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Research Article

OsHV-1 and notifiable protozoa in healthy *Crassostrea corteziensis* cultured in two distant areas of the Gulf of California

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ABSTRACT. Infectious diseases have been a major limiting factor for large scale production of oyster farming. Several factors have contributed to the emergence and spread of infectious diseases in all cultivation sites around the world. Therefore, the prevention and control of diseases has become a priority for the sustainability of global aquaculture. *Crassostrea corteziensis* is a native species that contributes to the Mexican Pacific oyster production and used in this study to assess seasonal simultaneous infective events between distant areas in the Gulf of California. The results of the molecular analysis showed a higher prevalence of *Perkinsus marinus* in the north area and *Marteilia refringens* in the south. OsHV-1 was only present in summer and autumn with low prevalence in the two areas. The histological analysis of the PCR-positive organisms presented alterations characteristic of infections. The presence of *M. refringens* in a new location on the Gulf of California suggests that this pathogen is already well established in the area and the dual presence of pathogens in *C. corteziensis* is reported for the first time.

Keywords: *Perkinsus marinus*, *Marteilia refringens*, *Crassostrea corteziensis*, OsHV-1, Gulf of California.

INTRODUCTION

Most of the worldwide oyster production is based on *Crassostrea gigas* since it has been successfully introduced to many countries, including Mexico (Chávez-Villalba, 2014). However, given the diversity of the genus *Crassostrea*, there are several species that are native to different places and with a certain degree of fishery production, aquaculture development or catch for local consumption. This is the case of *Crassostrea corteziensis* in the Pacific of the American Continent. Therefore, the global concerns about the pathological conditions of *C. gigas* are extended to other bivalve mollusks that are subject to some kind of exploitation in Mexico like the penshell *Atrina maura*, the mussel *Mytilus galloprovincialis*, the Catarina scallop *Argopecten ventricosus* and the pearl oyster *Pteria sterna* (Maeda-Martínez, 2008).

Moreover, the Gulf of California is a dynamic and highly structured system that has been subject of extensive oceanographic studies from physical, chemical, geological and biological points of view. The complexity of this marginal sea is such that 14 proposals have been made for regionalization (Petatán-Ramírez, 2015), and its rich primary and secondary productivity sustains important fishery resources and a strong aquaculture activity (Hidalgo-González & Álvarez-Borrego, 2001; Chávez-Villalba, 2014).

The bivalve aquaculture in Mexico is conducted almost exclusively on the Pacific coast of Baja California and the Gulf of California, and ranks fourth in Latin America after Chile, Brazil and Peru (Maeda-Martínez, 2008). While the culture of *C. gigas*, introduced to the country in the early 70's, depends on spat production in hatcheries and well standardized culture methods, the culture of *C. corteziensis* is carried

out at a more artisanal level and depends on the uptake of spat from the natural environment and in some few cases from hatcheries (Islas-Olivares *et al.*, 1978; Cáceres-Martínez *et al.*, 2010). However, like other bivalves, the high mortality in *C. corteziensis* during the larval and juvenile stage, is the main problem that limits the activity (Campa-Córdova *et al.*, 2011).

Among the possible causes of the massive mortalities is the emergence of infectious diseases due to a variety of pathogens, including protozoa parasites, bacteria and viruses (Vigneron *et al.*, 2004; Grijalva-Chon & Castro-Longoria, 2015) that have been extensively studied in some countries and listed by the World Organization for Animal Health (OIE, 2015). In the case of Latin America, some of them have already detected; however, very little of its effect on production is known (Cáceres-Martínez & Vásquez-Yeomans, 2008; Enríquez-Espinoza *et al.*, 2010; Grijalva-Chon *et al.*, 2015). In 1997, oyster massive mortalities were recorded for the first time in Mexico in the states of Sonora, Baja California and Baja California Sur in juvenile and early adult oyster (Maeda-Martínez, 2008) which caused production to behave erratically in subsequent years. Moreover, in the Gulf of California it has been detected ostreid herpesvirus type 1 or OsHV-1 (Vásquez-Yeomans *et al.*, 2004, 2010; Grijalva-Chon *et al.*, 2013), *Perkinsus marinus* (Cáceres-Martínez *et al.*, 2008, 2012; Enríquez-Espinoza *et al.*, 2010, 2015; Villanueva-Fonseca & Escobedo-Bonilla, 2013, Escobedo-Fregoso *et al.*, 2015) and recently *Marteilia refringens* (Grijalva-Chon *et al.*, 2015).

