

## INFECTION WITH OSTREID HERPESVIRUS 1 MICROVARIANTS

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### 1. Scope<sup>1</sup>

For the purpose of this chapter, infection with ostreid herpesvirus 1 microvariants is considered a viral infection of bivalve molluscs caused by ostreid herpesvirus 1 microvariants. Microvariants of OsHV-1 (ostreid herpesvirus 1) are genotypes of OsHV-1 that have sequence variations in a microsatellite locus upstream of the ORF4 and in ORF4 and ORF42/43 when compared with the reference sequence (accession number AY509253). The term “microvariants” is used in this chapter to refer to  $\mu$ Var and related variants. The term  $\mu$ Var is used to define a single variant presenting all the mutations reported by Segarra et al., 2010.

Mortality associated with OsHV-1 microvariants has been reported in the Pacific oyster, *Crassostrea gigas*, and in the Portuguese cupped oyster, *C. angulate*.

### 2. Disease information

#### 2.1. Agent factors

OsHV-1 is the aetiological agent of a contagious viral disease of Pacific oysters, also affecting other bivalve species. The genome of the virus was sequenced from infected Pacific oyster larvae collected in France in 1995 (Davison et al., 2005). As this specimen was the first to be described (through complete genome sequencing, accession number AY509253), it can be considered the reference type.

##### 2.1.1. Aetiological agent, agent strains

OsHV-1 particles have been purified from Pacific oyster larvae in France (Le Deuff & Renault, 1999) and were observed by transmission electron microscopy to be enveloped icosahedral with electron dense cores and a diameter around 120 nm. The intranuclear location of the virus particles, their size and ultrastructure are characteristic of members of the *Herpesvirales*.

The genome structure and sequence, and the capsid morphology (Davison et al., 2005) have been further studied in order to assess OsHV-1 phylogenetic status in relation to vertebrate herpesviruses. The entire virus DNA was sequenced (GenBank accession number AY509253) and OsHV-1 capsids appear structurally similar to those of other herpes viruses that have been studied (Davison et al., 2005). The virus was classified under the name *Ostreid herpesvirus 1* (OsHV-1).

Microvariants have been reported in Europe, Australia, New Zealand, and Asia (Dundon et al., 2011; Hwang et al., 2013; Jenkins et al., 2013; Lynch et al., 2012; Martenot et al., 2011; Paul-Pont et al., 2013a; Paul-Pont et al., 2013b; Peeler et al., 2012; Renault et al., 2012; Segarra et al., 2010; Shimahara et al., 2012).

##### 2.1.2. Survival outside the host

Maximum survival time outside the host is unknown.

Schikorski et al. (Schikorski et al., 2011a; Schikorski et al., 2011b) presented data on detection by real-time polymerase chain reaction (PCR) of OsHV-1  $\mu$ Var DNA in seawater following cohabitation experiments. The copy numbers of virus DNA in the water in the first 48 hours after injecting spat with virus reached  $1 \times 10^5 \text{ ml}^{-1}$ , and reached a maximum of  $1 \times 10^6 \text{ ml}^{-1}$  following infection of cohabiting oysters. The amount of infectious virus is unknown.

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1 NB: Version adopted by the World Assembly of Delegates of the OIE in May 2014.

### 2.1.3. Stability of the agent (effective inactivation methods)

The longest time for DNA detection in OsHV-1 released from macerated larvae and seeded into seawater was 22 days at 4°C and 12 days at 20°C (Vigneron et al., 2004). However, the relationship between detection of DNA in the PCR and infectivity of the virus is unknown. As a general rule, the survival of many aquatic animal viruses outside the host is greatest at lower temperatures.

As a herpesvirus, OsHV-1 may be assumed to be fragile outside its hosts. High temperature, chemicals or sunlight (UV) may destroy its lipid-containing envelope, capsid or DNA. However, it has been demonstrated that individual herpesvirus species may have different levels of stability to inactivation treatment. Inorganic salts such as Na<sub>2</sub>SO<sub>4</sub> present in seawater may stabilise herpesviruses (Wallis & Melnick, 1965).

### 2.1.4. Life cycle

Transmission is direct from host to host (Le Deuff et al., 1994; Schikorski et al., 2011a; Schikorski et al., 2011b). Recently, it was hypothesised that OsHV-1 is transmitted by vector particles in the water (Paul-Pont et al., 2013a).

