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First detection of Ostreid herpesvirus 1 in wild *Crassostrea gigas* in Argentina

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ABSTRACT

Ostreid herpesvirus 1 (OsHV-1) is a DNA virus of the genus Ostreavirus (*Malacoherpesviridae* family, Herpesvirales order). Worldwide, OsHV-1 and its microvariants have been associated with increased mortality of Pacific oysters, *Crassostrea gigas*. Adult asymptomatic oysters also have shown a high prevalence of viral infection. As a consequence, surveillance is needed to better describe OsHV-1 diversity, pathogenicity, clinical signs, and geographical distribution. We examined *Crassostrea gigas* sampled in October 2017 from the inner zone of the Bahía Blanca Estuary, Argentina, and found that 8 of 30 specimens (26.7%) presented macroscopic lesions in mantle tissues. Histological analysis revealed abnormal presentation of mantle epithelial cells and connective tissues. Conventional and real-time PCR conducted on the oyster samples revealed 70% to be positive for presence of OsHV-1 DNA. The nucleotide sequence of the amplicon obtained from one sample using the primer pair IA1/IA2 (targeting ORF 42/43) was 99% identical to OsHV-1 reference as well as µVar strains B and A (KY271630, KY242785.1), sequenced from France and Ireland. This finding represents the first detection of OsHV-1 DNA in a wild population of *C. gigas* in Argentina in association with gross mantle lesions.

1. Introduction

The Pacific oyster *Crassostrea gigas* (Thunberg, 1793) is one of the most successful marine invasive species and has been introduced to the intertidal and shallow subtidal areas of coastal marine environments of all continents except Antarctica (FAO, 2016). It displaces native species, modifies the community structure, and in several cases has spread pathogens in oyster populations (Arzul et al., 2017; Carrasco and Barón, 2010; Herbert et al., 2016). One of the main vectors for introduction and dispersal is aquaculture in protected bays and estuaries of temperate regions of the world (Ruesink et al., 2005). In Argentina, the species was intentionally introduced to Bahía Anegada (Buenos Aires Province, 40°S) in 1982. Although this activity was quickly abandoned, the introduction was followed by the discovery of wild populations in nearby intertidal environments (Escapa et al., 2004; Orensanz et al., 2001) as far as 100 km to the north at Bahía Blanca Estuary (dos Santos and Fiori, 2010), with further dispersion expected (Carrasco and Barón, 2010; Wörner et al., 2019; Carrasco et al., 2019). In 2002, Pacific oyster aquaculture was resumed under the Productive Harvest Program for aquaculture and, at present, there is a small industry and two oyster processing plants in San Blas and Los Pocitos, generating products for domestic markets and exports to Hong Kong and aiming to reach the European market (Burguener and Barón, 2017). However, while wild stocks are growing faster than expected, expanding north and south and causing a significant impact in the environment (Carrasco and Barón, 2010; dos Santos and Fiori, 2010), there is no monitoring program to detect pathogens circulating in the wild or in the farms.

Herpesvirus infections have been reported worldwide, often associated with mass mortality (Farley et al., 1972) or annual recruitment failures of bivalve species (Renault et al., 2001). The direct relationship between viral proliferation/transmission and environmental stress is influenced by physical and biological variables, an increase in oyster

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mortality typically being observed at high temperatures (Garcia et al., 2011; Martenot et al., 2015; Pernet et al., 2014, 2012; Petton et al., 2013, Renault et al., 2014a). The analysis of virus genomic sequences from different regions has shown variability, with recent notable mortality caused by a "µVar" and other virulent microvariants (Segarra et al., 2010; Renault et al., 2014b; OIE, 2018). Mineur et al. (2014) offered a broad perspective on the genetic variability of OsHV-1, reporting the greatest diversity of the virus in East Asia where multiple variants have been detected in wild populations of native oysters signs of disease, and characterizing genotypes associated with mass mortality in Europe.

Our goal was to evaluate the presence of *Ostreid herpesvirus 1*, primarily based on viral DNA detection by PCR, in a wild population of *C. gigas* in the Bahía Blanca Estuary, Argentina, in order to provide information about the presence of the virus and the health status of the oyster population.