The purpose of this study was to determine whether there is a temporary coupling of infectious events in distant cultures of *C. corteziensis* in the Gulf of California considering seasonal variation, presence and prevalence of *P. marinus*, *M. refringens* and OsHV-1.

MATERIALS AND METHODS

Oyster culture and sampling

In December, 2012, a lot of pathogen-free spat of *C. corteziensis* was obtained from the Northwest Center for Biological Research at La Paz, Baja California Sur, Mexico. Upon arriving at our laboratory, this lot was also analyzed for *P. marinus*, *M. refringens* and OsHV-1 as explained below. From this lot, two groups of 1000 spats each were formed to start cultures in Playa Manga, Sonora (27°58'40"N, 111°58'40"W) and Boca de Camichín, Nayarit (21°44'36"N, 105°29'30"W), hereinafter referred to as north and south areas respectively, and a distance between them of 1,220 km (Fig. 1). The spat lots were placed in mosquito net bags and then in suspended Nestier oyster boxes on sites that

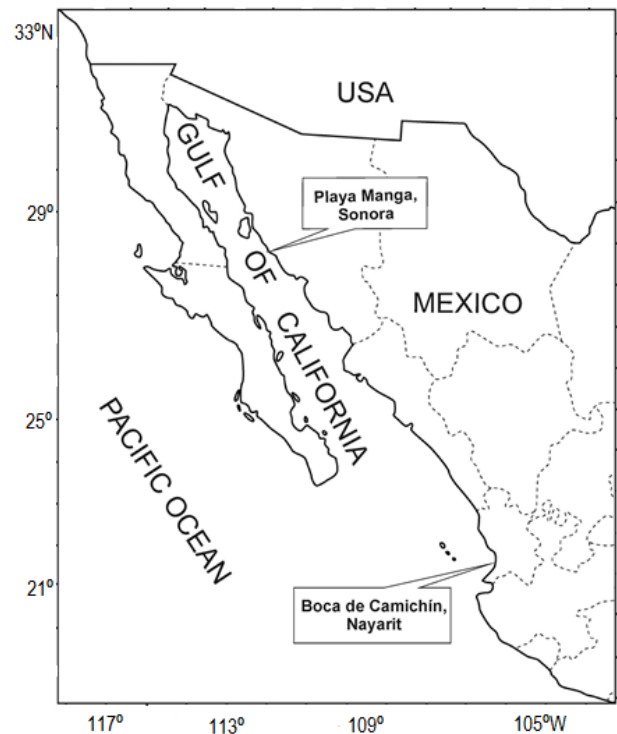


Figure 1. Location of the *Crassostrea corteziensis* culture sites in the states of Sonora and Nayarit, Mexico.

each oyster farm has for oyster cultures. There was help of oyster farmers for their necessary maintenance in both culture areas.

The culture lasted 15 months and 30 oysters per sample were taken in four sampling dates. The first sampling was in April 2013, the second one in June, the third one in October and the fourth one in March 2014. Data on surface temperature were also collected. The oysters were transported alive to the laboratory and shell length was recorded. Tissue samples from each oyster were taken and preserved in absolute ethanol for molecular analysis and Davidson's solution for histological analysis.

Pathogen diagnostics

DNA was extracted from the samples using 50 mg of tissue from a mixture of portions of mantle and gills. In the case of the initial spat, whole organisms were used, grouped into three pools of 20 individuals each. The DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN) following the manufacturer's instructions.

To detect OsHV-1, oligos C2: 5'-CTCTTTACCAT GAAGATACCCACC-3' and C6: 5'-GTGCACGGC TTACCATTTT-3' were used to amplify a region of 709 base pairs (bp) of the C region in the OsHV-1 reference genome, that codes for two proteins of unknown function (Arzul *et al.*, 2001). These oligos

also recognize different genotypic variants with sizes of amplicons from 529 bp to 723 bp (Arzul *et al.*, 2001; Segarra *et al.*, 2010; Martenot *et al.*, 2011, 2012; Grijalva-Chon *et al.*, 2013). PCR reactions were performed in volumes of 25 µL using Ready-to-Go PCR beads (GE Healthcare) with 100 ng of each oligo, 80 ng of DNA extract and PCR grade water. Thermocycler conditions were as recommended by Renault *et al.* (2000) and Renault & Novoa (2004) and Vigneron *et al.* (2004) and consisting of an initial denaturation at 94°C for two minutes, followed by 35 cycles of 94°C for a min, 50°C for 1 min and 72°C for 1 min. The final step was 72°C for 5 min.