## 2.2. Host factors

### 2.2.1. Susceptible host species

Mortality attributable to microvariants (Renault et al., 2012; Segarra et al., 2010) has been reported in the Pacific oyster and in the Portuguese cupped oyster (Arzul et al., 2013)

### 2.2.2. Susceptible stages of the host

Although the virus can be detected in all oyster stages, mortality due to microvariants mainly concern spat and juveniles.

### 2.2.3. Species or subpopulation predilection (probability of detection)

The Pacific oyster and in the Portuguese cupped oyster are naturally infected by microvariants. Young stages including larvae, spat and juveniles seem to be more susceptible to the infection. The virus is easier to detect in moribund animals than in apparently healthy animals.

### 2.2.4. Target organs and infected tissue

The infection-associated changes in spat/juveniles are mainly observed in connective tissues of all organs in which fibroblastic-like cells may exhibit enlarged nuclei with perinuclear chromatin (Arzul et al., 2002; Lipart & Renault, 2002; Renault et al., 1995; Schikorski et al., 2011a).

### 2.2.5. Persistent infection with lifelong carriers

Apparently healthy oysters, including adults, have been shown to be PCR-positive for OsHV-1 (Arzul et al., 2002; Moss et al., 2007; Sauvage et al., 2009). Pepin et al. (Pepin et al., 2008) showed that DNA copy numbers mg<sup>-1</sup> tissue were high (up to 10<sup>7</sup>) in oysters from populations with abnormal mortalities and low (lowest number detected 10<sup>1</sup>) in populations with no abnormal mortalities. A threshold viral copy number of 10<sup>4</sup> mg<sup>-1</sup> oyster tissue was proposed by Oden et al. (Oden et al., 2011) as being associated with mortality, an observation supported by Paul-Pont et al. (Paul-Pont et al., 2013b). Determining the levels of viral DNA in oysters by quantitative real-time PCR might be a way to differentiate between mechanical carriage of virus and low level of infection.

As the virus (DNA, protein or particles) has been detected in tissues of adult oysters, including the gonad (Arzul et al., 2002; Lipart & Renault, 2002), adults may be a source of infection for larvae or spat, particularly under stressful conditions, e.g. from high temperature (Le Deuff et al., 1996). However, what is not certain is whether true vertical transmission (transmission within the gametes) occurs or whether transmission is horizontal (Barbosa-Solomieu et al., 2005).

### 2.2.6. Vectors

The life cycle is direct. In a cohabitation challenge model using filtered seawater, no vector is required (Schikorski et al., 2011a; Schikorski et al., 2011b). A recent paper hypothesises that a microvariant is also transmitted by vector particles in the water column (Paul-Pont et al., 2013a).

### **2.2.7. Known or suspected wild aquatic animal carriers**

OshV-1 microvariant DNA has been recently detected in France and in Ireland in blue mussel, *Mytilus edulis*, and in *Donax trunculus*. However, in these cases, it remains unknown if these bivalve species are susceptible, resistant or may act as vector species.

## **2.3. Disease pattern**

### **2.3.1. Transmission mechanisms**

OshV-1 DNA ( $\mu$ Var) was detected by real-time PCR in the water surrounding dying Pacific oysters in the field.

Experimental transmission of  $\mu$ Var has been described by Schikorski et al. (Schikorski et al., 2011a; Schikorski et al., 2011b). Spat can be infected at 22°C following intramuscular injection of a filtered homogenate of naturally infected oysters, and also by cohabiting injected oysters with healthy oysters. Based on real-time PCR detection, results suggest that the virus may enter the digestive gland and haemolymphatic system, following which the virus was disseminated to other organs.

### **2.3.2. Prevalence**

Reported mortality rates and OshV-1 microvariants prevalence vary considerably between sites and countries and depend on a range of factors including the age of affected stocks (Lynch et al., 2012; Martenot et al., 2011; Peeler et al., 2012; Renault et al., 2012; Segarra et al., 2010).

### **2.3.3. Geographical distribution**

Microvariants have been reported associated with Pacific oyster mass mortalities in Europe (France, Ireland, Italy, The Netherlands, Spain, UK), Australia, New Zealand, and Korea, but is known to be detected elsewhere in the absence of oyster mortalities (e.g. Japan).

### **2.3.4. Mortality and morbidity**

Infection is often lethal for Pacific oyster spat and juveniles. Death usually occurs 1 week after infection, during or shortly after the warmest annual water temperatures (Friedman et al., 2005; Garcia et al., 2011; Renault et al., 1994b).

Infected larvae show a reduction in feeding and swimming activities, and mortality can reach 100% in a few days.

