Journal of Invertebrate Pathology
November 2017, Volume 150, Pages 45-53
http://dx.doi.org/10.1016/j.jip.2017.09.007
http://archimer.ifremer.fr/doc/00399/50997/

Haemocytes from *Crassostrea gigas* and OsHV-1: A promising *in vitro* system to study host/virus interactions

Morga Benjamin [1, *], Faury Nicole [1], Guesdon Stephane [2], Chollet Bruno [1], Renault Tristan [3]

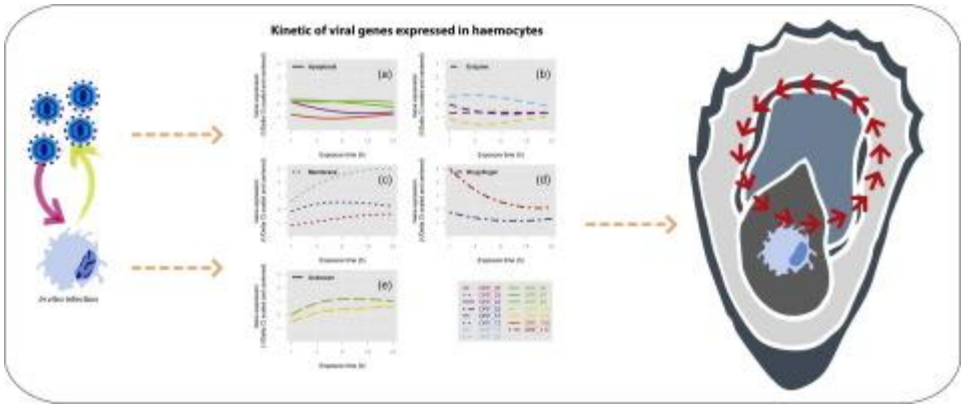
[1] Ifremer, RBE-SG2M-LGPMM, Station de La Tremblade, Avenue de Mus de Loup, F-17390 La Tremblade, France
[2] Ifremer, ODE-LERPC, Station de La Tremblade, Avenue de Mus de Loup, F-17390 La Tremblade, France
[3] Ifremer,PDG-RBE, Centre de Nantes, Rue de l'Ile d'Yeu, F-44311 Nantes, France

* Corresponding author : Benjamin Morga, email address : benjamin.morga@ifremer.fr

Abstract :

Since 2008, mass mortality outbreaks associated with the detection of particular variants of OsHV-1 have been reported in *Crassostrea gigas* spat and juveniles in several countries. Recent studies have reported information on viral replication during experimental infection. Viral DNA and RNA were also detected in the haemolymph and haemocytes suggesting that the virus could circulate through the circulatory system. However, it is unknown if the virus is free in the haemolymph, passively associated at the surface of haemocytes, or able to infect and replicate inside these cells inducing (or not) virion production. In the present study, we collected haemocytes from the haemolymphatic sinus of the adductor muscle of healthy *C. gigas* spat and exposed them *in vitro* to a viral suspension. Results showed that viral RNAs were detectable one hour after contact and the number of virus transcripts increased over time in association with an increase of viral DNA detection. These results suggested that the virus is able to initiate replication rapidly inside haemocytes maintained *in vitro*. These *in vitro* trials were also used to carry out a dual transcriptomic study. We analyzed concomitantly the expression of some host immune genes and 15 viral genes. Results showed an up regulation of oyster genes currently studied during OsHV-1 infection. Additionally, transmission electron microscopy examination was carried out and did not allow the detection of viral particles. Moreover, All the results suggested that the *in vitro* model using haemocytes can be valuable for providing new perspective on virus-oyster interactions.

Graphical abstract



Highlights

► This study is the first to show that OsHV-1 can initiate viral replication in haemocytes following an *in vitro* contact. ► The results showed that there were differential patterns of expression of viral genes over time. ► Our results open new ways of research in order to explore OsHV-1 pathogenesis. ► Haemocytes are critical to innate immunity and the virus evolved to perturb host defence.

I Introduction

Ostreid herpesvirus type 1 (OsHV-1), the causative agent of a disease producing major economic losses in the global Pacific oyster industry, is a member of the family *Malacoherpesviridae* from the order *Herpesvirales* (Le Deuff and Renault, 1999; Davison et al., 2005, Davison et al., 2009). OsHV-1 is a large enveloped virus that infects several bivalve species (Ostrea edulis, Pecten maximus, Ruditapes philippinarum) (Arzul et al., 2001a, Arzul et al., 2001b, Renault et al., 2001). Two OsHV-1 genotypes were initially reported in France, the reference type and a variant referred to as OsHV-1 Var (Arzul et al., 2001b and Renault et al., 2001). The presence of a third genotype, termed µVar (for "microvariant"), has been reported in France since 2008 in association with mass mortality outbreaks among French and European *C. gigas* (Segarra et al., 2010, EFSA 2010). Additional microvariants have been also reported since 2010 in New Zealand and Australia during mass mortality events affecting the Pacific oyster there (Jenkins et al., 2013; Keeling et al., 2014; Paul-Pont et al., 2013, 2014 ; Renault et al., 2012),

Control of OsHV-1 infection is considered as a key element to maintain the competitiveness and to increase the sustainability of the oyster industry. However, Pacific oysters like other marine molluscs present unique challenges in terms of health management (Renault, 2012). Strategies like vaccination that are currently used for other farmed animal species such as cattle and fish cannot be directly applied to Pacific oysters to protect them against the viral infection. A better understanding of interactions between the Pacific oyster and the virus may, however, reveal applicable and promising approaches to limit the harmful effects of these pathogens.

Early work with experimental infections results suggested that infective OsHV-1 particles could first enter the oyster host thought the digestive gland and the haemolymphatic system (Schikorski et al., 2011a). Virus particles could then be transported by haemolymph to the different organs before beginning an intense replication phase producing development of associated disease in target tissues

including mantle and gills (Schikorski et al 2011a, Corbeil et al., 2015, Segarra et al, 2016). These results opened new perspectives to study virus-oyster interactions.

Segarra et al. (2014a) recently developed a new tool to study the viral replication using a real time RT-PCR. They monitored expression of 39 OsHV-1 mRNAs in Pacific oyster spat (in mantle as a well-identified target organ) during an experimental infection at 0, 2, 4, 18, 26, and 42 h post-injection. As early as 2h post-injection some OsHV-1 mRNAs were detected and by 18 h post-infection all selected ORFs were expressed. Additionally, Corbeil et al (2015) developed an in situ hybridization technique allowing the detection of viral mRNA on histological sections and demonstrated the presence of viral mRNA in different tissues including the mantle.

Although haemocytes (the circulating cells present in haemolymph) play a key role in the immune response of molluscs (Fisher, 1986), few studies have investigated the immune response of *C. gigas* haemocytes against the virus OsHV-1 (Renault et al, 2011). Renault et al. (2011) explored the the haemocyte's response after *in vitro* contact with OsHV-1 using suppressive substractive hybridization in order to identify candidate genes involved in the host response to the viral infection. That study identified for the first time genes that could be involved in an interferon-like pathway, such as the IFI44 gene. Results were confirmed in various following studies (Green et al., 2015, Segarra et al., 2015). Another study found haemolymph from *C. gigas* to possess antiviral activity associated with the major haemolymph protein, cavortin (Green et al., 2014a, Green et al., 2014b). These data collectively show that the haemolymph and the haemocytes play a key role in the OsHV-1 infection. However, no study has yet directly investigated the replication of the virus in the haemocytes from *C. gigas*.

The main objective of the present study was to provide knowledge on OsHV-1 entry into haemocytes and viral replication in such cells after a viral challenge *in vitro* in haemolymph drawn from the adductor muscle of *C. gigas* spat. The kinetics of viral gene expression were also assessed, as already reported for vertebrate herpesviruses, with examination of early, intermediate early and late gene

expression. Transmission electron microscopy examination was used to search the *in vitro*-challenged haemocytes for the presence of viral particles.

This use of hemocytes *in vitro* experiment represents a novel approach to the study of an important contemporary problem in oyster health, and our study yielded fresh insight into the nature of the interaction between the virus and its oyster host. The objective of this work is to demonstrate whether haemocytes can be infected with the OsHV-1 virus.

2 Material and methods

2.1 Pacific cupped oysters

Pacific cupped oysters, *C. gigas*, were produced in March 2014 at Ifremer hatchery in Argenton, Brittany, France. Oysters were then reared at Ifremer facilities in Bouin, Vendée, France until September 2014 when the study began. Oysters 9 months old with an average size of 5 cm were used for this study.

2. 2. Haemolymph collection

Haemolymph was withdrawn from the adductor muscle sinus of oysters using a 1-ml syringe equipped with a needle (0.40 mm x 90 mm). To eliminate debris the haemolymph samples were filtered through 60-µm nylon mesh and held on ice to limit cell aggregation. The volume of haemolymph collected from each oyster was approximately 0.5 to 1 mL. Samples were pooled, and haemocyte counts were performed using a Malassez cell. Haemocyte concentration was adjusted to 1.10^6 cells ml^{-1} using 0.22 µm-filtered artificial sea water.