In the case of *Perkinsus* sp., the oligos PerkITS-85: 5'-CCGCTTTGTTTGGATCCC-3' and PerkITS-750: 5'-ACATCAGGCCTTCTAATGATG-3' were used (Casas *et al.*, 2002; Audemard *et al.*, 2004) to amplify 666 bp of the internal transcribed spacer of the ribosomal complex genes of all species of *Perkinsus*, except *Perkinsus qugwadi*. PCR reactions were in 25 µL using Ready-to-Go PCR beads with 30 ng of DNA, 10 ng of each oligo, and PCR grade water. Thermocycler conditions were reported by Enríquez-Espinoza *et al.* (2010) and consisted of an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

To detect *M. refringens*, a nested PCR was performed with the MT-1: 5'-GCCAAAGACAC GCCTCTAC-3' and MT-2: 5'-AGCCTTGATCACA CGCTTT-3' oligos (López-Flores *et al.*, 2004) to amplify a 525 bp fragment. The reactions were performed with Ready-to-Go PCR beads with 100 ng of DNA, 25 ng of each primer and PCR grade water in 12.5 µL volume. The thermocycler conditions were: 94°C for 5 min, 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min. The final extension was 72°C for 10 min. The nested reaction was made with MT-1B: 5'-CGCCACTACGACCGTAGCCT-3' and MT-2B: 5'-CGATCGAGTAAGTGCATGCA-3' oligos, with 1 µL of the first PCR reaction, 0.025 µg of each nested oligos, and PCR grade water in a 12.5 µL volume to amplify a 358 bp fragment. PCR conditions for nested PCR are the same as those used for the MT-1/MT-2 oligos. A negative control without DNA was included in all PCR runs.

All PCR products were visualized in 2% agarose gels, stained with Ethidium Bromide and digitally documented with a DNR MiniBis Pro system. To verify the identity of the PCR products described above, DNA sequences in both directions were obtained (Macrogen, Inc., South Korea) of some randomly selected amplicons. The sequences were edited using ChromasPro to remove ambiguities, and the identity was verified using

the basic local-alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI), USA.

Histology

The samples fixed in Davidson's solution were processed to obtain 5 µm sections and stained with the standard hematoxylin-eosin procedure to look for tissue changes or evidence of parasites (Bower *et al.*, 1994; Bondad-Reantaso *et al.*, 2001; OIE, 2015).

RESULTS

The surface temperature in the northern region showed a marked seasonality; from April to August 2013 the temperature was rising from 22.5°C to 32°C. Subsequently, the temperature dropped to 18.5°C in January 2014. Moreover, in the south, the temperature data were available from June 2013 where the maximum temperature was 28.9°C and the minimum value was in March 2014 with 23.7°C, so in this area the temperature was less fluctuating and warmer than in the north (Fig. 2).

The growth of organisms had differences between the study areas. In the first three sampling dates, cultured organisms in the south had greater size than the north, which was reversed in the last sampling (Fig. 3). Moreover, when comparing growth within each zone, in the north there was no significant difference in the sizes reached in the first two sampling dates but showed a large increase in size by the end of the study. This growth pattern was different in the south where a significant increase in size occurred in the first two samples then remained stable until the end.

PCR analyzes detected the expected amplicons for the three pathogens in several oysters of the two locations. Three amplicons obtained with MT-1B/MT-2B oligos were sequenced and found to be identical to each other; they showed an identity of 98 to 100% with the sequences reported for *M. refringens* by Grijalva-Chon *et al.* (2015) for the Gulf of California and 100% with AM292652 and AJ629353 sequences reported in GenBank for Spain. Five amplicons obtained with PerkITS85/PerkITS750 oligos were sequenced, four of which had 100% identity with GQ851511 sequence of *P. marinus* reported by Enríquez-Espinoza *et al.* (2010) for the Gulf of California. The fifth sequence had an identity of 99% with GQ861511 but had 100% identity with seven sequences reported in Genbank for *P. marinus*. Therefore, none of these sequences represented novel genotypes for both pathogens. Sequencing

