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In situ localization and tissue distribution of ostreid herpesvirus 1 proteins in infected Pacific oyster, *Crassostrea gigas*

Claire Martenot [a,*], Amélie Segarra [a], Laury Baillon [a], Nicole Faury [a], Maryline Houssin [c], Tristan Renault [b]

[a] *Ifremer (Institut Français de Recherche pour l'Exploitation de la Mer), Laboratoire de Génétique et Pathologie des Mollusques Marins, La Tremblade, France*
[b] *Ifremer, Département Ressources Biologiques et Environnement, Nantes, France*
[c] *LABEO Frank Duncombe, Caen, France*

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ABSTRACT

Immunohistochemistry (IHC) assays were conducted on paraffin sections from experimentally infected spat and unchallenged spat produced in hatchery to determine the tissue distribution of three viral proteins within the Pacific oyster, *Crassostrea gigas*. Polyclonal antibodies were produced from recombinant proteins corresponding to two putative membrane proteins and one putative apoptosis inhibitor encoded by ORF 25, 72, and 87, respectively. Results were then compared to those obtained by *in situ* hybridization performed on the same individuals, and showed a substantial agreement according to Landis and Koch numeric scale. Positive signals were mainly observed in connective tissue of gills, mantle, adductor muscle, heart, digestive gland, labial palps, and gonads of infected spat. Positive signals were also reported in digestive epithelia. However, few positive signals were also observed in healthy appearing oysters (unchallenged spat) and could be due to virus persistence after a primary infection.

Cellular localization of staining seemed to be linked to the function of the viral protein targeted. A nucleus staining was preferentially observed with antibodies targeting the putative apoptosis inhibitor protein whereas a cytoplasmic localization was obtained using antibodies recognizing putative membrane proteins. The detection of viral proteins was often associated with histopathological changes previously reported during OsHV-1 infection by histology and transmission electron microscopy. Within the 6 h after viral suspension injection, positive signals were almost at the maximal level with the three antibodies and all studied organs appeared infected at 28 h post viral injection. Connective tissue appeared to be a privileged site for OsHV-1 replication even if positive signals were observed in the epithelium cells of different organs which may be interpreted as a hypothetical portal of entry or release for the virus. IHC constitutes a suited method for analyzing the early infection stages of OsHV-1 infection and a useful tool to investigate interactions between OsHV-1 and its host at a protein level.

1. Introduction

For several decades, mass mortality outbreaks of the Pacific oyster, *Crassostrea gigas*, have been regularly reported throughout the world and consequently dramatic losses in production were recorded, generating a decline in the oyster farming industry. Spat mortality events are most often associated with the detection of ostreid herpesvirus 1 (OsHV-1) which is the type species within the genus *Ostreavirus* and the *Malacoherpesviridae* family (Davison et al., 2009, 2005; Le Deuff and Renault, 1999). From 2008 onwards, OsHV-1 variants had been reported and a variant called µVar was mainly detected along the French coast (Martenot et al., 2012, 2011; Renault et al., 2014, 2012; Segarra et al., 2010). The variant µVar is characterized by 26 mutations in two regions of the viral genome: the C region (ORF 4/5), and an IAP region (ORF 42/43) as reported by Segarra et al. (2010). OsHV-1 reference type and variants have been detected in France (Le Deuff and Renault, 1999; Nicolas et al., 1992; Renault and Lipart, 1998), Ireland (Clegg et al., 2014; Lynch et al., 2012; Peeler et al., 2012), Spain (Aranguren et al., 2012; Da Silva et al., 2008; Roque et al., 2012), Italy (Domeneghetti et al., 2014; Dundon et al., 2011), Portugal (Batista et al., 2014), South Korea (Hwang et al.,

* Corresponding author at: Avenue de Mus de Loup, 17390 La Tremblade, France.
E-mail address: claire.martenot@ifremer.fr (C. Martenot).

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2013; Jee et al., 2013), United States (Burge et al., 2011, 2006; Friedman et al., 2005), Mexico (Grijalva-Chon et al., 2013), Australia (Jenkins et al., 2013; Paul-Pont et al., 2014, 2013a,b), and New Zealand (Bingham et al., 2013; Keeling et al., 2014; Renault et al., 2012; Whittington et al., 2015) during mortality outbreaks or without oyster mortality. Juveniles (less than 18 months old) were more impacted by mortality associated with OsHV-1 detection than older ones (oysters more than 18 months) (Francois et al., 2009; Renault, 2006) even if OsHV-1 DNA or proteins were found at different stages of development (Arzul et al., 2002; Martenot et al., 2012, 2011). OsHV-1 DNA was also detected in different oyster species (Comps and Cochennec, 1993; Hine et al., 1998; Hine and Thorne, 1997), clams (Renault et al., 2001a,b), and scallops (Arzul et al., 2001), suggesting a broad host range, such as with pseudorabies virus (Aujeszky's disease) (Flamand et al., 1998).