2.3. OsHV-1 suspension

Initial tissue homogenates were prepared using ten experimentally infected animals as previously reported (Schikorski et al., 2011a). Oysters were opened by removing the upper valve. Gills and mantle of these animals were then dissected and pooled together in a 50-mL sterile tube. All subsequent dilutions were made using 0.22 µm-filtered artificial seawater (ASW). The total mass of tissues was weighed and 10 volumes of 0.22 µm-filtered ASW were added to the tube (9 mL of seawater per g of tissues). Tissues were then homogenized on ice using an Ultraturax mixer (3 × 5 s). After centrifugation (1000 *g*, 5 min, 4°C), supernatant was placed in a new tube and diluted by addition of 4 volumes of 0.22 µm-filtered ASW. Finally, the clarified tissue homogenate was filtered consecutively in sterile conditions using syringe filters at 5-µm, 2-µm, 0.45-µm and 0.22-µm pore sizes (Millipore, Billerica, USA).

Control, OsHV-1-free tissue homogenate. To produce control, virus-free homogenate, the protocol described above was applied to oysters found to be negative for the presence of OsHV-1 DNA by real time PCR.

2.4. Experimental design

Haemocytes (1×10^6 cells mL^{-1}, 5 mL) were incubated with OsHV-1 suspension (2.5 mL, 10^5 OsHV-1 copies/µL) under low agitation at 19°C and analysed after 1, 4, 8, 18 and 24 h of *in vitro* virus exposure. The control consisted of haemocytes (1×10^6 cells mL^{-1}, 5mL) incubated in presence of oyster tissue homogenate considered to be OsHV-1 free (2.5 mL, see above). The whole experiment was carried out twice in duplicate. To all samples, 350 µL of antibiotic mix was added with 4 mg mL^{-1}

152 streptomycin, 11.6 mg mL^{-1} penicillin, 5.1 mg mL^{-1} neomycin, 3.3 mg mL^{-1} erythromycin, and 0.1µL
153 mL^{-1} nystatin.

154

155 **2.5 DNA extraction from haemocytes**

156 Haemolymph (7.5 mL) was centrifuged for 10 min at 1500 *g*, and DNA was extracted from the
157 haemocyte pellet. DNA extraction was performed using a QIAamp Tissue Mini Kit (QIAgen) according
158 to the manufacturer's protocol.

159

160 **2.6 OsHV-1 DNA quantification**

161 Real time quantitative PCR was performed in duplicate using a Mx3005P Thermocycler sequence
162 detector (Agilent). Amplification reactions were each performed in a total volume of 20 µL. Each well
163 contained 5 µL DNA from sea water or 5 µg DNA total from oyster mantle, 10 µL of Brilliant III Ultra-
164 Fast SYBR®Green PCR Master Mix (Agilent), 2 µL of each primer OsHVDP For (forward) 5'-
165 ATTGATGATGTGGATAATCTGTG-3' and OsHVDP Rev (reverse) 5'-GGTAAATACCATTGGTCTTGTTCC-3'
166 (Webb et al., 2007) at the final concentration of 550 nM each, and 1 µL of distilled water. Real time
167 PCR cycling conditions were as follows: 3 min at 95°C followed by 40 cycles of amplification at 95°C
168 for 5 s and 60°C for 20 s. The results were expressed as a Log10 of virus OsHV-1 copy number of viral
169 DNA/µL of DNA extract.

170 OsHV-1 quantification data were analyzed with XLSTAT-Pro® 2014.5.03 software (Addinsoft; Paris,
171 France). Results were expressed as means ± standard error. A two-way analysis of variance (ANOVA)
172 followed by Fisher post-tests were used to analyze differences between means of virus DNA amounts
173 (DNA copy number) quantified during the kinetic experiment.

174

175 **2.7 Total RNA extraction**

176 Total RNA was extracted using TRIZOL® Reagent™ (Ambion®) according to the manufacturer's
177 recommendations. Total RNA was treated with Turbo™ DNAse (Ambion®) to remove genomic DNA.
178 After DNAse treatment, a second RNA extraction using TRIZOL was carried out. RNA quality and
179 quantity were determined using a NanoDrop 2000 (Thermo Scientific). First-strand cDNA synthesis
180 was performed using the SuperScript® III First-Strand Synthesis System (Invitrogen) with 500 ng of
181 RNA used. A No RT (No Reverse Transcription) was performed after RNA extraction using real time
182 PCR in order to control absence of oyster and/or virus genomic DNA using EF primers (Table 1) and
183 the OsHVDP For/OsHVDP Rev primers.

184

185 **2.8 OsHV-1 gene expression**

186 To study viral gene expression, 15 viral genes were selected based on protein functions or structures
187 of related proteins among the 124 ORFs of OsHV-1 (Davison et al., 2005). These belonged to 5 groups
188 or families of genes. Among genes selected, 11 were previously studied by Segarra et al. (2015a).
189 Four genes were added for this study (Table 1). Real-time quantitative RT PCR was used to study the
190 expression of the 15 viral genes using the previously described protocol with 5 µL of cDNA dilution
191 (1/30) instead of genomic DNA. Elongation factor alpha (EF1 alpha) was chosen to normalize the viral
192 gene expression. The Ct difference between the viral gene and EF1 alpha provided the relative
193 expression level of the viral gene.

194 Viral gene expression levels were calculated for each sample with formula:

195 Delta Ct=Ct ORF-Ct Elongation factor alpha

196 The levels of gene expression (delta Ct) of the initial array data were transformed by the inverse
197 function *1/delta Ct*. This allowed easier interpretation thanks to positive link between this indicator

198 (1/delta Ct) and the level of gene expression. Discrimination of gene expression was based on the
199 results of a principal component analysis (PCA). This analysis was based on the average of replicates
200 per exposure time (centered and reduced). This data configuration by exposure time (case of PCA)
201 allowed determination of the relative increase of the gene expression between treatments for each
202 exposure time. The classification of genes based on their expression at the different exposure times
203 was performed by the k-means method. The choice of the optimal number of groups (clusters) was
204 determined by comparing the sum of squared errors (SSE) for different groups of numbers (clusters)
205 tested. The graphs and statistical analyzes were performed using R software (R development Core
206 Team, 2014).

207

208 **2.9 Immune gene from *C. gigas* gene expression in haemocytes**

209 The relative expression of two genes from *C. gigas* spat was studied during assays. The relative
210 quantification value (ratio R) was calculated using the method described by Pfaffl: $R = [(E_{target})^{\Delta CT\ target(control-sample)}]/[(E_{EF\alpha-1})^{\Delta CT\ EF\alpha-1\ (control-sample)}]$. The efficiency of each primer pair was determined by
212 constructing a standard curve from serial dilutions. These 2 genes from the Pacific oyster were
213 myeloid differentiation factor 88 (MyD88) and interferon-induced protein 44 (IFI44), selected based
214 on previous studies (Renault et al., 2011, Segarra et al., 2015) showing that their expression was
215 significantly increased during OsHV-1 infection.

216

217 **2.10 Transmission electron microscopy examination**

218 Haemocyte suspensions (110^6 cells) were centrifuged at 500 *g* for 8 min at 4°C and supernatant was
219 removed. Samples were fixed in 3% glutaraldehyde solution for 1 day at 4°C. Cells were washed 3
220 times with 0.4 M cacodylate buffer and post-fixed with a solution of 1% osmium tetroxide for 1 h at
221 4 C. Cells were washed twice again in 0.4 M cacodylate buffer. After dehydration in successive baths

of ethanol, and treatment with 2 baths of propylene oxide, samples were progressively impregnated and embedded in Epon. After polymerization at 60°C, semi-thin sections were cut to 1-µm thickness for quality control and then to 80–85 nm for examination on Leica Ultracut (EM UC6), with the ultra-thin sections floated onto copper EM grids and stained with uracil acetate/ lead citrate (Lewis and Knight, 1977). The sections were examined using a transmission electron microscope (JEOL-JEM 1000) at 80 kV.

3 Results

3.1 OsHV-1 DNA detection in haemocytes

During the *in vitro* assays, the quantification of OsHV-1 DNA was carried out by sampling at 1, 4, 8, 18 and 24 h after virus contact (Figure 1). Average virus DNA amounts were 3.56×10^5 at T0, 1.3×10^6 at 1 h, 1.5×10^6 at 4 h, 1.27×10^6 at 8 h, 2.3×10^6 at 18 h and 2.3×10^6 at 24 h viral DNA copies/µL of total DNA extracted from the haemolymph pellets assumed to represent haemocytes. Virus DNA amounts at 1, 4, 8, 18 and 24 h after virus contact were significantly different T0 ($p=0.001$-0.010). The differences corresponded to an increase of virus DNA, the virus DNA quantity being 4-fold greater than T0 at 4 h and 8 h and 8-fold greater at 18 h and 24 h.