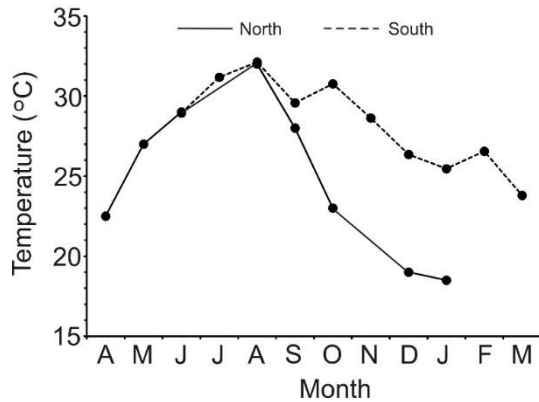


Figure 2. Monthly variation in surface water temperature (point values) in two culture areas of *Crassostrea corteziensis* in the Gulf of California.

of the amplicons obtained with C2/C6 oligos was unsuccessful probably due to denaturation.

According to the PCR results, there were large variations in prevalence. In the first sample, the north area did not show any pathogen while in the south most oysters (83.3%) showed no pathogens (Fig. 4). From there, the frequency of healthy organisms diminished to practically be absent by the end of the study.

M. refringens and *P. marinus* had a higher prevalence in the north area. The highest prevalence of *M. refringens* was in the third sampling where it was present in 46.7% of the organisms and even at the end of the study it had a significant prevalence (40%). In the case of *P. marinus*, the prevalence presented two major pulses, the first in the second sampling, where it was present in 40.7% of the organisms, and the second and most important one was by the end of the study, present in 96.7% of organisms. In this northern area, the prevalence of OsHV-1 was not important because the highest prevalence was 7.4%.

In the case of the southern area, *M. refringens* did not reach the same levels of prevalence than in the north, since while in the north the highest prevalence was in the third sampling, in the south the minimum value was at that time, with only 3.3% of infected organisms. In this area, the highest prevalence was presented by the end of the study with 30% of infected organisms, and an important observation is that this pathogen was present from the first sampling. Regarding *P. marinus*, it presented prevalence values that were increasing throughout the study. This pathogen was present from the first sample with low values, but by the second sampling, the prevalence had reached 56.7% and ended up with 96.7% of infected organisms in the last sampling. With regard to OsHV-1, as in the northern area, it also ranked third in importance and was present only in the second and third

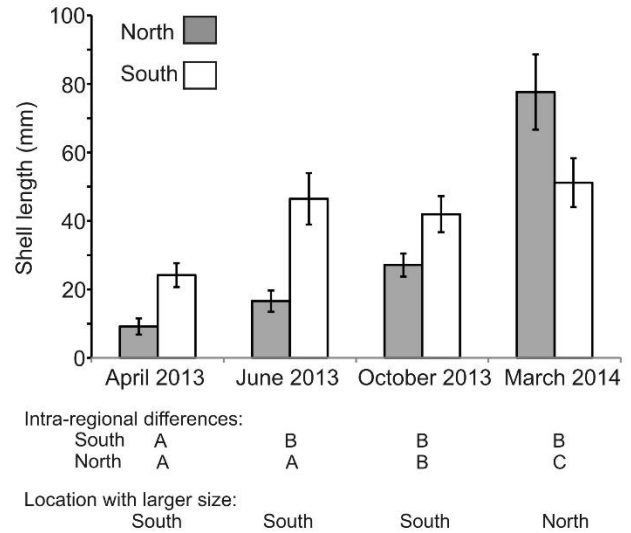


Figure 3. Size of *Crassostrea corteziensis* grown in two areas of the Gulf of California. Same letter in the sampling comparison within each area indicate no significant differences.

