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Short Communication

Molecular evidence of the protozoan parasite *Marteilia refringens* in *Crassostrea gigas* and *Crassostrea corteziensis* from the Gulf of California

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ABSTRACT. The search for exotic pathogens related to the outbreaks and in surveillance samplings of the Mexican oyster farms, is a recent activity achieved by academic institutions and state committees for Aquatic Animal Health, with remarkable results. In samples of *Crassostrea gigas* collected through December 2009, January 2010 and November 2010, and of *C. corteziensis* in September 2011, the protozoan *Marteilia refringens* was detected for the first time in the Gulf of California. The carrier oysters were from cultures without abnormal mortality rates, whereby, the use of histology, *in situ* hybridization and transmission electron microscopy studies are necessary to determine if *M. refringens* has become established in the Gulf of California oyster cultures. Detection of *M. refringens* is of great concern to the global oyster farming industry.

Keywords: *Marteilia refringens*, *Crassostrea gigas*, *Crassostrea corteziensis*, Gulf of California.

Evidencia molecular del parásito protozoario *Marteilia refringens* en *Crassostrea gigas* y *Crassostrea corteziensis* del Golfo de California

RESUMEN. La búsqueda de patógenos exóticos relacionados con brotes de enfermedades y en muestreos de vigilancia de las granjas ostrícolas de México es una actividad reciente, realizada por instituciones académicas y comités estatales de sanidad acuícola, con resultados notables. En muestras de *Crassostrea gigas* colectadas en diciembre 2009, enero 2010 y noviembre 2010 y de *C. corteziensis* en septiembre 2011, se detectó por PCR el protozoario *Marteilia refringens* por primera vez en el Golfo de California. Los ostiones portadores provenían de cultivos sin mortalidades anormales, por lo cual, el uso de histología, hibridación *in situ* y microscopía electrónica de transmisión son necesarios para determinar si *M. refringens* se ha establecido en los cultivos de ostras del Golfo de California. La detección de la presencia de *M. refringens* es de gran preocupación para la industria ostrícola.

Palabras clave: *Marteilia refringens*, *Crassostrea gigas*, *Crassostrea corteziensis*, Golfo de California.

The oyster's culture along the Mexican Pacific coast began nearly forty years ago, and for almost twenty years the oyster culture run without major problems, until massive mortalities were observed since the end of the 1990's until 2009. The quest for a pathogen had shown the evidence of the presence of the ostreid herpesvirus 1 (OsHV-1) (Vásquez-Yeomans, 2004, 2010; Grijalva-Chon *et al.*, 2013) and the protozoan *Perkinsus marinus* (Cáceres-Martínez *et al.*, 2008; Enríquez-Espinoza *et al.*, 2010; Escobedo-Fregoso *et al.*, 2015), which is endemic of the Atlantic coast.

In aquatic cultured species many pathogens are not specific and infect a wide range of related host species. In mollusks, several protozoan species seriously threaten the cultures, and because of the emergence of exotic diseases of great concern to aquaculture farmers, countries had implemented strict regulations for trading live organisms or frozen commodities to avoid its spread.

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However, the previous trade of infected broodstock, spat, or juveniles, before these regulatory rules were in effect, affected not only the established cultures but also wild populations.

Marteilia refringens is a protozoan of great concern to the mollusk aquaculture, mainly in Europe, as it is responsible for the Aber disease that causes mass mortalities in *Ostrea edulis*. This parasite also has the ability to infect several bivalve species; therefore, survey studies in areas of mollusk culture are of worldwide interest. The OIE (2009) listed the susceptible host species, vectors, and carriers for this protozoan, but *Crassostrea gigas* and *C. corteziensis* were not included in any category. Thus, the aim of this study was to investigate the occurrence of *M. refringens* in two oyster species cultured in the Gulf of California.

During December 2009 through November 2010, 30 specimens of adult *C. gigas* (10.35 ± 1.82 cm length) were monthly collected (n = 360) in La Cruz coastal lagoon, Sonora, Mexico (28°48'87''N, 111°55'03''W). The oysters were transported to the Laboratory of Molecular Ecology at the Sonora University. Tissues of digestive gland and gills were dissected using sterile instruments for every oyster and immediately fixed with 95% ethanol. Additionally, 19 tissue samples of *C. corteziensis* cultured during September 2011, from La Paz, Baja California Sur, Mexico (24°08'13''N, 110°25'37''W) at more than 530 km south of La Cruz, were included in the current study.