### **2.3.5. Environmental factors**

Mortality outbreaks associated with the detection of microvariants are more frequent during summer, which might suggest a link between seawater temperature and viral infection. High seawater temperatures appear to be one of the potential factors influencing OshV-1 infection. However, microvariants associated mortality does not occur consistently when water temperature is permissive. In Australia the seasonal risk factors are less certain and the temperature effects appear to be different to those in Europe (Paul-Pont et al., 2013b).

Moreover, stressful conditions particularly rearing techniques seem to favour viral infection. During summer, if oyster transfers occur this may result in virus transmission.

## **2.4. Control and prevention**

### **2.4.1. Vaccination**

Not applicable, although Pacific oysters are known to have an inducible antiviral immune response capability (Green & Montagnani, 2013; Renault et al., 2011).

### **2.4.2. Chemotherapy**

None.

### **2.4.3. Immunostimulation**

Not applicable.

#### **2.4.4. Resistance breeding**

Based on recent data, it has been demonstrated that Pacific oyster families are less susceptible to OsHV-1 including  $\mu$ Var (Degremont, 2011; Sauvage et al., 2009).

#### **2.4.5. Restocking with resistant species**

In France a project of restocking with selected Pacific oysters is on-going and the first results showed less mortality when these oysters were placed in the field (Degremont et al., com pers.). In Australia, restocking with Sydney rock oysters and flat oysters is occurring.

#### **2.4.6. Blocking agents**

None.

#### **2.4.7. Disinfection of eggs and larvae**

None.

#### **2.4.8. General husbandry practices**

Biosecurity practices may be successfully applied in confined and controlled facilities such as hatcheries and nurseries in order to protect the facility and the surrounding environment from the introduction of the virus.

In artificial rearing conditions (mollusc hatchery/nursery), OsHV-1 outbreaks may therefore be controlled through quarantine and hygiene measures including virus inactivation through treatments such as water filtration and ultraviolet irradiation (between 3 and 30 mJ cm<sup>-2</sup>). However, it is necessary to keep in mind that reduction of virus load depends on the initial titre and the virus reduction capacity of the techniques used for inactivation.

Moribund and dead oysters should be removed and destroyed whenever feasible. Equipment used in an infected zone should not be sent and used in a non-affected zone without adequate cleaning and disinfection.

### **3. Sampling**

#### **3.1. Selection of individual specimens**

Live or moribund individuals should be sampled.

#### **3.2. Preservation of samples for submission**

For histology, the best preservative is Davidson's AFA, but 10% buffered formalin, 10% seawater formalin or other standard histology fixatives are also acceptable. For PCR assays, samples must be preserved in 95–100% ethanol, a suitable nucleic acid preservation reagent or kept frozen (–80°C).

#### **3.3. Pooling of samples**

Pooling of samples may be acceptable under some circumstances, however, the impact on sensitivity and design prevalence must be considered.

#### **3.4. Best organs or tissues**

For histology, sections through the visceral mass that include digestive gland, gill and mantle are used. For PCR, a section of mantle tissue or combined gill and mantle tissue is best.

#### **3.5. Samples/tissues that are not suitable**

Gonad tissues may be not reliable for PCR assays because of the presence of inhibitors.

## **4. Diagnostic methods**

### **4.1. Field diagnostic methods**

#### **4.1.1. Clinical signs**

Infection by OsHV-1 microvariants may cause an acute disease. Animals are likely to die within a few days of manifesting clinical signs of the disease. Clinical signs may be dead or gaping bivalves but these are not specific to infection with OsHV-1 microvariants.

#### **4.1.2. Behavioural changes**

Infected hosts may be slow to close their valves when disturbed but these behavioural changes are not specific to infection with OsHV-1.

### **4.2. Clinical methods**

#### **4.2.1. Gross pathology**

Clinical signs may be dead or gaping bivalves but these clinical signs are not specific to infection with OsHV-1.

#### **4.2.2. Clinical chemistry**

None.

#### **4.2.3. Microscopic pathology**

See Section 4.2.6.

#### **4.2.4. Wet mounts**

Not applicable.

#### **4.2.5. Smears**

Not applicable.

#### **4.2.6. Fixed sections**

The most consistent features of infection with OsHV-1 are nuclear changes including hypertrophy, nuclear margination and pycnosis. The infection-associated lesions in spat are mainly observed in connective tissues in which fibroblastic-like cells exhibit enlarged nuclei with perinuclear chromatin. Highly condensed nuclei (apoptosis features) were also reported in other cells interpreted as haemocytes. These cellular abnormalities are not associated with massive haemocyte infiltration.