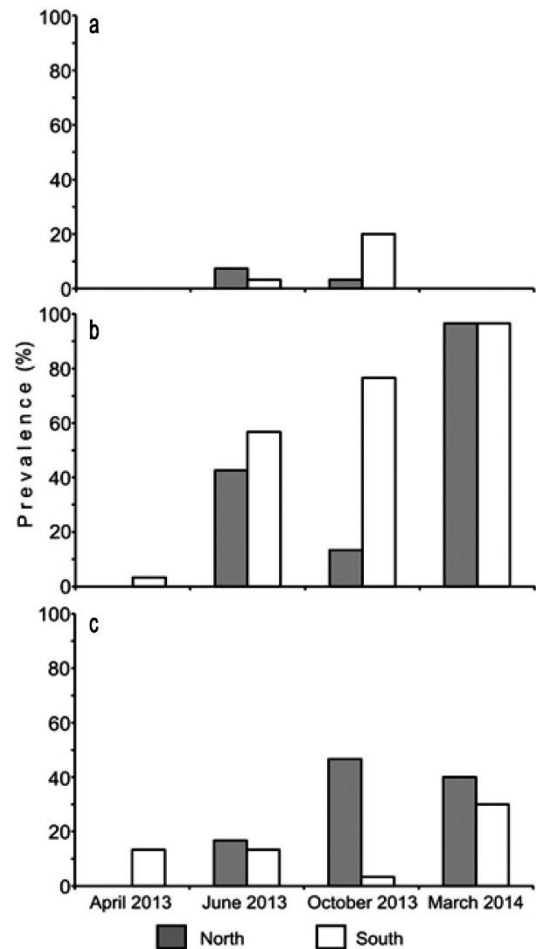


Figure 4. Pathogen prevalence in *Crassostrea corteziensis* grown in two areas of the Gulf of California. a) OsHV-1; b) *Perkinsus marinus*; c) *Marteilia refringens*.

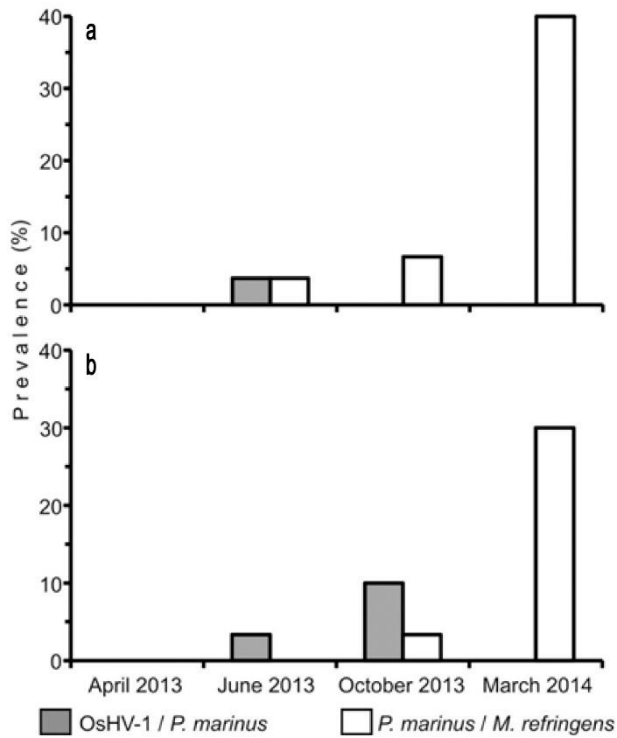


Figure 5. Prevalence of dual pathogen presence in *Crassostrea corteziensis* grown in two areas of the Gulf of California. a) North, b) South.

sampling, although here it had a greater presence in the third sampling, detected in 20% of organisms.

One aspect that is evident from the above descriptions is that there were oysters with two kinds of pathogens starting from the second sampling date (Fig. 5). In the north, the dual infection OsHV-1/*P. marinus* was detected in the second sampling with a prevalence of 3.7%. However, the combination *P. marinus*/*M. refringens* was present from the second sampling and its prevalence was increased to 40% by the end of the study. Moreover, in the case of the south area, the dual infection OsHV-1/*P. marinus* was present at the second sampling with 3.3% prevalence and in the third sampling with 10%. Also in this sampling, dual infection *P. marinus*/*M. refringens* was present with a low prevalence of 3.3% which increased to 30% in the last sampling. In neither of the two study areas, was the dual presence OsHV-1/*M. refringens* found. At the end of the study, the highest prevalence of *P. marinus*/*M. refringens* was present in both places. Eighty percent of the histological sections of samples that were PCR-positive to *M. refringens* and *P. marinus* presented evidence of their presence but not both in the same oyster (Fig. 6a), and the oysters infected with OsHV-1 presented the typical gill erosion associated to this virus (Figs. 6b-6c).