2. Materials and methods

Thirty *C. gigas* specimens were randomly collected from the inner intertidal zone of the Bahía Blanca Estuary, Buenos Aires Province, in October 2017 and live-preserved in an ice box for 24 h until processing. At the laboratory, the oysters were clinically evaluated and sizes were recorded. All specimens were opened and the mantle and connective tissue were observed under a dissecting microscope. Pieces of mantle were frozen at −80 °C for further OsHV-1 molecular analysis, and the rest of the body was fixed in Davidson's solution (Shaw and Battle, 1957) for 24 h before transfer to 70% ethanol to conduct a gross histopathological analysis following processing by routine histological procedures. Transverse 5–7 µm-thick sections obtained from each specimen were stained with hematoxylin and eosin and were examined under a binocular bright field microscope with a magnification of 40X and 100X (DM 2500 LED, Leica Microsystems) and photographed with a DFC 310X Leica digital camera to capture high-resolution images.

DNA was extracted from 50 mg frozen mantle tissue of all 30 individuals using a QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol. Elution of DNA was performed with 100 µl of distilled water. PCR assays were conducted using three primer pairs, all in duplicate, a strategy useful to determine variation in three genomic regions (Renault et al., 2012). The three gene regions were amplified via PCR in a cocktail containing 0.5 µl of template DNA, 5 µl dNTPs (2 mM), 5 µl 10x Taq buffer, 0.5 µl each primer (100 ng/µl), 3 µl MgCl (25 mM), 35.5 µl distilled water, and 0.5 µl Taq DNA polymerase (5 unit/µl; InbioHighway). According to Renault et al (2012) the expected size of gene fragment for the ORF35, −36, −37, and −38 genes are 989 bp, 384 bp, or no amplification. These fragments were amplified using the Del 36-37F2 (5-ATACGATGCGTCGGTAGAGC-3) and Del 36-37R (5-CGAGAACCCCATTCCTGTAA-3) primers. A second gene, ORF-43 (607 bp), was amplified using the primer pairs IA1/IA2 (5-CGCGGTTCATATCCAAAGTT-3 and 5-AATCCCCATGTTTCTTGCTG-3, respectively (Segarra et al., 2010b). The last fragment, ORF-4 (709 bp), was amplified using the primer pair C2/C6 (5-CTCTTTACCATGAAGA TACCCACC-3 and 5-GTGCACGGCTTACCATTTTT-3, respectively (Segarra et al., 2010b). PCR was conducted in an Applied Biosystems, Verity 96 well Thermocycler with the following cycling condition: 94 °C for 2 min followed by 40 cycles of amplification at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and final extension at 72 °C for 5 min.

PCR amplified products were assessed by electrophoresis on 1.5% agarose gels, purified using an InbioHighway DNA purification kit, and evaluated by electrophoresis; all amplicons were sent for sequencing at the IDEAus-CONICET DNA Sequencing Laboratory (CCT CONICET CENPAT, Chubut, Argentina). A control consisting on two positive OsHV-1 DNA samples isolated in France were supplied by Dr. Renault (Ifremer) and used to optimize the conventional PCR. Sequence identity was verified by comparison with those listed in the GenBank database using the BLAST algorithm. Seven samples found to be positive by conventional PCR were also screened for Ostreid herpesvirus by qPCR in order to confirm the presence of OsHV-1. These samples were randomly selected but samples of four oysters which presented macroscopic mantle abnormalities were incorporated. Real time quantitative PCR was performed in duplicate using a Mx3005 P Thermocycler sequence detector (Agilent). Amplification reactions were each performed in a total volume of 20 µl. Each well contained 5 µl DNA from sea water or 5 ng DNA total from oyster mantle, 10 µl of Brilliant III Ultra-Fast SYBR®Green PCR Master Mix (Agilent), 2 µl of each primer OsHVDP For (forward) 5′-ATTGATGATGTGGATAATCTGTG-3′ and OsHVDP Rev (reverse) 5′-GGTAAATACCATTGGTCTTGTTCC-3′ (Webb et al., 2007) at the final concentration of 550 nM each, and 1 µl of distilled water. Real time PCR cycling conditions were as follows: 3 min at 95 °C followed by 40 cycles of amplification at 95 °C for 5 s and 60 °C for 20 s. Assays included a standard curve and a negative control (5 µl of distilled water instead of the 5 µl of simple DNA). Results were expressed in viral DNA copies of total DNA for oyster mantle samples.