Histological examination of the animal is not sufficient to identify infection with herpesvirus. Whilst Cowdry type A inclusions (eosinophilic intranuclear inclusions with perinuclear chromatin) are typical of many herpesvirus infections they are not a diagnostic feature of herpesvirus infections of oysters (Arzul et al., 2002). Cowdry type A inclusions have never been reported following histological examination of infected Pacific oysters in France (Renault et al., 1994a; Renault et al., 1994b). Moreover, intranuclear inclusion bodies were not observed, although there was other cellular/nuclear pathology, in association with OsHV-1 infections in oysters in Mexico (Vasque-Yeomans et al., 2010) or USA (California) (Friedman et al., 2005).

#### **4.2.7. Electron microscopy/cytopathology**

See Section 4.3.1.1.4.

### 4.3. Agent detection and identification methods

#### 4.3.1. Direct detection methods

##### 4.3.1.1. Microscopic methods

###### 4.3.1.1.1. Wet mounts

Not applicable.

###### 4.3.1.1.2. Smears

Not applicable.

###### 4.3.1.1.3. Fixed sections

###### 4.3.1.1.3.1. Samples to be taken

Live or moribund oysters.

###### 4.3.1.1.3.2. Technical procedure

Sections of tissue that include mantle, digestive gland, gills and adductor muscle should be fixed for 24 hours in 10% formaldehyde fixatives such as Davidson's AFA or other suitable fixative followed by normal processing for paraffin histology and staining with haematoxylin and eosin. Observations are made at increasing magnifications up to  $\times 400$ .

###### 4.3.1.1.3.3. Positive controls

These are recommended and are available from the Genetics and Pathology Laboratory, Ifremer, La Tremblade, France. Positive controls are tissue sections from  $\mu$ Var-infected oysters.

###### 4.3.1.1.3.4. Levels of validation

###### 4.3.1.1.3.4.1. Specificity and sensitivity

Whatever is the genotype, specificity is very low, and sensitivity is good for moderate- to high-intensity infections, but low for low-intensity infections.

###### 4.3.1.1.3.4.2. Gold standard

None.

###### 4.3.1.1.3.5. Interpretation of results

A positive result is the occurrence of cell abnormalities in tissue sections: Fibroblastic-like cells exhibiting enlarged nuclei with perinuclear chromatin. Highly condensed nuclei are also reported in other cells interpreted as haemocytes. These cellular abnormalities are not associated with massive haemocyte infiltration.

In susceptible host species, within the known range for OsHV-1 microvariants, a positive result is presumptive evidence of viral infection only and should be confirmed by species-specific PCR, *in-situ* hybridisation (ISH) or DNA sequencing.

###### 4.3.1.1.3.6. Availability of commercial tests

No commercially available tests.

###### 4.3.1.1.4. Electron microscopy/cytopathology

Transmission electron microscopy can be used to confirm the presence of viral particles in infected animals.

Tissue samples (containing connective tissue such as mantle) for examination by electron microscopy should be fixed using 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer and post-fixed in 1% (w/v) osmium tetroxide, washed in 0.1 M cacodylate buffer (3  $\times$  10 minutes), dehydrated in a graded series of ethanol (70%, 1  $\times$  10 minutes; 95%, 2  $\times$  15 minutes; 100%, 3  $\times$  20 minutes), washed in propylene oxide

(2 × 15 minutes), pre-infiltrated in 50% propylene oxide/50% Epon resin (1 hour), infiltrated in 100% Epon resin (1 hour) and then embedded in Epon resin.

Virus replication mainly takes place in fibroblastic-like cells throughout connective tissues especially in mantle, labial palps, gills and digestive gland (Renault et al., 1994b; Renault et al., 1995; Schikorski et al., 2011a). Virogenesis begins in the nucleus of infected cells where capsids and nucleocapsids are observed. Viral particles then pass through the nuclear membrane into the cytoplasm and enveloped particles are released at the cell surface. Intranuclear and cytoplasmic capsids present a variety of morphological types including electron lucent capsids, toroidal core-containing capsids, and brick-shaped core-containing capsids.

#### 4.3.1.2. Agent isolation and identification

##### 4.3.1.2.1. Cell culture/artificial media

To date, attempts to culture the virus in both vertebrate and invertebrate cell lines and in primary oyster cell cultures have been unsuccessful.