3.2 OsHV-1 gene expression in haemocytes

243 OsHV-1 RNA transcripts were detected in *C. gigas* haemocytes (haemolymph pellets) using RT-qPCR
244 for the 15 selected viral genes. The selected ORFs are mostly involved in various known biological
245 functions, coding for apoptosis proteins (ORF42, 87, 99 and 106), enzymes (ORF 20, 57, 75 and 100),
246 ring finger proteins (ORF53 and 117), and membrane proteins (ORF 25, 72 and 80), with two others
247 corresponding to uncharacterized proteins (ORF 82 and 104). Viral mRNAs were detectable from one
248 hour after contact for all the selected viral genes. Figure 2 illustrates averaged changes in gene
249 expression by exposure time. The selected representation allows visualization of the variability of the
250 expression level of genes according to the time of exposure to the virus. Some genes were highly
251 expressed, such as ORF 80, ORF 82 or ORF 104. Others were not, like ORF 100, ORF 106 or ORF 53.
252 One, ORF 117, reached a lower peak level but did so earlier in the challenge.

253 The results of the PCA (Figure 3) showed that essential inertia of this analysis (total variance near
254 95%) was supported by axis 1 (≈85%) and to a lesser extent by axis 2 (≈10%) (Figure 3a). Whatever
255 the exposure time, the expression level was reported by axis 1 (Figure 3b) with the highest values on
256 the left (all the arrows pointing toward the left). This axis illustrated the quantitative aspect of the
257 experiment. Axis 2 took into account the effect of exposure time: a top gradient down appeared with
258 times of exposure, weak and strong, in top and bottom, respectively.

259 Thanks to K-means analysis of the PCA outputs, previous quantitative and kinetic considerations of
260 experimental results allowed gene discriminations into 6 groups (Figure 4): (i) genes with low
261 expression expressed rather late, ORF 20, ORF 25, ORF 100 and ORF 10; (ii) genes with intermediate
262 expression expressed rather late, ORF 72, ORF 82 and ORF 104; (iii) genes with low expression
263 expressed rather early, ORF 42, ORF 53 and ORF 57; (iv) genes with intermediate expression
264 expressed rather early, ORF 75, ORF 87 and ORF 99; (v) a gene with intermediate expression
265 expressed very early, ORF 117; and (vi) a gene with high expression expressed very late, ORF 80
266 (Figure 4).

In another way, Figure 5 showed relative changes of viral gene expression at each exposure time. Some genes, including ORF 117, tended to be quickly expressed, from one hour, with expression subsequently decreasing (Figure 5). Other genes such as ORF 82, ORF 80, ORF 72 and ORF 25 were gradually expressed during the time course of the experiments. These three last ORFs, whose curves presented an increase over time (in all cases up to 8 hours), were associated with membrane proteins (Figure 5c). Apoptosis and enzyme genes were unchanged, they expressed at the same level (figure 5a and 5b).

Immune gene expression of *C. gigas* during an *in vitro* experiment

A significant increase in mRNA levels was reported for IFI44 and MyD88 at several times after infection *in vitro* between control and haemocytes in contact with the virus (Figures 6a and 6b). MyD88 gene expression was up-regulated at 4 hpi (hour post infection) in haemocytes in contact with the virus ($R = 9.18$) compared to control ($R = 3.8$) ($p < 0.05$), and at 8 hpi in haemocytes in contact with the virus ($R = 7.10$) compared to control ($R = 3.5$) ($p < 0.05$) (Figure 6a). IFI44 gene expression was up-regulated at 8 hpi in haemocytes in contact with the virus ($R = 1.67$) compared to control ($R = 0.56$) ($p < 0.05$), at 18 hpi in haemocytes in contact with the virus ($R = 4.85$) compared to control ($R = 0.24$) ($p < 0.05$), and 24 hpi in haemocytes in contact with the virus ($R = 3.935$) compared to control ($R = 0.35$) ($p < 0.05$) (Figure 6b).

Transmission electron microscopy examination

Although cell structures appeared to be well preserved (Figure 7), TEM examination did not permit the identification of viral capsids and enveloped particles in haemocytes 18 h and 24 h after virus contact.

4 Discussion

The objective of the present work was to study if OsHV-1 is able to penetrate and initiate viral replication in haemocytes following *in vitro* contact with the virus.

In this context, *in vitro* assays were undertaken putting haemocytes in contact with a viral suspension to better understand the potential role of these cells in infection with OsHV-1. It was chosen to work under *in vitro* conditions using circulating cells collected from haemolymph to avoid complex interactions. The protocol previously developed by Renault et al. (2011) was adapted and modified. *In vitro* viral contact assays were performed over a 24-hour period. In the course of these *in vitro* assays a quantification of the viral DNA was carried out and the expression of viral genes was assessed according to the work of Segarra et al. (2014a) and Martenot et al., (2017). Moreover, observations by transmission electron microscopy were carried out in order to research viral particles.

The results showed an increase of the amounts of viral DNA over time, beginning by 1 h post virus contact and becoming even more significant at 18 h and 24 h. Associated with these results was the detection of the viral transcripts as soon as 1 h after virus contact with an increase in viral transcripts over time. The association of viral DNA and RNA in cells collected from haemolymph suggested that the virus replicated in haemocytes. However, differences in terms of viral transcript amounts varied depending of the analyzed ORFs.

During their productive cycle, vertebrate herpesviruses exhibit a strictly regulated temporal cascade of gene expression that can be divided into three main stages: immediate-early (IE), early (E), and late (L). Herpesvirus genes have traditionally been classified kinetically on the basis of individual expression studies in cell cultures (Honess et al., 1974). Different studies have been performed to define the expression of early and/or late genes and contributed to the classification and functional characterization of viral genes. Genome-wide microarray and reverse transcription quantitative (RT-q)PCR expression studies have been performed for several mammalian herpesviruses belonging to the family *Herpesviridae (*Stingley et al., 2000, Martinez-Guzman et al., 2003, Dittmer et al., 2005, Tombácz et al., 2009, Wagner et al., 2002, Ebrahimi et al., 2003, Aguilar et al., 2005, Aguilar et al., 2006). In channel catfish virus (ictalurid herpesvirus 1, IcHV1) the expression kinetics of a limited number of open reading frames (ORFs), namely ORF3, ORF5, ORF5/6, ORF6, ORF8A/9, ORF9, ORF12/13, ORF39 and ORF46 (Huang et al., 1998, Silverstein et al., 1998, Silverstein et al., 1995), has been studied in cell culture by northern blot analyses. Transcriptional regulation of the 14 ORFs in the terminal direct repeat of the genome has also been analyzed by northern blot analysis in cell culture (Stingley et al., 2000*)* and *in vivo (*Stingley et al., 2003*)*. For this small number of IcHV1 ORFs, temporal expression patterns similar to that of mammalian herpesviruses were demonstrated. In addition, transcription in cell culture of 20 ORFs in koi herpesvirus (cyprinid herpesvirus 3, CyHV3) was demonstrated by RT-PCR (Dishon et al., 2007*)*. Segarra et al (2014a) initiated some work in this field, assessing the transcription of 39 OsHV-1 genes during experimental *in vivo* infections. Although a few of genes were expressed early after virus injection, it remained difficult to define clear kinetics of virus gene expression (Segarra et al., 2014a; 2014b). Such results could be due to the use of the mantle to assess viral gene expression. The cells of this organ could be infected non-concomitantly, obscuring the dynamics of the virus cycle. Indeed, cell lines are generally used to monitor replication of the virus under *in vitro* conditions (Beurden et al., 2013), but there are no bivalve cell lines. In this context, haemocytes that can be easily collected from haemolymph can be of interest to decipher

336 the viral cycle and kinetics of viral gene expression keeping in mind that those could be different
337 depending of cell types.

338 Although haemocytes and haemolymph were considered to play a role in the development of OsHV-
339 1 infection in Pacific oysters by some authors (Schikorsky et al., 2012b, Segarra et al., 2015, Segarra
340 et al., 2016), replication of OsHV-1 has never been demonstrated in haemocytes, the oyster defense
341 cells. Segarra et al. (2016) hypothesized that the virus could be transported by haemocytes because,
342 using *in situ* hybridization, they observed strong labelling in the heart and the haemolymphatic
343 system with positive cells interpreted to be haemocytes, which could suggest a place of replication
344 and dissemination of the virus through the haemolymphatic system to all compartments of the
345 oyster. Similarly, the first transcriptomic work carried out on *C gigas* and OsHV-1 was performed
346 between haemocytes and the virus (Renault et al., 2011). The results obtained made it possible to
347 show for the first time immune genes involved in the response to OsHV-1 infection. These genes
348 have since been widely used as markers of OsHV-1 infection (Green et al., 2014a, Green et al., 2015,
349 Segarra et al 2014b).