3. Results

Table 1 summarizes results obtained using different analysis techniques. Mean size of the stock analyzed was 90 ± 28 mm, and all specimens appeared healthy before being opened. Clinical observations showed macroscopic lesions in the mantles of 8 of 30 oysters and the mantle tissue appeared thinner than that of apparently healthy animals. Multiple edematous lesions of variable size (c.a., 0.1–1 cm) filled with colorless liquid were noted in two oysters. Multiple rounded ulcerative lesions with thickened edges and translucent centers where the mantle looked thinner than normal were present in six oysters; some lesions appeared to be empty vesicles and others contained a minimal amount of translucent liquid (Fig. 1).

Histopathological analysis of oysters that were OsHV-1 positive by PCR revealed diffuse hemocyte infiltration in the connective tissue of the mantle in four of eight specimens, with diapedesis of hemocytes across mantle epithelia and disruption of the connective tissue (Fig. 2b–d). Hemocytes were swollen with a high nucleus to cytoplasm ratio. Intranuclear-like inclusions were observed in mantle epithelial cells and connective tissue of the mantle (not shown). Non-specific

Table 1
Oyster disease diagnostic results using different techniques, including OsHV-1-specific PCR assays.

Evaluation	Details		Positive/Total tested
Clinical analysis	Macroscopic abnormalities	Irregular structures	**8/30**
Histology	Microscopic abnormalities	Irregular tissue observation	**4/8**
Conventional PCR	36–37R IA1/IA2 C2/C6	ORF 35, −37, −38 ORF −42, −43 ORF 4	**21/30**
qPCR	qPCR	test 1 test 2	**6/6** **7/7**

