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

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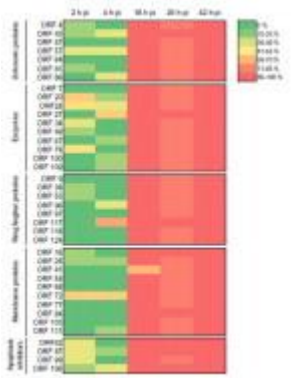
Abstract:

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.

Graphical abstract



Highlights

► We studied the detection and expression kinetics of 39 OsHV-1 genes. ► Viral RNA detection would allow much better understanding on the viral cycle. ► OsHV-1 mRNAs were detected during the early hours of infection, 2 and 4 hpi. ► Viral mRNAs were all detected at 18 hours post infection.

Keywords : *Crassostrea gigas* ; OsHV-1 ; Viral gene expression ; Inhibitors of Apoptosis ; Real time PCR ; Elongation Factor

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; Arzul et al., 2001; Segarra et al., 2010; Garcia et al., 2011). 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 ORF 100 (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 hours post injection). Relative expression was also performed on viral genes detected at all times. Thus, 8 viral genes were analysed 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.

2. Material and methods

2.1. Animals

A pool of Pacific oysters *Crassostrea 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, n° 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 hours 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 5L of filtered seawater (1µm) 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 to 100mg) 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 *Crassostrea 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 analysed 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 (Figure 1). (i) The first group consisted of 7 ORFs encode unknown proteins (ORF 4, ORF43, ORF47, ORF 57, ORF64, ORF81 and ORF 86) 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 (ORF 7, ORF 20, ORF 24, ORF 27, ORF 34, ORF 49, ORF 67, ORF 75, ORF 100 and ORF 109), (iii) one family of Ring-finger genes with 8 members (ORF 9, ORF 38, ORF 53, ORF 96, ORF 97, ORF117, ORF 118, ORF 124), (iv) one family of genes predicted to encode membrane proteins (with 10 members: ORF 16, ORF 25, ORF 41, ORF 54, ORF 68, ORF 72, ORF 77, ORF 84, ORF 103 and ORF 111), (v) one family whose products

were related to inhibitors of apoptosis (four members containing BIR domains: ORF 42, ORF 87, ORF 99, ORF 106) (Table 2). Thirty-nine specific primer pairs were designed on a unique gene sense or antisense and validated by real time PCR (Table 2). Standard curves were performed for each primer pair using serial dilutions of total DNA and PCR efficacy ($E=10^{(-1/slope)}$) was calculated thanks to these curves (Rasmussen, 2001). Relative expression was performed using ORFs detected at all times. Thus, 8 viral ORFs were normalized using the selected oyster housekeeping gene of *Crassostrea gigas*, EF (GenBank, Accession No. AB122066) identified as the most stable one in the present study. Infected individuals collected at 2 h post-infection were used as calibrators. The relative quantification value (ratio R) was calculated using the method described by Pfaffl, 2001: $R= [(E_{target})^{\Delta CT\ target(control-sample)}]/ [(E_{EF\alpha-1})^{\Delta CT\ EF\alpha-1\ (control-sample)}]$. All amplification obtained by real time PCR was validated by sequencing using the PRISM® 3130 XL-Avant Genetic Analyzer with a 36 cm capillary array and POP 7 polymer (data not shown).

2.7. Data analysis

CTs were calculated with the Stratagene Mxpro software 4.0. To determine the expression stability of housekeeping gene candidates 2 excel applets were used, *Genorm* (Vandesompele et al., 2002) and *Normfinder* (Andersen et al., 2004). These programs calculate the gene expression normalization factor and determine the most stable internal controls. *Genorm* algorithm assumed that expression variation of the ratio of two ideal reference genes is identical in all samples. Based on pairwise variation of reference candidate genes, the stability measure (M value), which reflects instability in expression levels of one gene, is calculated. M values < 1.5 are considered as stable reference genes. The main goal of the *Normfinder* program is to determine the expression stability of candidate references for all samples according

to their intra- and inter-group variation. *NormFinder* provides a direct measure for expression variation of each gene under a given set of experimental conditions. Based on this algorithm, genes with the lowest average expression stability values will be top ranked.

Results for relative expression for the 8 selected ORFs were expressed as mean ± standard error. A Mann-Whitney test was used to analyze OsHV-1 gene expressions between each time. Correlations between the viral DNA detection and the viral transcript detection were tested with the Spearman's nonparametric rho (Rs) using XLSTAT software (version 2013).

3. Results

Pacific oysters injected with the OsHV-1 suspension demonstrated high mortality rates in the 3 experiments (Figure 2), whereas oysters injected with sterile artificial seawater showed no mortality (Figure 2). The cumulative mortality rate reached 10.5% at 42 hours post injection, 40.8% after 66 hours and 63% at the end of the experiments (90 hours). Virus DNA quantification was performed for each individual collected during one experiment. No virus DNA was detected in control oysters. The first detection of virus DNA was observed 2 hours post injection. Individual OsHV-1 DNA amounts ranged from 0 to 7.5, 0 to 5 x 10^1, 2.3 x 10^3 to 2.1 x 10^5, 1.7 x 10^1 to 1 x 10^6 and 1 x 10^3 to 1.2 x 10^6 DNA copies per ng of total DNA at 2 h, 4 h, 18 h, 26 h and 42 h pi respectively (Figure 3).

