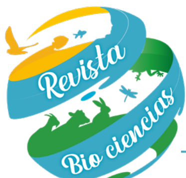

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Case report/ Reporte de caso

Ostreid Herpesvirus 1 in pacific lion-paw scallop *Nodipecten subnodosus* from Baja California Sur, Mexico.

Virus tipo herpes de los ostreidos en almeja mano de león en Baja California Sur, México.

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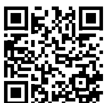
ABSTRACT

The present study is the first report of the presence of Ostreid Herpesvirus 1 (OsHV-1) in the pacific lion-paw scallop *Nodipecten subnodosus* from Baja California Sur, Mexico, collected from 2010 to 2012. The retrospective study was conducted to evaluate if the OsHV-1 was implicated in the population decline of 2010. OsHV-1 was detected by PCR, while viral particles were quantified by qPCR. OsHV-1 detection was conducted by PCR amplification of three different genome regions used to identify OsHV-1 variants. The phylogenetic analysis revealed the position of *N. subnodosus* OsHV-1 among OsHV-1 samples previously collected in different countries and bivalves, where the OsHV-1 of *N. subnodosus* was different from OsHV-1 µVar. Similarity analyses showed differences between the OsHV-1 of *Crassostrea gigas* and the OsHV-1 of *N. subnodosus* from Mexico, however, the sequence was identical to that of OsHV-1 from France 1993. This study contributes to understanding the OsHV-1 genetic diversity and identifies the potential virus host or species involved in the viral cycle.

KEY WORDS: Oyster's diseases, *Nodipecten subnodosus*, viral polymorphism, bivalves, OsHV-1.



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RESUMEN

Este estudio reporta por primera vez la presencia del herpesvirus de los ostreidos tipo 1 (OsHV-1) en la almeja *Nodipecten subnodosus* de Baja California Sur, México, colectada de 2010 a 2012. Este reporte se fundamenta en un estudio retrospectivo para evaluar si OsHV-1 estuvo implicado en el decaimiento de la población de *N. subnodosus* a partir del 2010. El virus fue detectado por PCR y las partículas virales fueron cuantificadas mediante qPCR. La detección de OsHV-1 de *N. subnodosus* fue realizada amplificando con PCR tres regiones diferentes del genoma utilizadas para describir las variantes de OsHV-1. Se realizó un análisis filogenético para mostrar la posición de OsHV-1 de *N. subnodosus* en muestras de OsHV-1 colectadas anteriormente en diferentes países y bivalvos, obteniendo que OsHV-1 of *N. subnodosus* es diferente a OsHV-1 µVar. Los análisis de similitud mostraron diferencias entre OsHV-1 de *Crassostrea gigas* y el OsHV-1 de *N. subnodosus* de México, sin embargo, la secuencia fue idéntica al OsHV-1 de Francia 1993. Este estudio contribuye a comprender la diversidad genética de OsHV-1 e identificar el hospedero potencial del virus o las especies involucradas en el ciclo viral.

PALABRAS CLAVE: Enfermedades de ostreidos, *Nodipecten subnodosus*, polimorfismo viral, bivalvos, OsHV-1

Introduction

The pacific lion-paw scallop *Nodipecten subnodosus* fishery is an important economic activity in the Ojo de Liebre lagoon, located in the North of Baja California Sur (BCS) state, Mexico. The population of *N. subnodosus* has displayed a critical population decrease due to unknown causes, suggesting overexploitation and a low reproductive rate as potential explanations (González-Ortiz *et al.*, 2017). However, pathogens present in the area are a factor to consider, and the present study was conducted to detect the Ostreid Herpesvirus (OsHV-1) in *N. subnodosus* since the oyster *C. gigas* is also cultured in the Ojo de Liebre lagoon, which was introduced from other countries. OsHV-1 has been widely spread, and detected in different bivalves such as oysters, clams, mussels, and scallops (Arzul *et al.*, 2001a); however, the highest mortalities are observed in *C. gigas* (Renault *et al.*, 1994). In BCS, the presence of OsHV-1 has not been reported; however, in the neighboring state of Baja California and La Cruz coastal lagoon of Sonora, OsHV-1 has been detected in *C. gigas* (Vasquez-Yeomans *et al.*, 2004; Martínez-García *et al.*, 2020).