Several molecular diagnostic assays were developed to screen for OsHV-1 including real-time PCR based on TaqMan® and SYBR® Green chemistry (Burge et al., 2011; Martenot et al., 2010; Pepin et al., 2008; Webb et al., 2007), conventional PCR (Arzul et al., 2002; Barbosa-Solomieu et al., 2004; Batista et al., 2007; Renault et al., 2000), real-time reverse transcription PCR (Burge and Friedman, 2012; Martenot et al., 2015; Segarra et al., 2014), propidium monoazide (PMA) real-time PCR (Moreau et al., 2015), and *in situ* hybridization (ISH) (Arzul et al., 2002; Corbeil et al., 2015; Lipart and Renault, 2002). Histology associated to ISH or immunohistochemistry (IHC) assay allows studying the virus distribution in numerous organs of the same animal based on positive signals detection albeit this approach is longer than PCR. In addition, histological changes are subtle and difficult to detect in OsHV-1 infections and consequently ISH and IHC are powerful approaches to better understand this disease. Transcriptomic approaches (Jouaux et al., 2013; Rosani et al., 2014; Segarra et al., 2014) were conducted to better understand the viral cycle, especially the gene expression sequence (Segarra et al., 2014) but there are no data related to the viral protein expression and distribution during an experimental infection in seed and juvenile oysters. Previously, Arzul et al. (2002) detected OsHV-1 proteins in cells localized in connective tissue of gills, mantle, adductor muscle, and the gonad in asymptomatic *C. gigas* adults using antibodies produced against the entire virus. Only 30% of OsHV-1 proteins have putative function obtained using protein prediction software and 70% of the OsHV-1 genome correspond to proteins of unknown function presenting no homology with protein sequences found in biological data banks (i.e. Genbank).

This study details the first time that an IHC assay has been used to localize and describe the distribution of three viral proteins in OsHV-1 infected spat during an experimental infection and in unchallenged spat produced in hatchery. In this context, three polyclonal antibodies were produced by ProteoGenix (Schiltigheim, France) from three recombinant proteins and targeted two putative structural proteins (class I membrane proteins) and one putative apoptosis inhibitor containing a "baculovirus inhibition of apoptosis protein repeat" (BIR) domain, encoded by ORF 25, 72, and 87, respectively. IHC results were then compared with those obtained by *in situ* hybridization (ISH) performed on the same oysters targeting ORF4/5 region encoding two proteins of unknown function (Corbeil et al., 2015; Lipart and Renault, 2002). The comparison between methods of virus detection at the DNA, RNA, and protein level, and the analysis of immunohistochemistry results by a biometric method called Landis and Koch numeric scale constitutes an innovative approach. In addition, the present study constitutes the first report of the viral protein detection at different times post OsHV-1 infection in controlled conditions with three different polyclonal antibodies within same individuals.

2. Materials and methods

2.1. Biological material

The biological material selected for the present study, was previously used to describe the tissue distribution of OsHV-1 RNA and DNA during a viral challenge by *in situ* hybridization (Segarra et al., 2016) in order to realize a complementary and global approach on the same oysters at different expression level. The spat used were hatchery-produced, nine months old and measured around three centimeters (Segarra et al., 2016). The DNA and the RNA probes targeted the C region (ORF 4 encoding a protein of unknown function) and the ORF 87 (encoding a putative apoptosis inhibitor), respectively (Segarra et al., 2016). These data were compared with those obtained in the present work by IHC to investigate the specificity of antibodies.

A viral suspension prepared from experimentally infected oysters, was injected in the adductor muscle of C. gigas spat (10^8 viral DNA copies per oyster). All oysters received thus the same amount of OsHV-1 DNA at the same time (Schikorski et al., 2011a,b). Six spat were collected at 2 h, 6 h, and 28 h post viral injection for further analysis.

C. gigas spat (NSI 01/15) produced in 2014 at the Ifremer hatchery located in Argenton (Brittany, France) and then reared at the Ifremer facilities in Bouin (Vendée, France) were used as unchallenged control for immunohistochemistry and the *in situ* hybridization assays.

2.2. Polyclonal antibody production

Two putative membrane proteins encoded by ORF 25 and ORF 72 and one putative apoptosis inhibitor encoded by ORF 87, were selected to analyze the tissue distribution of viral proteins during an experimental infection. Based on a protein sequence analysis, the two first proteins might correspond to structural proteins located on the surface of the viral envelope, and therefore play a key role in the interaction between the virus and the host. This interaction especially occured in the earliest stage of infection during the attachment and the entry of the virus into host cell. The second targeted viral protein was a putative apoptosis inhibitor which might alter host immune response. Segarra et al. (2014) showed that mRNA corresponding to these three ORFs were expressed at high levels by 28 h post viral challenge and we hypothesized that the proteins might be strongly produced, increasing the ability to detect them. Moreover, these mRNA were expressed early during OsHV-1 infection in experimental conditions.

Polyclonal antibodies targeting proteins encoded by ORFs 25, 72, and 87 were produced by ProteoGenix (Schiltigheim, France). Briefly, the partial cDNA of each ORF was cloned in pET-43.1a vector in order to express the protein with His tag in N-terminal position (cloning strategy: NdeI/XhoI). After purification of the three recombinant proteins, each one was then injected to two different rabbits. The post-immune sera of the six rabbits were individually collected and polyclonal antibodies were separately purified using protein A affinity chromatography (ProteoGenix).

2.3. Antibody treatment using proteins extracted from non-challenged oysters

Polyclonal antibodies against viral proteins were treated using proteins extracted from non-challenged oysters (considered as non-infected animals) to reduce non-specific epitope interactions, i.e. binding of primary antibodies to oyster proteins. All oyster tissues were mashed with TRIS EDTA buffer pH 8.3 and 10 µL