350 The expression levels of two host genes, Myeloid differentiation 88 (MyD88) and Interferon induced
351 protein 44 (IFI44) genes, were also analyzed during the *in vitro* assays. It was previously shown that
352 the expression of these genes was modulated in *C. gigas* haemocytes after a contact with OsHV-1
353 (Renault et al., 2011). MyD88 transcripts were up-regulated at 4 h and 8 h. Segarra et al. (2014b)
354 showed that the expression level of MyD88 was positively correlated with viral DNA amounts. IFI44
355 was highly up-regulated at 8hpi and continued to increase at 24hpi. The detection of increasing levels
356 of IFI44 transcripts in infected haemocytes was thus concomitant with the detection of increasing
357 amounts of OsHV-1 DNA and RNA by real-time PCR. As previously reported by Renault et al (2011)
358 and Segarra et al. (2014b), expression of the MyD88 and IFI44 genes was enhanced in the present
359 work confirming that these genes are markers of interest with regard to viral infection. It should be
360 recalled that Segarra et al (2014b) proposed that if the overexpression of the MyD88 gene could

361 have a negative prognostic value (a high level of transcripts is associated with high mortality rates
362 under experimental conditions), that of the gene IFI44 rather seemed associated with a real ability to
363 defend against the viral infection.

364 A principal component analysis was undertaken to demonstrate whether there was a differential
365 expression of viral genes over time. Differential expression level was reported. ORF 117, encoding an
366 unknown protein, was expressed very early and then its level of expression decreased. Conversely, a
367 weak expression of the ORF 80 (encoding a putative membrane protein) was reported early after
368 virus contact although a very strong expression was observed after 18 h and 24 h. Comparable to
369 mammalian and fish herpesviruses, most of the early and early-late genes encode proteins involved
370 in viral DNA replication, enzymes involved in nucleic acid metabolism (Beurden et al., 2013). Most of
371 the late genes encode structural proteins such as membrane proteins, capsid proteins and
372 glycoproteins (Stingley et al 2003, DeeAnn Martinez-Guzman et al., 2003). However in
373 Gammaherpesvirus, Martinez-Guzman et al.(2003) showed that one glycoprotein and one tegument
374 protein peaked relatively early at 8 hpi and remained high.

375 Although viral DNA and RNA detection suggested that viral replication occurred in oyster haemocytes
376 maintained *in vitro*, transmission electron microscopic examination did not result in identification of
377 viral particles. From these results several questions arise. Is the virus able to produce complete viral
378 particles in haemocytes? There can be not an expression of all ORFs which results in an incomplete
379 viral cycle in this cell type, and that opens new questions on the role of the haemocyte (persistence
380 or productive cycle). OsHV-1 infection of oyster haemocytes may result in an abortive infection, with
381 no viral particle production, as occurs when MDV or HSV-1 infects macrophages (Morahan et al.,
382 1989; Tenney & Morahan, 1991; Barrow et al., Wu et al., 1993; Barrow et al. 2003). Does the
383 complete viral replication occur only in a very small number of cells rendering the observation of viral
384 particles quite hazardable?

385

Conclusion

For the first time, we have shown that a virus can initiate viral replication in haemocytes following an *in vitro* contact. We also studied kinetics of expression of some viral genes using RT-qPCR. The results showed that there were differential patterns of expression of viral genes over time. One gene, ORF 117, was identified as very early expressed, other genes as intermediately expressed. A gene with a high level of expression, represented by ORF 80, was expressed quite late. These results showed that OsHV-1 gene transcription is effective in Pacific oyster haemocytes maintained in *in vitro* conditions. Our results open new ways of research in order to explore OsHV-1 pathogenesis. First, haemocytes are critical to innate immunity and the virus evolved to perturb host defence. Second, and as hypothesized previously (Segarra et al., 2016), haemocytes are excellent candidate cells for transporting OsHV-1 to target organs including mantle and gills during the earliest stages of pathogenesis. Haemocytes could be 'carrier cells' responsible for transporting OsHV-1 to target organs such as heart, mantle, and gills during the earliest stages of pathogenesis. Third, in vitro infection could be used to determine the kinetic of the viral gene during his replication using a global approach using the NGS sequencing.

Acknowledgments

The authors wish to thank the Ifremer hatchery team (LGPMM) in La Tremblade and the nursery team (LSPC) in Bouin for the production of Pacific oysters. Sandy Picot and Claire Martenot are thanked for their technical support. The authors are very grateful to Dr. Ryan Carnegie and Dr. Claire Martenot for revising the manuscript. This work received financial support from the European

409 projects VIVALDI (H2020 n°678589) and from the Ifremer's Scientific Direction (Projet direction
410 scientifique) and the EU funded project.

411

Aguilar, J.S., Ghazal, P., Wagner, E.K., 2005. Design of a herpes simplex virus type 2 long oligonucleotide-based microarray: global analysis of HSV-2 transcript abundance during productive infection. Methods Mol. Biol. 292, 423-448.

Aguilar, J.S., Devi-Rao, G.V., Rice, M.K., Sunabe, J., Ghazal, P., Wagner, E.K., 2006. Quantitative comparison of the HSV-1 and HSV-2 transcriptomes using DNA microarray analysis. Virology 348, 233-241. http://dx.doi.org/10.1016/j.virol.2005.12.036.

Arzul, I., Renault, T., Lipart, C., 2001a. Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission. Dis. Aquat. Organ. 46, 1–6. http://dx.doi.org/10.3354/dao046001.

Arzul, I., Renault, T., Lipart, C., Davison, A.J., 2001b. Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. J. Gen. Virol., 82, 865–870.

Arzul, I., Renault, T., Thébault, A., Gérard, A., 2002. Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. Virus Res. 84, 151–160. http://dx.doi.org/10.1016/S0168-1702(02)00007-2.

Barrow, A.D., Burgess, S.C.., Baigent, S.J., Howes, K., Nair, V.K., 2003. Infection of macrophages by a lymphotropic herpesvirus: a new tropism for Marek's disease Virus. J. Gen. Virol., 84, 2635-2645. http://dx.doi.org/10.1099/vir.0.1920660.

van Beurden, S.J., Peeters, B.P., Rottier, P.J., Davison, A.J., Engelsma, M.Y., 2013. Genome-wide gene expression analysis of anguillid herpesvirus 1. BMC Genomics 6, 14:83. http://dx.doi.org/1471-2164/14/83.

Corbeil, S., Faury, N., Segarra, A., Renault, T. 2015. Development of an in situ hybridization assay for the detection of ostreid herpesvirus type 1 mRNAs in the Pacific oyster, Crassostrea gigas. J. Virol. Methods 211, 43-50. http://dx.doi.org/10.1016/j.jviromet.2014.10.007.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2009. The order Herpesvirales. Arch. Virol. 154, 171–177. http://dx.doi.org/10.1007/s00705-008-0278-4.

Davison, A.J., Trus, B.L., Cheng, N., Steven, A.C., Watson, M.S., Cunningham, C., Le Deuff, R.M., Renault, T., 2005. A novel class of herpesvirus with bivalve hosts. J. Gen. Virol. 86, 41–53. http://dx.doi.org/10.1099/vir.0.80382-0.

Dishon, A., Davidovich, M., Ilouze, M., Kotler, M., 2007. Persistence of cyprinid herpesvirus 3 in infected cultured carp cells. J. Virol. 81, 4828-4836. http://dx.doi.org/10.1128/JVI.02188-06.

Dittmer, D.P., Gonzalez, C.M., Vahrson, W., DeWire, S.M., Hines-Boykin, R., Damania, B., 2005. Whole-genome transcription profiling of rhesus monkey rhadinovirus. J. Virol. 79 (13): 8637-8650. 10.1128/JVI.79.13.8637-8650.2005.

Ebrahimi, B., Dutia, B.M., Roberts, K.L., Garcia-Ramirez, J.J., Dickinson, P., Stewart, J.P., Ghazal, P., Roy, D.J., Nash, A.A., 2003. Transcriptome profile of murine gammaherpesvirus-68 lytic infection. J. Gen. Virol. 84, 99-109. http://dx.doi.org/10.1099/vir.0.18639-0.

Efsa, 2015. Oyster mortality 13(6), 4122 (p. 59). http://dx.doi.org/10.2903/j.efsa. 2015.4122.

Fisher, W.S., 1986. Structure and functions of oyster hemocytes, in: Brehélin D.M. (Ed.), Immunity in Invertebratres., Springer, Berlin Heidelberg, pp. 25–35.

Green, T.J., Benkendorff, K., Robinson, N., Raftos, D., Speck, P. 2014a. Anti-viral gene induction is absent upon secondary challenge with double-stranded RNA in the Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol. 39, 492–497. http://dx.doi.org/10.1016/j.fsi.2014.06.010.

Green, T.J., Robinson, N., Chataway, T., Benkendorff, K., O'Connor, W., Speck P., 2014b. Evidence that the major hemolymph protein of the Pacific oyster, Crassostrea gigas, has antiviral activity against herpesviruses. Antiviral Res. 110, 168–174. http://dx.doi.org/10.1016/j.antiviral.2014.08.010.

