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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

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A B S T R A C T

Ostreid herpesvirus-1 (OsHV-1) is responsible for massive mortality events in commercially farmed Pacific oysters (*Crassostrea gigas*) in Australia, New Zealand, Europe and the USA. Economic losses have been severe in many countries since 2008, associated with a strain known as OsHV-1μ-var. Despite intensive studies of the virus itself, there is almost no information on its detection in natural seawater, how it is spread over wide geographic distance in water or on how it is transmitted from oyster to oyster *via* seawater. The aim of the current work was to (1) assess and compare several centrifugation methods in order to detect OsHV-1 in natural seawater samples using real-time quantitative PCR, in such a way that large numbers of samples could be processed efficiently and (2) assess the potential for particulate attachment of OsHV-1 using filtration. Compared to testing unprocessed seawater samples, centrifugation of seawater at 1000 × *g* for 20 min with testing of the pellet improved OsHV-1 detection rates by two fold. Results suggest that OsHV-1 may be attached to particles large enough to be pelleted at low *g*-force, as well as in the form of small particles, free virus or free viral DNA. Filtration of seawater using low protein binding filters could not be used to assess OsHV-1 particle attachment, due to interactions between particles, free virus or free viral DNA and the membranes.

1. Introduction

Ostreid herpesvirus-1 (OsHV-1) is a member of the family *Malacoherpesviridae* within the order *Herpesvirales* (ICTV, 2013). OsHV-1 has been identified as one of the key pathogens causing summer mortalities in the Pacific oyster (*Crassostrea gigas*) in Europe (Le Deuff and Renault, 1999; Renault et al., 2000; Roque et al., 2012), Australia (Jenkins et al., 2013; Paul-Pont et al., 2013), New Zealand (Keeling et al., 2014) and the US (Burge et al., 2006, 2007). In 2010 the *C. gigas* industry generated the highest contribution (650,000 tonnes–US$ 1.14 billion) to the worldwide production of oysters in aquaculture (4.3 million tonnes–US$4.3 billion) (FAO, 2010), and losses due to OsHV-1 have been severe (EFSA, 2010; Keeling et al., 2014; Paul-Pont et al., 2014).

Summer mortally syndrome was first identified in adult oysters in Japan in the early 1960s (Mori, 1979), with involvement of OsHV-1 confirmed in juvenile mortality events over the following decades in several countries (Renault et al., 2000). Spat and juvenile oysters are worst affected by OsHV-1 with mortalities of between 40 and 100% typically seen, however all age and size classes are known to be susceptible to the disease (Arzul et al., 2002; Garnier et al., 2007; Schikorski et al., 2011b; Paul-Pont et al., 2013). In 2008 a new strain known as OsHV-1μ-var emerged and has been responsible for mass mortalities in Europe, Australia and New Zealand (Renault and Arzul, 2001; Keeling et al., 2014; Paul-Pont et al., 2014). Despite the dramatic impact that OsHV-1 has had on these highly valuable stocks, little information exists on the mechanisms of transmission and spread of the disease in open marine environments (Renault and Novoa, 2004; Garcia et al., 2011; Paul-Pont et al., 2013). In fact such questions have mostly been overlooked (Paul-Pont et al., 2013) and as a consequence there is little knowledge about methods to detect OsHV-1 in seawater.

It has been suggested that seawater may act as a medium in the horizontal transmission of OsHV-1 (Sauvage et al., 2009) and previous studies in small aquaria by Schikorski et al. (2011a) demonstrated that OsHV-1 could be transmitted by cohabitation of infected oysters with healthy oysters. Recent studies by Paul-Pont et al. (2013) have demonstrated a non-random and highly clustered distribution of Pacific oyster mortalities in the field. This led to an hypothesis about attachment of the virus to particles

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with planktonic characteristics, either live organisms or non-viable or inorganic particles. It is well known that viruses, like other microbes, attach to particles in their environment through complex reversible and irreversible interactions, and so their fate and transport is associated with those particles (Tufenkji, 2007). Pathogens, including bacteria and viruses, present in aquatic environments have been shown to become adsorbed onto the surfaces of suspended particles, which enhances pathogen transmission, stability and survival (Bitton, 1975; Sakoda et al., 1997; Tang et al., 2011). Thus the possibility that OsHV-1 can attach to particles in seawater is worthy of investigation and may provide options for detection.

