Aquaculture 450 (2016) 412–421

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Stability of Ostreid herpesvirus-1 (OsHV-1) and assessment of disinfection of seawater and oyster tissues using a bioassay

Paul Hick *, Olivia Evans, Richard Looi, Chloe English, Richard J. Whittington

Faculty of Veterinary Science, The University of Sydney, Camden, NSW 2570, Australia

ARTICLE INFO

Article history:
Received 17 July 2015
Received in revised form 19 August 2015
Accepted 21 August 2015
Available online 28 August 2015

Keywords:
Ostreid herpesvirus-1
Bioassay
Disinfection
Oyster
Crassostrea gigas

ABSTRACT

Microvariant genotypes of *Ostried herpesvirus-1* (OsHV-1 μVar) have recently emerged as a cause of epizootic mortality in Pacific oysters (*Crassostrea gigas*) in many countries. Measures that can reduce the spread of the virus are required to decrease the incidence and distribution of the disease at the local and global scales. Disease management strategies and biosecurity plans require data describing the stability of OsHV-1 in the environment and the methods required for effective disinfection of the virus. A bioassay using intramuscular injection of 10 month old oyster spat had a limit of detection of 3.6×10^3 copies of OsHV-1 genome per oyster. This was 10-fold more sensitive compared to immersion challenge of 5 month old spat, even though the oysters were exposed to a greater volume of inoculum over a period of 2 h. OsHV-1 remained infectious in seawater for 2 days at 20 °C and in wet or dry, non-living oyster tissues for at least 7 days at 20 °C. OsHV-1 was inactivated by: commercial multipurpose disinfectants used according to label directions (Virkon-S, Dupont; quaternary ammonium preparation, Livingston); sodium hydroxide (20 g/L 10 min), iodine (0.1% 5 min) and formalin (10% v/v 30 min); and physical measures including heating to 50 °C for 5 min and exposure to a high dose of ultraviolet light. Ineffective disinfectant treatments were: heating to 42 °C for 5 min, and alkaline detergent (2000 ppm, 10 min) (Pyroneg, Johnson Diversey). Sodium hypochlorite (50 ppm available chlorine, 15 min) inactivated OsHV-1 in relatively clean seawater, but this treatment was not effective after addition of protein, 10% v/v foetal bovine serum. A concentration of 200 ppm available chlorine for 15 min did not inactivate OsHV-1 in oyster tissue. This study provides information that will assist in modelling the disease risk posed by OsHV-1 and data that are necessary to devise effective biosecurity strategies to control the spread of OsHV-1 in a variety of situations. The potential role of fomites in the spread of the virus, and recurrence of disease in endemically infected areas were highlighted by the persistence of infectivity in non-living oyster tissues.

Statement of relevance: Data on the duration of infectivity and effective disinfection of OsHV-1 in filed relevant preparations is needed for improved disease control.

1. Introduction

Ostreid herpesvirus-1 (OsHV-1) has gained international prominence as a pathogen of the Pacific oyster (*Crassostrea gigas*). The greatest concern is with the microvariant genotype (OsHV-1 μVar) which was first identified as the cause of unusually severe and widespread disease in Pacific oysters in France in 2008 (Segarra et al., 2010). This genotype was characterised by several polymorphisms in the regions that were sequenced, open reading frame (ORF) 4 and ORF43, and a deletion in a microsatellite region upstream of ORF4, compared to the reference genotype. Several genotypes closely related to OsHV-1 μVar have since been associated with severe disease outbreaks (Martenot et al., 2012, 2013; Renault et al., 2012). To encompass the sequence variation amongst highly pathogenic genotypes of OsHV-1, the World Organisation for Animal Health adopted as the definition of a microvariant a deletion in the microsatellite region upstream of ORF4 (OIE, 2014).

Retrospective studies revealed that OsHV-1 μVar replaced the reference genotype and became the dominant cause of Pacific oyster mortality in both Ireland and France in 2008 (Morrissey et al., 2015; Renault, et al., 2012). Subsequently, the global distribution has increased with announcement of disease of Pacific oysters attributed to OsHV-1 μVar in Spain and the Netherlands (Gittenberger et al., 2015; Roque et al., 2012). Disease caused by OsHV-1 μVar first occurred in the southern hemisphere in Australia and New Zealand in 2010 (Jenkins et al., 2013; Keeling et al., 2014). A wider distribution of OsHV-1 μVar was indicated through surveillance of apparently healthy oysters in Italy, Japan and Portugal (Batista et al., 2015; Shimahara et al., 2012). Acute viral necrosis virus, the cause of a epizootic mortality in Chinese scallops (*Chlamys farreri*) was identified as another variant of OsHV-1 and was found to be widespread in surveys of several bivalve species in China (Bai et al., 2015; Ren et al., 2013).

* Corresponding author at: Faculty of Veterinary Science, The University of Sydney, 425 Werombi Road, Camden, NSW 2570, Australia.
E-mail address: paul.hick@sydney.edu.au (P. Hick).

http://dx.doi.org/10.1016/j.aquaculture.2015.08.025
0044-8486/

In addition to an expanding geographic range, epizootic mortality events associated with OsHV-1 μVar have recurred every summer in the affected growing regions (Bingham et al., 2013; Degremont, 2013). An important component of the disease control response is biosecurity to restrict the range of OsHV-1 which requires understanding transmission pathways. Widespread distribution of oyster spat of French origin was associated with local and international spread of OsHV-1 (Dundon et al., 2011; Peeler et al., 2012; Roque et al., 2012). Methods for spread of the virus to more distant locations may include biofouling of boats and shipping ballast water (Paul-Pont et al., 2014). Unlike several other affected countries, the distribution of OsHV-1 in Australia has been limited to two geographically proximate estuaries (Paul-Pont et al., 2014). This may reflect a rapid, vigorous and sustained biosecurity response that included cessation of movement of live Pacific oysters and farming equipment out of the affected regions (Cameron et al., 2011). However, further understanding about the mechanisms for transmission and spread of OsHV-1 is required for effective containment measures (Paul-Pont et al., 2013a).

Disinfection procedures are important components of disease control, and general guidelines for aquatic animal health are available from the OIE (OIE, 2009). However, interpretation and implementation of this document by industry and regulatory authorities require pathogen specific information. *Ostried herpes virus 1* is the type species of the genus *Ostreavirus* within *Malacoherpesviridae*, a family in the order *Herpesvirales* (King et al., 2011) This virus is morphologically similar to other herpesviruses, with an icosahedral capsid 116 nm in diameter surrounded by a complex lipid envelope (Davison et al., 2005). Its double stranded DNA genome of 207 kb contains many features of the genomic organisation of other herpesviruses (Davison et al., 2005; Renault et al., 1994). The presence of an envelope implies greater susceptibility to degradation in the environment relative to non-enveloped viruses (Quinn, Markey, 2001). This was demonstrated for the aquatic herpesviruses, Koi herpesvirus (KHV) and *Oncorhynchus masou* virus (salmonid herpesvirus, OMV) (Kasai et al., 2005; Yoshimizu et al., 2005). However, in the absence of a permissive cell line for growth *in-vitro*, such data has been more difficult to obtain for molluscan herpesviruses. A bioassay was used to determine that *Haliotid herpesvirus 1* (abalone herpesvirus, AbHV) remained infectious after 1 day in seawater at 15 °C but not at 25 °C (Corbeil et al., 2012). Further, AbHV was shown to be inactivated by iodine and a non-ionic surfactant preparation. OsHV-1 in a tissue homogenate retained infectivity for experimentally infected spat for less than 72 h at 16 °C and <36 h at 25 °C (Martenot et al., 2015). Detailed data on the duration that OsHV-1 retains infectivity in seawater and tissues is required to inform hydrodynamic dispersion models for prediction and monitoring of disease (Pande et al., 2015).

The aims of this study were to determine the stability of OsHV-1 in seawater and oyster tissue to inform studies of viral transmission and spread, including spread modelling, and the efficacy of disinfection measures that could be applied for disease control. For these purposes, a bioassay using live oysters was optimised to identify infectious OsHV-1 with maximum sensitivity.