Green, T. J., Rolland, J-L., Vergnes, A., Raftos, D., Montagnani ,C., 2015. OsHV-1 countermeasures to the Pacific oyster's anti-viral response. Fish & Shellfish Immunology *, 47(1), 435-443 . http://doi.org/10.1016/j.fsi.2015.09.025 ,*

Honess, R.W., Roizman, B., 1974. Regulation of herpesvirus macromolecular synthesis, I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14, 8-19.

Huang, S., Hanson, L.A., 1998. Temporal gene regulation of the channel catfish virus (Ictalurid herpesvirus 1). J. Virol. 72, 1910-1917.

Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S.A., Gu, X., Read, A., Go, J., Dove, M., O'Connor, W., Kirkland, P.D., Frances, J., 2013. Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 µ-var) in Crassostrea gigas (Pacific oysters) in Australia. Dis. Aquat. Organ. 105, 109-126. http://dx.doi.org/10.3354/dao02623.

Keeling, S.E., Brosnahan, C.L., Williams, R., Gias, E., Hannah, M., Bueno, R., McDonald, W.L., Johnston, C., 2014. New Zealand juvenile oyster mortality associated with ostreid herpesvirus 1-an opportunistic longitudinal study. Dis. Aquat. Organ. 109, 231–239. http://dx.doi.org/103354/dao02735.

Le Deuff, R.M., Renault, T., 1999. Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. J. Gen. Virol. 80, 1317–1322.

Lipart, C., Renault, T., 2002. Herpes-like virus detection in infected *Crassostrea gigas* spat using DIG-labelled probes. J. Virol. Methods 101, 1–10.

Martenot, C., Segarra, A., Baillon, L., Faury, N., Houssin, M., Renault, T. 2016. In situ localization and tissue distribution of ostreid herpesvirus 1 proteins in infected Pacific oyster, *Crassostrea gigas*. J. Invertebr. Pathol. 136, 124-135. http://doi.org/10.1016/j.jip.2016.04.002.

Martenot, C., Gervais, O., Chollet, B., Houssin, M., Renault, T., 2017. Haemocytes collected from experimentally infected Pacific oysters, Crassostrea gigas: Detection of ostreid herpesvirus 1 DNA, RNA, and proteins in relation with inhibition of apoptosis. Plos One, 12(5), e0177448 (1-19) . http://doi.org/10.1371/journal.pone.0177448

Martinez-Guzman, D., Rickabaugh, T., Wu, T.T., Brown, H., Cole, S., Song, M.J., Tong, L., Sun, R., 2003. Transcription program of murine gammaherpesvirus 68. J. Virol. 77, 10488-503.

Morahan, P.S., Mama, S., Anarki, F., Leary, K., 1989. Molecular localization of abortive infection of resident peritoneal macrophages by herpes simplex virus type 1. J. Virol. 63, 2300–2307.

Paul-pont, I., Dhand, N.K., Witthington, R.J., 2013. Influence of husbandry practices on OsHV-1 associates mortality of Pacific oysters *Crassostrea gigas*. Aquaculture 412-413, 202-214. http://dx.doi.org/10.1016/j.aquaculture.2013.07.038.

Paul-pont, I., Evans, O., Dhand, N.K., Rubio, A., Coad, P., Whittington, R.J., 2014. Descriptive epidemiology of mass mortality due to Ostreid herpesvirus-1 (OsHV-1) in commercially farmed Pacific oysters (*Crassostrea gigas*) in the Hawkesbury River estuary, Australia. Aquaculture 422–423. http://dx.doi.org/10.1016/j.aquaculture.2013.12.009.

Renault, T., 2012. Pacific Cupped Oyster, Crassostrea gigas, Mortality Outbreaks and Infectious Disease, in: Qin, J.G. (Ed.), Oysters Physiology, Ecological distribution and Mortality. Nova Science Publishers Inc., New York, pp. 203-225.

Renault, T., Lipart, C., Arzul, I., 2001. A herpes-like virus infecting Crassostrea gigas and Ruditapes philippinarum larvae in France. J. Fish Dis. 24, 369-376. http://dx.doi.org/10.1046/j.1365-2761.2001.00300.x.

Renault, T., Moreau, P., Faury, N., Pepin, J.F., Segarra, A., Webb, S., 2012. Analysis of clinical Ostreid herpesvirus 1 (Malacoherpesviridae) specimens by sequencing amplified fragments from three virus genome areas. J. Virol. 86, 5942-5947. http://dx.doi.org/10.1128/JVI.06534-11.

Renault, T., Faury, N., Barbosa-Solomieu, V., Moreau, K., 2011. Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, Crassostrea gigas, challenged with Ostreid herpesvirus 1. Dev. Comp. Immunol. 35, 725-735. http://dx.doi.org/*10.1016/j.dci.2011.02.004.*

Schikorski, D., Faury, N., Pepin, J.F., Saulnier, D., Tourbiez, D., Renault, T., 2011a. Experimental ostreid herpesvirus 1 infection of the Pacific oyster Crassostrea gigas: kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. Virus Res. 155, 28–34. http://dx.doi.org/10.1016/j.virusres.2010.07.031.

Schikorski, D., Renault, T., Saulnier, D., Faury, N., Moreau, P., Pepin, J., 2011b. Experimental infection of Pacific oyster Crassostrea gigas spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. Vet. Res. 42, 27. http://dx.doi.org/10.1186/1297-9716-42-2

Segarra, A., Pépin, J.F., Arzul, I., Morga, B., Faury, N., Renault, T., 2010. Detection anddescription of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, Crassostrea gigas, in France in 2008. Virus Res. 153, 92–99. http://dx.doi.org/10.1016/j.virusres.2010.07.011.

Segarra, A., Baillon, L., Faury, N., Tourbiez, D., Renault, T., 2016. Detection and distribution of ostreid herpesvirus 1 in experimentally infected Pacific oyster spat. J. Invertebr. Pathol. 133, 59–65. http://dx.doi.org/10.1016/j.jip.2015.11.013.

Segarra, A., Faury, N., Pepin, J.F., Renault, T. 2014a. Transcriptomic study of 39 ostreid herpesvirus 1 genes during an experimental infection . J. Invertebr. Pathol. 119, 5-11 . http://dx.doi.org/10.1016j.jip.2014.03.02.

Segarra, A., Mauduit, F., Faury, N., Trancart, S., Dégremont, L., Tourbiez, D., Haffner, P., Barbosa-Solomieu, V., Pépin, J.F., Travers, M.A., Renault, T. 2014b. Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1 . Bmc Genomics , 15, 1-13. http://dx.doi.org/10.1186/1471-2164-15-580.

Segarra, A., Faury, N., Pépin, J.F., Renault, T., 2014. Transcriptomic study of 39 ostreid herpesvirus 1 genes during an experimental infection. J. Invertebr. Pathol. 119, 5-11. http://dx.doi.org/10.1016/j.jip.2014.03.002.

Silverstein, P.S, Bird, R.C, van Santen, V.L, Nusbaum, K.E, 1995. Immediate-early transcription from the channel catfish virus genome: characterization of two immediate-early transcripts. J. Virol. 69, 3161-3166.

Silverstein, P.S., van Santen, V.L., Nusbaum, K.E., Bird, R.C., 1998. Expression kinetics and mapping of the thymidine kinase transcript and an immediate-early transcript from channel catfish virus. J. Virol. 72, 3900-3906.

Stingley, R.L., Gray, W.L., 2000. Transcriptional regulation of the channel catfish virus genome direct repeat region. J. Gen. Virol. 81, 2005-2010. http://dx.doi.org/10.1099/0022-1317-81-8-2005

Stingley, R.L., Griffin, B.R., Gray, W.L., 2003. Channel catfish virus gene expression in experimentally infected channel catfish, Ictalurus punctatus (Rafinesque). J. Fish Dis. 26, 487-493. http://dx.doi.org/10.1046/j.1365-2761.2003.00484.

Stingley, S.W., Ramirez, J.J., Aguilar, S.A., Simmen, K., Sandri-Goldin, R.M., Ghazal, P., Wagner, E.K., 2000. Global analysis of herpes simplex virus type 1 transcription using an

oligonucleotide-based DNA microarray. J. Virol. 74, 9916-9927. http://dx.doi.org/10.1128/JVI.74.21.9916-9927.2000.

Tenney, D.J., Morahan, P.S., 1991. Differentiation of the U937 macrophage cell line removes an early block of HSV-1 infection. Viral Immunol. 4, 91–102.

Tombácz, D., Tóth, J.S., Petrovszki, P., Boldogkoi, Z., 2009. Whole-genome analysis of pseudorabies virus gene expression by real-time quantitative RT-PCR assay. BMC Genomics, 10, 491-10. http://dx.doi.org/10.1186/147162164-10-491.

Wagner, E.K., Ramirez, J.J., Stingley, S.W., Aguilar, S.A., Buehler, L., Devi-Rao, G.B., Ghazal, P., 2002. Practical approaches to long oligonucleotide-based DNA microarray: lessons from herpesviruses. Prog. Nucleic Acid Res. Mol. Biol. 71, 445-491.

Wu, L., Morahan, P.S., Leary, K., 1993. Regulation of herpes simplex virus type 1 gene expression in nonpermissive murine resident peritoneal macrophages. J. Leukoc. Biol. 53, 61–65.