The relative expression level of 4 oyster genes was studied over mantle tissue during the experimental infection of Pacific oyster, *Crassostrea gigas*, by OsHV-1. Thus, the most popular tools of *Genorm* and *Normfinder* were used to aid the selection of suitable reference gene from among the candidates. Those two programs evaluate gene expression stability using different algorithms and consider genes with low expression stability values to be more stably expressed than those with high values. According M values, ranking from the least to the most stable one out of the 4 genes was as follows: Actin (M= 0.87), GAPDH (M=0.71), L5 (M= 0.6) and EF (M=0.6) with *Genorm* (data not shown). Stability index were follows: Actin (0.63), GAPDH (0.4), L5 (0.38) and EF (0.29) with *Normfinder* (data not shown). Thus, the most stable gene reported was EF with the lowest value from both softwares.

Based on protein functions, structures and on families genes described by Davison et al. (2005), 39 ORFs belonging to 5 groups/families of genes were selected in this study (Figure 1). Although viral transcripts of some OsHV-1 ORFs were detected by real time RT PCR in few individuals as soon as 2 h and 4 h pi at low quantities, viral transcripts were mainly quantifiable from 18 h pi. More precisely, 18 viral transcripts were detected in few individuals at 2 h (ORF 4, ORF 16, ORF 20, ORF 24, ORF 25, ORF 34, ORF 38, ORF 42, ORF 43, ORF 49, ORF 53, ORF 57, ORF 72, ORF 75, ORF 81, ORF 87, ORF 99 and ORF 106) and 16 ORFs at 4h (ORF 20, ORF 24, ORF 25, ORF 27, ORF 43, ORF 57, ORF 67, ORF 72, ORF 86, ORF 87, ORF 96, ORF 100, ORF 106, ORF 109, ORF 111 and ORF 117) in all groups (Figure 4). Moreover, the DNA viral quantity was correlated with each viral transcript during the experimental infection ($Rs= 0.81, \pm 0.1, p<0.0001$).

Only the apoptosis inhibitor group (BIR with four members: ORF 42, ORF 87, ORF 99 and ORF 106) has all genes detected 2 h pi in a few of individuals (Figure 5).

A relative expression analysis was performed for ORFs detected at each time of collection. Thus, 8 virus genes were selected. They belong to 4 groups/families: (i) unknown proteins with ORF 43 and ORF 57, (ii) enzyme group with ORF 20 and ORF 24, (iii) menbrane protein group with ORF 25 and ORF 72 and, (iv) apoptosis inhibitors with ORF 87 and ORF 106 (Figure 6). No ORFs belonging to ring finger protein groups were detected at all times. Eight genes presented a relative expression significantly different between 4 h pi and 18 h pi ($p=0.05$). At 4 h pi, ORF 24 and ORF 25 were down regulated compared at 2 h pi. After this time, all ORFs were up regulated, especially ORF 87. However, the expression level between 18, 26 and 42 h pi were variable. ORF 72 was more expressed at 18 h pi compared at 26 and 42 h pi. Nevertheless, the relative expressions of these 8 ORFs were not significantly different between 18, 26 and 42 h pi.

4. Discussion

High mortality rates and viral DNA detection in Pacific oyster spat after intramuscular injection confirmed active virus replication in experimentally infected animals as previously reported (Schikorski et al., 2011). In the present study we selected injection protocol rather than cohabitation in order to contaminate all animals at the same time. To study viral gene expression, animals were mainly collected before mortality occurred in order to detect viral transcripts as early as possible and to avoid degradation of such transcripts in dead or dying individuals.

Our study is the first to demonstrate OsHV-1 RNA detection early after exposure. Main differences in terms of RNA detection between virus genes were observed at 2 and 4 h pi. Moreover, virus transcripts were detected from 18 h pi for the 39 genes monitored in this study. This result was correlated with virus DNA quantity detected by real time PCR with high amounts detected from 18 h pi.

A few of studies were previously reported on OsHV-1 gene expression. The first detection of viral RNA was reported for the ORF 4 (unknown protein) in juveniles and adult oysters 48 h post-infection (Renault et al., 2011). Another study was performed based on ORF 100 (viral DNA polymerase) transcripts (Burge and Friedman, 2012). Burge and Friedman (2012) detected viral mRNA on day 1 and showed that DNA polymerase expression increased during the early days of infection in *Crassostrea gigas* larvae. Here, ORF 100 transcripts were detected at 18 h post-infection in oyster spat. DNA polymerase is essential for the replication of viral genomes during herpesvirus infections. Previous studies of the transcript of HSV-1 DNA polymerase gene (UL30) have classified it as a E gene (early gene) (Wobbe et al., 1993; Yager and Coen, 1988). Recently, Beurden et al. (2013) studied the genome-wide gene

expression of anguillid herpesvirus 1 and reported that DNA polymerase (ORF 55) was also classed as an early gene. Apoptosis inhibitor genes (ORF 42, ORF 87, ORF 99 and ORF 106) were also detected as early as a 2 h post infection in a few of individuals. Typically, apoptosis inhibitors contain one or more BIR domains and often a carboxy-terminal Ring finger. Ring finger proteins have been shown to play a key role in transregulatory functions in vertebrate herpesviruses (Cohen and Nguyen, 1998; Moriuchi et al., 1994). The Ring finger domain of ICP0 and homologs from alphaherpesviruses are required for the activation of quiescent genomes blocking silencing of viral DNA (Everett et al., 2010; Gu and Roizman, 2009). Ince et al. (2008) showed that CIV Apoptosis inhibitor should be classified as an immediate-early CIV gene.