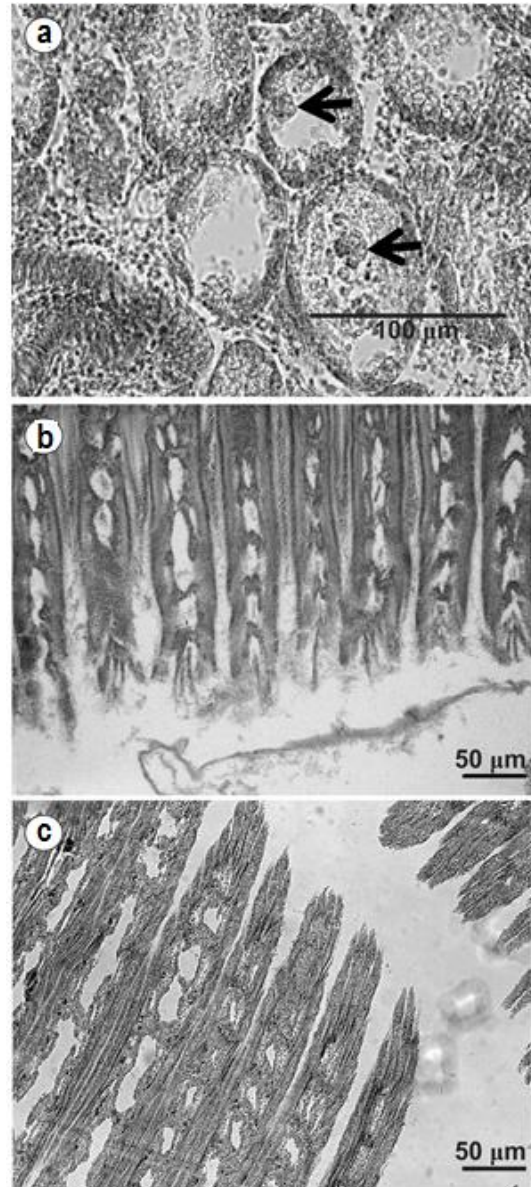


Figure 6. Histological tissue sections of *Crassostrea corteziensis* depicting a) developing spores in digestive gland in a PCR-positive to *Marteilia refringens*, b) gill damaged in a PCR-positive to OsHV-1, c) gill of a healthy oyster.

DISCUSSION

The growth of the organisms was notoriously different between the two culture localities, which can be coupled to the different physical, chemical and biological conditions of each area. Unfortunately, we do not have estimates of available food for oysters, but the temperature may be a causal part of the differences found because the low growth achieved in the south, where a smaller range of temperature fluctuation was present, was evident. This contrasted sharply with the

one obtained in the north area. Hidalgo-González & Álvarez-Borrego (2001) analyzed the seasonal differences in phytoplankton biomass between the north and south areas of the Gulf of California and found that there is a general trend of increased productivity in the north compared to the south. The high temperature to which the oysters are exposed in the Gulf of California not only influences the growth rate but also triggers defense mechanisms against extreme temperatures typical of this area, as demonstrated by Valenzuela-Castillo *et al.* (2015).

Recently, the presence of *M. refringens* in *C. gigas* and *C. corteziensis* cultivated in Bahía de Kino, Sonora, and Bahía de La Paz, Baja California Sur has been reported (Grijalva-Chon *et al.*, 2015), furthermore our results in Nayarit show that this pathogen is already well established in the Gulf of California. This pathogen causes large mortalities in *Ostrea edulis* in Europe and some studies have shown that the temperature of 17°C directly influences the proliferation and transmission of the protozoan, followed by the outbreak of the disease (Carrasco *et al.*, 2015). In our samples, the highest prevalence of *M. refringens* occurred in the northern area, where the temperature reached lower levels although in the south it was present throughout the year. Nevertheless, it is important to note that, as in the study of Grijalva-Chon *et al.* (2015), the oysters were from cultures without mortality or evident problematic condition, implying that both oyster species may be asymptomatic carriers of this protozoan. The *M. refringens* sequences obtained from the PCR amplicons are not a new genotype because they have a 100% identity with the one reported by Grijalva-Chon *et al.* (2015) from *C. corteziensis* (JQ066725), which in turn has a 100% identity with the European sequence AM292652.

The first relevant pathogen detected in shellfish farming of Northwest Mexico was OsHV-1 (Vásquez-Yeomans *et al.*, 2004, 2010), which had been responsible for mortalities in cultures of California since the early years of the nineties decade in the past century (Friedman *et al.*, 2005). In Europe, it has been reported that favorable temperatures for infection are 16-24°C (EFSA AHAW Panel, 2015). However, given the particular conditions of the Gulf of California, the presence of OsHV-1 was detected when the water temperature was above 23°C and up to 30°C, during summer-autumn.