2. Materials and methods

2.1. Oysters and their husbandry

Triploid Pacific oysters were obtained from a commercial hatchery in Tasmania, Australia and certified free of OsHV-1 by the competent authority. If not used immediately as spat they were held under commercial growing conditions in the Shoalhaven River, New South Wales, which is considered free of OsHV-1 (Animal Health Australia, 2011); oysters (n = 30) from this batch were sampled randomly and tested negative for OsHV-1 prior to the trial.

Two different age groups were used. At the start of the trial older spat were approximately 5 cm shell length and either 9 or 10 months old; younger spat were 4.64 +/− 0.7 mm shell length and 5 months old. They were transported to a physical containment level 2 aquatic facility at the University of Sydney. Oysters were maintained in aquaria with artificial seawater (ASW; Red Sea Salt) at 30 parts per thousand salinity at 20 +/− 1.5 °C. The older spat were maintained in 10–15 L aquaria and younger spat in 1 L plastic containers. Individual aquaria did not share water, and lids were used to reduce cross contamination. Aeration was continuous and a full water exchange was undertaken every 48 h. Oysters were fed a maintenance ration of a commercial algae concentrate (Shellfish Diet 1800, Reed Mariculture) in one session each day.

2.2. Source of infective OsHV-1

Freshly grown OsHV-1 was produced in 2014 immediately prior to each experiment by propagation in donor oysters. The stock virus was prepared as a 0.2 μm filtered 10% w/v tissue homogenate in sterile artificial seawater from naturally infected oysters that were sampled from the Georges River in 2011, as described by Paul-Pont et al. (2013a) and stored at −80 °C. Older spat were injected using a 25 g needle into the adductor muscle with 0.1 mL of a final dilution of 1% w/v of the OsHV-1 infected, homogenised tissue in sterile ASW. Material containing infective OsHV-1 was prepared from a pool of mantle and gill 54 h after challenge of these oysters (n = 15). Tissues were minced with a scalpel blade and then homogenised by stomaching (MiniMix, Crown Scientific) with a 2.5% volume of ASW to make a tissue paste that was further processed as described below. A tissue homogenate prepared from frozen oysters without OsHV-1 infection was injected into donor oysters (n = 6) for the production of negative control water and oyster tissue paste using the same procedure. The water used to maintain these donor oysters and their tissues was used without further storage, requiring the procedure to be repeated after the virus stability experiment for the disinfection studies.

2.3. Stability of OsHV-1

Preparations containing OsHV-1 were incubated in the dark at 20 °C in a refrigerated incubator (Thermoline). The following preparations were examined:

OsHV-1 in wet oyster tissue: After storage the tissue paste was homogenised further by stomaching to a final concentration of 10% w/v in sterile ASW. This homogenate was centrifuged at 1000 *g* for 10 min at 4 °C; the supernatant was filtered to 0.2 μm using syringe driven filters (Minisart, Sarturius) then used in the study.

OsHV-1 in dry oyster tissue: Multiple 1 g portions of the oyster tissue paste were spread thinly on the surface of sterile petri dishes and allowed to dry in a refrigerated incubator in the dark at 20 °C until required. A sample of the dried tissue was removed from the petri dish immediately prior to each time point to test for OsHV-1. The tissue was reconstituted in sterile ASW, made up to 10% w/v tissue homogenate, based on the weight of tissue before drying. This homogenate was centrifuged at 1000 *g* for 10 min at 4 °C; the supernatant was filtered to 0.2 μm.

OsHV-1 in seawater: An aliquot of the seawater which housed the donor oysters used to amplify freshly propagated OsHV-1 was retained and filtered to 0.45 μm. The concentration of OsHV-1 was increased by adding 5% v/v of the 10% filtered tissue homogenate prepared from the mantle and gill of the donor oysters.

Negative controls. Negative control wet and dry oyster tissue and seawater preparations were prepared according to the same methods, but using the mantle and gill tissues of oysters injected with an oyster tissue homogenate that did not contain OsHV-1.

Aliquots were removed at intervals for up to 7 days and tested immediately by qPCR for OsHV-1 and using the bioassay described below. The seawater preparation was tested without further dilution; tissue suspensions were further diluted to 1% w/v in sterile ASW prior to challenge to avoid toxic effects of tissue components on the oysters in the bioassay (data not shown).

2.4. Disinfection of OsHV-1

Disinfection of seawater was performed in a 10 mL volume while treatment of tissue was in a 3 mL volume. Procedures were performed at 20 °C in polypropylene laboratory tubes (Falcon) with minimal exposure to light, unless stated otherwise. Buffer exchange was performed after chemical treatments to remove residual disinfectants prior to the bioassay. A regenerated cellulose 30 000 molecular weight cut-off (MWCO) membrane centrifuge device (Amicon Ultra-15 Ultracel, Merck Millipore) was prepared by pre-rinsing the membrane with ultrapure water (Milli-Q, Millipore). Up to 10 mL of the OsHV-1 preparation was loaded and the device was centrifuged at 4000 *g* for 5 min at 4 °C. The filtrate was discarded and an equal volume of ASW was added to the retentate before repeating the centrifugation. The retentate (containing OsHV-1) was recovered from the membrane and made up to the original volume with sterile ASW. Loss or inactivation of OsHV-1 during the buffer exchange was evaluated using a positive control (OsHV-1 preparation without any disinfection treatment). The quantity of OsHV-1 genome before and after each buffer exchange procedure was assessed by qPCR. The potential for residual toxicity from chemical disinfection to impact the bioassay, despite buffer exchange, was evaluated by performing each chemical treatment on the negative control oyster tissue preparation.

2.4.1. OsHV-1 in seawater

The OsHV-1 in seawater preparation was prepared with 0.45 μm filtered seawater from the holding tanks with infected donor oysters with the addition of 5% v/v of a 0.2 μm filtered tissue homogenate (10% w/v) to increase the concentration of OsHV-1. The treatments were:

- Treatment 1. *Positive control*. Untreated OsHV-1 in seawater held at 4 °C for the duration of the other treatments.
- Treatment 2. *Positive control with buffer exchange*. Untreated OsHV-1 in seawater subject to buffer exchange.
- Treatment 3. *Heat*: A 1 mL aliquot was incubated at 42 °C for 5 min in a hybridisation oven (Thermo).
- Treatment 4. *Ultraviolet light*: The preparation was exposed to UV irradiation for 10 min in a white plastic reagent reservoir (Vistalab Technologies, catalogue number 3054-1000). A dose of >1000 mW/cm^2 at 254 nm was provided at a distance of 15 cm from 2× Sankyo Denki G15T8 15 W germicidal lamps rated at 4.9 W output each, in the UVC range.
- Treatment 5. *Chlorine*. Sodium hypochlorite (125 g/L, Formula Chemicals) was added to a final concentration of 50 ppm available chlorine. The suspension was mixed thoroughly; the duration of contact was 15 min.
- Treatment 6. *Chlorine with high organic load*. A final concentration of 50 ppm available chlorine was prepared as for Treatment 5. Organic load was provided by addition of 10% v/v foetal bovine serum to the OsHV-1 in seawater preparation. The suspension was mixed thoroughly; the duration of contact was 15 min.
- Treatment 7. *Virkon*. The powdered product (Virkon-S DuPont) was freshly prepared in water according to directions of the manufacturer for a 10% w/v solution. This was added to the OsHV-1 preparation to a final concentration of 1% w/v. The preparation was mixed thoroughly; the duration of contact was 15 min.

After these treatments the OsHV-1 in seawater preparations were tested using a bioassay immediately and without further dilution.