618

619 Table 1: List of primer for viral gene expression

ORFs	Forward	Reverse	Efficiency (%)	Amplicon (bp)	Protein
ORF20	ctggctgttctgccatttcc	agacagcggcaaggtgatgt	97.8	213	Ribonucleotide reductase small subunit
ORF25	ctcgccaaaggtcgtatcca	ccacaagggtgaattccatgtt	98.7	200	Membrane protein
ORF42	gcaggcataacaggtgagca	tgagaggcgtgacagggaat	99.9	205	Apoptosis inhibitor
ORF53	ccgaaaaaccagggactgga	tgggcgggaagtagatcgtt	98.5	197	Ring finger protein
ORF57	ttaccagcaccgagcaggat	tcgccgcttttatccaacac	99.2	150	chloride channel
ORF72	acctccccgtcaatggtatga	tccaccacacccctacaatca	94.7	180	Membrane protein
ORF75	atgatctgcgccactctggt	tgtgcctgaaggatgtgcaa	100	186	dUTPase (Enzyme)
ORF80	aagaggatttgggtgcacag	ttgcatcccaggattatcag	98.5	166	Membrane protein
ORF82	atgcagaccaccatgtttga	ccgagagccttaacaccaag	99	200	Unknown
ORF87	cacagacgacatttccccaaa	aaagctcgttcccacattggt	98.7	196	Apoptosis inhibitor
ORF99	ggtggaggtggctgttgaaa	ccgactgacaacccatggac	96.3	200	Apoptosis inhibitor
ORF100	accaggaccacgcctttgat	cccgcctttccataaattgg	100.6	197	DNA polymerase
ORF104	gggagagcttagggaaatgg	atttaccttcgggagccact	100.5	158	Unknown
ORF106	tctggcatccaacctccaaa	tcagcctatgacgaggcaatg	100.8	200	Putative apoptosis inhibitor
ORF117	aatttcccgcctctgtgctt	tgatgacggaagtggcaaca	98	200	Ring finger protein

620

621

Figure caption

Figure 1; Histogram of copy number of viral DNA OsHV-1 at different times of sampling 1, 4, 8, 18 and 24h post inoculation. The significant differences between the T0 and the others times are shown by ★ (★for p=0.01 and ★★ for p=0.001).

Figure 2 ; Heatmap of viral gene expression in haemocytes after an *in vitro* infection.

Figure 3 ; PCA on expressions of viral RNA OsHV-1 from the Pacific oyster hemocytes after different times of exposure to the virus chart of eigenvalues (a) of variable representation, different exposure times haemocyte virus (b).

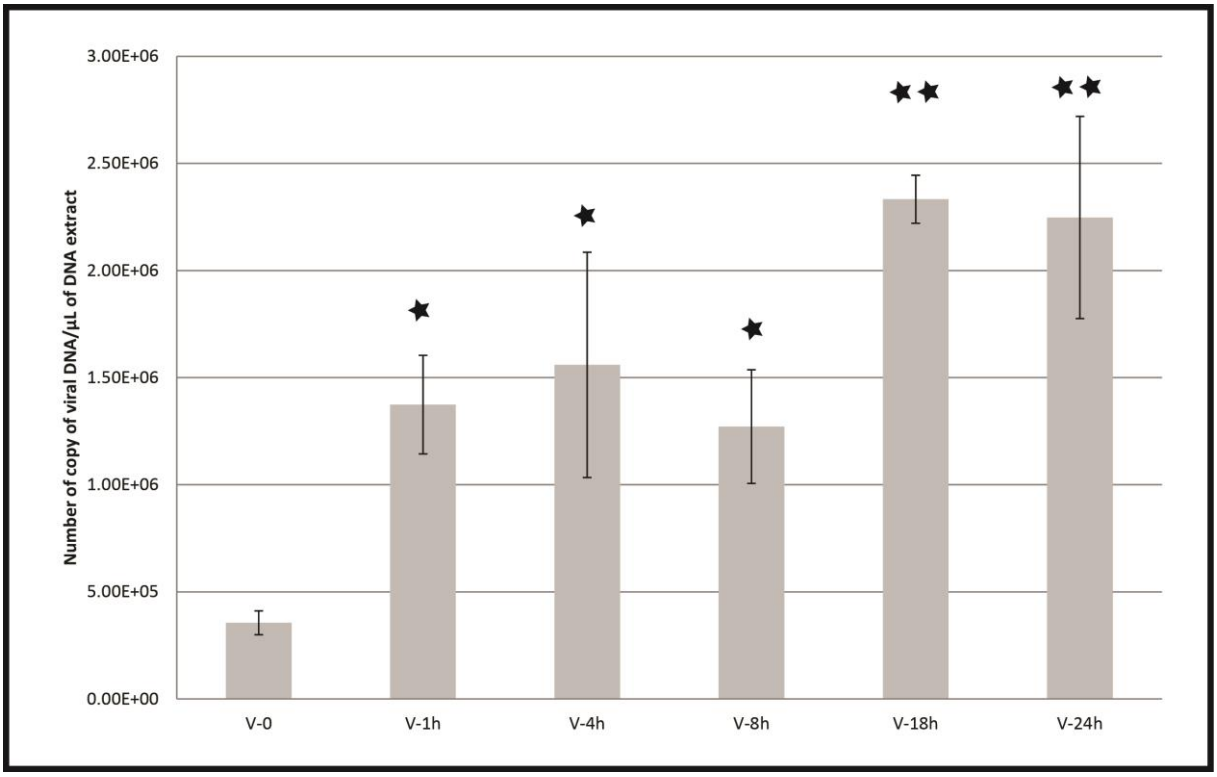
Figure 4 ; Qualitative and kinetic discrimination of genes expression into 6 groups by the method of k-means (of the result sets PCA).

Figure 5 ; Evolution of relative gene expression at different exposure times. (5a) Apoptosis genes, (5b) Enzyme genes, (5c) Membrane genes, (5d) Ring-fingers genes, (5e) Unknown genes

Figure 6 ; Relative expression of immune gene of C. gigas. 6a MyD88 expression in infected haemocyte. 6b IFI44 expression in infected haemocyte. The significant differences between the control and infected haemocytes at each time ★ (★for p=0.01 and ★★ for p=0.001).

Figure 7 ; Infected haemocyte from *C. gigas* observed after 24h of contact with OsHV-1 by transmission electron microscopy.

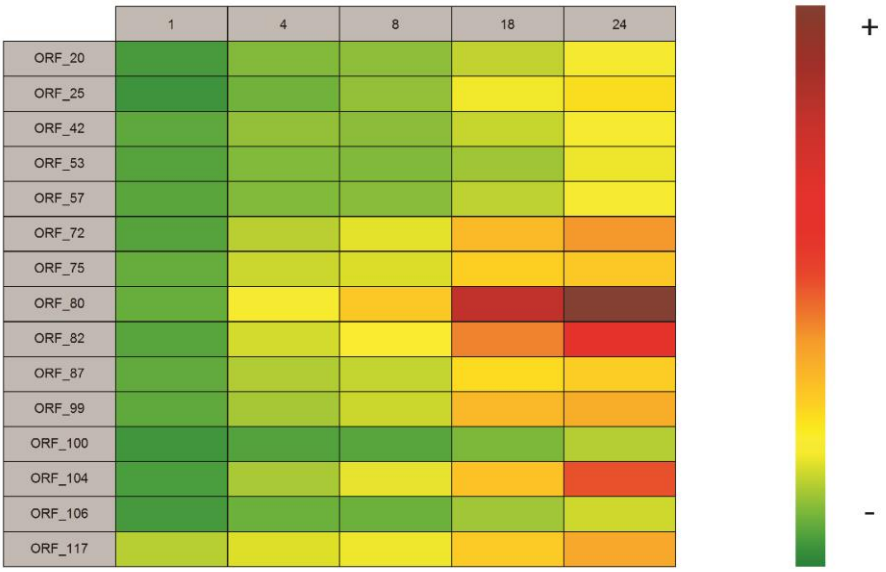
Table 1: List of primer for viral genes expression