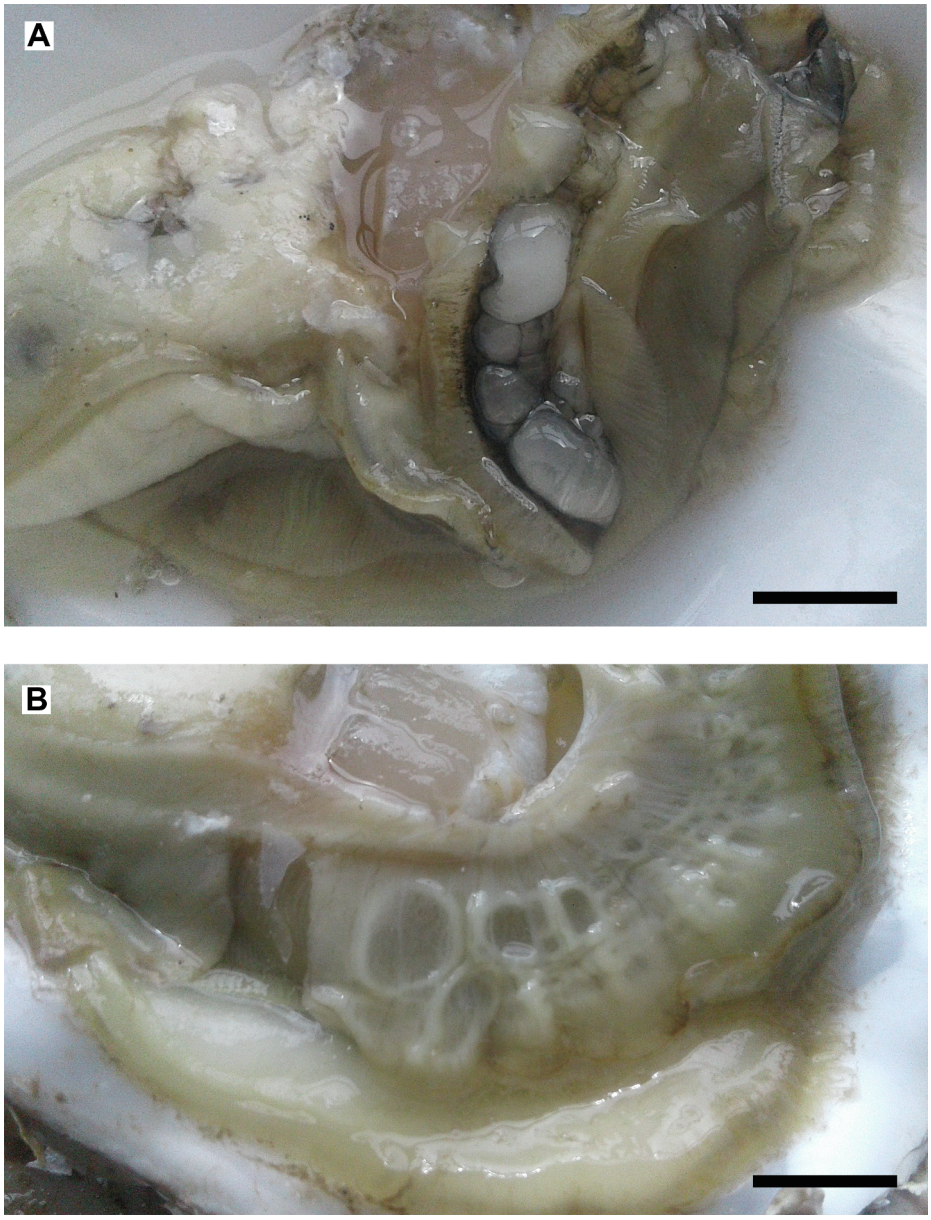

Fig. 1. Macroscopic morphology of mantle lesions in *Crassostrea gigas*. (A) Multiple vesicular lesions in the mantle. (B) Multiple ulcerative lesions, with thickened edges and translucent centers. Scale bars: 1 cm.

nuclear changes such as pyknosis were also observed (Fig. 2d).

Duplicates of all samples were analyzed by conventional PCR using three sets of primer pairs targeting three virus genome regions: C2/C6 (ORF-4), IA1/IA2 (ORF-42 and 43) and Del 36-37R (ORF35, −36, −37 and −38). Of the samples, 21 of 30 (70%) were positive, including those belonging to eight specimens with macroscopic abnormalities in mantle tissue (Table 1). The amplicons of the three PCR primer pairs were similar to the expected sizes (~700, 600 and 900 bp, for primers C2/C6, IA1/IA2 and Del 36-37F2-R, respectively). The seven samples tested using qPCR were positive (Table 1) with low virus genome copies (1.022×10^1–3.548×10^1 viral DNA copies/μL of total DNA extracted). Positive samples by conventional and qPCR (n = 7) were used for sequencing, only one sequence being recovered in reference to the IA1/IA2 primer pair (GenBank Accession number MK610098). Alignment analysis of 572-bp product revealed 99% identity (100% query cover) with the genotype of reference (AY509253.2) and μVar variant strains B and A (KY271630, KY242785.1, Burioli et al., 2017), both isolated during mortality events in *C. gigas* in France and Ireland.

4. Discussion

Herpesviruses have been associated with high mortality in larvae and juveniles of several marine mollusc species, including the Pacific oyster *C. gigas*, resulting in high losses worldwide (Hine et al., 1992; Renault et al., 1994b, 1994a; Vásquez-Yeomans et al., 2010). Our study, based on the detection of viral DNA, provides the first report on the presence of OsHV-1 in wild populations of *C. gigas* in Argentina, and the second in South America after its recent detection in introduced *C. gigas* and native *C. brasiliana* on the coast of Santa Catarina, Brazil (Mello et al., 2018). The search for the virus was performed after the detection of clinical abnormalities in mantle tissues while processing the animals (Fig. 1a and b). Histological examination of oysters indicated the presence of lesions that may result from an OsHV infection because we observed the occurrence of diapedesis of hemocytes across mantle epithelia and pyknosis of epithelial cells of the mantle (Fig. 2). The macro- and microscopic lesions we found are consistent with those reported in some previous studies (Arzul et al., 2017; Cáceres-Martínez