##### 4.3.1.2.2. Antibody-based antigen detection methods

Specific antibodies have been developed (Arzul et al., 2002). However, they are not currently available for diagnostic purposes.

##### 4.3.1.2.3. Molecular techniques

At present there are a number of different PCR methods available for the detection of OsHV-1. These include both conventional and real-time PCRs (Martenot et al., 2010; Pepin et al., 2008; Renault et al., 2000).

*Samples to be taken:* Live or moribund molluscs. Larvae (100–200 mg), spat (100–200 mg) or 2–3 mm<sup>2</sup> tissue pieces are excised aseptically from mantle, placed into 1.5 ml tubes, preserved in 95° alcohol or kept frozen (–80°C). Dissecting utensils should be flamed between samples to prevent cross-contamination.

##### 4.3.1.2.3.1. Samples to be taken

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##### 4.3.1.2.3.2. Conventional PCR assays

Conventional PCR assays have been used successfully to detect OsHV-1 DNA in bivalves and different primer pairs have been designed (see Batista et al., 2007 for a review).

Two pairs of primers (A3/A4 and A5/A6) were designed and used to detect virus DNA in Pacific oyster larvae and spat via nested PCR (Renault et al., 2000). The specificity of these primer pairs was evaluated using DNA from Pacific oyster as well as DNA from vertebrate herpesviruses; 500 fg of virus DNA extracted from purified particles was routinely detected. The one-step PCR assay with the A3/A4 primer pair not only allowed amplification of OsHV-1 DNA but also the detection of a variant of this virus in Pacific oyster and Japanese carpet shell (*R. philippinarum*) larvae (Arzul et al., 2002).

Other primers were then designed including C2/C6. The combination of primer pairs A3/A4 and A5/A6 allowed less PCR amplification than C2/C6 (21.4% vs 32.4%) when the same larval samples were analysed (Renault & Arzul, 2001). C2/C6 primer pair systematically allowed the detection of 1 fg of purified viral DNA (Renault et al., 2004). A detection limit of 10 fg of purified viral DNA for both primer pairs C13/C5 and Gp3/Gp4 has been reported (Vigneron et al., 2004). As little as 1 pg and 10 pg allowed the C9/C10 and the OsHVDPFor/OsHVDPRev primer pairs, respectively, to detectably amplify a specific product (Webb et al., 2007).

Although PCR specificity has been assessed for some of the primer pairs used to detect virus DNA (see above), this has not been done for all designed primer pairs. Moreover, the amplification conditions that have been used in PCR assays using different primer pairs were based on the conditions optimised for A3/A4 and A5/A6 (Renault et al., 2000). An experimental procedure scheme used for the detection of OsHV-1 DNA by conventional PCR has been proposed by Batista et al., 2007.

4.3.1.2.3.3. *OsHV-1* specific SYBR<sup>®</sup> Green<sup>2</sup> PCR assay (Pepin et al., 2008)

Fifty mg of larvae/spat/mantle tissue are ground in 50 µl double-distilled water using a disposable piston. The crushed tissues are diluted six-fold and clarified at 10,000 *g* for 5 minutes. One hundred µl recovered supernatant are treated using a commercial DNA tissue kit (QIAGEN - QIamp tissue mini kit<sup>®</sup>) according to the manufacturer's protocol. Final elution of the DNA is performed with 100 µl TE buffer. The DNA is stored at -20°C. Prior to PCR, DNA concentrations can be measured by absorbance at 260 nm. According to total DNA concentration measured in samples, they are diluted in order to obtain 20 ng total DNA per PCR reaction.

Three sets of primers can be used targeting three regions of viral DNA: (ORF4, ORF88 and ORF99). Primer pairs B4/B3 (Arzul et al., 2001; ORF99 encoding a BIR protein) and C9/C10 (Barbosa-Solomieu et al., 2004; ORF4) were previously designed for single PCR, whereas the Gp4/Gp7 primer pair (ORF88 encoding a class I membrane protein) was assessed for qPCR. The primer pairs B4/B3, C9/C10 and Gp4/Gp7 yield PCR products of 207, 197 and 85 bp, respectively.