Studies have suggested that viruses in water tend to be far less concentrated than viruses in other matrices, such as infected oyster tissue and sediments, typically requiring some form of concentration to enhance detection (Paul et al., 1993; Katayama et al., 2002; Haramoto et al., 2009; Schikorski et al., 2011a). Methods such as ultrafiltration, tangential flow filtration, ultracentrifugation, precipitation, and adsorption of viruses onto positively or negatively charged membranes, can all be used to concentrate viruses from water samples (Katayama et al., 2002; Haramoto et al., 2009; Honjo et al., 2010; Shinohara et al., 2011; Suzuki et al., 2011). However these methods are not practical when large numbers of samples need to be tested, as is the case for epidemiological studies on OsHV-1. Nor can these methods be easily utilised in the assessment of potential virus association to particulates of varying sizes, as many of these methods require or result in virus dissociating from matter within the sample matrix.

The aim of the current work was to (1) assess and compare several simple centrifugation methods in order to detect OsHV-1 in natural seawater samples using real-time quantitative PCR (qPCR), in such a way that large numbers of samples could be processed efficiently and (2) assess the potential for particulate attachment of OsHV-1 using a simple filtration methodology. Low speed centrifugation was found to improve OsHV-1 detection rate, and suggested that OsHV-1 may exist in association with particles as well as free in seawater. Filtration was found to be an unsuitable method for assessing potential OsHV-1 particulate attachment.

2. Materials and methods

2.1. Seawater samples

Samples were collected from Woolooware Bay in the Georges River estuary 16 km south of Sydney, NSW, Australia and the Hawkesbury River estuary 40 km north of Sydney, NSW Australia between November 2012 and May 2013, at times when mortalities due to OsHV-1 were observed in farmed *C. gigas* in each estuary. Samples were collected using three different methods: (i) a 11.00 μm mesh plankton net with a 200 mL removable collection jar (Allied Filter Fabrics, Hornsby, Australia), towed from a boat. Artificial seawater was used to wash material caught inside the net down into the collection jar. Collection jars were removed, sealed with lids, shaken to ensure the contents were well mixed and a subsample of 120 mL was then transferred into a sterile polycarbonate screw cap jar; (ii) a horizontal beta bottle Van Dorn style water sampler (The Environmental Collective, ENVCO Global, Auckland, New Zealand); subsurface samples were collected into plain polyethylene bottles of ≥1 L capacity and (iii) submerging a ≥1 L capacity clean, polyethylene bottle approximately 200 mm below the surface of the water and allowing the bottle to fill with water. Samples were placed into an insulated box (esky) for transport to the laboratory. All collection jars and nets were rinsed thoroughly with artificial seawater between tows, and disinfected with an oxidising disinfectant (1% Virkon® Antec International, Sydney, Australia) between each trip to the river.

2.2. Water centrifugation protocol

As OsHV-1 may be associated with particles in seawater, centrifugation may be a useful method to concentrate virus prior to examination by qPCR. Plain unprocessed seawater was compared to pelleted seawater samples in Experiment 1, and pelleted seawater samples to supernatant in Experiment 2.

2.2.1. Experiment 1

A total of 248 water samples were used for the analysis of unprocessed water samples compared to the pellet of the same water samples after low speed centrifugation. All samples used in this analysis were kept for less than 24 h at 4 °C after collection and prior to processing. Samples were shaken vigorously for 30 s to resuspend particles. An aliquot of 1 mL was removed and placed into a 1.5 mL screw cap tube containing 0.2 g silica beads (Daintree Scientific, St. Helens, Australia) and these tubes were kept at −80 °C until further analysis (unprocessed sample). Fifteen millilitres of the remaining sample was then poured into a 15 mL polypropylene tube (Falcon) and centrifuged at 1000 × *g* for 20 min (Allegra® 12X-R, Beckman Coulter®, Lane Cove, Australia). This volume/tube was chosen for convenience. The supernatant was decanted to leave 2 mL of supernatant and the pellet, which were then shaken vigorously for 30 s to resuspend the pellet. A 1 mL aliquot of the pellet was stored in a 1.5 mL screw cap tube containing 0.2 g silica beads at −80 °C until further analysis (pellet sample).

