NOTE

The apparent disappearance of oyster herpes virus type 1 (OsHV-1) from the Ebro Delta, Spain

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Abstract
The Ebro Delta oyster culture industry has a history of repeated mortality episodes. Surveillance to monitor mortality events has determined that OsHV-1 has been replaced by OsHV-1 μvar. The collected data provides evidence suggestive of genetic selection for this new virus strain and its mechanism of thermal induction.

Infections by ostreid herpesvirus (OsHV) in oysters have been documented in various countries and dispersal of the virus is thought to be largely due to transport of live animals for the purpose of cultivation (Arzul et al., 2001a; 2001b; ICES, 2004; Davison et al., 2005; Friedman et al., 2005). Host specificity is not highly restricted, as various species have been documented with herpes infections including Pacific oyster (*Crassostrea gigas*), American oyster (*C. virginica*), Portuguese oyster (*C. angulata*), European flat oyster (*Ostrea edulis*), Australian flat oyster *O. (= Tiostrea) chilensis*, Manila clam (*Ruditapes philippinarum*), carpet shell clam (*R. decussatus*), and scallop (*Pecten maximus*) (ICES, 2004).

Viral genomes are the fastest evolving biological entities due in a large part to the higher error rate of their polymerases (Duffy et al., 2008). A higher error rate inherent in a particular DNA polymerase should make error prone regions of a genome, such as tandem repeats or introns, even more likely to drift over short periods of time. Furthermore, abrupt changes in viral genomes can occur through recombination during co-infections of different strains (Filée, 2013). How these genetic changes affect the progress of the infection and influence the host response will in turn influence to a great degree which genomes are selected and transmitted.

For more than a decade, mortalities of Pacific oysters (*C. gigas*) have been occurring in Catalonia in the Ebro Delta, the most important shellfish culture area in the Spanish Mediterranean; although reports of mortalities associated with OsHV have also been reported from as far north as Ireland (Peeler et al., 2012). At our

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institute, surveillance for ostreid herpesvirus in oysters was increased in 2005 due to recurrent mortality events and a need to better understand the cause of these episodes. Beginning in 2008, Pacific oyster farming areas in very different ecosystems along the entire French coastline experienced greatly increased mortalities. According to reports (Segarra et al., 2010), mortality rates varied from 40% to 100%, and affected mostly juveniles. Coinciding with these mortality events in France, high mortality rates were experienced in the Ebro Delta region where the majority of oyster seed is from France. The precise aetiology for these mortalities remains under investigation.

In the context of this work, we followed the presence of various parasites, some vibrio bacteria, and OsHV over a period of several years. In regards to ostreid herpesvirus in the Ebro Delta region of Catalonia, OsHV-1 was detected for the first time by nested PCR in 2005 at a very low prevalence. Surveillance for the presence of ostreid herpesvirus has been continuous for the past eight years within the framework of different projects. During this time frame, different DNA-based diagnostic methods have been applied: PCR, sequencing, or PCR-RFLP (Aranguren et al., 2012). The ultimate goal of these surveillance measures has been to gain more insight into the aetiology of the mortalities to aid in alleviating them. We present here a brief summary of some of these genetic analyses collected from 2005 to 2013. The objective of this work is to report for the Mediterranean coast of Spain what has been observed for the Atlantic side of the Iberian Peninsula: the apparent disappearance of OsHV-1 and replacement with the µvar strain; and to provide some suggestions for new areas of investigation in regard to the thermal sensitivity of this important oyster pathogen.

Oysters were collected during different seasons of the year as part of three different projects: one regional (OSTROMORT: 2007-2010), one national (HERPEMOL: 2011-2012) and one international (BIVALIFE: 2011-2013). Within each project the sampling schedules had some overlap, but all covered the peak of the mortality events in the spring, as well as pre- and post-mortality periods in their respective years. Sampling was performed in two distinct sites, Fangar Bay and Alfacs Bay, and approximately 2,500 oysters were tested between 2005 and 2013. There is *C. gigas* culture in both bays but it is more dominant in Fangar Bay. Sampling was performed approximately bimonthly, and 30 animals were sampled from each of multiple production sites within each bay. Depending on the project and year the number of sites varied, and details of the epidemiological aspects of this work are currently being analysed (Rodgers et al., in preparation). OsHV DNA surveillance was initiated in 2005 and beginning in 2007 a sampling scheme was implemented to better understand the dynamics of the mortality events. Oyster samples were initially collected from March to September, targeting periods associated with mortality episodes: before (March/April), during (May/June), and after (July-September). The sampling schedule was extended in 2013 to include January and November sample dates to observe more data during cold weather months.