The OsHV-1 genome is integrated by 124 ORFs (Davison *et al.*, 2005; Gallardo-Ybarra *et al.*, 2019), and different target areas are used for virus diagnosis. ORF 35−38 and ORF 42−43 regions are polymorphic areas, whereas ORF 100 is less polymorphic (Davison *et al.*, 2005);

however, ORF 4 is the best polymorphic region to describe viral diversity (Renault *et al.*, 2012, De la Re-Vega *et al.*, 2017). Phylogenetic analyses based on ORF 4 showed differences between the strain from France 1993 and strains from France 2005−2012 (Martenot *et al.*, 2015), and even allowed OsHV-1 µVar identification (Segarra *et al.*, 2010). This work describes the OsHV-1 presence in BCS Mexico for the first time and compares the phylogeny between *C. gigas* and *N. subnodosus*.

Material and methods

OsHV-1 Diagnosis

OsHV-1 diagnosis was conducted in 15 scallops from 2010, 15 from 2011, 66 from 2012, and 44 from 2013. A pool of gills and mantle of each adult *N. subnodosus* from Ojo de Liebre Lagoon, BCS, Mexico was sampled. Gills were sampled since it is the virus replication site in *C. gigas* and the mantle since the virus is inactive in *C. gigas* (Gallardo-Ybarra *et al.*, 2019). DNA was extracted following the QIAamp DNA protocol (Qiagen, Hilden. Germany). Mantle and gills samples (10 mg) from each tested individual were homogenized with 100 µL of lysis buffer ATL and 20 µL of proteinase K and were incubated at 56 °C for 1 h. The pellet was recovered after centrifugation (8000 rpm), and 200 µL of ethanol 96 % was incorporated. The pellet was washed with 500 µL of AW1 buffer and filtered with mini spin columns at 8000 rpm, and 500 µL of AW2 buffer was added and centrifuged. Obtained DNA was resuspended with distilled water.

The 709 bp of the ORF 4 was amplified with primers C2/C6 (Arzul *et al.*, 2001b), the 607 bp of the ORF 42−43 was amplified with primers IA2−IA1 (Segarra *et al.*, 2010), and ORF 35−38 was amplified with primers Del 36−37F/Del 36−37R (Renault *et al.*, 2012) with two expected amplicons: of 989 bp or 384 bp. PCR reactions were performed in a total volume of 50 µL, with 2.5 U (0.5 µL) of Taq DNA GoldStar polymerase, 5 µL 10× Taq DNA polymerase buffer (Eurogentec, Seraing, Belgium), 1.5 mM $MgCl_2$ except for Del 36−37F/Del 36−37R with 2 mM $MgCl_2$, 0.05 mM of each dNTP, 1 µM of each primer, and 100 ng of DNA. After DNA heating for 3 min at 94 °C, 42 cycles were carried out followed by a final elongation step of 10 min at 72 °C. Each of the 35 cycles consisted of a DNA melting step at 94 °C for 1 min, a primer annealing step for 1 min at 58 °C (55 °C for IA2/IA1), and a primer elongation step at 72 °C for 90 s. Expected fragments were purified with gel extraction kit Montage (Merck Millipore, MA, USA) and sequenced by ABI PRISM®3130 XL-Avant genetic analyzer (Thermo Fisher Scientific, MA, USA).

Quantification of OsHV-1 by qPCR

The OsHV-1 copies were quantified in 10 samples (10 scallops) with OsHV-1 (Table 1). PCR reactions were carried out in 20 µL containing 10 µL of Brilliant® SYBR Green PCR master mix, 25 ng of DNA, 5 µM of primers DPF/DPR (Webb *et al.*, 2007), and water. Amplicons were amplified in a qPCR system Mx3000p (Agilent Technologies, Santa Clara, CA, USA) in the genetics and pathology laboratory, at IFREMER, France. Thermal conditions were: 95 °C for 3 min, 40 cycles at 95 °C for 5 s, and 60 °C for 20 s; the melting temperature curve was at 95 °C for

1 min, 60 °C for 30s and 95 °C 30s. The number of OsHV-1 copies/ng of total DNA was compared with the standard curve values of OsHV-1 µVar (HQ842610).