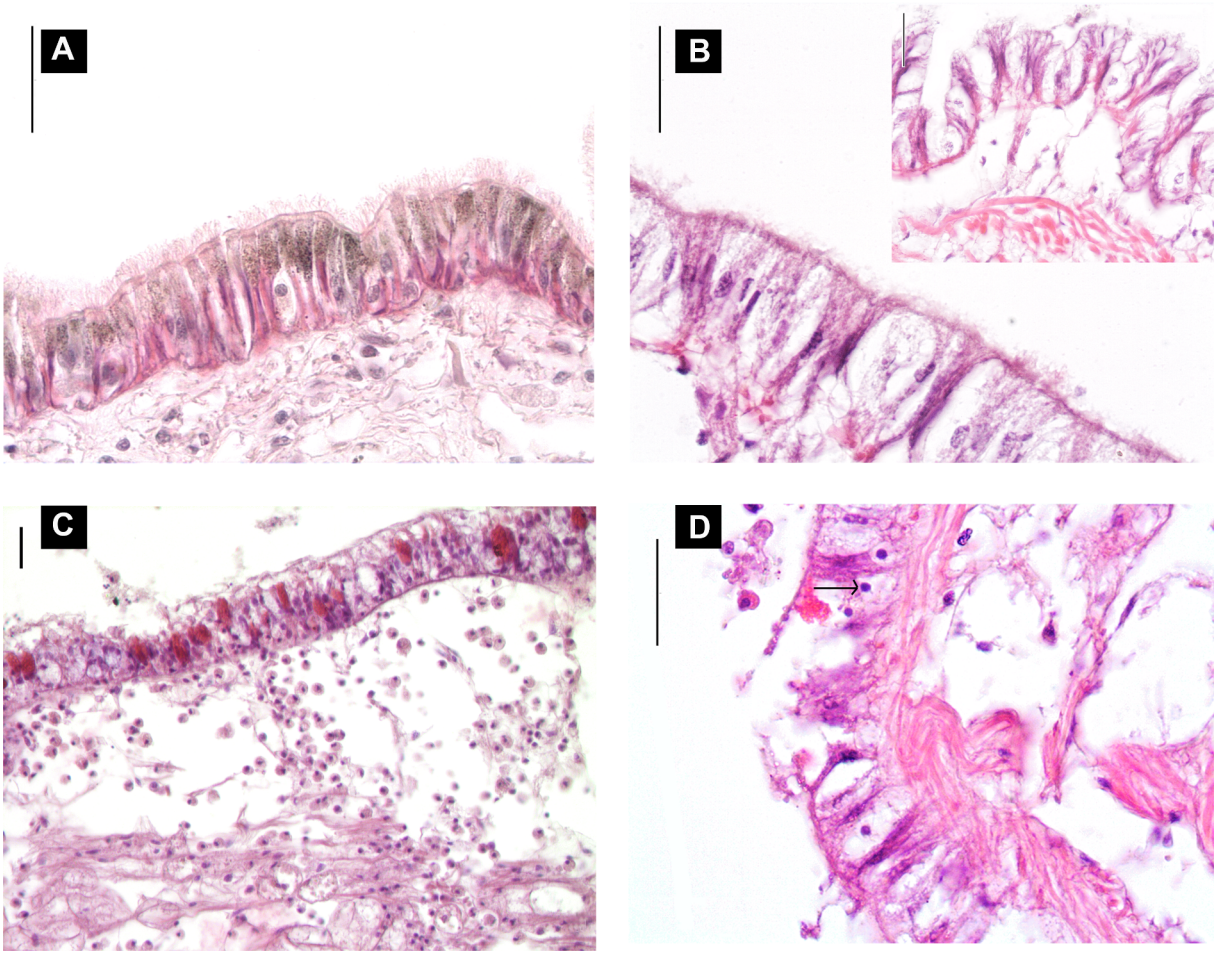

Fig. 2. Microscopic morphology of lesions in *Crassostrea gigas*. Hematoxylin and eosin stained. (A) Portion of normal mantle from health oyster 1000X. (B) Mantle of an oyster with vesicular lesions 1000X. Vacuolization of the epithelial cell is observed. Note the disarrangement of the connective tissue and loss of the ciliature of the mantle epithelial. (C) Diffuse hemocyte infiltration in the connective tissue (400X) and (D) Condensed hyperbasophilic nuclei pyknosis (arrowheads) in mantle epithelium 1000X. B, C and D were OsHV-1 positive by PCR. Scale bars: 20 µm.

et al., 2018; Meyers et al., 2010; Vásquez-Yeomans et al., 2010). While such mantle lesions may be associated with bacterial infection or protozoan diseases causing other signs such as dark pustules on inner oyster shell, mantle recession, emaciation, gapping, flat or raised lesions on the body surfaces of the mantle (Elston et al. 1987; Friedman et al. 1991; Sanil et al. 2010), none of these alterations or etiological agents were observed in *C. gigas* (Fig. 2) we collected.

While most of the oysters appeared to be in healthy condition, 70% were apparently infected with Ostreid herpesvirus 1 (Genbank accession number MK610098) based on viral DNA detection by PCR. At the adult stage of oyster development the virus may be present without clinical signs or mortality in the population (Arzul et al., 2017, 2002; Lipart & Renault, 2002; Dundon et al., 2011). Moreover, viral replication and transmission has been detected in the absence of mortality (de Kantzow et al., 2016; Petton et al., 2013; Renault et al., 2014a). Because most mortality events are observed when seawater temperature rises to 16–24 °C, many authors suggest the existence of a temperature threshold above which the replication and transmission of the virus increase, causing high mortality rates (Jenkins et al., 2013; Oden et al., 2011; Paul-Pont et al., 2013; Petton et al., 2013; Renault et al., 2014a). The physiological response of invertebrates to infection by pathogens is directly impacted by the surrounding environment (Burge et al., 2017). In this work, the temperature at the