First detection of herpesvirus variant (OsHV-1μvar) in Pacific oysters (Crassostrea gigas) in Spain and development of a rapid method for its differential diagnosis

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Abstract
Ostreid herpesvirus-1 (OsHV-1) infections have been reported around the world associated with mortalities of the Pacific oyster, Crassostrea gigas. Recently, a new genotype called OsHV-1μvar was described associated with important mortalities of C. gigas in France and has been considered a dangerous emerging pathogen for oysters.

In the present work we describe for the first time the presence of this new genotype OsHV-1μvar in Spain associated to mortalities of C. gigas. Moreover, we have developed a specific polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) assay to identify OsHV-1μvar and distinguish it from the reference herpesvirus genotype. We propose this technique as a useful method to provide a rapid identification of the OsHV-1μvar in epizootiological studies and disease control programmes.

World bivalve production has increased substantially in the last fifty years. Pacific oyster (Crassostrea gigas) is one of the most important mollusc species produced around the world. China is the top producing country with 3.9 million tonnes in 2006, followed by Japan, South Korea, and France (FAO, 2008). Episodes of increased mortality have been reported in all areas of Pacific oyster production since the 1950’s. Herpes-like virus and herpesvirus infections have been associated with mortalities in larvae, spat and juveniles of C. gigas around the world, most notably in the USA (Farley et al., 1972; Friedman et al., 2005; Burgue et al., 2006), New Zealand (Hine et al., 1992), Australia (Hine and Thorne, 1997), Mexico (Vásquez-Yeomans et al., 2004) and France (Renault et al., 1994). Herpes-like viruses were also described in other bivalves such Ostrea angasi (Hine and Thorne, 1997) or Ruditapes philippinarum, Pecten maximus and O. edulis (Comps and Cochenne, 1993; Renault et al., 2000; Arzul et al., 2001; Renault et al., 2001).

Different diagnostic methods including conventional polymerase chain reaction (PCR), in situ hybridization, immunohistochemistry and
real-time PCR have been developed to detect oyster herpes virus (Arzul et al., 2002; Lipart and Renault, 2002; Batista et al., 2007; Pepin et al., 2008; EU Commission Regulation 175/2010; Martenot et al., 2010).

Recently a new oyster herpes virus genotype, namely OsHV-1μvar, has been described as associated with important mortalities of spat and juveniles of *C. gigas* in France (Segarra et al., 2010). According to these authors and also the EU directives (Council Directive 2006/88/EC; EU Commission Regulation 175/2010), there is an urgent need to identify this emergent pathogen because it produces a serious disease situation. Epizootiological studies are being carried out but it is important to have an easy and rapid method to confirm the detection of OsHV-1μvar that can be available to all laboratories involved in herpes virus disease control programmes.

In Spain, several batches of *C. gigas* from two locations in Spain (Asturias in the north and Cádiz in the south) were analyzed and the associated mortality levels estimated by the growers in the field. In Asturias, two different cultured batches of *C. gigas* (*n* = 200) both presenting high mortalities in August, 2010 were analyzed. The first batch had *C. gigas* of 15-20 mm suffering 86 % mortality and the second batch consisted of *C. gigas* (20-48 mm) suffering 78 % mortality. Five months later (December, 2010) a batch of 75 *C. gigas* of 5-6 cm that had survived the initial disease outbreak were also analyzed. From the second location (Cádiz) one sample (*n* = 30) of *C. gigas* of 10-20 mm from natural beds was analyzed in October, 2011 (the mortality rate is unknown). Culture of *C. gigas* in Asturias is conducted in off-bottom culture trays, where the seed are kept in mesh bags attached by ropes to wood frames located in the low intertidal zone.

Oysters were harvested and sent the same day to the laboratory under refrigerated conditions. Once in the laboratory, oysters were opened aseptically and DNA was extracted with phenol-chlorophorm. In Asturias, when oysters were smaller than 3 cm, pools consisting of 2 *C. gigas* were prepared but adults were analyzed individually. Oysters from the southern location (Cádiz) were all individually analyzed.

OsHV-1 specific PCR analysis of the 275 oyster samples (3 batches) collected from Asturias was performed using two primer pairs. The first one, C2/C6, amplified a 709 bp fragment (Renault and Arzul, 2001) and the second one, CF/CR, proposed for differentiating OsHV-1μvar, amplified a 157 bp of OsHV-1μvar and 173 bp of OsHV-1 (EU Commission Regulation 175/2010).

PCR analysis was carried out in a final volume of 25 μl containing 2.5 U of Taq DNA polymerase (Roche Diagnostic S.L), 10 x reaction buffer (Roche Diagnostic S.L), 2.5 mM MgCl₂ 0.05 mM of each dNTP, 10 μM of each primer and 1 μl of DNA. The PCRs were carried out with a denaturing step of 95°C for 5 min followed by 35 cycles of 95°C 1 min, 50°C 1 min, 72°C 1 min and by a final elongation step of 10 min at 72°C. Negative controls consisted of 1 μl of distilled water. Plasmid OsHV-1 reference DNA kindly supplied by the European Union Reference Laboratory for Mollusc Diseases (Ifremer, La Tremblade, France) was also amplified as positive control. PCR products were resolved in 2% agarose in TAE buffer gels (w/v), stained with ethidium bromide. A 100 bp ladder was
used as a size marker (Invitrogen Life Technologies™).

