Journal of Invertebrate Pathology 119 (2014) 5–11

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Transcriptomic study of 39 ostreid herpesvirus 1 genes during an experimental infection

Amélie Segarra [a], Nicole Faury [a], Jean-François Pépin [b], Tristan Renault [a,*]

[a] Ifremer (Institut Français de Recherche pour l'Exploitation de la Mer), Unité Santé, Génétique et Microbiologie des Mollusques (SG2M), Laboratoire de Génétique et Pathologie des Mollusques Marins (LGPMM), Avenue de Mus de Loup, 17390 La Tremblade, France
[b] Ifremer, Laboratoire Environnement Ressources des Pertuis Charentais (LERPC), Avenue de Mus de Loup, 17390 La Tremblade, France

ARTICLE INFO

Article history:
Received 20 December 2013
Accepted 18 March 2014
Available online 27 March 2014

Keywords:
Crassostrea gigas
OsHV-1
Viral gene expression
Inhibitors of apoptosis
Real time PCR
Elongation factor

ABSTRACT

Massive mortality outbreaks have been reported in France since 2008 among Pacific oysters, *Crassostrea gigas*, with the detection of a particular OsHV-1 variant called µVar. Virus infection can be induced in healthy spat in experimental conditions allowing to better understand the disease process, including viral gene expression.

Although gene expression of other herpesviruses has been widely studied, we provide the first study following viral gene expression of OsHV-1 over time. In this context, an *in vivo* transcriptomic study targeting 39 OsHV-1 genes was carried out during an experimental infection of Pacific oyster spat. For the first time, several OsHV-1 mRNAs were detected by real-time PCR at 0 h, 2 h, 4 h, 18 h, 26 h and 42 h post-injection. Several transcripts were detected at 2 h post-infection and at 18 h post-infection for all selected ORFs. Quantification of virus gene expression at different times of infection was also carried out using an oyster housekeeping gene, Elongation factor.

Developing an OsHV-1-specific reverse transcriptase real time PCR targeting 39 viral gene appears a new tool in terms of diagnosis and can be used to complement viral DNA detection in order to monitor viral replication.

1. Introduction

Herpesviruses are widely distributed among vertebrates and have been also reported in invertebrates. A herpesvirus, called ostreid herpesvirus 1 (OsHV-1), has been identified in different bivalve species causing massive mortality outbreaks especially in Pacific oyster, *Crassostrea gigas,* larvae and spat (Garcia et al., 2011; Segarra et al., 2010; Arzul et al., 2001; Renault et al., 1994). In this context, tools have been developed such as immunohistochemistry (Le Deuff et al., 1995), *in situ* hybridization (Lipart and Renault, 2002; Renault and Lipart, 1998), polymerase chain reaction (Renault et al., 2000; Renault and Lipart, 1998) and quantitative polymerase chain reaction (Pepin et al., 2008) in order to detect OsHV-1. The virus genome was completely sequenced (GenBank accession number AY509253) and encodes at least 124 genes (Davison et al., 2005). However, more than 70% genes encode putative proteins presenting no homology with proteins from databases (Davison et al., 2005).

Many *in vitro* studies have been performed in order to define virus gene transcription for several mammalian herpesviruses using chemicals including cyclohemixide, as inhibitors of gene expression (Rubins et al., 2008; Honess and Roizman, 1975, 1974). Currently, no bivalve cell lines are available and no *in vitro* studies of OsHV-1 gene expression can be performed. Despite these constraints, 2 studies reported OsHV-1 gene expression targeting a single virus gene, ORF4 (protein of unknown function) (Renault et al., 2011) or ORF100 (DNA polymerase) (Burge and Friedman, 2012).

Recently, techniques including reverse transcription real time PCR, microarray or RNAseq have been used to study virus gene expression (Beurden et al., 2013; Rossetto et al., 2013; Tombácz et al., 2009; Rubins et al., 2008). In view of the low number of genes encoded by the virus, we selected the RT-qPCR technique to confirm OsHV-1 mRNA presence. For the first time, 39 OsHV-1 mRNAs were studied and detected in Pacific oyster spat during an experimental infection (0, 2, 4, 18, 26, and 42 h post-injection). Relative expression was also performed on viral genes detected at all times. Thus, 8 viral genes were analyzed using a host housekeeping gene. For this purpose, the stability of four commonly used housekeeping genes in real time quantitative gene expression studies was first determined in *C. gigas* spat challenged by OsHV-1.

* Corresponding author. Fax: +33 5 46 76 26 11.
E-mail address: Tristan.Renault@ifremer.fr (T. Renault).

http://dx.doi.org/10.1016/j.jip.2014.03.002
0022-2011/

2. Materials and methods

2.1. Animals

A pool of Pacific oysters *C. gigas* 7 month old was used in the present study. Oysters were produced in March 2011 at Ifremer facilities in La Tremblade (Charente Maritime, France) during the course of the Bivalife EU funded project (FP7, No. 266157, 2011–2014).

2.2. Defining mortality kinetics in experimentally infected Pacific oysters

Forty oysters were "anaesthetized" during 4 h in a solution containing magnesium chloride (MgCl2, 50 g/L) in seawater (1 v)/distilled water (4 v) (Namba et al., 1995). In order to perform a maximum of sampling the first day post-infection and before mortality, several dilutions of a viral suspension at 1.5×10^6 copies of viral DNA/μL were tested: 1.5×10^6, 5×10^4 (1/25) or 1.2×10^3 (1/125) copies of viral DNA/μL. Based on mortality records (data not shown), 100 μL of a OsHV-1 (μVar genotype, (Segarra et al., 2010)) suspension at 1.2×10^3 copies of viral DNA/μL were injected into the adductor muscle. Then, of 20 oysters using a 1 mL syringe (Schikorski et al., 2011) and placed in a tank with 5 L of filtered seawater (1 μm) at 22 °C. The bacterial content of the viral suspension was tested before each challenge by plating on Marine agar. A negative control consisted of 20 oysters intra-muscularly injected with 100 μL of sterile artificial seawater and placed in another tank.