B4: 5'-ACT-GGG-ATC-CGA-CTG-ACA-AC-3'

B3: 5'-GTG-GAG-GTG-GCT-GTT-GAA-AT-3'

C9: 5'-GAG-GGA-AAT-TTG-CGA-GAG-AA-3'

C10: 5'-ATC-ACC-GGC-AGA-CGT-AGG-3'

Gp4: 5'-GGC-GTC-CAA-ACT-CGA-TTA-AA-3'

Gp7: 5'-TTA-CAC-CTT-TGC-CGG-TGA-AT-3'

The C9/C10 primer pair yield reliable parameters for qPCR with *OsHV-1* DNA, as well as the B3/B4 primer pair, which show closely similar parameters with a slightly lower *E* value (96.3%). The Gp4/Gp7 primer pair is less efficient (*E* = 91.3%) and less sensitive ( $\geq 50$  copies  $\mu\text{l}^{-1}$ ). The primer pair C9/C10 appears to be the most sensitive and efficient.

An additional primer pair DPFor/DPRev can be also used producing a 197 bp product (ORF100, DNA polymerase).

DPFor: 5'-ATT-GAT-GAT-GTG-GAT-AAT-CTG-TG-3'

DPRev: 5'-GGT-AAA-TAC-CAT-TGG-TCT-TGT-TCC-3'

Targeting different segments of *OsHV-1* DNA is important in order to define more precisely viral genotypes. Although ORF4 is an interesting candidate to describe diversity because virus polymorphism has been already reported in this area, ORF100 (DNA polymerase) appears to be less polymorphic.

All amplification reactions are performed in a total volume of 25 µl with 96-microwell plates. Each well (25 µl) contains 5 µl extracted DNA dilution (sample) or *OsHV-1* DNA (positive control), 12.5 µl Brilliant III Ultra-Fast SYBR<sup>®</sup> Green QPCR Master Mix or FullVelocity<sup>®</sup> Master Mix (Stratagene), 2.5 µl each diluted primer (final concentration 200 nM) and 2.5 µl distilled water. Thermal cycle conditions are: 1 cycle of pre-incubation at 95°C for 10 minutes; 40 cycles of amplification at 95°C for 30 seconds (15 seconds with FullVelocity<sup>®</sup> Master Mix), 60°C for 45 seconds (30 seconds with FullVelocity<sup>®</sup> Master Mix) and 72°C for 45 seconds with Brilliant<sup>®</sup> Master Mix; and melting temperature curve analysis at 95°C for 60 seconds, 60°C for 30 seconds and 95°C for 30 seconds. Real time PCR analysis should be performed in triplicate with 5 µl sample dilutions as DNA template or a viral DNA control.

Absolute quantitation of copies of *OsHV-1* DNA (copies  $\mu\text{l}^{-1}$ ) is carried out by comparing CT (threshold cycle) values obtained with the standard curve, using the Thermocycler software. Each experiment includes a positive DNA control (*OsHV-1* genomic DNA for absolute quantitation) and blank controls (NTC, no template control consisting of deionised sterile water). PCR efficiency (*E*) is calculated from standard curves as the percentage of template molecules that is doubled during each cycle ( $[10^{(-1/\text{slope})} - 1] \times 100$ ), with requirements that it fell into the range 95–105% and that the coefficient of determination (*R*<sup>2</sup>) is >0.98. In order to allow detection of non-specific products, a dissociation

2 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.



protocol (melt curve) takes place after the amplification cycles. The temperature at which SYBR® Green fluorescence is generated by the double-stranded amplicon dissociation is recorded.

Regarding the test's sensitivity, it is considered that it can detect systematically 4 DNA copies  $\mu\text{l}^{-1}$ . The dynamic range for the qPCR was estimated from several standard curve assays, and a linear relationship was obtained between input copy number of the viral DNA template and CT value for over 5 log 10 dilutions. It was possible to quantitate OsHV-1 DNA copy numbers at least from 10 to  $5 \times 10^6$  copies  $\mu\text{l}^{-1}$ .

#### 4.3.1.2.3.4. OsHV-1 specific TaqMan® PCR assay (Martenot et al., 2010)

The target was the B region of the OsHV-1 genome, which encodes a putative apoptosis inhibitor (Arzul et al., 2001). Primer pairs and two TaqMan® probes were designed to detect simultaneously the target gene and an internal control (IC). The IC was a synthesised sequence containing at each end the forward OsHV1BF (5'-GTC-GCA-TCT-TTG-GAT-TTA-ACA-A-3') and reverse B4 (5'-ACT-GGG-ATC-CGA-CTG-ACA-AC-3') primers. The B4 primer used for the TaqMan PCR was the same as that published by Pepin et al., 2008.