The total genomic DNA from the samples was isolated with the QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN) and PCR was carried out with Ready-to-Go PCR beads (GE Healthcare). The nested PCR was performed with primers and PCR conditions reported by López-Flores *et al*. (2004) and López-Flores (2003) that target the ribosomal DNA intergenic spacer (rDNA IGS). The first reaction was run with 125 ng DNA and 25 ng of each primer in a total volume of 12.5 µL using PCR-grade water to amplify a 525 base-pair amplicon. The primer sequences were MT-1 5'-GCCAAAGACA CGCCTCTAC-3' and MT-2 5'-AGCCTTGATCACA CGCTTT-3'. The PCR conditions were, an initial denaturalization at 94°C for 5 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final step of 72°C for 10 min. The nested reaction was made with Ready-to-Go PCR beads in 12.5 µL of total volume with 0.5 µL of the first reaction and 0.025 µg of each primer to amplify a 358 base-pair amplicon. The nested primers were MT-1B 5'-CGCCACTAC GACCGTAGCCT-3' and MT-2B 5'-CGATCGAGTA AGTGCATGCA-3', and the PCR conditions were, an initial denaturalization at 94°C for 5 min, 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final step of 72°C for 10 min. DNA of *Ostrea edulis* infected with *M. refringens* type O and corresponding to sequence AM292652 of the GenBank was used as positive control; samples without DNA were included as negative controls.

Finally, the PCR products were visualized on 2% agarose gels stained with ethidium bromide. To verify the identity of the PCR products, only two amplicons obtained from *C. gigas* and the two from *C. corteziensis* were sequenced in both senses with primers MT-1B and MT-2B and the chromatograms were revised with ChromasPro v. 1.5 (Technelysium). The resulting sequences were analyzed using the basic local-alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI), USA and a multiple sequencing alignment was also done using ClustalX (Thompson *et al.*, 1997) with some *M. refringens* sequences reported in GenBank.

In this survey, the majority of the sampled organisms were diagnosed as negative to the parasite; however, *M. refringens* was detected in four different organisms by nested PCR (1.1%) of the total number of *C. gigas* analyzed and two organisms of *C. corteziensis* (10.5%). The positive samples of Bahia de Kino, Sonora, were collected in December 2009 (n = 1), January 2010 (n = 1) and November 2010 (n = 2). In accordance with López-Flores *et al*. (2004), a single DNA amplicon of 358 base pair (bp) was obtained from the samples diagnosed as positive (Fig. 1).

Two DNA amplicons from each geographical region were sequenced and analyzed (GenBank accession numbers JQ066723-JQ066726). The BLAST analysis matched 60 *M. refringens* entries with 94-100%

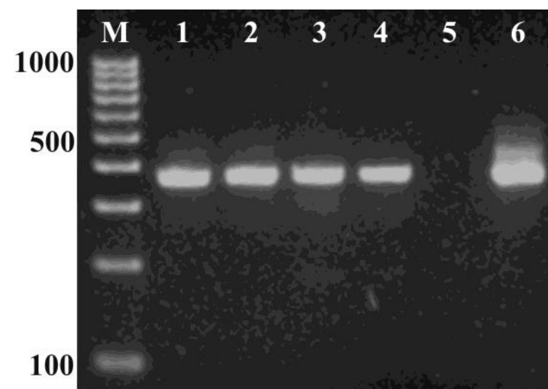

Figure 1. Nested PCR amplicons (358-bp) of rDNA IGS agarose gel. M: DNA size marker. Lanes 1-2: amplicons obtained from *Crassostrea gigas* tissue. Lanes 3-4: amplicons obtained from *C. corteziensis* tissue. Lane 5: negative control. Lane 6: positive control of *Ostrea edulis* infected with *Marteilia refringens*.

identity and coverage of 100% for most of the entries. These sequences also matched partially with three sequences corresponding to a new *Marteilia* species (JN820090-JN820092), but with coverage of 60 to 88% and identities of 80 to 82%. The alignment of sequences showed that *M. refringens* from *C. gigas* has more substitutions than those from *C. corteziensis,* when compared to the European AM292652 sequence (Fig. 2).

Before the first massive mortalities at the end of the 1990s, there was no strict control to prevent the exchange of farmed oysters from different culture sites, and there are no official figures regarding the movement of organisms between farms or geographic areas. All oyster farmers remember that a batch of *Crassostrea virginica* was stocked in the Gulf of California more than 10 years ago but there are not official data to support that information. In a recent study, Escobedo-Fregoso *et al.* (2015) made a phylogenetic analysis that suggests the Atlantic coast origin of the *P. marinus* from the Mexican Pacific coast and his would support the version of the translocation of oysters from the Atlantic to the Pacific, carrying not only *Perkinsus* but *Marteilia*. Furthermore, there is evidence that *C. gigas* can carry some primary stages of *M. refringens* without being seriously affected; so *C. gigas* is considered as resistant to infection with this parasite species (OIE, 2009; Berthe, 2004). This would explain the low prevalence of *M. refringens* in *C. gigas* samples. Nevertheless, a PCR analysis can detect the presence of a pathogen, but this not necessarily implies a real infection (Burreson, 2008), and therefore an extensive study including histology, *in situ* hybridization or transmission electron microscopy must prove that *C. gigas* and *C. corteziensis* are susceptible species for *M. refringens* infections. Another important aspect of the OIE (2014) is the self-declaration of freedom from *M. refringens* for countries or zones and its repercussion over importations and exportations of live animals or commodities. Until the *C. gigas* and *C. corteziensis* susceptibility is resolved, the presence of *M. refringens* in some locations of the Gulf of California is of great concern to the oyster culture industry of the region.