Mortality was monitored during 90 h after injection and percentages of cumulative mortality were daily defined for both conditions (oysters infected with OsHV-1 or injected with sterile artificial seawater). Dead oysters were removed from tanks during the time course of the experiment. The experiment was performed successively 3 times.

2.3. Studying housekeeping genes and virus gene expression in OsHV-1 experimentally infected Pacific oysters: experimental design

One hundred and twenty oysters were first "anesthetized" during 4 h as previously described. One hundred μL of viral suspension were injected into the adductor muscle of 60 oysters. A negative control consisted of 60 oysters intra-muscularly injected with 100 μL of sterile artificial seawater. Sixty negative control oysters were then randomly distributed in 3 tanks and 60 experimentally infected oysters placed in 3 other tanks supplied with 5 L of filtered seawater (1 μm) at 22 °C.

Several sampling times were determined based on previously defined mortality kinetics after experimental virus infection. The sampling points were 0 h, 2 h, 4 h, 18 h and 26 h post-injection before mortality occurred and at 42 h post-injection during mortality outbreak. At each time and for each condition, 3 oysters were collected in each tank (9 individuals per time/condition) and 2 pieces of mantle were sampled from each individual. A piece of mantle (50–100 mg) was disposed in a tube containing 1 mL of TRIZOL® Reagent™ (Ambion®) and frozen at −80 °C for further RNA extraction. Another piece of mantle was directly frozen at -20 °C for further total DNA extraction. Mantle was selected in the present study for real time RT PCR analysis as this organ has already been showed as a site of interest for viral DNA detection (Schikorski et al., 2011; Sauvage et al., 2009; Arzul et al., 2002).

2.4. DNA extraction

Total DNA extraction was performed from a mantle fragment from each collected sample. The DNA extraction was performed with QiAamp tissue mini kit® (QIAgen) according to the manufacturer's protocol. Elution was performed in 100 μL of buffer AE provided in the kit.

2.5. Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRIZOL® Reagent™ (Ambion®) according to the manufacturer's recommendation. Total RNA was treated with Turbo™ DNAse (Ambion®) to remove genomic DNA. The RNA quality and quantity were determined using NanoDrop 2000 (Thermo Scientific) and Bioanalyser 2100 (Agilent). First-strand cDNA synthesis was carried out using the SuperScript ® III First-Strand Synthesis System (Invitrogen) using 8000 ng of RNA treated. A No RT was performed after each DNAse treatment using real time PCR in order to control absence of oyster and/or virus genomic DNA.

2.6. Real time PCRs and relative expression

Real time quantitative PCR was performed in duplicate using a Mx3000 Thermocycler sequence detector (Agilent). All forward and reverse primers used in the present study were designed using primer3 software (Koressaar and Remm, 2007; Untergasser et al., 2012) and synthesised by Eurogentec. Using the same biological material the detection and quantification of OsHV-1 DNA was first carried out using a previously published real time PCR protocol (Pepin et al., 2008). In a second step, real time quantitative RT PCR was used in order (i) to select and validate a suited oyster housekeeping gene from *C. gigas* spat during OsHV-1 experimental infection and (ii) to study viral gene expression. Amplification reactions were performed in a total volume of 20 μL to study oyster housekeeping genes and viral gene expression. Each well contained 5 μL of cDNA dilution (1/30), 10 μL of Brillant® SYBR® Green III PCR Master Mix (Agilent), 2 μL of each primer (3 μM) and 1 μL of distilled water. Real time PCR cycling conditions were as follow: 3 min at 95 °C, followed by 40 cycles of amplification at 95 °C for 5 s, 60 °C for 20 s. Melting curves were also plotted (55–95 °C) in order to ensure that a single PCR product was amplified for each set of primers. In all cases negative controls (without cDNA) were included to rule out DNA contamination.

Concerning selection of a suited oyster housekeeping gene, the expressed sequence tags (EST) of 4 candidate genes were obtained from a subtracted cDNA library of *C. gigas* and four primer pairs were designed (Table 1). Individual samples of infected or non-infected oyster spat were collected and analyzed at 0 h, 2 h, 4 h, 18 h, 26 h and 42 h post-infection.

To study virus gene expression, 39 genes were selected based on protein functions or structures of related proteins among the 124 ORFs of OsHV-1 (Davison et al., 2005) and belongs to 5 groups/families of genes (Fig. 1). (i) The first group consisted of 7 ORFs encode unknown proteins (ORF4, ORF43, ORF47, ORF57, ORF64, ORF81 and ORF86) whose 2 ORFs already used to differentiate virus specimens (Renault et al., 2012; Segarra et al., 2010), (ii) 10 genes encoding enzymes or proteins presenting known viral domains (ORF7, ORF20, ORF24, ORF27, ORF34, ORF49, ORF67, ORF75, ORF100 and ORF109), (iii) one family of Ring-finger genes with 8 members (ORF9, ORF38, ORF53, ORF96, ORF97, ORF117, ORF118, ORF124), (iv) one family of genes predicted to encode membrane proteins (with 10 members: ORF16, ORF25, ORF41, ORF54, ORF68, ORF72, ORF77, ORF84, ORF103 and ORF111), (v) one family whose products were related to inhibitors of apoptosis (four members containing BIR domains: ORF42, ORF87, ORF99, ORF106)