2.4.2. OsHV-1 in tissue

The OsHV-1 in tissue preparation was a 0.2 μm filtered 10% w/v homogenate of mantle and gill tissue from oysters 48 h after challenge by injection. The tissue suspension was thoroughly mixed immediately after the addition of the chemical disinfectants. The treatments evaluated for disinfection of OsHV-1 in tissue were:

- Treatment 8. *Positive control*. Untreated OsHV-1 in oyster tissue held at 4 °C for the duration of the other treatments.
- Treatment 9. *Positive control with buffer exchange*. Untreated OsHV-1 in oyster tissue subject to buffer exchange.
- Treatment 10. *Heat*. A 1 ml aliquot incubated at 50 °C for 5 min in a hybridisation oven (Thermo).
- Treatment 11. *Chlorine 100 ppm*: Sodium hypochlorite (125 g/L) was added to a final concentration of 100 ppm available chlorine; the duration of contact was 15 min.
- Treatment 12. *Chlorine 200 ppm*: Sodium hypochlorite (125 g/L) was added to a final concentration of 200 ppm available chlorine; the duration of contact was 15 min.
- Treatment 13. *Virkon 1%*: The appropriate volume of the 10% w/v stock solution prepared for Treatment 7 was added to OsHV-1 in tissue for a final concentration of 1% v/v; the duration of contact was 15 min.
- Treatment 14. *Iodine*. 10% povidone iodine equivalent to 1% available iodine (Betadine antiseptic liquid, FH Faulding & Co) was added to the OsHV-1 in tissue for a final concentration of 0.1% available iodine; the duration of contact was 5 min.
- Treatment 15. *Detergent product*. An alkaline detergent cleaning agent, Pyroneg (Johnson Diversey) was added to the OsHV-1 in tissue for a final concentration of 2 ml per litre; the duration of contact was 10 min.
- Treatment 16. *Sodium hydroxide*. A stock solution of NaOH (Sigma-Aldrich) was prepared in water and an appropriate volume was added to the tissue suspension for a final concentration of 20 g/L; the duration of contact was 10 min.
- Treatment 17. *Formalin*. A 36.5–38% solution of formaldehyde in water (Sigma-Aldrich) was used at a final dilution of 1/25 v/v in the tissue suspension; the duration of contact was 30 min.
- Treatment 18. *Quaternary ammonium compound*. A commercial product containing the cationic surfactant alkyl dimethyl benzyl ammonium chloride (General Disinfectant, Livingstone International) was used at a final dilution of 1/25 v/v in the tissue suspension; the duration of contact was 10 min.

The same chemical disinfection procedures were applied to the negative control oyster tissue homogenates, the buffer exchange procedure was performed and the disinfected material was tested for toxic effects in the bioassay.

The disinfected oyster tissue preparations were tested immediately using the bioassay described below. A further 10-fold dilution of each preparation in sterile ASW was made after treatment, immediately prior to injection or immersion challenge of oysters, resulting in a final concentration representing approximately 1% w/v for tissue components that otherwise may be potentially toxic in the bioassay when present in a more concentrated form.

2.5. Bioassays

Oysters were relaxed by immersion in 50 g/L $MgCl_2$ in freshwater for up to 4 h prior to challenge.

2.5.1. Immersion

Relaxed oysters were rinsed briefly in ASW then transferred to a 1.5 L plastic container with 100 mL of inoculum for younger spat or 250 mL of inoculum for older spat and held for 2 h with intermittent gentle mixing and no aeration. The oysters and inoculum were then transferred to the maintenance aquarium. Bioassays using immersion of younger spat were conducted in duplicate.

2.5.2. Injection

A 100 μL aliquot of the inoculum was injected into the adductor muscle of 10 month old spat using a tuberculin syringe with a 25 g needle; oysters were then transferred to the maintenance aquarium.

Oysters were monitored for mortality each day by assessing the clearance of feed from the water and by testing the response of the valve to light touch and compression. Dead oysters were removed and stored at −80 °C. The trial was terminated 7 days after immersion in, or injection with OsHV-1 preparations and the remaining live oysters were removed and stored at −80 °C.

The number of copies of the OsHV-1 genome was estimated by qPCR in the virus preparations before and after treatment. The number of copies of the OsHV-1 genome in oysters at the time of death and in surviving oysters 7 days after challenge was estimated in gill and mantle tissue for older spat or in pools of multiple entire individuals for younger spat.

2.6. Detection and quantification of OsHV-1 by qPCR

Younger spat were tested in pools (0.3–0.5 g); they were homogenised in 1.5 mL molecular biology grade water (Ultrapure™) in with 2 mL conical screw-cap tubes (Molecular BioProducts) with 2 × 3 mm stainless steel balls (Aussie Sapphires). Older spat were tested individually; a 0.1 g pool of mantle and gill tissue was homogenised in 1 mL of water with 0.4 g of zirconia-silica beads (Biospec Products, Daintree Scientific). Bead beating was performed at frequency 30 for 2 min, repeated after 180° rotation of the tubes (TissueLyser II, Qiagen). The tubes were centrifuged at 900 *g* for 10 min. Nucleic acids were purified from 50 μL of each homogenate using the MagMax-96 Viral Isolation Kit (Ambion) following the manufacturer's recommended protocol on a magnetic particle processor (MagMax Express-96, Ambion).

The number of copies of the B-region of the OsHV-1 genome was determined relative to a plasmid standard described by Paul-Pont et al. (2013b) using a real-time PCR (qPCR) assay adapted from Martenot et al. (2010). Samples were tested in duplicate 25 μL reactions prepared in AgPath-ID qPCR master mix (Life Technologies) using a Mx3000P Multiplex Quantitative PCR System (Stratagene) according to the method described by Paul-Pont et al. (2013b).

2.7. Interpretation of bioassay results

A positive bioassay result was defined by replication of OsHV-1. This was indicated by: mortality of some oysters and a quantity of OsHV-1 ≥ 1.0×10^4 genome copies/mg of tissue; or when there was no mortality, the amount of OsHV-1 in the tissues tested was demonstrably greater than the amount introduced to the treatment group for the untreated inoculum. This was indicated by a concentration of OsHV-1 in tested tissues > 100 times the concentration in the inoculum. The bioassay was considered inconclusive if low quantities of OsHV-1 DNA were detected in oyster tissues 7 days after exposure. The bioassay using immersion of younger spat was defined as positive if at least 1 replicate was positive as defined above.

3. Results

3.1. Sensitivity of the bioassay formats

Injection of 10 month old spat was the most sensitive method of detecting OsHV-1; a 10^{-4} dilution of a preparation of OsHV-1 in seawater resulted in mortality in both replicates, in two separate titration experiments (Table 1). The quantity of OsHV-1 administered at this dilution was 3.6×10^4 genome copies based on quantification of the stock solution (3.6×10^6 OsHV-1 genome copies/μL seawater), the dilution rate (of 10^{-4}) and injection of 0.1 mL. The limit of detection in the bioassay using immersion of 5 month old spat was 10-fold less; spat were immersed in a dilution of the inoculum containing 3.6×10^3 OsHV-1 genome copies/μL in a total volume of 100 ml of seawater (Table 1a). When using 10 month old spat, injection was also superior to immersion for detection of infective OsHV-1 (Table 1b). The higher sensitivity of injection of older spat compared to immersion of younger spat was also demonstrated when the two methods were used concurrently to identify the duration of infectivity of OsHV-1 (Table 3b). For this reason, the bioassay model using injection of 10 month old spat was used to test disinfection methods.

An immersion challenge of spat that had not been relaxed in $MgCl_2$ and were challenged by immersion with or without feeding was not used because of inferior sensitivity compared to a bioassay using relaxed oysters (data not shown).

3.2. Stability of OsHV-1

The initial quantity of OsHV-1 was $>10^6$ genome copies per μL or mg for the seawater and wet oyster tissue preparations, respectively. Following storage at 20 °C, the amount of OsHV-1 genome that was detected in seawater by qPCR declined by 5 logs over the first 24 h and then was undetectable by 5 days (Fig. 1). In contrast, the decline in the oyster tissue preparation at 20 °C was much slower; at day 7 the number of OsHV-1 genome copies/mg was 1.4×10^4 for wet tissue and 8.4×10^5 for dry tissue.