A major concern regarding viral gene expression is the choice of an internal reference gene control. Several studies on viral gene expression used a host gene as an internal control (Tombácz et al., 2009; Liu and Yang, 2005). The four selected housekeeping genes are involved in ribosomal metabolism (L5), cytoskeleton structure (Actin), protein metabolism (GAPDH) and elongation factor (EF), respectively. These genes are frequently used as housekeeping genes in mollusks (Du et al., 2013; Morga et al., 2010; Siah et al., 2008). Both algorithms gave EF gene as the most stable housekeeping gene when *Crassostrea gigas* spat were challenged by OsHV-1. EF1 was among the most frequently used housekeeping genes in several marine species (Morga et al., 2010; Araya et al., 2008). However, other housekeeping genes of interest have been also identified in *C. gigas* depending of experimental designs (Du et al., 2013; Green and Montagnani, 2013). The validation of EF as housekeeping gene allowed the study of viral expression genes by the relative expression method. The 8 ORFs detected at each time were selected

and their relative expression was not significantly different between 18, 26 and 42 h pi, this result could be explained by a rapid cycle of replication of OsHV-1. As other herpesviruses, OsHV-1 could be able to replicate in less than 24 hours (Reichelt et al., 2009; Smith and Harven, 1973). ORF 87 (belonging to Apoptosis inhibitor group) was especially up regulated after 4 h pi. This result suggests an important role of IAPs in virus replication and disease development. IAPs may help the virus to limit the cellular apoptotic response.

Currently, it was not possible to affiliate the 39 OsHV-1 ORFs according to categories (immediate early, early or late genes) as described widely in vertebrate herpesvirus. Nevertheless, this study also demonstrates that OsHV-1 is able to replicate at 2 h pi in oyster spat based on RNA and DNA detection.

Finally, detection of a few of virus mRNAs before 18 h post-injection may also suggest a limit of the use of the real time RT PCR technique. As this technique targeted a particular gene, it is important to mention that the number of copies for a particular transcript could be lower than the number of copies of viral DNA in an animal at a particular collection time. Moreover, no precise information is available at a cellular level concerning viral replication sites. Mantle was selected in the present study for real time RT PCR analysis as it has already been showed as a site of viral DNA detection (Schikorski et al., 2011; Sauvage et al., 2009; Arzul et al., 2002). However, some differences in terms of genes expression could exist depending of targeted tissues or cells. Finally, similarly to virus DNA detection, an inter-individual variability of viral gene expression was observed between oysters for a same time point. Such differences could be partly explained by susceptibility differences between individuals. The use of bi-parental oysters as a source of infected material

could be more appropriate to study the expression of OsHV-1 genes in *C. gigas*. An approach is in progress in the laboratory.

Conclusions

We studied the detection and expression kinetics of 39 OsHV-1 genes by real-time PCR at 0 h, 2 h, 4 h, 18 h, 26 h and 42 h post infection. For the first time, OsHV-1 mRNAs were detected during the early hours of infection and more precisely at 2, 4 and 18 h pi. Viral RNA detection by real-time PCR could be an additional tool in order to acquire a better understanding on the viral cycle, viral gene expression and spread of the virus. Although the results reported in the present study suggest that some OsHV-1 genes are expressed at 2h further studies are needed to complete these first results.

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Figure caption

Figure 1: Distribution of OsHV-1 genes according 5 putative protein groups: Unknown proteins, Enzymes, Ring finger proteins, Membrane proteins and Apoptosis inhibitors. Colors represent the OsHV-1 gene percentage, black and black shades denote 39 ORFs selected in this study and all ORFs of OsHV-1 respectively. These groups were described previously by Davison et al., 2005.

Figure 2: Percent cumulative mortality of *Crassostrea gigas* spat after injection challenge with OsHV-1. Bars represent SD of experimental infections (n=3).

Figure 3: Virus DNA detection by real time quantitative PCR in oyster spat after intramuscular injection with OsHV-1. (Error bars represent SD, n=9 individuals at each collection time).

Figure 4: Heatmap illustrating 39 viral gene transcripts at 2 h, 4 h, 18 h, 26 h and 42 h by real time PCR. Colors represent the infected individual percentage, green and red denote no and all individuals positive respectively (n=9 individuals analysed at each collection time).

Figure 5: Histogram of detection mRNA viral in 1/CT for all infected samples for ORFs belonging apoptosis inhibitors family A: ORF 42, B: ORF 87, C: ORF 99 and D: ORF 106) in *Crassostrea gigas* during experiment trial. Bars represent SD (n=2). (n=9 individuals analysed at each collection time).

Figure 6: Relative expression of the 8 viral gene transcripts (normalized to EF) in *Crassostrea gigas* mantle after infection with OsHV-1 using time 2 h pi as calibrator. Values are means of three replicates and bars represent SE (n= 9). * Mann Whitney test *($p<0.05$)*.