2.2.2. Experiment 2

A total of 248 water samples, distinct from those in Experiment 1, were used in the analysis of supernatant compared to the pellet of the water samples after low speed centrifugation. All samples used in this analysis were frozen at −20 °C prior to processing. Samples were thawed overnight at room temperature and shaken vigorously for 30 s to resuspend particles. An aliquot of 15 mL was poured into a 15 mL polypropylene tube and centrifuged at 1000 × *g* for 20 min. A 1 mL aliquot of the supernatant was removed and placed into a 1.5 mL screw cap tube containing 0.2 g silica beads (supernatant sample). The remaining supernatant was decanted to leave 2 mL of supernatant and the pellet. The tube was shaken vigorously for 30 s to resuspend the pellet and a 1 mL aliquot was removed and placed into a 1.5 mL screw cap tube containing 0.2 g silica beads (pellet sample). Tubes were frozen at −80 °C until further analysis.

2.3. Experiment 3 water filtration

As OsHV-1 may be associated with particles in water, filtration may provide useful information with regard to OsHV-1 particle attachment or association, as well as the size range of these particles.

Twenty-five water samples, distinct from those in Experiments 1 and 2, were used in this analysis. All samples were kept for less than 24 h at 4 °C after collection and prior to processing. Processing of each sample occurred according to the procedure illustrated in Fig. 1. Briefly a 1 mL aliquot of the original water sample was taken and put into a 1.5 mL screw cap tube containing 0.2 g silica beads (control sample). Fifteen millilitres of water was filtered both in series and individually. For series filtration samples were filtered sequentially through a 5.00 μm, 0.45 μm surfactant free cellulose acetate membrane (Sartorius Minisart®, Stedim Biotech, Sydney, Australia) and a 0.22 μm polyethersulfone membrane (MILLEX®-GP, Millipore, Bayswater, Australia) sterile, single use, low protein binding syringe filter unit and 1 mL of each filtrate was collected and stored at −80 °C until further analysis. For individual filter analysis water samples were passed through either a 5.00 μm, a 0.45 μm or a 0.22 μm sterile single use low protein binding syringe filter unit and 1 mL of each filtrate was collected and stored at −80 °C until further