Sequence analysis

Multiple sequence alignment was evaluated with CLUSTAL W software, and all sequences were cut according to the reference sequence AY5093253. Phylogenetic distances of the region ORF 4 were calculated with the Tamura Nei model, and the region ORF 35-38 and ORF 42-43 were evaluated with the model Tamura-3-parameter using MEGA 6. Phylogenetic trees were evaluated with 10,000 bootstraps repetitions.

Results and discussion

OsHV-1 detection

The ORF 42−43, and ORF 35−38 regions of the Ostreid Herpesvirus, were detected in only 10 samples of *N. subnodosus* (Table 1). The ORF 35−38 amplicon size was 384 bp, this length corresponds to the deletion of 605 bp in the genome of OsHV-1 µVar (HQ842610). The amplicon of 989 bp of OsHV-1 was not detected, suggesting that the virus detected in *N. subnodosus* has the characteristic of OsHV-1 µVar. However, the similitude analysis of the ORF 35−38 region of Ostreid herpesvirus of *N. subnodosus* revealed 100 % identity to OsHV-1 from France, Japan, Portugal, and New Zealand (KT429193, KT429194, KT429195, and JN800252), even at 99 % of coverage and 100 % identity to *Chlamys farreri* acute viral necrosis virus (GQ153938), and 98 % of coverage showed 99 % of identity to OsHV-1 µVar (KF185077). The results with this region indicate high similarity to OsHV-1 and OsHV-1 µVar; however, ORF 35−38 depicted low polymorphism to evaluate variants.

On the other hand, the similitude analyses of the ORF 42−43 region showed 99 % identity to both the complete genome of OsHV-1 (AY509253) and *C. farreri* acute viral necrosis virus. The similarity to the OsHV-1 µVar (KU864508 and KU864508) is 99 % with 91 % of coverage. The results showed high similarity to OsHV-1 and OsHV-1 µVar, a reason to analyze the more polymorphic region ORF 4.

Although the ORF 42−43 and ORF 35−38 regions of OsHV-1 were detected in 10 samples, ORF 4 was detected only in one sample (Table 1). The inconsistency between results is due to the higher ORF4 polymorphism (Arzul *et al.*, 2001b) compared to the ORF 35−38 and ORF 42−43. According to the similitude analysis, the sequence of region ORF 4 of Ostreid herpesvirus from *N. subnodosus* has the highest similitude (97 % identity, and 96 % coverage) to OsHV-1 from France 1993 (JN800065), and only 87 % of coverage showed 98 % of identity to OsHV-1 µVar of *C. gigas* from France (KF185073). In addition, 87 % of coverage showed 97 % identity to *C. farreri* acute viral necrosis virus (GQ153938).

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The analyses with the three regions of the virus of *N. subnodosus,* showed high similarity to OsHV-1, followed by the acute viral necrosis virus causative of outbreaks in the Chinese scallop *C. farreri* (Ren *et al*., 2013), and to OsHV-1 µVar of *C. gigas.* However, the analyses with the most polymorphic region ORF4, suggest the highest similitude with OsHV-1.

Table 1. Diagnostic of OsHV-1 in samples of *N. subnodosus* analyzed by qPCR and PCR.