The seawater preparation was tested using the bioassay with immersion of 5 month old spat at each time point. At day 0 there was a positive result with >50% mortality (Table 2). The decline in quantity of OsHV-1 that was detected by qPCR was reflected in the bioassay results, as mortality declined with increasing storage time of the seawater. Although below the limit of quantification by qPCR, the remaining OsHV-1 present in the sample after 24 and 48 h at 20 °C was determined to be infectious using the immersion bioassay. The absence of infectious OsHV-1 at 7 days was confirmed using both immersion and injection bioassays.

The wet oyster tissue homogenate initially contained 3.6×10^6 OsHV-1 genome copies/μL with a decline to 1.4×10^4 copies per μL after 7 days at 20 °C (Fig. 1). The dry tissue contained $>10^5$ genome copies per μL after reconstitution as 10% w/v filtered tissue homogenate at each time it was tested up to 7 days at 20 °C. Infectious OsHV-1 was shown to be present in these preparations by either the immersion or injection bioassay format at each time point tested over the 7 day period (Table 3). The result of each positive bioassay showed very large quantities of the OsHV-1 genome in tissues indicating viral infection and replication in spat of both ages.

3.3. Disinfection of OsHV-1

There were 3.5×10^2 OsHV-1 genome copies/μL in the preparation of seawater used for disinfection studies resulting in each oyster being challenged by injection of 3.5×10^4 genome copies (Table 4). A positive bioassay result for this preparation, with and without buffer exchange, was indicated by amplification of large quantities of OsHV-1 and 100% mortality (n = 9). Treatment of this preparation with 1% Virkon and UV light inactivated the OsHV-1, but heating to 42 °C was ineffective.

Table 1
The sensitivity of different bioassay formats for detection of infectious OsHV-1.

(a) The sensitivity of challenge of spat by immersion compared to challenge of juvenile oysters by injection.

Inoculum	Method	Dilution of inoculum	Quantity OsHV-1[a]	Replicate	n	Cumulative mortality (%)	Dead oysters		Live oysters at day 7		Bioassay outcome
							qPCR	Quantity OsHV-1[a]	qPCR	Quantity OsHV-1[a]	
Seawater containing OsHV-1	Immersion 5 month spat	10^{-2}	3.6×10^4	1	24	50	Positive	1.6×10^6	Positive	1.7×10^6	Positive
				2	20	70	Positive	1.9×10^6	Negative	-	Positive
		10^{-3}	3.6×10^3	1	24	4	Sample not available	n/a	Inconclusive	BLOQ	Inconclusive
				2	24	71	Positive	1.0×10^7	Positive	1.1×10^6	Positive
		10^{-4}	3.6×10^2	1	22	0	No sample	–	Negative	–	Negative
				2	27	0	No sample	–	Inconclusive	BLOQ	Inconclusive
	Injection 10 month spat	10^{-2}	3.6×10^4	1	8	63	Positive (n = 5)	$> 1.0 \times 10^6$	Positive	1.9×10^3–2.5×10^4	Positive
				2	8	63	Positive (n = 5)	$> 1.6 \times 10^6$	Positive (n = 3)	1.8×10^2–6.4×10^3	Positive
		10^{-3}	3.6×10^3	1	8	63	Positive (n = 5)	8.0×10^4–6.0×10^5	Positive (n = 3)	3.5×10^2–4.1×10^3	Positive
				2	8	88	Positive (n = 7)	5.0×10^4–1.6×10^6	Positive (n = 1)	2.5×10^6	Positive
		10^{-4}	3.6×10^2	1	8	50	Positive (n = 4)	2.2×10^2–2.5×10^6	Positive (n = 3) Negative (n = 1)	BLOQ - 3.0×10^2	Positive
				2	8	13	Positive (n = 1)	8.7×10^5	Positive (n = 4) Negative (n = 3)	BLOQ - 3.5×10^5	Positive
Negative control seawater	Immersion 5 month spat	Neat	0	1	24	0	No sample	–	Negative	–	Negative
				2	25	0	No sample	–	Negative	–	Negative
	Injection 10 month spat	Neat	0	1	8	0	No sample	–	Negative (8/8)	–	Negative

(b) A comparison of the sensitivity of challenge by immersion compared to injection of juvenile oysters.

Inoculum	Method	Dilution of inoculum	Quantity OsHV-1[a]	n	Cumulative mortality (%)	Dead oysters		Live oysters at day 7		Bioassay outcome
						qPCR	Quantity OsHV-1[a]	qPCR	Quantity OsHV-1[a]	
OsHV-1 in dilute oyster tissue homogenate	Immersion 10 month spat	$1/10^{-3}$	3.6×10^3	7	0	No sample	–	Negative (n = 7)	–	Negative
		$1/10^{-4}$	3.6×10^2	7	0	No sample	–	Negative (n = 7)	–	Negative
		$1/10^{-5}$	3.6×10^1	7	0	No sample	-	Negative (n = 7)	–	Negative
	Injection 10 month spat	$1/10^{-3}$	3.6×10^3	7	86	Positive (n = 6)	4.0×10^4–4.0×10^6	Positive (n = 1)	2.6×10^3	Positive
		$1/10^{-4}$	3.6×10^2	7	14	Positive (n = 1)	2.3×10^6	Positive (n = 1) Negative (n = 5)	1.4×10^4	Positive
		$1/10^{-5}$	3.6×10^1	7	0	No sample	n/a	Negative (n = 7)	–	Negative
Negative control oyster tissue homogenate	Immersion 10 month spat	Neat	Negative	8	0	No sample	n/a	Negative (n = 8)	–	Negative
	Injection 10 month spat	Neat	Negative	7	0	No sample	n/a	Negative (n = 7)	–	Negative

[a] OsHV-1 genome copies/mg tissue or μL seawater.

Sodium hypochlorite (50 ppm available chlorine) inactivated the OsHV-1, but addition of protein as 10% FBS to the preparation prior to treatment with this dose of chlorine protected the virus from inactivation.

The preparation of oyster tissue used for disinfection studies contained 2.1×10^5 genome copies/μL (Table 5). This preparation was positive in a bioassay in which each oyster was challenged with 2.1×10^6 genome copies when 0.1 mL of a 10% dilution of the tissue was injected. Treatment of the tissue preparation at 50 °C for 5 min inactivated OsHV-1. Disinfection was also achieved using Virkon, the quaternary ammonium compound, sodium hydroxide, formalin and iodine. Treatment with the detergent or sodium hypochlorite at 100 and 200 ppm available chlorine were ineffective.

There was no mortality due to negative control oyster tissue after disinfection treatments (Table 5b). Further, these oysters had no evidence of clinical signs of adductor muscle weakness or reduced feed clearance compared to unchallenged control oysters. This indicated that the buffer exchange procedure was sufficient to prevent any toxic effects of the chemicals on the oysters that might have interfered with the outcome of the bioassay by preventing detection of OsHV-1 replication.

4. Discussion

OsHV-1 is a pathogen of great economic significance globally, but limited data are available on its stability or disinfection (OIE, 2014).