649

650 Figure 1

651



652

653 Figure 2

654

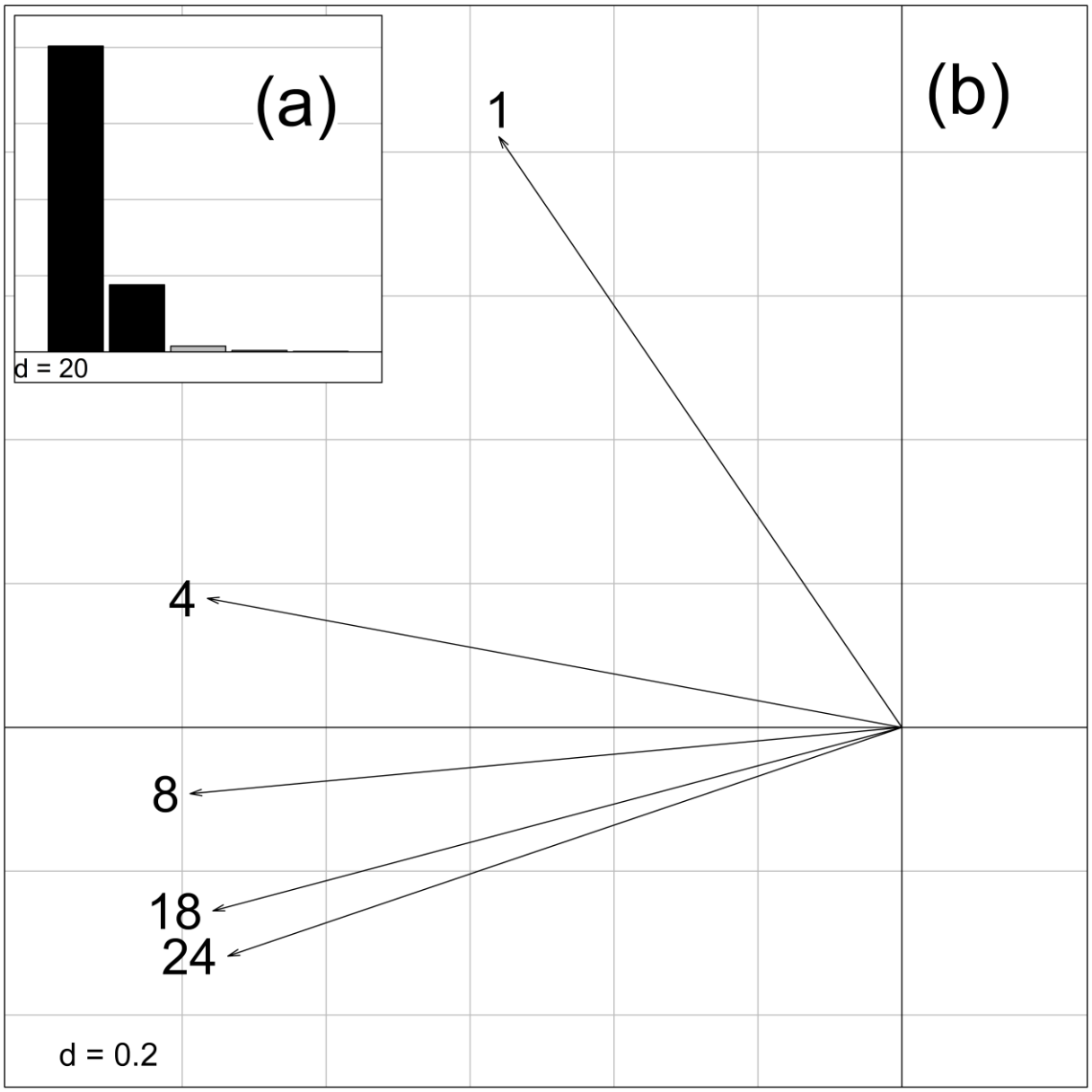
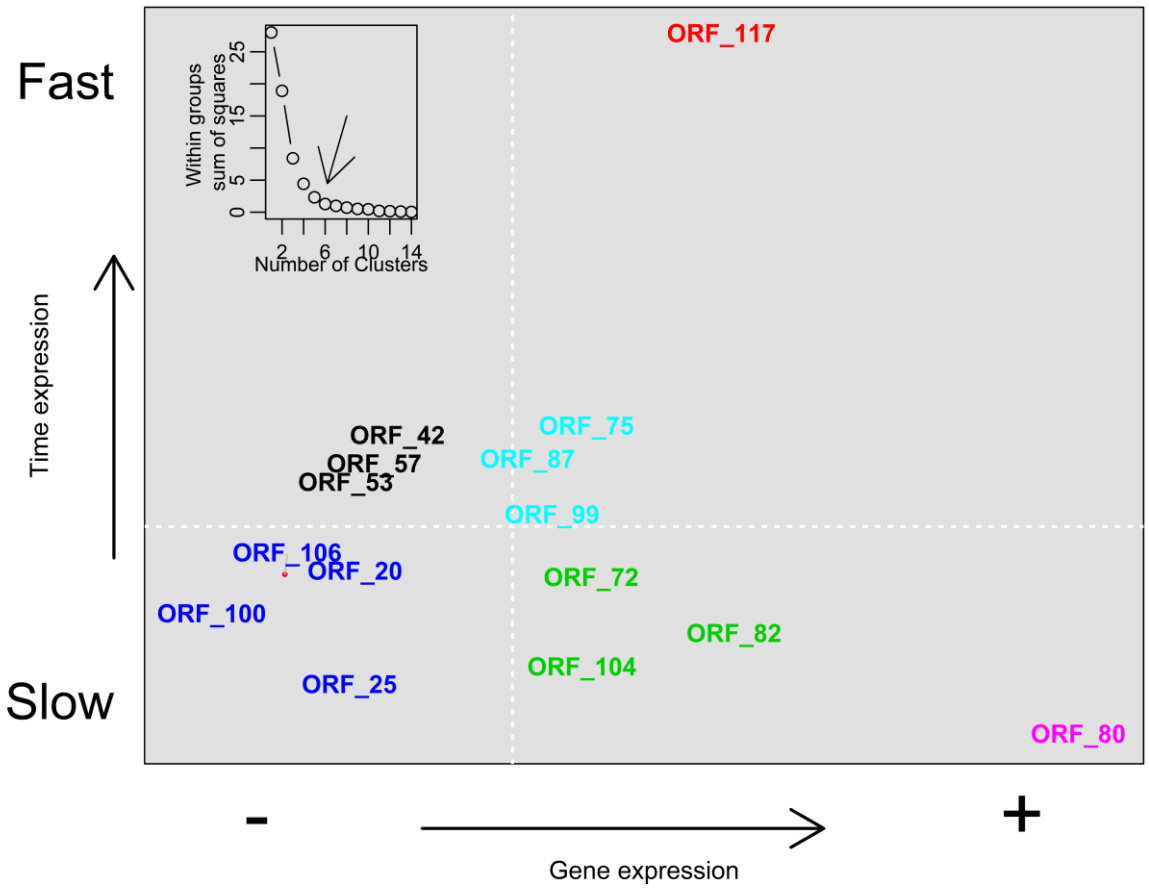