Sample name	Sampling date	qPCR ORF100 DPF/DPR		PCR		
		No. of Ct	Viral copies/ ng of total DNA	ORF4 C2/C6 (709 bp)	ORF 42−43 IA1/IA2 (607 bp)	ORF 35−38 Del 35−38 (989 bp, or 384 bp)
A1	November 2012	38.07	2.10	Not detected	+	+ (384 bp)
A2		37.81	2.56	+	+	+ (384 bp)
A3		36.26	8.29	Not detected	+	+ (384 bp)
B4	August 2011	38.68	1.32	Not detected	+	+ (384 bp)
B5		37.18	4.14	Not detected	+	+ (384 bp)
B6		38.96	1.07	Not detected	+	+ (384 bp)
B7		38.88	1.13	Not detected	+	+ (384 bp)
B8		38.06	2.11	Not detected	+	+ (384 bp)
C9	June 2010	Not detected	Not detected	Not detected	+	+ (384 bp)
C10		39.90	5.24E-001	Not detected	+	+ (384 bp)

Phylogeny of Ostreid Herpesvirus

According to phylogenetic results, the ORF 4 sequences of *Ostreid herpesvirus* were grouped into two main clades (Fig. 1). The first group had OsHV-1 sequences from Asia, Oceania, France 2008−2012, USA San Diego (MW504462), and the OsHV-1 µVar sequence of oysters from France (HQ842610). The second clade grouped sequences of OsHV-1 of *C. gigas* from France in 2003, USA in 2007, and Mexico in 2011 (Grijalva-Chon *et al*., 2013), and the reference sequence AY509253, which is 100 % similar to OsHV-1 of *C. gigas* cultivated in the La Cruz estuary, Sonora, Mexico in 2017−2018 (Martínez-Garcia *et al*., 2020), and with *C. gigas* from Morua estuary, in Kino Bay, Sonora, Mexico (De-la-Re-Vega *et al*., 2017). However, the OsHV-1 of *N. subnodosus* from Mexico 2012 (OQ716803) and the sequence of *C. gigas* of France 1993 (JN800065) were grouped in a subclade of Table 1. Diagnostic of OsHV-1 in samples of *N. subnodosus* analyzed by qPCR and PCR.

The high similitude of the OsHV-1 of *N. subnodosus* to OsHV-1 from France 1993, and the divergence with strains of France 2008−2012, suggest that OsHV-1 of *N. subnodosus* is not a recent introduction to Mexico. Although in Mexico the virus was reported in *C. gigas* oysters collected in 2011, the alignment of ORF 4 of OsHV-1 of *N. subnodosus* (OQ716803) has a deletion of 21 bp in the microsatellite region compared to the sequence of *C. gigas* from Mexico (JF894308), considering different genotypes between the OsHV-1 of *N. subnodosus* and *C. gigas* from Mexico (JF894308).

The neighbor-joining analysis of the ORF 42−43 region of OsHV-1 detected in *N. subnodosus* (OQ716804, Figure 1), revealed a divergent clade of OsHV-1 μVar. The OsHV-1 detected in *N. subnodosus* was grouped with the sequence of the OsHV-1 reference genome, OsHV-1 of France 1993, and with *Chlamys farreri* acute viral necrosis. The phylogeny of the ORF 35−38 region of OsHV separated the virus of *N. subnodosus* in a different clade of the OsHV-1 μVar (KF185077). The phylogeny with the three regions of OsHV discards that the virus of *N. subnodosus* is the variant μVar causing the highest mortalities in France.