sampling site in October 2017 (mean monthly SST: 14.8 °C) could have limited viral replication and consequently resulted in the lack of clinical signs in most of the specimens. On the other hand, it has also been observed that low salinity (e.g., 10‰) reduces the replication and infectivity of the virus and the mortality generated compared to that in higher salinity (15–35‰) (Fuhrmann et al., 2018). The sampling area covered in our study is subject to the typical salinity dynamics of temperate bays and estuaries; it is characterized by high variability, ranging from 16 to 41‰ (mean: 33‰) (Piccolo and Perillo, 1990; Freije et al., 2008; Berasategui et al., 2018). No further insight is possible based on the available salinity data. Food availability is another important environmental factor to consider when evaluating possible stressors favoring or limiting virus prevalence and its effects on oysters. High-quality food (diatoms) decreases the risk of mortality in the first feeding stages of *C. gigas* (Pernet et al., 2014). Also, an interesting feature to note is that the presence of the toxic dinoflagellate *Alexandrium catenella* influences the oyster's immune response to viral infection, reducing viral prevalence (Lassudrie et al., 2016, 2015). Although we did not analyze food resources, previous studies show that diatoms dominate the phytoplankton annual cycle in the Bahía Blanca estuary (Gayoso, 1998), with a winter diatom bloom (July-early to September) at the inner zone of estuary (Popovich and Marcovecchio, 2008). Although high phytoplankton availability and relatively low water temperature may have acted as moderating factors for virus pathogenicity, further studies are needed to confirm this hypothesis.

This is the first study reporting the detection of Ostreid herpesvirus 1 DNA in Argentinean populations of *C. gigas*. New field samplings are needed to understand the annual variability of OsHV-1 in wild stock of the species along the coast of Argentina in order to characterize how

virus infection correlates with atmospheric and biological variables and their influence on oyster mortality.

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