained thus: it can occur because of low infection levels, with <1 parasite 20 mg^{-1} tissue, and because of the patchy distribution of parasites in host tissues (Renault 2008). Our observed discrepancies in RFTM and PCR results at Estero Santa Barbara and Estero Morua can be explained by the infection level: at Estero La Cruz the infection level was 44-fold and 25-fold greater than that at Estero Santa Barbara and Estero Morua, respectively.

The true *Perkinsus* species identity was confirmed with the DNA sequence of the ITS of the ribosomal RNA (rRNA) gene complex. ITS sequences are preferred over non-transcribed spacer (NTS) sequences because of their ability to detect more species (Renault 2008, OIE 2009). The distribution of *P. marinus* comprises the East and Gulf coasts of the USA (Villalba et al. 2004); however, its presence along the Pacific coast of Mexico was recently reported in the pleasure oyster *Crassostrea corteziensis* in Nayarit by Cáceres-Martínez et al. (2008). The origin of this parasite on the Mexican Pacific coast is unknown but the movement of oysters in and out of the Gulf of California and acquisition of infected spat from abroad may contribute to the spread of the infection. The presence of *P. marinus* in *C. gigas* in the Gulf of California implies that the wild mollusc species in the area could also be infected. If this is true, the eradication of *P. marinus* in *C. gigas* cultures is an impossible task and oyster farmers only have the option to follow good culture practices to minimize the impact of the illness.

Mollusc species have different responses to a pathogenic protozoan infection, and environmental factors such as temperature and salinity are involved in the infective process (Burreson et al. 1994, Chu 1996, La Peyre et al. 2006). Perkinsosis is an illness associated with warm waters (La Peyre et al. 2006), which is a characteristic of the Gulf of California, where surface water temperatures on oyster farms reaches 30 to 32°C in summer. However, because of the wide range of salinities and temperatures that can be found along the >1000 km long Gulf of California, it is necessary to determine which areas have a greater risk for *Perkinsus marinus* infections.

The massive mortality events in the Gulf of California could be generated by a complex environment-host-parasite interaction, so that the contribution of *Perkinsus marinus* in those events should be reviewed in the light of the possible presence of other parasitic species and the wide environmental scenarios along the Gulf of California associated with its geographic

characteristics and seasonal variability. Continuous monitoring of cultured oyster and wild clam species is necessary to determine the prevalence of the pathogen and the infection level following OIE (2009) recommendations.

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