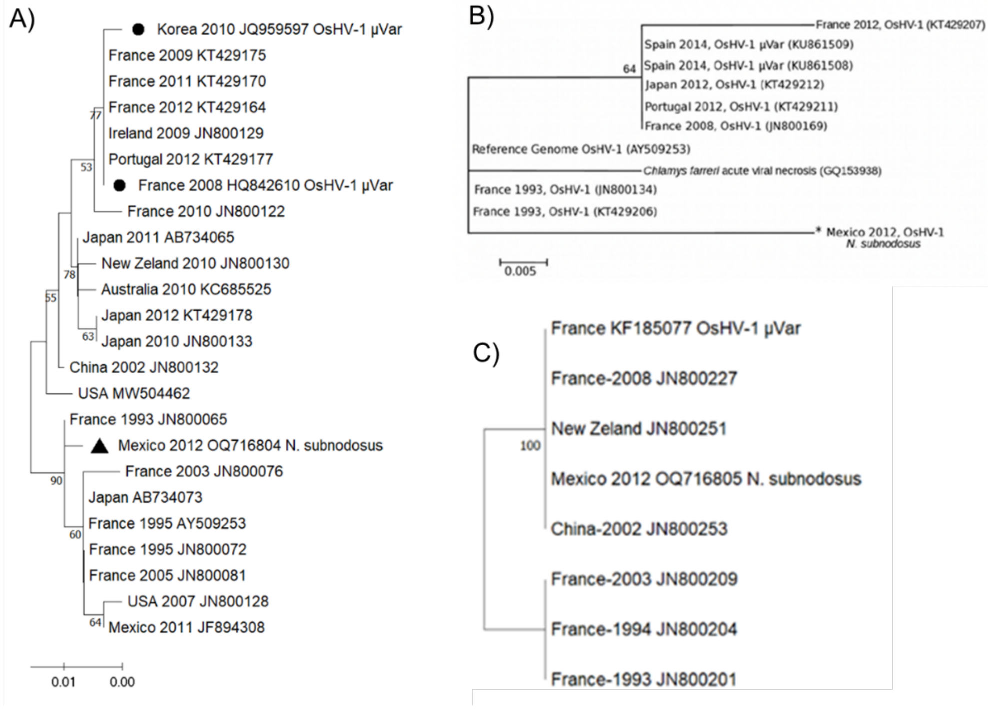

Figure 1. Phylogenetic tree of the region.

A) ORF4 of the OsHV-1. Analyses were evaluated with Neighbor-joining algorithm supported by 10,000 bootstraps repetitions based on the Kimura-2-parameter model. B) Phylogenetic tree of the region ORF 42-43 and C) ORF35-38 of the OsHV-1, evaluated with Neighbor-joining algorithm supported by 10,000 bootstrap repetitions based on the Tamura-3-parameter model.

Viral load in *N. subnodosus*

The analyses of OsHV-1 by qPCR detected 9/10 positive samples, however, the viral load was low, and was not even detectable in one sample that showed amplification of ORF 42−43 and ORF 35−38, associated with the difference in the amplification region by qPCR. Reports of viral loads below 50 copies/mg of tissue in *C. gigas* did not cause mass die-offs in France (Pepin *et al.*, 2008), and viral loads of 2.6×10^4 copies/ng of total DNA were found in dead *Ostrea edulis* (López Sanmartín *et al.*, 2016). The low viral load found in *N. subnodosus* does not cause die-offs in adult scallops, and viral transmission could be attributable to *C. gigas* cultured in the lagoon. However, the evaluation of OsHV-1 was analyzed in live scallops and maybe those were not infected. Hence, year-round monitoring will clarify the load viral influenced by environmental factors.

In conclusion, the Ostreid herpesvirus of *N. subnodosus* from Ojo de Liebre lagoon, BCS, Mexico, is a different strain from OsHV-1 μVar. The high similarity between OsHV-1 of *N. subnodosus* and OsHV-1 from France 1993 indicates that this virus was not recently introduced to BCS, Mexico, and there is a risk of outbreaks spreading to other susceptible species as *C. gigas*, which is cultured in the area. The low viral load detected in our study suggests that *N. subnodosus* adults act as asymptomatic hosts of OsHV-1 and probably a reservoir

Based on the results presented here, the possibility of OsHv-1 as the cause of the high mortality and the decrease of *N. subnodosus* populations must be discarded. However, the animals sampled were only the survivors, and the detection may be associated with a selection of resistant organisms, for that is recommended seasonal monitoring.

Author contribution

“Work conceptualization, CEF, RVJ. Development of the methodology, AQAA, JAHG. Analysis of results, AQAA, CEF, JAHG, RVJ. Manuscript writing and preparation, AQAA, CEF, GMC. Writing, revising, and editing, CEF, RVJ. Fundraising, project manager, CEF, RVJ.

All authors of this manuscript have read and accepted the published version of it.

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