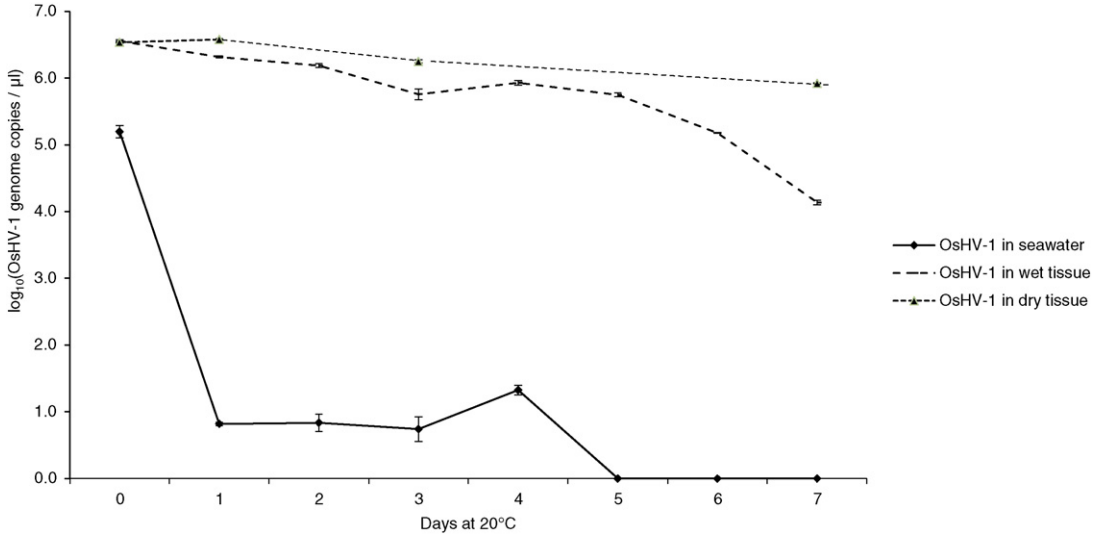

Fig. 1. Quantity of OsHV-1 genetic material present in water and wet and dry oyster tissue samples at 20 °C measured over a period of 1 week. The wet oyster tissue was stored as a tissue homogenate in sterile seawater. The dry oyster tissue was rehydrated and prepared as a 10% w/v (original weight) tissue homogenate at the time of testing. Data are the number of OsHV-1 genome copies/mg of tissue or µL of seawater (log_{10} transformed, average and standard deviation for qPCR of single OsHV-1 preparations).

Studies to measure the inactivation of viruses provide essential information for control of human diseases and diseases of economically important farmed animals, but are difficult and complex to conduct (Sattar and Springthorpe, 2001). The absence of an *in-vitro* culture method to titrate viruses severely inhibits such studies of viral diseases of crustaceans and shellfish. However, a precedent for use of a bioassay to test the virucidal activity of chemicals for abalone herpesvirus was provided by Corbeil et al. (2012). In the present study, an optimised bioassay provided a sensitive method of detecting infectious virus for the purposes of assessing the stability and disinfection of OsHV-1.

4.1. Stability and persistence of OsHV-1

The persistence of infectious OsHV-1 in seawater for 48 h, but not for 72 h is consistent with previous findings for infected tissue homogenates stored at 16 °C and 25 °C (Martenot et al., 2015). The duration of persistence of OsHV-1 in the present study may have been increased because of protection from UV light and because the titre of virus was higher than is found in seawater in a disease outbreak situation (Evans et al., 2014). However, there was no opportunity for the virus to attach to particles present in seawater which might provide protection against degradation in field conditions (Paul-Pont et al., 2013a). This information is relevant for hydrodynamic models to predict the spread of OsHV-1 (Pande et al., 2015). The data also informs disease management practices such as the ageing of water before use or discharge from a hatchery (Whittington et al., 2015).

The OsHV-1 present in dried and moist tissue from acutely infected oysters remained infectious for at least one week at 20 °C, and the end point for viral infectivity was not determined. The concentration of OsHV-1 in infected tissues was such that an infectious dose could be present in a very small volume of dried tissue. These concentrations of virus were typical of those recorded in field outbreaks, where up to 10^7 OsHV-1 genome equivalents/ mg are often reported (Oden et al., 2011; Paul-Pont et al., 2013b). Infectious OsHV-1 in non-living tissues

Table 2
Stability of OsHV-1 in seawater at 20 °C.

Inoculum	Method	Storage time (days)	Quantity OsHV-1[a]	Replicate	n	Cumulative mortality (%)	Dead oysters		Live oysters at day 7		Bioassay outcome
							qPCR	Quantity OsHV-1[a]	qPCR	Quantity OsHV-1[a]	
Seawater containing OsHV-1	Immersion 5 month spat	0	3.4×10^6	1	23	57	Positive	5.1×10^4	Positive	7.6×10^5	Positive
				2	24	50	Positive	1.8×10^6	Positive	BLOQ	Positive
		1	BLOQ (6.6×10^0)	1	20	0	No sample	–	Positive	1.7×10^4	Positive
				2	20	25	Positive	3.0×10^5	Positive	4.8×10^5	Positive
		2	BLOQ (7.5×10^0)	1	20	5	Positive	3.8×10^5	Positive	4.8×10^6	Positive
				2	20	5	Positive	4.7×10^4	Negative	–	Positive
		3	BLOQ (6.5×10^0)	1	24	4	Negative	–	Negative	–	Negative
				2	24	4	Positive	BLOQ	Negative	–	Negative
		5	0 (Negative)	1	20	5	Not tested	–	Negative	–	Negative
				2	19	5	Not tested	–	Negative	–	Negative
		7	0 (Negative)	1	22	0	No sample	–	Negative	–	Negative
				2	21	0	No sample	–	Negative	–	Negative
	Injection 10 month spat	7	0 (Negative)	1	8	0	No sample	–	Negative (n = 8)	–	Negative
Negative control seawater	Immersion 5 month spat	0	0	1	24	0	No sample	–	Negative	–	Negative
				2	25	0	No sample	–	Negative	–	Negative
		7	0	1	20	0	No sample	–	Negative	–	Negative
				2	21	0	No sample	–	Negative	–	Negative
	Injection 10 month spat	7	0	1	8	0	No sample	–	Negative (n = 8)	–	Negative

BLOQ: below the limit of quantification. A positive result for OsHV-1 by qPCR, but below the range of quantification of the assay.
[a] OsHV-1 genome copies/mg tissue or µL seawater.

Table 3
Stability of OsHV-1 in oyster tissue stored at 20 °C. Tissues were either stored wet (as a 1/10 w/v tissue homogenate in sterile seawater) or were allowed to dry and tested after preparing a 1/10 w/v (original weight) tissue homogenate after the storage time.

(a) Wet oyster tissue (stored as a tissue homogenate) at 20 °C.

Inoculum	Method	Storage time (days)	Quantity OsHV-1[a]	Replicate	n	Cumulative mortality (%)	Dead oysters		Live oysters at day 7		Bioassay outcome
							qPCR	Quantity OsHV-1[a]	qPCR	Quantity OsHV-1[a]	
OsHV-1 in oyster tissue	Immersion 5 month spat	0	3.6×10^6	1	25	8	Positive	3.3×10^7	Negative	–	Positive
				2	26	38	Positive	2.1×10^7	Positive	3.5×10^6	Positive
		1	2.1×10^6	1	20	10	Positive	4.6×10^6	Negative	–	Positive
				2	20	45	Positive	2.0×10^7	Positive	3.3×10^6	Positive
		3	5.9×10^5	1	32	9	Positive	6.7×10^7	Positive	2.7×10^6	Positive
				2	26	12	Positive	2.0×10^6	Negative	–	Positive
		7	1.4×10^4	1	20	15	Positive	1.5×10^6	Negative	–	Positive
				2	22	14	Positive	2.5×10^7	Negative	–	Positive
	Injection 10 month spat	7	1.4×10^4	1	8	88	Positive (n = 7)	2.6×10^3–1.9×10^7	Positive (n = 1)	9.4×10^2	Positive
Negative control (OsHV-1 free oyster tissue)	Immersion 5 month spat	0	Negative	1	25	0	No sample	–	Negative	–	Negative
		1	Negative	1	22	0	No sample	–	Negative	–	Negative
		3	Negative	1	25	0	No sample	–	Negative	–	Negative
		7	Negative	1	22	0	No sample	–	Negative	–	Negative
	Injection 10 month spat	7	Negative	1	8	0	No sample	–	Negative (8/8)	–	Negative