Figure 1

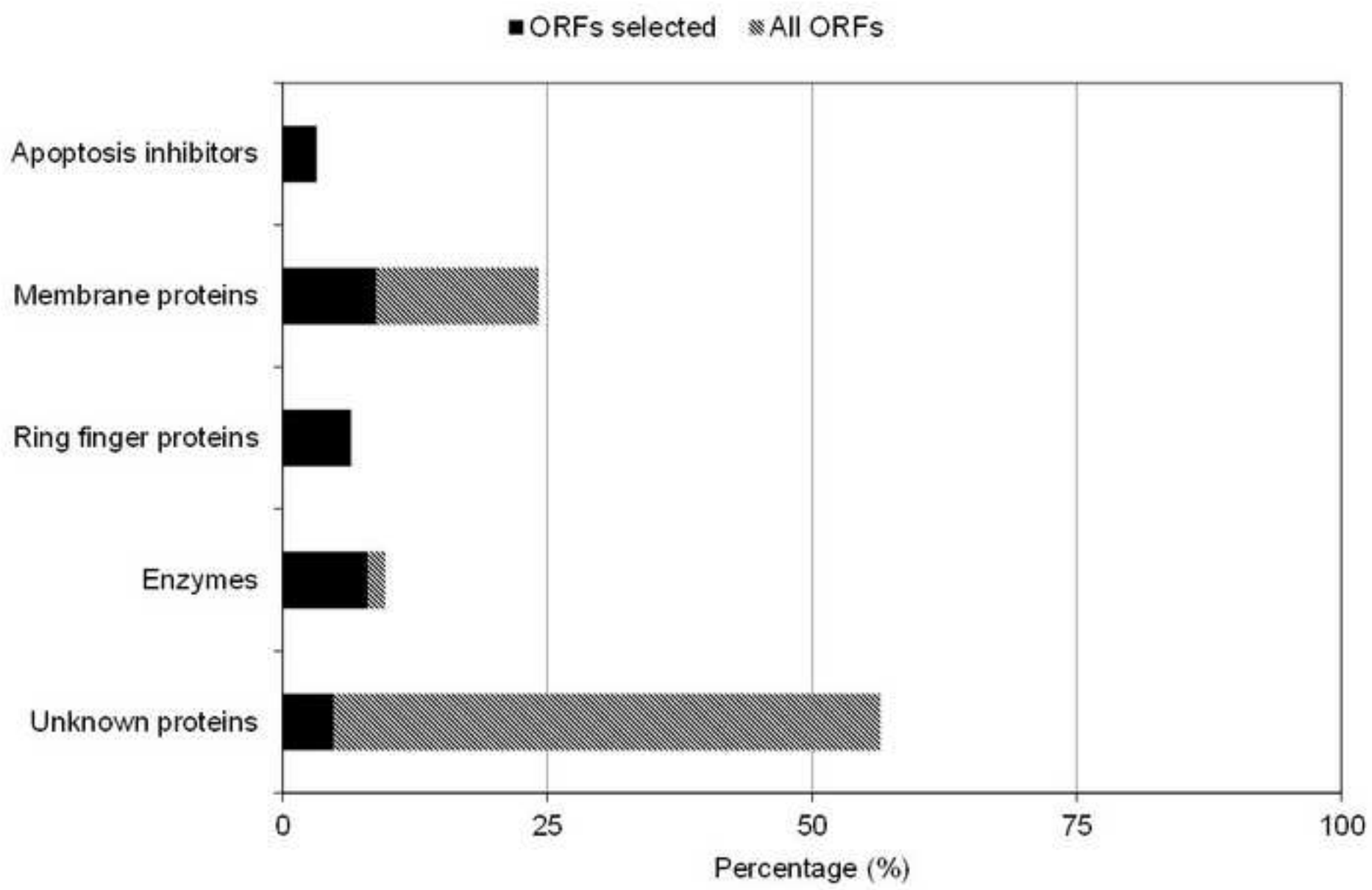

Figure 2

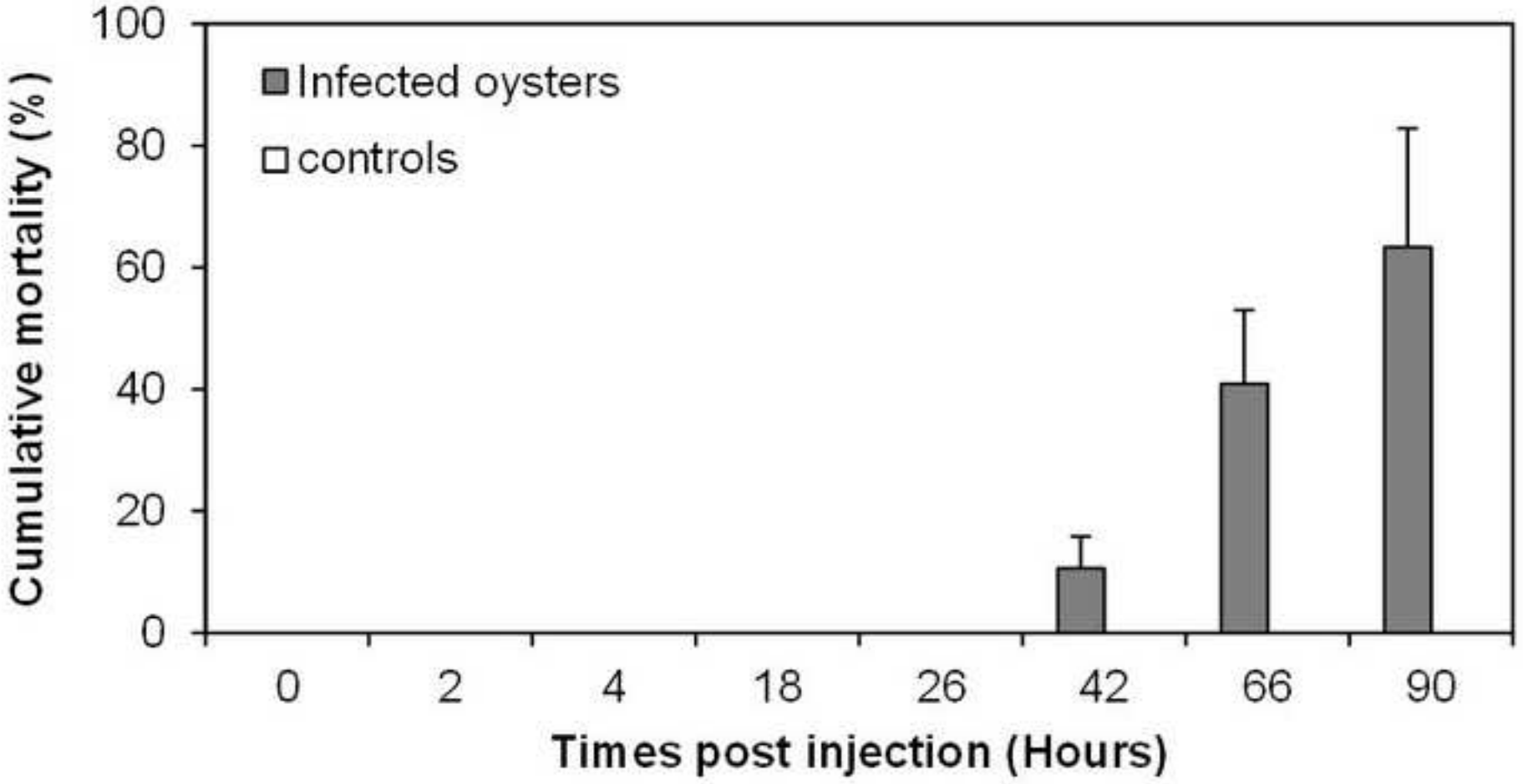

Figure 3

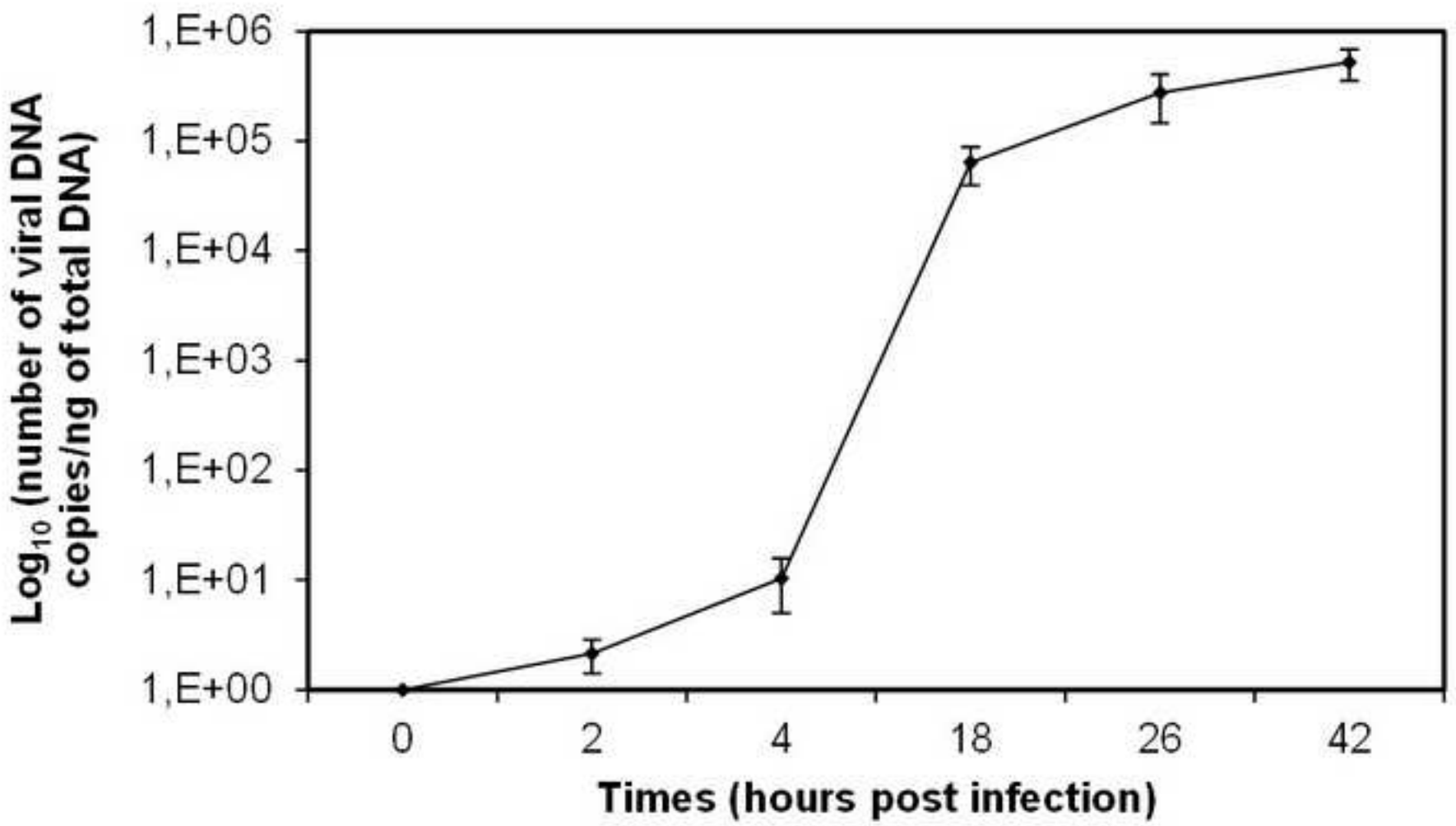

Figure 4

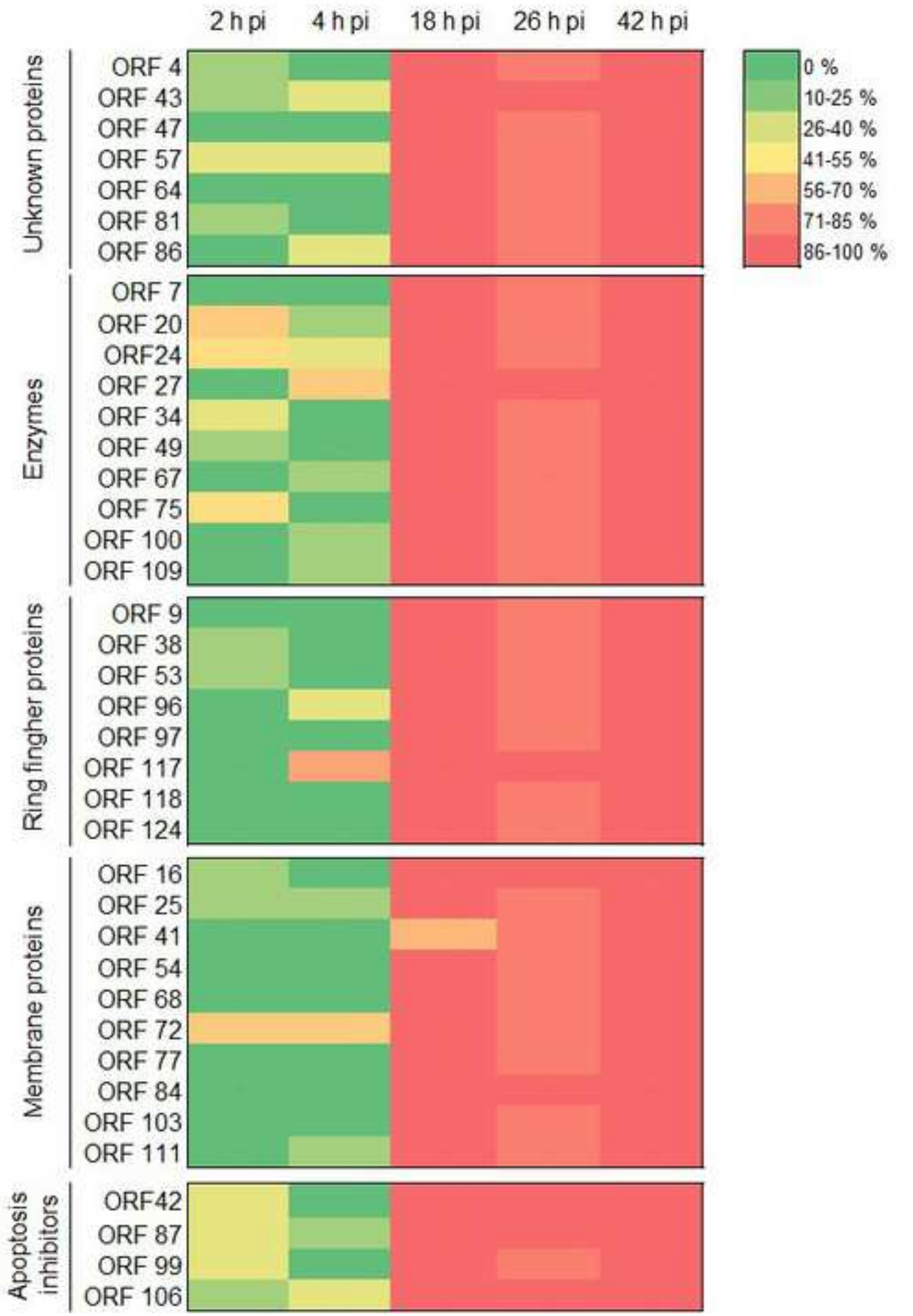

Figure 5

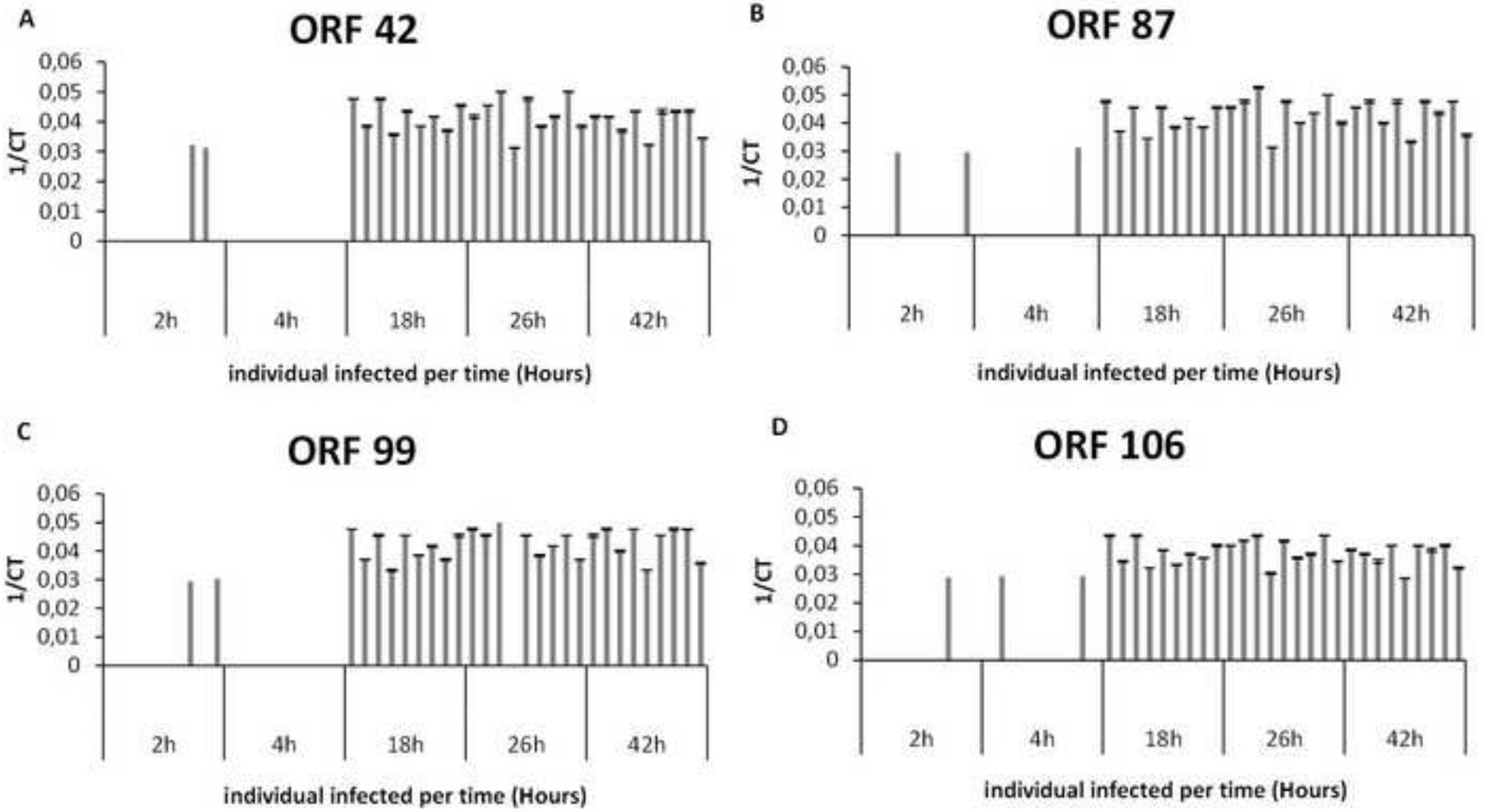