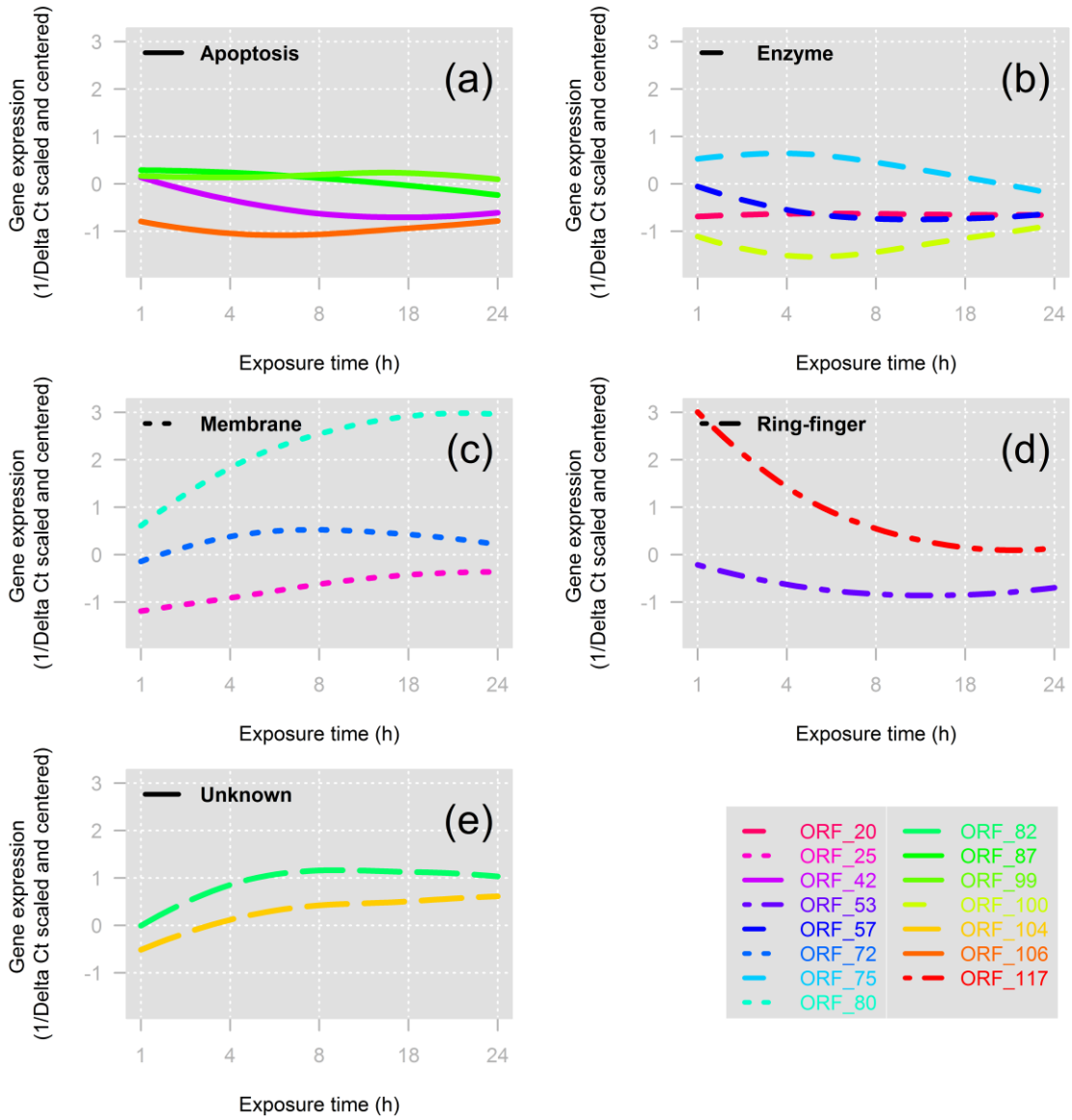
Figure 3



658

659 Figure 4

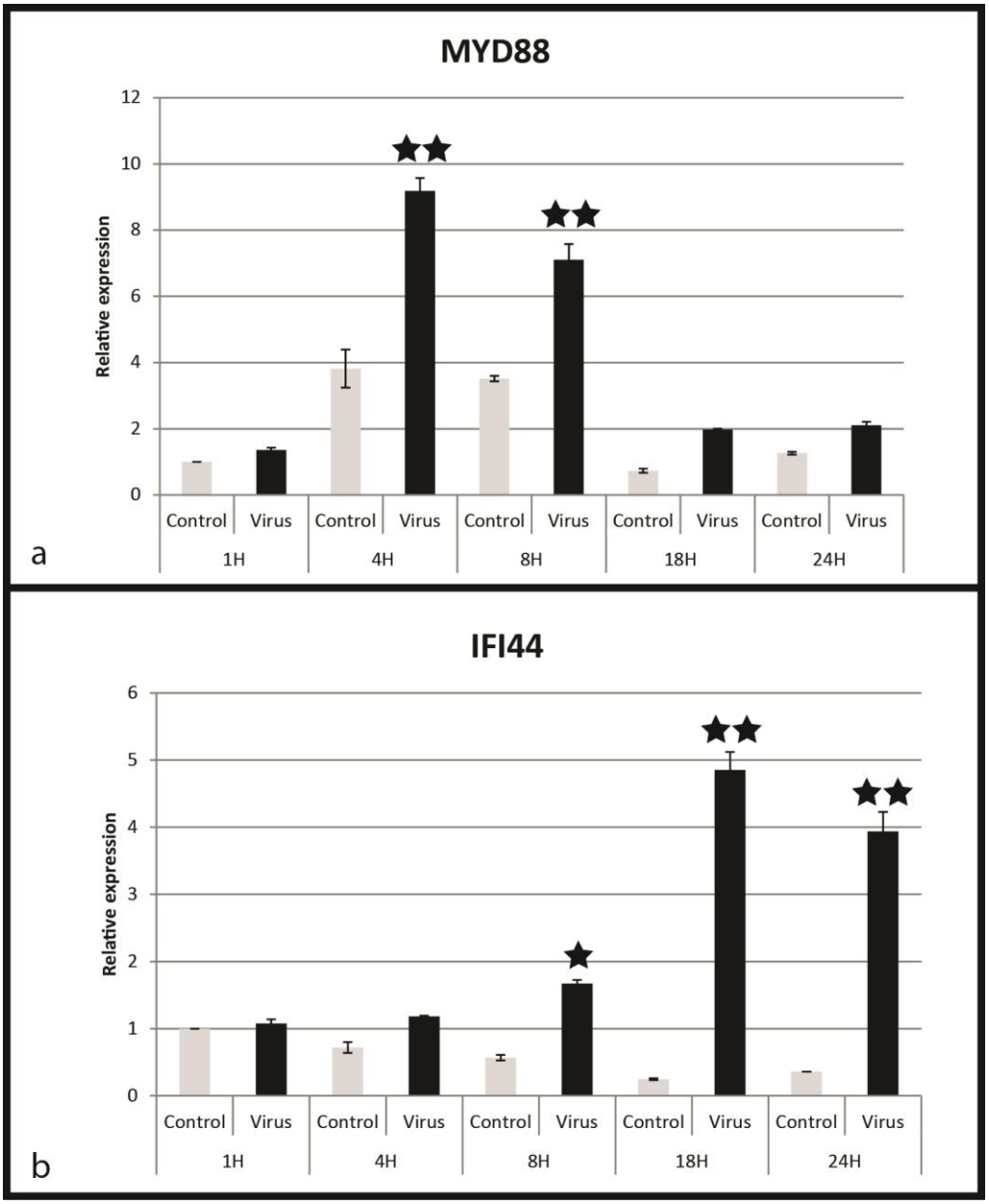
660



661

662 Figure 5

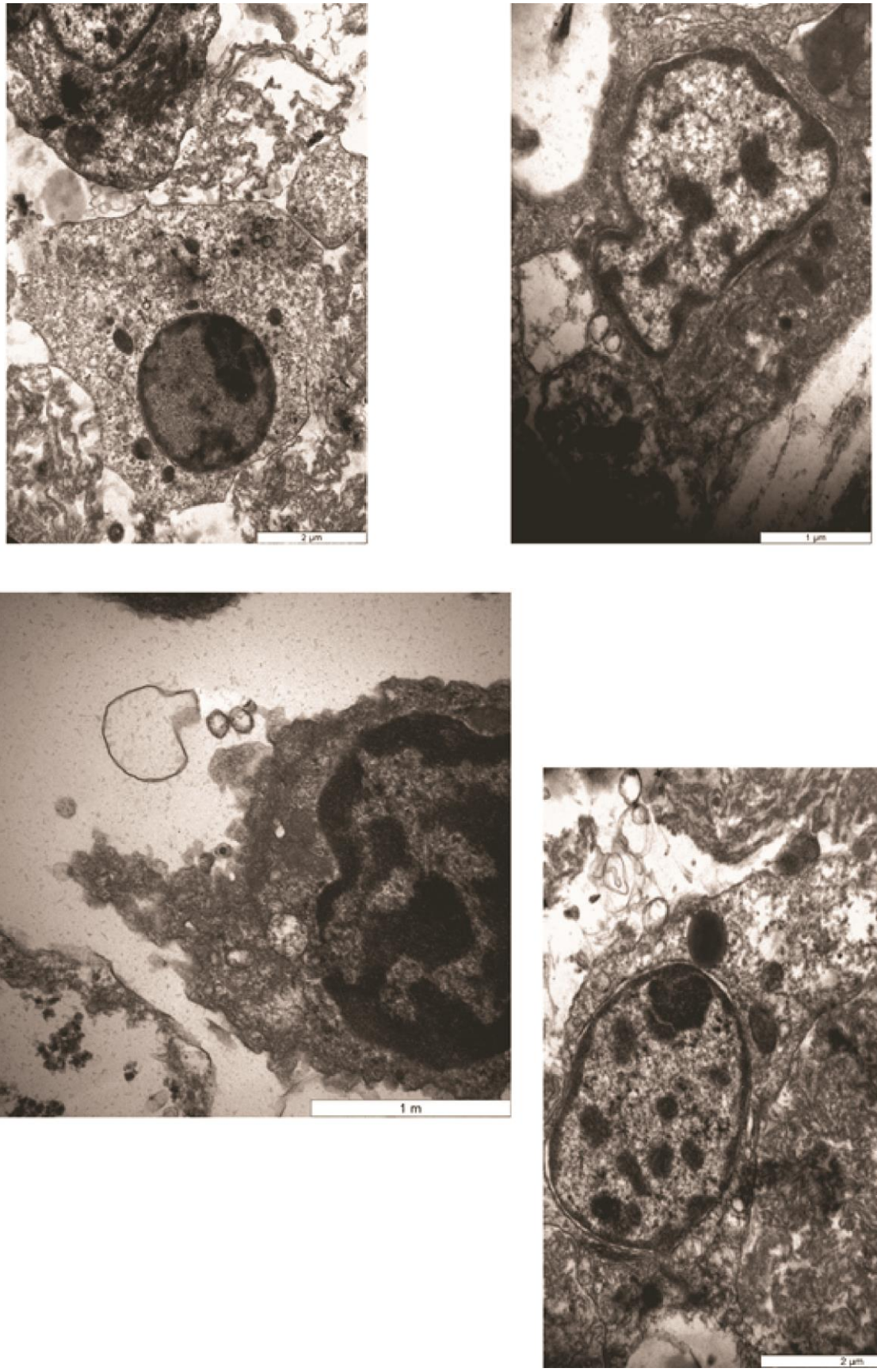
663



664

665 Figure 6

666



667

668 Figure 7