The amplification of the targeted region and IC was performed by using the OsHV1BF and B4 primers. The B (5'-TGC-CCC-TGT-CAT-CTT-GAG-GTA-TAG-ACA-ATC-3') and the IC (5'-ATC-GGG-GGG-GGG-GGT-TTT-TTT-TTT-ATC-G-3') probes were labelled at the 5' end with the fluorescent reporter dyes TxR and FAM, respectively, and at the 3' end with an appropriate quencher (BHQI or BHQII).

The reaction mixture contained 12.5  $\mu\text{l}$  premix ExTaq® 2× Takara® (Lonza, Verviers, Belgium), 0.5  $\mu\text{l}$  each primer (20  $\mu\text{M}$ ), 0.5  $\mu\text{l}$  TaqMan® probes (10  $\mu\text{M}$ ) and 9  $\mu\text{l}$  water. Two  $\mu\text{l}$  DNA sample was added to 23  $\mu\text{l}$  reaction mixture. The amplification was performed in two stages under the following conditions: 1 cycle of 95°C for 10 seconds, followed by 40 cycles of amplification at 95°C for 5 seconds, 60°C for 20 seconds. The virus quantitation was carried out by comparison with standard curve values.

The protocol for quantifying OsHV-1 in Pacific oysters based on a SYBR® Green real-time PCR was first developed (Pepin et al., 2008). Martenot et al. (Martenot et al., 2010) developed an alternative protocol based on TaqMan® chemistry. The quantitation limits were 1000 and 18  $\text{UG mg}^{-1}$  of tissues for the SYBR® Green-based method and the TaqMan® method, respectively, and the latter protocol has a detection limit of 6  $\text{UG mg}^{-1}$  of tissues. Comparing the two protocols using DNA samples obtained from 210 spat, the kappa index (0.41) indicated a moderate concordance between the protocols, according to the measures of Landis and Koch. All samples that were positive by the reference protocol were also positive by the alternative protocol. Of the 76 samples that were negative by the reference protocol, 49 were positives by the alternative protocol. Although these results may suggest that the alternative protocol can be more sensitive than the reference protocol, formal validation is needed. A protocol based on TaqMan® chemistry is under development and validation for the detection of virus specimens or variants presenting the deletion reported in the microsatellite upstream from the ORF4 area for microvariants (Pepin et al., pers. comm.).

#### 4.3.1.2.3.5. OsHV-1 specific in-situ hybridisation

The *in-situ* hybridisation (ISH) procedure described here uses a digoxigenin (DIG)-labelled DNA probe to detect OsHV-1 in formalin-fixed, paraffin-embedded tissue (Arzul et al., 2002; Lipart & Renault, 2002). This assay can detect the generic and microvariants but cannot distinguish between genotypes.

Sections of tissue that include mantle, digestive gland, gills and adductor muscle should be fixed for 24 hours in Davidson's AFA or other suitable fixative and processed using standard procedures for histological examination.

Thick tissue sections on silane-prep™ slides are dewaxed in xylene (2 × 5 minutes), treated in absolute ethanol (2 × 5 minutes) and air dried at room temperature (15 minutes). Sections are then permeabilised with proteinase K (100  $\mu\text{g ml}^{-1}$  in distilled water) for 30 minutes at 37°C in a humid chamber. Proteolysis is stopped by one 3-minute wash in 0.1 M Tris, 0.1 M NaCl buffer (pH 7.5) at room temperature. Sections are dehydrated in 95° ethanol for 1 minute, absolute ethanol for 1 minute and air dried (15 minutes).

A prehybridisation step is carried out with pre-hybridisation buffer (50% formamide, 10% dextran sulfate, 4 × SSC [0.06 M  $\text{Na}_3\text{citrate}$ , 0.6 NaCl, pH 7], 250  $\mu\text{g ml}^{-1}$  yeast tRNA and 10% Denhart) for 30 minutes at 42°C in a humid chamber. The prehybridisation buffer solution is replaced with 100  $\mu\text{l}$  hybridisation buffer solution containing 50  $\mu\text{l}$  digoxigenin-labelled probe (5 ng  $\mu\text{l}^{-1}$ ) and 50  $\mu\text{l}$  hybridisation buffer (50% formamide, 10% dextran sulfate, 4× SSC, 250  $\mu\text{g ml}^{-1}$  yeast tRNA and 10% Denhart). Slides are

covered with plastic coverslips (Polylabo, France). DIG-labelled probes are synthesised from OsHV-1 genomic DNA (100 pg per reaction) by incorporation of digoxigenin-11-dUTP (Boehringer Mannheim, Germany) during conventional PCR. The primer pair C1/C6 is used:

C1: 5'-TTC-CCC-TCG-AGG-TAG-CTT-TT-3'

C6: 5'-GTG-CAC-GGC-TTA-CCA-TTT-TT-3'

Target DNA and digoxigenin-labelled probe are denatured at 95°C for 5 minutes and the hybridisation is carried out overnight at 42°C in a humid chamber.