Approximately 50 mg of tissue was collected and subjected to DNA extraction (Qiagen Kit) and quality of extracted DNA was evaluated by spectrophotometry. Aliquots of 50 ng of

DNA were amplified using primers C2 and C6 (Arzul et al., 2001a). They were then analysed further by RFLP using Mfe I (Aranguren et al. 2012) and/or bidirectional sequencing using the same primers as those used in the original amplification (Sistemas Genomicos LLC, Valencia).

During many of the pre and post mortality periods OsHV detection was 0%. The first OsHV-1 positive samples were detected in the Ebro Delta in 2005. The sequences obtained from those samples showed close affinity to the type strain OsHV-1. Subclones of positive PCR amplicons from 2005, 2009, and 2010 were sequenced, but only samples obtained in 2005 revealed diversity within the simple sequence repeat (SSR) region [ACT]n, now described as the H10 microsatellite (Renault et al. 2014). There was a lapse in sampling during 2006. In 2007 no positive OsHV infections were detected although 109 animals were tested. In the subsequent years (2008-2013) it was only the OsHV-1 µvar genotype that was found. We also demonstrated this using the PCR-RFLP as a faster diagnostic assay which discriminates between the two genotypes. This same chronological pattern of progression of sequence types from the H10 region was also observed in a genotyping study of OsHV in France (Renault et al., 2014).

Oyster culture in the Ebro Delta region began on a commercial scale in the 1950's, with a later introduction (1980's) of the Pacific oyster (*C. gigas*). Spring/summer mortalities have been a problem in many regions of the world (Degremont et al., 2010). In the Mediterranean, summer is typically a period of stress for these animals because of high water temperatures, reduced oxygen levels, and a high potential for toxic algae blooms. These multiple stressors make aetiology unclear. However, after implementing more detailed surveillance methods in 2005 for detection of viral DNA, the ostreid herpesvirus type strain was detected, but at a very low prevalence. The severity of the mortality episodes became notably more severe beginning in 2008 (Roque et al., 2012), and coincided with extreme mortality rates throughout the French littoral region (Segarra et al., 2010; Martenot et al., 2011). Although our data set is lacking samples for 2006, the only genotype for the ORF 4 region of OsHV-1 detectable since 2008 has been that of microvar as observed previously in France between 2008 and 2010 (Martenot et al., 2011). It is also an observation somewhat remarkable when considering that in another study Renault and colleagues (2012) found ORF 4 to be the most polymorphic region analysed among a sample group of clinical isolates.

The microvar strain of OsHV-1 is characterised in part by several mutations near the open reading frame 4 (ORF 4) in the C region of the genome (Segarra et al., 2010) (Figure 1). The inability to detect the type strain (OsHV-1) in the past eight years may be due to natural genetic drift, recombination, or the *de novo* introduction of the µvar strain, which has displaced the previously existing type-strain. Regardless of the cause, the stability of the copy number within the H10 region and conservation of the flanking indel mutations is interesting in that it suggests this is an area of the genome that is now under some type of positive selection pressure. The [ACT] copy number and flanking indel mutations currently found could alter the 5′ untranslated region (UTR) of ORF 4 in a way which alters thermal stability thereby relieving secondary structure in the 5′ UTR which

may help ribosome scanning for initiation of translation (Babendure et al., 2006). Secondary structure in the 5´UTR, upstream from the ATG start codon, has been shown to have a significant impact on the efficiency of ribosome recruitment and translation initiation (Babendure et al., 2006; Yun and Sherman, 1995). As oysters are poikilothermic animals thermal stability of the 5´UTR of any transcript are likely to be directly influenced by changing environmental conditions. The induction of OsHV-1 μvar replication seems to have a threshold for thermal activation when water temperatures approach 16ºC (Petton et al., 2013) which occurs in May-June in this part of the Mediterranean coinciding with mortalities. The timing of virus induction is somewhat later in Ireland, in July, as might be expected for more northern latitudes (Peeler et al., 2012).