(b) Oyster tissue allowed to dry at 20 °C

Inoculum	Method	Storage time (days)	OsHV-1 (copies/µl)	Replicate	n	Cumulative mortality (%)	Dead oysters		Live oysters at day 7		Bioassay outcome
							qPCR	Quantity OsHV-1[a]	qPCR	Quantity OsHV-1[a]	
OsHV-1 in oyster tissue (prepared from dried tissue)	Immersion 5 month spat	0	1.6×10^5	1	25	36	Positive	4.3×10^1	Positive	7.2×10^5	Positive
				2	24	21	Positive	1.5×10^6	Positive	2.2×10^5	Positive
		1	3.8×10^6	1	20	30	Positive	7.5×10^5	Positive	1.7×10^1	Positive
				2	20	25	Positive	2.6×10^6	Positive	6.9×10^2	Positive
		3	1.8×10^6	1	26	15	Positive	9.0×10^5	Positive	8.0×10^5	Positive
				2	24	13	Positive	4.9×10^5	Negative	–	Positive
		7	8.4×10^5	1	20	0	No sample	–	Negative	–	Negative
				2	21	0	No sample	–	Negative	–	Negative
	Injection 10 month spat	7	8.4×10^5	1	8	63	Positive (n = 5)	9.2×10^5–4.3×10^6	Negative (n = 3)	BLOQ	Positive
Negative control (dried oyster tissue)	Immersion 5 month spat	0	0	1	25	0	No sample	–	Negative	–	Negative
		1	0	1	19	0	No sample	–	Negative	–	Negative
		3	0	1	22	0	No sample	–	Negative	–	Negative
		7	0	1	20	0	No sample	–	Negative	–	Negative
	Injection 10 month spat	7	0	1	8	0	No sample	–	Negative (n = 8)	–	Negative

[a] OsHV-1 genome copies/mg tissue or µL seawater

present on surfaces such as boots, clothing, boats, cultivation equipment and debris on oyster shell may be relevant to pathogen spread. Fomites need to be considered in addition to translocation of live oysters to contain the distribution of OsHV-1 over long distances. Furthermore, recurrent outbreaks at endemic locations could be due to release of infective virus from residues of oyster tissue remaining on farm equipment, in sediments, or on other structures, animals and plants. This needs to be examined through field surveys, and the maximum duration of infectivity of OsHV-1 in dried oyster tissues needs to be determined through further laboratory experimentation.

Previously, filtered tissue homogenates from oysters infected with OsHV-1 were found to retain infectivity for 2–3 days at 16 °C and 25 °C and for 3 months at 4 °C, but not when frozen at −20 °C; OsHV-1 retained infectivity in oyster tissues stored at −80 °C (Martenot et al., 2015; Paul-Pont et al., 2015). The tissue preparations in this study provided a matrix, quantity of virus and conditions that were as relevant as possible to a disease outbreak situation in the field (Jouaux et al., 2013; Paul-Pont, et al., 2014).

4.2. Disinfection and inactivation of OsHV-1

This is the first study to report data for physical and chemical inactivation of OsHV-1. The need for effective disinfection to prevent disease spread is highlighted by the duration that OsHV-1 remained infectious outside of a living host. Previously, generic disinfection measures were inferred from studies of similar viruses, and conservatively high doses of disinfectants were recommended, such as 1% chlorine (NSW DPI, 2014).

Several physical and chemical procedures presumed to be suitable for disinfection of herpesvirus in a marine environment were effective. Examples were a commercial product used according to the directions of the manufacturer (Virkon), and generic chemicals at anticipated virucidal concentrations (sodium hydroxide at 20 g/L or 0.1% iodine). There were also some exceptions, for example, a non-ionic surfactant inactivated AbHV (Corbeil et al., 2012), but the alkaline detergent used in this study did not inactivate OsHV-1. The temperature resistance of OsHV-1 was identified as being greater than 42 °C, with 50 °C for 5 min being sufficient to inactivate this virus. Hot water baths with 5 min contact time may provide a relatively practical and environmentally safe approach to disinfection in the field. The high dose of UV used in this study confirmed the efficacy of the similarly high UV exposure reported by Schikorski et al. (2011). In practice, quality assurance of UV disinfection of aquaculture water is difficult to achieve due to problems with measurement of dose, flow rates and durations of exposure, and shading of infectious particles due to turbidity and other factors., However, UV disinfection was shown to be unnecessary in 5 µm filtered seawater in a field setting, filtration alone being sufficient to prevent mortality of spat (Whittington et al., 2015).

Table 4
Disinfection of OsHV-1 in seawater assessed using a bioassay in conjunction with qPCR. The concentration of OsHV-1 in the seawater before treatment was 3.5 × 102 OsHV-1 genome copies/μL.

Treatment ID	Disinfection method				Bioassay result				
	Treatment	Dose/description	Buffer exchange	Quantity OsHV-1[a] (after treatment)	n	Mortality (%)	qPCR oysters		Outcome
							No. positive	Quantity OsHV-1[a] (Range)	
1	No treatment	Positive control	No	1.7×10^2	6	100	6	1.4×10^5–2.9×10^6	Positive
2	No treatment	Positive control	Yes	1.9×10^2	3	100	3	3.0×10^5–9.5×10^5	Positive
3	heat	42 °C 5 min	No	9.1×10^1	6	83	6	9.3×10^2–1.2×10^6	Positive
4	UV irradiation	>1000 mW/cm^2 10 min	No	1.4×10^1	5	0	0	0	Negative
5	Sodium hypochlorite	50 ppm available chlorine 15 min	Yes	1.8×10^2	5	0	0	0	Negative
6	Sodium hypochlorite	10% v/v foetal bovine serum + 50 ppm available chlorine 15 min	Yes	2.2×10^2	5	100	5	1.0×10^4–1.7×10^6	Positive
7	Virkon	1% w/v 15 min	Yes	0	4	0	0	0	Negative

[a] OsHV-1 genome copies/mg tissue or μL seawater.

Importantly, this study identified that sodium hypochlorite (chlorine), a widely available and commonly used disinfectant, was not effective at a concentration of 200 ppm. Inactivation of the aquatic herpesviruses, Koi herpesvirus and *O. masou* virus with as little as 1 ppm chlorine for 20 min has been reported (Kasai et al., 2005; Yoshimizu et al., 2005). Inactivation of viral pathogens in healthcare settings often uses 200 ppm available chlorine with up to 5000 ppm used when disinfection more resistant pathogens such as the spores of *Clostridia* spp. is required (Rutala and Weber, 2008). In a marine environment, available chlorine derived from calcium hypochlorite did not

Table 5
Disinfection of OsHV-1 in oyster tissue at 20 °C (as a 10% w/v tissue homogenate in seawater containing 2.1×10^5 genome copies/μL).

(a) The result of a bioassay and qPCR to determine the effectiveness of the chosen disinfection methods

Treatment ID	Disinfection method				Bioassay result				
	Treatment	Dose/description	Buffer exchange	Quantity OsHV-1[a] (after treatment)	n	Mortality (%)	qPCR oysters		Outcome
							No. positive	Quantity OsHV-1[a] (range)	
8	No treatment	Positive control OsHV-1	No	2.1×10^5	6	100	6	3.3×10^4–1.1×10^6	Positive
9	No treatment	Positive control OsHV-1 after buffer exchange	Yes	9.5×10^4	8	100	8	5.7×10^4–4.4×10^6	Positive
10	Heat	50 °C 5 min	No	6.3×10^4	8	0	0	0	Negative
11	Sodium hypochlorite	100 ppm available chlorine 15 min	Yes	4.5×10^4	8	100	8	1.8×10^5–1.4×10^6	Positive
12	Sodium hypochlorite	200 ppm available chlorine 15 min	Yes	3.5×10^4	8	100	8	4.1×10^3–1.1×10^6	Positive
13	Virkon	1% w/v (Virkon-S, DuPont) 10 min	Yes	1.2×10^2	6	0	0	0	Negative
14	Iodine	0.1% available iodine 5 min	Yes	6.5×10^4	6	0	0	0	Negative
15	Alkaline detergent	0.2% Pyroneg (JohnsonDiversey) 10 min	Yes	3.4×10^4	6	100	6	1.0×10^5–9.2×10^5	Positive
16	Sodium hydroxide	20 g/L NaOH 10 min	Yes	9.8×10^2	6	0	0	0	Negative
17	Formalin	10% v/v 30 min	Yes	2.2×10^4	6	0	0	0	Negative
18	Quaternary ammonium compound (QAC)	1/25 v/v commercial QAC preparation (Livingston) 10 min	Yes	3.1×10^4	6	0	0	0	Negative