After hybridisation, coverslips were removed carefully and slides were washed for 10 minutes in 1 × SSC (0.2% BSA) at 42°C. Specifically bound probe was detected using a peroxidase-conjugated mouse IgG antibody against digoxigenin (Boehringer Mannheim, Germany) diluted 1:250 in 1 × PBS (1 hour at room temperature). Unbound peroxidase-conjugated antibody was removed by six washes in 1 × PBS (5 minutes). Diaminobenzidine (DAB) tetrahydro-chloride was diluted in 1 × PBS (0.7 mg ml<sup>-1</sup>). The colour solution was added to tissue sections (500 µl) and incubated at room temperature in the dark for 20 minutes. The reaction was stopped with two 1 × PBS washes. Slides were stained for 20 seconds in Unna Blue (RAL, France) followed by ethanol dehydration and mounted in Eukitt via xylene.

Specific dark brown intra-cellular staining is indicative of the presence of viral DNA.

Thirty Pacific oyster adults have been analysed using three different techniques: PCR, ISH and immunochemistry, in order to detect OsHV-1 in subclinical individuals (Arzul et al., 2002). PCR and ISH allowed detection of oyster herpes virus DNA in 93.3% and 86.6%, respectively, of analysed oysters while polyclonal antibodies allowed detection of viral proteins in 76.6% of analysed adult oysters.

#### 4.3.1.2.4. Agent purification

OsHV-1 can be purified from infected animals using a previously developed technique (Le Deuff & Renault, 1999).

### 4.3.2. Serological methods

None applicable.

## 5. Rating of tests against purpose of use

Should perinuclear chromatin be observed by histology, electron microscopy at least should be undertaken to identify any virus-like particles present and demonstrate their location within cells. Viruses observed by EM should be described as e.g. herpesvirus-like until further investigations are done to provide further evidence of the identity of the virus. As different herpesviruses are morphologically similar, a virus should only be described as OsHV-1 if it had been shown to have identity with the latter virus using OsHV-1 specific primers or probes.

For OsHV-1, the demonstration of the presence of intracellular viral structural and non-structural proteins, OsHV-1 specific messenger RNA, and virions constitute evidence for replication, but detection of viral DNA by PCR alone does not.

As an example, the methods currently available for targeted surveillance and diagnosis are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose.

**Table 5.1. Methods for surveillance and diagnosis**

	Targeted surveillance			Presumptive diagnosis			Confirmatory diagnosis		
	Larvae	Juveniles	Adults	Larvae	Juveniles	Adults	Larvae	Juveniles	Adults
<b>Gross signs</b>	d	d	d	d	d	d	d	d	d
<b>Bioassay</b>	d	d	d	d	d	d	c	c	d
<b>Histopathology</b>	d	d	d	d	d	d	d	d	d
<b>Transmission EM</b>	d	d	d	d	d	d	b	b	b
<b>Antibody-based assays</b>	d	d	d	d	d	d	d	d	d
<b>In-situ DNA probes</b>	c	c	c	c	c	c	b	b	b
<b>PCR</b>	a	a	a	a	a	a	b	b	b
<b>qPCR</b>	a	a	a	a	a	a	b	b	b
<b>Sequence</b>	d	d	d	d	d	d	a	a	a
EM = electron microscopy; PCR = polymerase chain reaction; qPCR = real-time PCR.									

## 6. Test(s) recommended for targeted surveillance to declare freedom from OsHV-1 infection

PCR and real-time PCR are recommended.

## 7. Corroborative diagnostic criteria

### 7.1. Definition of suspect case

A suspect case of infection with microvariants is a case of mortality of susceptible species associated with detection of OsHV-1 by PCR or qPCR. Infection can exist without evidence of mortality.

### 7.2. Definition of confirmed case

A case of infection with ostreid herpesvirus 1 microvariants is confirmed when detection by histology, transmission electron microscopy, or PCR is followed by sequencing leading to sequences consistent with the definition of microvariants.

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**NB:** There is not currently an OIE Reference Laboratory for infection with ostreid herpesvirus 1 microvariants (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: <http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).