Figure 6

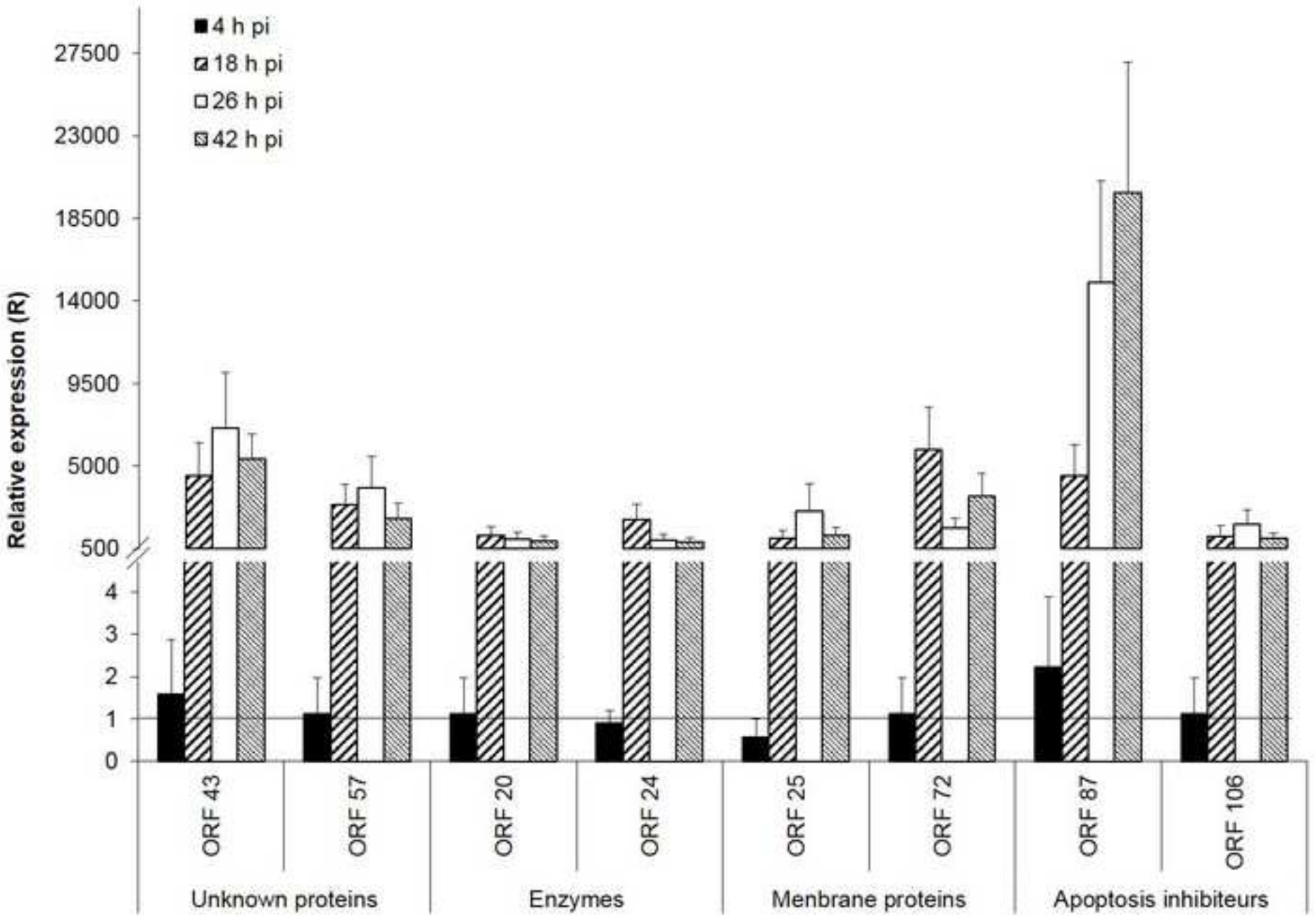

Table 1: Candidate genes housekeeping selected

Gene name	Accession number	Function	Forward	Reverse	Amplicon (bp)	Efficiency (%)
Actin	FJ669295	Cytoskeleton structure protein	CACCAACTGGGATGACATGG	AGGGACAGTACGGCCTGGAT	198	97.5
Elongation factor 1-alpha (EF)	AB122066	Translation eukaryotic factor	AGTCACCAAGGCTGCACAGAAAG	TCCGACGTATTTCTTTGCGATGT	200	98.8
Glyceraldehyde-3-phosphatedehydrogenase (GAPDH)	AJ544886	Glycolytic enzyme	CATCATCTCTGCCCCCTCAG	TGGCTGTGTACGCATGGACT	194	96.8
Ribosomal protein (L5)	AJ563456	Ribosome subunit	CCCATGAGCTGCCTCGATAC	GCACCTGAATGCACCTGGTT	194	97

Table 2: 39 Open Reading Fragments (ORFs) selected among 124 ORFs of OsHV-1.