(b) Chemical disinfection methods performed on an oyster tissue preparation without OsHV-1 to determine the effect of chemical treatments on the bioassay

Treatment ID	Disinfection method				Bioassay result				
	Treatment*	Dose/description	Buffer exchange	Quantity OsHV-1[a] (after treatment)	n	Mortality (%)	qPCR oysters		Outcome
							No. positive	Quantity OsHV-1[a] (Range)	
19	Chemical control	Sodium hypochlorite, 200 ppm available chlorine 15 min	Yes	0	6	0	0	–	Negative
20	Chemical control	Commercial QAC preparation (Livingston) 1/25 v/v 10 min	Yes	0	3	0	0	–	Negative
21	Chemical control	Formalin solution 10% v/v 30 min	Yes	0	3	0	0	–	Negative
22	Chemical control	Alkaline detergent 0.2% (Pyroneg, JohnsonDiversey), 10 min	Yes	0	3	0	0	–	Negative
23	Chemical control	Iodine 0.1% available (Betadine antiseptic, FH Faulding & Co) 5 min	Yes	0	3	0	0	–	Negative
24	Chemical control	Virkon-S (DuPont) 1% w/v 10 min	Yes	0	3	0	0	–	Negative
25	Chemical control	Sodium hydroxide 20 g/L 10 min	Yes	0	3	0	0	–	Negative

[a] OsHV-1 genome copies/mg tissue or μL seawater.

inactivate AbHV at a concentration of 20 ppm when assessed by an injection challenge, although the results of immersion bioassay suggested that the infectivity of virus in the preparation was reduced (Corbeil et al., 2012). In the present study, a dose of sodium hypochlorite calculated to provide 50 ppm available chlorine inactivated OsHV-1 in relatively clean seawater. However, as expected, the presence of organic matter in the form of 10% foetal bovine serum inhibited the virucidal activity of this treatment. Sodium hypochlorite at the intended concentration of 200 ppm available chlorine was ineffective for disinfection of naturally infected oyster tissue. However, the organic matter in the tissue did not prevent the activity of heat, the oxidative disinfectant Virkon and several other chemical treatments. These treatments are therefore also expected to be efficacious in water with moderate organic load.

The data from this study will assist farmers and regulators to interpret the general guidelines often provided for disinfection so that specific decontamination protocols can be recommended for specific pathogens and in each unique aquaculture setting (Department of Agriculture Fisheries and Forestry, 2008). This document indicates the need to follow precise instructions for use of disinfectants, and to measure their activity. For example, treatment of water for all pathogens required an initial concentration of 30 ppm available chlorine with a residual of 5 ppm after 24 h. The use of sentinel oysters to evaluate the success of decontamination procedures will be informed by the format of bioassays described in this study.

4.3. A bioassay for OsHV-1

Infection models for OsHV-1 have been described that use injection or cohabitation (Paul-Pont et al., 2015; Schikorski et al., 2011). For this study, the bioassay that was most fit-for-purpose based on analytical sensitivity used injection of 10 month old spat to detect infectious OsHV-1. The limit of detection was 3.6×10^4 copies of the OsHV-1 genome per mL of inoculum. Notwithstanding, many of the copies of the genome detected by PCR were likely to have been virus replication intermediates from the host cell rather than infectious virions. A greater volume of the inoculum was used for exposure by immersion but this did not result in an improved limit of detection for the bioassay. However, the immersion bioassay provided useful information about the infectivity of OsHV-1 by a route that might more closely resemble natural transmission. Differential mortality between challenged and control oysters was evident. Mortality alone was not a reliable indicator of replication of OsHV-1 and histolopathological examination was not expected to provide a reliable method for identifying disease caused by OsHV-1 (Jenkins et al., 2013; Keeling et al., 2014). Thus qPCR was necessary to provide specificity to the bioassay and it potentially increased its sensitivity by identifying subclinical infection. Quantification of OsHV-1 DNA provided unequivocal evidence of virus replication in most cases, as the number of copies of the genome in the samples was in excess of the total amount added to the system at the time of inoculation, or else no OsHV-1 was detected. At the lowest doses in the titration of OsHV-1 by immersion of spat, an inconclusive bioassay result the detection of trace quantities of OsHV-1 DNA. The method for detecting viral RNA described by Martenot et al. (2015) provides a direct method of demonstrating replication of OsHV-1 that does not require quantification, and is applicable in bioassay studies.

5. Conclusions

The role of fomites as well as live oysters must be considered to control the spread of OsHV-1 because a high titre of OsHV-1 can persist in the tissues of dead oysters, even after drying, for periods of at least one week. The persistence of an infectious dose of OsHV-1 in seawater for 2 but not 3 days informs hydrodynamic models for the distribution of OsHV-1 and water treatment for hatcheries. Disinfection of tissues and water can be achieved with relatively inexpensive methods including heat and commercial or generic chemicals. Disinfection protocols need to consider the material that is to be disinfected. The disinfectant chlorine at commonly recommended concentrations was not effective for inactivation of OsHV-1 in the presence of modest quantities of organic matter.

Acknowledgements

This study was funded by the Australian Government through Fisheries Research and Development Corporation (FRDC Project No. 2012/032) and by the University of Sydney. Oysters used in this study were donated by Scott Parkinson, Shellfish Culture Tasmania. Oysters were grown under commercial conditions prior to use by Leon and Angela Reipsamen, Goodnight Oysters, New South Wales. Expert technical assistance was provided by Alison Tweedie, Vickie Patten, Craig Kristo and Stuart Glover. Serge Corbeil is thanked for useful discussions.