The C2/C6 amplified PCR products were ligated into pGEM-T easy vector by T/A cloning following the manufacturer’s instructions and transformed into *E. coli* One Shot TOP 10F’ Chemically Competent cells (Invitrogen Life Technologies™). Transformed cells were screened by PCR and products of five positive clones were sequenced twice from both ends. RFLP analysis was conducted with C2/C6 PCR products which were digested with 1 μl of FastDigest® MfeI (MunI) restriction enzyme (Fermentas Inc.) for 5 min in a final reaction volume of 30 μl at 37ºC. Digested products were analysed on 2 % agarose in TAE buffer gels (w/v).

Herpes virus was not detected in oyster samples of 20-48 mm size collected from Asturias. However, in the batch of oysters of 15-20 mm, 1/50 was positive. A band of approximately 700 bp was detected when the amplification was conducted with the primer set C2/C6 (Figure 1A) and a band of approximately 160 bp was detected with the pair of primers CF/CR (Figure 1b). Herpes virus was not detected from the adult oysters sampled in December. All sequences corresponding to the amplifications with primers C2/C6 (PCR products and clones) were identical to each other but showed differences (nucleotide additions, several substitutions and deletions) to the sequence of the OsHv-1 reference isolate (Figure 2). However, the sequence data indicates that the sequence of 695 bp obtained from a sample collected from Asturias corresponded to the new variant OsHV-1μvar (Segarra et al., 2010).

The C2/C6 products and positive clones were digested using the MfeI restriction enzyme. The reference OsHv-1 was not digested by the enzyme because it did not present the target sequence of the MfeI (CAATTG) (Figure 2), however, all the analyzed samples displayed the same restriction profile that consisted of two bands, one of 194 bp and another one of 501 bp (Figure 1C).

**Figure 1.** PCR results of the positive oyster samples. A: Amplification with the C2/C6 primers of reference OsHv-1 (R) and the Spanish sample (1). B: Amplification with the CF/CR primers of reference OsHv-1 (R) and the Spanish sample (1). “r” is the product of the amplification of a cloned OsHv-1μvar product (provided by the European Union Reference Laboratory of Mollusc Diseases, Ifremer, La Tremblade, France). C: Restriction profiles with the enzyme MfeI of the positive PCR product of the Spanish sample (1), of the cloned products (1a, 1b, 1c) or of the reference OsHv-1 (R), amplified with C2/C6 primers. M: 100 bp marker; -: negative control.
In order to test the reliability of the aforementioned rapid PCR method for the differential diagnosis of ostreid herpesvirus-1, analysis of C. gigas from the southern location (Cádiz) was performed using only the C2/C6 pair of primers and MfeI restriction enzyme as previously described. A band of approximately 700 bp was detected in 21/30 samples. A/g286er restriction analysis with MfeI enzyme it was determined that 4/21 samples corresponded to the herpes virus reference biotype and 17/21 samples to the herpes virus μvar biotype. The diagnosis of all samples were confirmed by DNA sequencing (results not included).

There are several different PCR methods available for the detection of OsHV-1, such as conventional PCRs with the primer sets C2-C4, C2-C6 (Batista et al., 2007) as well as real-time methods (Pepin et al., 2008; Martenot et al., 2010; Ren et al., 2010; Schikorski et al., 2011). However, only the sequencing of the products obtained by amplification with primers C2-C6 can be used to identify the OsHV-1μvar strain definitively (Segarra et al., 2010). A new primer pair was published in EU Commission Regulation 175/2010; the CF-CR primers, that could be used to distinguish herpesvirus genotypes based on the different mobility of the amplified products in agarose gel. However, as it can be seen in Figure 1B, the differentiation is not conclusive and can lead to an erroneous interpretation especially when co-infection of two genotypes occurs in the same sample (there is only a difference of 16 bp).

In this study we describe a RFLP analysis using the MfeI restriction enzyme, that distinguishes
between the herpesvirus reference genotype and OsHv-1μvar, and could be used as a preliminary screening tool before sequencing the PCR product. At present, a vast amount of research activity is being focussed on the use of real-time PCRs, however, such PCRs are relatively expensive and they are not available to all laboratories involved in mollusc disease monitoring programmes.

The detection of OsHv-1μvar in Spain, although apparently with a very low prevalence in the northern location, confirms the widespread distribution of OsHv-1μvar infection in European bivalves. This highlights the importance of developing improved, reliable, simple and rapid diagnostic methods that can help in the control of diseases that could be spread by bivalve movements and importations between countries.

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References


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