ORFs	Forward	Reverse	Efficiency (%)	Amplicon (bp)	Protein
ORF4	tacaccggcctctttcagga	cgaattgttcactgcccaca	96.1	217	Unknown protein
ORF7	cgatagcgagggagacgatg	ccgtgtgttccattctgcaa	100.8	198	Primase(Enzyme)
ORF9	ccatcctcgttgggggtaat	cgtggcatgtgagcagagag	98.9	189	Ring finger protein
ORF16	tccaaacccagacccagctt	ccaaagagcaggccagggta	102.4	148	Membrane protein
ORF20	ctggctgttctgccatttcc	agacagcggcaaggtgatgt	97.8	213	Ribonucleotide reductase small subunit
ORF24	ccgccaaatgctctctcttc	tggaattgagggagccatga	98.6	200	Primase (Enzyme)
ORF25	ctcgccaaaggtcgtatcca	ccacaagggtgaattccatgtt	98.7	200	Membrane protein
ORF27	accgcccatggtgctaattc	cctgcaaggccaagtatgga	97	189	dUTPase (Enzyme)
ORF34	cttggacaaatgcatcccaaa	tggcacccacattcaaggtt	100.7	183	dUTPase (Enzyme)
ORF38	ttttgccatcgctcttcgtc	tgccctgtttgcagagaacc	101.5	164	Ring finger protein
ORF41	aaccgtgtgtcccaccattc	accgcagtgcaactccaatc	102	183	Membrane protein
ORF42	gcaggcataacaggtgagca	tgagaggcgtgacagggaat	99.9	205	Apoptosis inhibitor
ORF43	tgaggataccagacccagacaagt	tcccaacctcttgcatcacc	98.8	199	Unknown protein
ORF47	gcgccaaaatggtagtggaa	tccttggcaggtcctgtgat	98.6	202	Unknown protein
ORF49	acccaaagtcggtggggtaa	cggagatttgcggtgtacca	102.1	191	Helicase (Enzyme)
ORF53	ccgaaaaaccagggactgga	tgggcgggaagtagatcgtt	98.5	197	Ring finger protein
ORF54	taaccatgggcaccgaagac	acatctcgccgctttgtcac	95	193	Membrane protein
ORF57	ttaccagcaccgagcaggat	tcgccgcttttatccaacac	99.2	150	Unknown protein
ORF64	gtacccaatgtggcagtgacaa	tttgatacggagacggggaag	102	200	Unknown protein
ORF67	gatgacggcattggtgaggt	cctgtgttgcggcttggtta	98.5	195	Helicase (Enzyme)
ORF68	ttggtaccggccattgacat	ggaaccgagtttgccacctt	102.7	185	Membrane protein
ORF72	acctccccgtcaatggtatga	tccaccacacccctacaatca	94.7	180	Membrane protein
ORF75	atgatctgcgccactctggt	tgtgcctgaaggatgtgcaa	100	186	dUTPase (Enzyme)
ORF77	cctgcattggcagtcggtat	tgcctgtgtggatgtcgaag	103.2	189	Membrane protein
ORF81	tggcacagtacacagcaccag	agaccccgccaaacgtctat	95.7	182	Unknown protein
ORF84	catctgtgctgtcatcgtcca	tgtacacctcggtgcaggtttt	97.6	186	Membrane protein
ORF86	tgaaaccagacatggacacacc	ccccgcagttatagcccaat	98.0	185	Unknown protein
ORF87	cacagacgacatttccccaaa	aaagctcgttcccacattggt	98.7	196	Apoptosis inhibitor
ORF96	caccataccggtggtgtcct	tgttgcgagtcatgctctgg	100.9	180	Ring finger protein
ORF97	acatcttcccccatgccatac	acgcatgttccagccatacc	103.6	196	Ring finger protein
ORF99	ggtggaggtggctgttgaaa	ccgactgacaacccatggac	96.3	200	Apoptosis inhibitor
ORF100	accaggaccacgcctttgat	cccgcctttccataaattgg	100.6	197	DNA polymerase
ORF103	gaggcattggcagcaagtgt	gctggcttctcgtcttggaat	99.9	199	Membrane protein
ORF106	tctggcatccaacctccaaa	tcagcctatgacgaggcaatg	100.8	200	Putative apoptosis inhibitor
ORF109	agtggccatgagtgccagat	ccacaacgcctgggtatgat	102.3	165	ATPase subunit (Enzyme)
ORF111	cttccttggccaacgcctat	gcgttagacattcccgacca	104	179	Membrane protein
ORF117	aatttcccgcctctgtgctt	tgatgacggaagtggcaaca	98	200	Ring finger protein
ORF118	ccggaaggtcgtcagggtta	caccttcgccacccgtatta	95.7	183	Ring finger protein
ORF124	aagtccagcccacaacatgg	actggcgagacgtcattggt	98	197	Ring finger protein