In studies of OsHV-1 infections in California in 2002 and 2003 using PCR detection Friedman and coauthors (Friedman et al., 2005) found the primer pair C1/C6 did not amplify viral DNA while C2/C6 did amplify the target sequence. This implies a deletion in the upstream region from ORF 4 whose coding sequence originates quite near the C2 primer binding site (Figure 1). The deletions upstream of the C2/C6 region in strains of OsHV-1 observed in France (Arzul et al., 2001a) and California (Friedman et al., 2005) may have acted to shorten the distance between the cap site of the mRNA and the ATG start codon (Figure 1) thereby enhancing translation efficiency, as seen in deletion mutation studies in yeast (Yun et al., 1995). Also of possible relevance are the observed indels flanking the SSR region upstream of the translation initiation codon. Any deletion may influence secondary structure of the 5´ UTR and/or thereby the positioning of the ribosome. Additionally, a BLAST analysis of the coding sequence of ORF 4 indicates it contains a conserved protein domain for a DNA polymerase (although it does not encode the viral DNA polymerase), suggesting a function involving binding of nucleic acid and virus replication. In recent work on the transcriptome of the virus during experimental infection, ORF 4 was shown to be highly expressed early in the infection cycle in susceptible oyster families (Segarra et al., 2014) which is in accordance with this idea but the precise function of this gene is still unknown.

While the mechanism of gene regulation and the overall control of viral thermal induction presented in this note is speculation, it is based on analysis of observed genetic changes and these ideas fit within well-supported previously described genetic regulatory mechanisms; it also suggests an interesting area of investigation into the physical mechanisms of gene activation for this virus. When the variation of the H10 microsat region seen in previous years is taken into consideration, it appears very likely that this hypervariable region is now under some type of selective pressure. Although absence of proof is not proof of absence, it appears that OsHV-1 has disappeared from the Ebro Delta. Further research should be conducted to understand more clearly the gene regulatory mechanisms controlling the thermal induction of replication for this virus, and to understand fully the significance of this conserved region of the OsHV-1 genome. Site-directed mutagenesis together with in vitro transcriptional analyses may be helpful in understanding more specifically the functional regulation of this genomic region and its influence on the thermal sensitivity described for this virus.

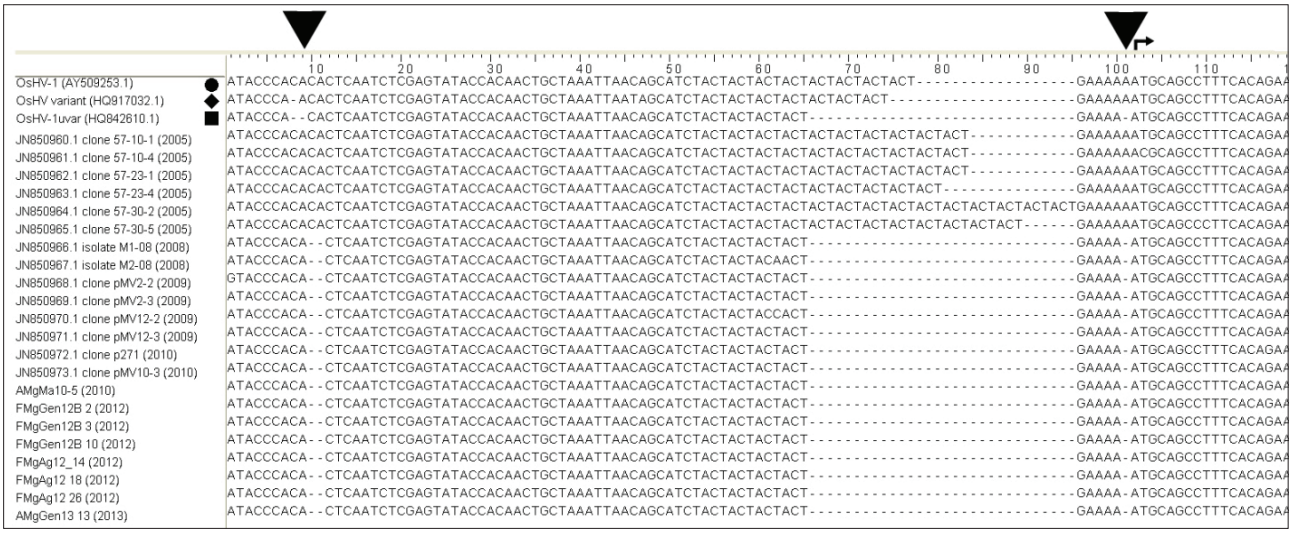

Figure 1. Examples of sequence data from infected *C. gigas* samples from various lots collected between 2005 - 2013. Data show a sudden change in sequence that became fixed after 2008. The top three sequences are published sequences (GenBank) from the type strain OsHV-1 (●), OsHV-1 variant (◆) and the OsHV µvar (■) which are shown for comparison. Arrowheads indicate conserved indel mutations flanking the SSR region. Small arrow in upper right indicates the location of the start codon for open reading frame 4.

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