The OIE (2009) recommends the use of primers Pr4 and Pr5 (Le Roux *et al.*, 2001) for detection of *M. refringens*, but the primers designed by López-Flores *et al.* (2004) were used in this study because of their higher specificity and sensitivity. The OIE (2009) mentions that although those primers are more

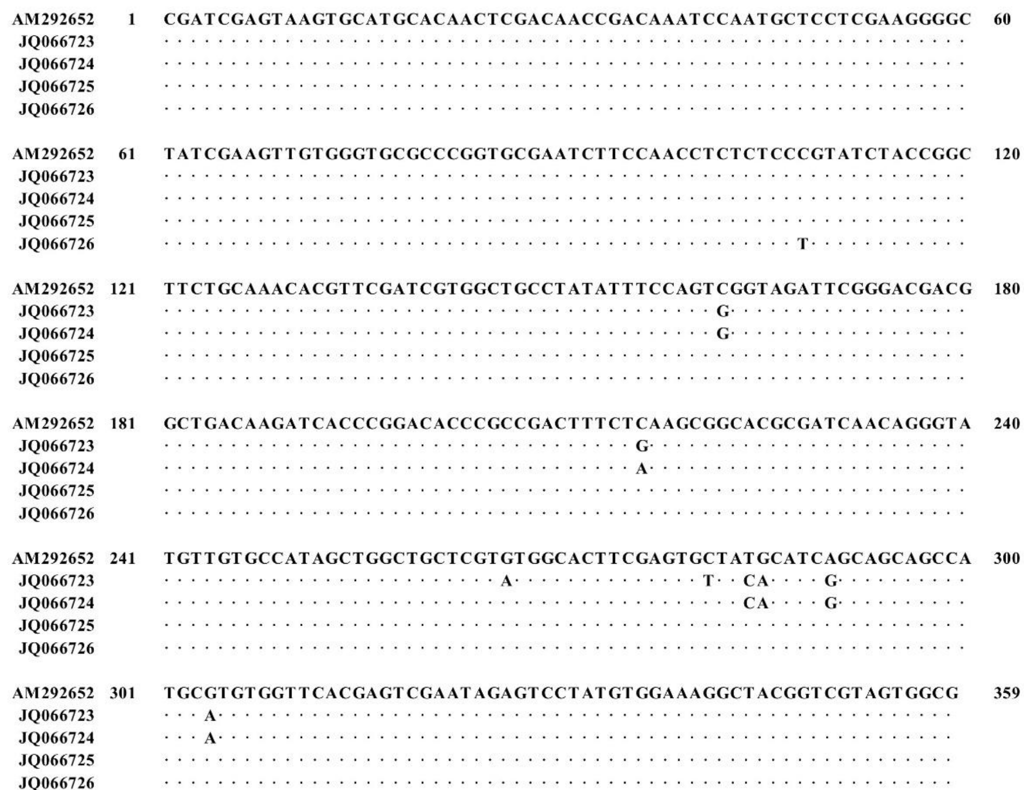

Figure 2. Nucleotide sequences of the 359-bp PCR product of *Marteilia refringens* from *Crassostrea gigas* (JQ066723 and JQ066724) and *C. corteziensis* (JQ066725 and JQ066726) and comparison with GenBank sequence AM292652. Dots represent identical bases to the AM292652 sequence.

sensitive, a thorough study for the evaluation of its specificity is still necessary; however Carrasco *et al*. (2012), found the new *M. refringes* type C infecting *Cerastoderma edule* in Europe for the first time by using the primers designed by López-Flores *et al*. (2004).

The sampled oysters come from cultures without abnormal mortalities of the same condition that Grijalva-Chon *et al*. (2013) describes for oysters with OsHV-1 in the same location and, fortunately, the prevalence of *M. refringens* DNA in the sampled months is low. All this requires an extensive study that includes wild mollusk species to determine the genetic variability of *M. refringens* in the Gulf of California, species susceptibility, and possible relationships among genotypes and host native species. The presence of OsHV-1, *P. marinus* and now *M. refringens* DNA in *C. gigas* and the native *C. corteziensis* clears up doubts, at least in part, about the possible pathogens involved in the massive mortality events that threatened cultures some years ago. Although there may be other pathogens that may jeopardize the survival of oyster species at different stages, such as the presence of some *Vibrio* bacteria and other protozoan species, the relevance of this study lies in identifying pathogen species that are notifiable to the World Organization for Animal Health (OIE) and which had not been previously reported in the eastern Pacific.

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