References

Animal Health Australia, 2011. Animal Health Surveillance Quarterly Report. 16 (3) pp. 8–9.
Bai, C.M., Wang, C.M., Xia, J.Y., Sun, H.L., Zhang, S., Huang, J., 2015. Emerging and endemic types of Ostreid herpesvirus 1 were detected in bivalves in China. J. Invertebr. Pathol. 124, 98–106.
Batista, F.M., Lopez-Sanmartin, M., Grade, A., Morgado, I., Valente, M., Navas, J.I., Power, D.M., Ruano, F., 2015. Sequence variation in ostreid herpesvirus 1 microvar isolates detected in dying and asymptomatic *Crassostrea angulata* adults in the Iberian Peninsula: insights into viral origin and spread. Aquaculture 435, 43–51.
Bingham, P., Brangenberg, N., Williams, R., Andel, M.v., 2013. Marine and freshwater investigation into the first diagnosis of ostreid herpesvirus type 1 in Pacific oysters. Surveillance. Ministry for Primary Industries, Wellington, New Zealand, p. 40.
Cameron, A., Crane, M.S.J., (facilitators), 2011. Final Report: OsHV-1 µVar International Workshop, Cairns, Queensland, Australia 9–10 July 2011. AusVet Animal Health Services and Fisheries Research and Development Corporation, Canberra.
Corbeil, S., Williams, L.M., Bergfeld, J., Crane, M.S.J., 2012. Abalone herpes virus stability in sea water and susceptibility to chemical disinfectants. Aquaculture 326, 20–26.
Davison, A.J., Trus, B.L., Cheng, N.Q., 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.
Degremont, L., 2013. Size and genotype affect resistance to mortality caused by OsHV-1 in *Crassostrea gigas*. Aquaculture 416, 129–134.
Department of Agriculture Fisheries and Forestry, 2008. Operational Procedures Manual — Decontamination (Version 1.0), Australian Aquatic Veterinary Emergency Plan (AQUAVETPLAN). Australian Government Department of Agriculture, Fisheries and Forestry, Canberra, ACT.
Dundon, W.G., Arzul, I., Omnes, E., Robert, M., Magnabosco, C., Zambon, M., Gennari, L., Toffan, A., Terregino, C., Capua, I., Arcangeli, G., 2011. Detection of Type 1 Ostreid Herpes variant (OsHV-1 mu var) with no associated mortality in French-origin Pacific cupped oyster *Crassostrea gigas* farmed in Italy. Aquaculture 314, 49–52.
Evans, O., Paul-Pont, I., Hick, P., Whittington, R.J., 2014. A simple centrifugation method for improving the detection of Ostreid herpesvirus-1 (OsHV-1) in natural seawater samples with an assessment of the potential for particulate attachment. J. Virol. Methods 210, 59–66.
Gittenberger, A., Voorbergen-Laarman, M.A., Engelsma, M.Y., 2015. Ostreid herpesvirus OsHV-1 muVar in Pacific oysters *Crassostrea gigas* (Thunberg 1793) of the Wadden Sea, a UNESCO world heritage site. J Fish Dis.
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 mu-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis. Aquat. Org. 105, 109–126.
Jouaux, A., Lafont, M., Blin, J.-L., Houssin, M., Mathieu, M., Lelong, C., 2013. Physiological change under OsHV-1 contamination in Pacific oyster *Crassostrea gigas* through massive mortality events on fields. BMC Genomics 14, 590.
Kasai, H., Muto, Y., Yoshimizu, M., 2005. Virucidal effects of ultraviolet, heat treatment and disinfectants against Koi herpesvirus (KHV). Fish Pathol. 40, 137–138.
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. Org. 109, 231–239.
King, A.M., Lefkowitz, E., Adams, M.J., Carstens, E.B., 2011. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Science, Burlington.
Martenot, C., Oden, E., Travaille, E., Malas, J.P., Houssin, M., 2010. Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster *Crassostrea gigas*. J. Virol. Methods 170, 86–89.
Martenot, C., Fourour, S., Oden, E., Jouaux, A., Travaille, E., Malas, J.P., Houssin, M., 2012. Detection of the OsHV-1 mu Var in the Pacific oyster *Crassostrea gigas* before 2008 in France and description of two new microvariants of the Ostreid Herpesvirus 1 (OsHV-1). Aquaculture 338, 293–296.
Martenot, C., Travaille, E., Lethuillier, O., Lelong, C., Houssin, M., 2013. Genome exploration of six variants of the Ostreid Herpesvirus 1 and characterization of large deletion in OsHV-1 mu Var specimens. Virus Res. 178, 462–470.

Martenot, C., Denechere, L., Hubert, P., Metayer, L., Oden, E., Trancart, S., Travaillee, E., Houssin, M., 2015. Virulence of Ostreid herpesvirus 1 mu Var in sea water at 16 degrees C and 25 degrees C. Aquaculture 439, 1–6.

Morrissey, T., McCleary, S., Collins, E., Henshilwood, K., Cheslett, D., 2015. An investigation of ostreid herpes virus microvariants found in *Crassostrea gigas* oyster producing bays in Ireland. Aquaculture 442, 86–92.

NSW DPI, 2014. POMS Oyster Equipment Movement Protocol. Second edition. New South Wales Department of Primary Industry Prime Fact 1287 (www.dpi.nsw.gov.au/factsheets accessed 12-6-2015.).

Oden, E., Martenot, C., Berthaux, M., Travaille, E., Malas, J.P., Houssin, M., 2011. Quantification of ostreid herpesvirus 1 (OsHV-1) in *Crassostrea gigas* by real-time PCR: determination of a viral load threshold to prevent summer mortalities. Aquaculture 317, 27–31.

OIE, 2009. Chapter 1.1.3 Methods for disinfection of aquaculture establishments. Manual of Diagnostic Tests for Aquatic Animals (World Organisation for Animal Health) (Accessed at: http://www.oie.int/international-standard-setting/aquatic-manual/access-online/.).

OIE, 2014. Chapter 2.4.9 Infection with Ostreid herpesvirus 1 microvariant (version adopted May 2014). Manual of Diagnostic Tests for Aquatic Animals (World Organisation for Animal Health) (Accessed at: http://www.oie.int/international-standard-setting/aquatic-manual/access-online/.).

Pande, A., Acosta, H., Brangenberg, N.A., Keeling, S.E., 2015. Design of a detection survey for Ostreid herpesvirus-1 using hydrodynamic dispersion models to determine epidemiological units. Prev. Vet. Med. 119, 80–84.

Paul-Pont, I., Dhand, N.K., Whittington, R.J., 2013a. Spatial distribution of mortality in Pacific oysters *Crassostrea gigas*: reflection on mechanisms of OsHV-1 transmission. Dis. Aquat. Org. 105, 127–138.

Paul-Pont, I., Dhand, N.K., Whittington, R.J., 2013b. Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. Aquaculture 412, 202–214.

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, 146–159.

Paul-Pont, I., Evans, O., Dhand, N.K., Whittington, R.J., 2015. Experimental infections of Pacific oyster *Crassostrea gigas* using the Australian ostreid herpesvirus-1 (OsHV-1) microVar strain. Dis. Aquat .Org. 113, 137–147.

Peeler, E.J., Reese, R.A., Cheslett, D.L., Geoghegan, F., Power, A., Thrush, M.A., 2012. Investigation of mortality in Pacific oysters associated with Ostreid herpesvirus-1 mu Var in the Republic of Ireland in 2009. Prev. Vet. Med. 105, 136–143.

Quinn, P., Markey, B., 2001. Disinfection and disease prevention in veterinary medicine. In: Block, S.S. (Ed.), Disinfection, Sterilization, and Preservation. Lippincott Williams & Wilkins, Philadelphia, PA, p. 1481.

Ren, W.C., Chen, H.X., Renault, T., Cai, Y.Y., Bai, C.M., Wang, C.M., Huang, J., 2013. Complete genome sequence of acute viral necrosis virus associated with massive mortality outbreaks in the Chinese scallop *Chlamys farreri*. Virol. J. 10.

Renault, T., Cochennec, N., Le Deuff, R.-M., Chollet, B., 1994. Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. Bull. Eur. Assoc. Fish Pathol. 14, 64–66.

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.

Roque, A., Carrasco, N., Andree, K.B., Lacuesta, B., Elandaloussi, L., Gairin, I., Rodgers, C.J., Furones, M.D., 2012. First report of OsHV-1 microvar in Pacific oyster (*Crassostrea gigas*) cultured in Spain. Aquaculture 324, 303–306.

Rutala, W.A., Weber, D.J., 2008. Guideline for Disinfection and Sterilization in Healthcare Facilities. Centres for Disease Control and Prevention, Atlanta (GA 30333).

Sattar, S., Springthorpe, S., 2001. Methods of Testing the Virucidal Activity of Chemicals. Lippincott Williams & Wilkins, Philadelphia, PA.

Schikorski, D., Renault, T., Saulnier, D., Faury, N., Moreau, P., Pepin, J.F., 2011. Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. Vet. Res. 42.

Segarra, A., Pepin, J.F., Arzul, I., Morga, B., Faury, N., Renault, T., 2010. Detection and description 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.

Shimahara, Y., Kurita, J., Kiryu, I., Nishioka, T., Yuasa, K., Kawana, M., Kamaishi, T., Oseko, N., 2012. Surveillance of type 1 Ostreid Herpesvirus (OsHV-1) variants in Japan. Fish Pathol. 47, 129–136.

Whittington, R.J., Hick, P.M., Evans, O., Rubio, A., Alford, B., Dhand, N., Paul-Pont, I., 2015. Protection of Pacific oyster (*Crassostrea gigas*) spat from mortality due to ostreid herpesvirus 1 (OsHV-1 μVar) using simple treatments of incoming seawater in land-based upwellers. Aquaculture 437, 10–20.

Yoshimizu, M., Shimizu, T., Kasai, H., 2005. Survivability of koi herpesvirus (KHV) in environmental water as well as physical and chemical inactivation. Fish Pathol. 40, 203.