OsHV-1 primarily affects the oysters in their larval and juvenile stage (Arzul *et al.*, 2002), so mortality events are often associated with the early stages of cultivation. Nonetheless, in larger organisms, changes in the structure of the gills have been reported (Vásquez-Yeomans *et al.*, 2010) and confirmed in the

present study. Recently, a new highly virulent variant (OsHV-1 μ var) has wreaked havoc in European oyster cultures (Segarra *et al.*, 2010) but so far there are no reports of this variant in the Americas. As to *M. refringens* in this study and the report by Grijalva-Chon *et al.* (2013) for OsHV-1, the apparently healthy pre-adult and adult *C. corteziensis* may actually be a reservoir for this pathogen.

P. marinus also has a connection with the seasonal temperature variation, so the presence of this pathogen has its maximum intensity in autumn and the lowest at the beginning of spring (Burreson & Ragone-Calvo, 1996). In the Gulf of California, Cáceres-Martínez *et al.* (2008) reported this protozoan in *C. corteziensis*, and Enríquez-Espinoza *et al.* (2010) in *C. gigas*. Enríquez-Espinoza *et al.* (2015) mention that this pathogen was found throughout the year in cultures of *C. gigas* and wild black clam *Chionista fluctifraga* with fluctuating prevalence and without mortality events. This pathogen has proven to be the most prevalent one in the south area where the higher temperature was presented compared to the north. The presence in the south area since the first sampling, and the increasing prevalence in subsequent surveys, suggests a large population of *P. marinus* established in that area, but at the end of the study the prevalence was 96% in both locations. Escobedo-Fregoso *et al.* (2015) reported high prevalence of *P. marinus* in *C. corteziensis* in two locations of Nayarit during the summer, indicating a match with our high number of carrier oysters, so it will not be uncommon to find high prevalence values anywhere in the Gulf of California.

Villanueva-Fonseca & Escobedo-Bonilla (2013) evaluated the presence of *Perkinsus* sp. in Sinaloa, an intermediate location between our study areas, using the Ray's fluid thioglycollate medium (RFTM), and recorded high prevalence in cultures of *C. gigas* during September and October. With the same technique, Enríquez-Espinoza *et al.* (2015) reported high prevalence values in *C. gigas* cultivated in Bahía Kino, Sonora. While there may be a discrepancy when comparing prevalence obtained by RFTM and those obtained by PCR, high values obtained with both methods indicate a significant increase in recent years.

Dual infections in oyster species have been reported in areas that historically have had a long history of surveillance, for example in *C. virginica* on the east coast of the United States with dual infections of *Haplosporidium nelsoni* and *P. marinus* (Ragone-Calvo *et al.*, 2003), similarly in *Crassostrea hasar* with *P. olsenii* and *P. marinus* in Brazil (Da Silva *et al.*, 2014). The oyster cultures in the Gulf of California begin with pathogen-free spat according to regulation, but the Gulf of California waters harbor pathogens that

are of global concern, does not guarantee a pathogen-free culture. Dual infections OsHV-1/*P. marinus* and *P. marinus*/*M. refringens* found in *C. corteziensis* have not been reported in the literature. In an advanced culture, as evidenced in the last sampling, dual infections can occur in a significant number of individuals, the most common being the simultaneous presence of the two protozoans on the same host.

The surface circulation of the Gulf of California is extremely complex as it is the result of the interaction of winds, tides, water density, heat exchange and confluence of water masses associated with the California Current, the Costa Rica Coastal Current and the North Equatorial Current at certain times of the year (Marinone & Lavin, 1997; Petatán-Ramírez, 2015), as well as the presence of cyclonic eddies in summer and anticyclonic eddies in winter (Soto-Mardones *et al.*, 1999). All this dynamism complicates defining a regionalization (Petatán-Ramírez, 2015) which is reflected in the different infective pattern seen in *C. corteziensis* when comparing both culture areas. Nevertheless, given the diversity of species of bivalve mollusks present in the Gulf of California (Fischer *et al.*, 1995) it is necessary to ensure continuous monitoring that includes several species of mollusks and allow early detection of OsHV-1 μ var, still absent in the Gulf of California.

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