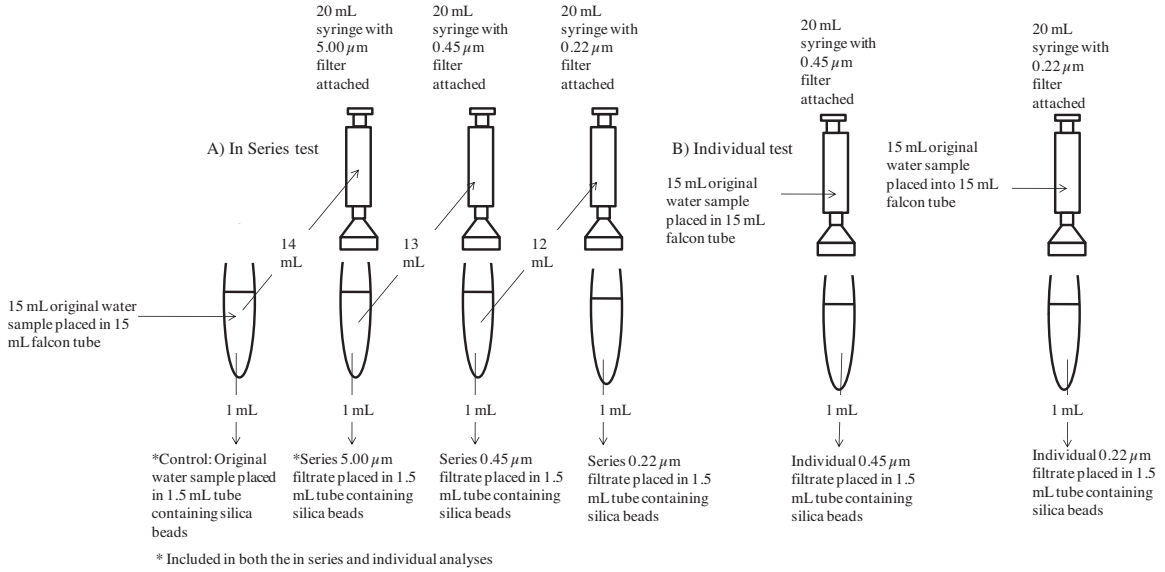

Fig. 1. Schematic representation of filtration process for (A) in series and (B) individual sample examination for Experiment 3.

analysis. All filter units (used for individual and series filtration) were kept and stored in individual 70 mL screw cap jars at −80 °C until further processing of the membranes. All work benches, biosafety cabinet surfaces and pipetting equipment were disinfected with 200 ppm sodium hypochlorite solution both before and after filtration to eliminate the potential for cross contamination.

The filter membrane was manually removed from the partially thawed filter unit using laboratory tweezers and placed into a 1.5 mL screw cap tube containing 1 mL of Ultrapure™ distilled water (Invitrogen™, Life Technologies™, Mulgrave, Australia) and 0.2 g silica beads. Tubes were frozen at −80 °C until further analysis. All equipment was disinfected and rinsed in both hot and cold water prior to use and in between each membrane removal.

A pilot study utilising 5 water samples, previously confirmed positive by qPCR, was conducted prior to Experiment 3. Results of this trial suggested OsHV-1 could be detectable at all stages of the filtration analysis, and that membranes would be positive by qPCR most often where corresponding filtrates were also positive. This suggested that the virus detected may be present in a range of forms, including free virus, flocculated or aggregated virus, virus adsorbed to particles, or various viral components, including free DNA. Based on these results, and the more efficient nature of testing filtrates compared to membranes, filter membranes in Experiment 3 were tested only if the corresponding filtrates tested positive by qPCR at any stage of the analysis.

2.4. Homogenisation and DNA extraction

All 1.5 mL tubes containing samples and silica beads were subjected to the same homogenisation procedure to separate the virus from particles in the sample. Tubes were thawed and placed into a bead-beating machine (Fastprep®-24 System, MP Biomedical, Seven Hills, Australia) for 15 s at a speed of 6.5 m/s. Samples were then clarified by centrifugation at 1000 × *g* for 10 min in a microcentrifuge (Heraeus® Biofuge® Pico, Thermo Electron Corporation, Asheville, USA), and supernatants were stored at −80 °C until DNA extraction.

DNA extraction was performed using a 5X MagMAX™-96 Viral RNA Isolation Kit (Ambion®, Life Technologies™, Mulgrave, Australia) and MagMAX™ Express 96 magnetic particle processor (Applied Biosystems™, Life Technologies™, Mulgrave, Australia) according to manufacturer's instructions for a 50 μL sample volume protocol using the AM1836 deep-well standard programme (Ambion®, Life Technologies™, Mulgrave, Australia). However a sample volume of 200 μL was used for all extractions. Nucleic acid preparations were stored at −20 °C until qPCR analysis.

2.5. Polymerase chain reaction

Two real-time qPCR methods were used to analyse the DNA extracts. The first assay, used to analyse all samples in Experiment 1, was a Taqman® assay adapted from an assay developed by Martenot et al. (2010). An AgPath-ID One-Step RT-PCR kit (Life Technologies™, Mulgrave, Australia) was used. The reaction contained 12.5 μL of 2× real-time qPCR buffer, 5.425 μL of nuclease free water, 0.225 μL of 100 μM OsHV1BF forward primer (5′- GTC GCA TCT TTG GAT TTA ACA A -3′), 0.225 μL of 100 μM OsHV1B4 reverse primer (5′- ACT GGG ATC CGA CTG ACA AC-3′), 0.625 μL of 10 μM OsHV-1 probe (5′-6FAM-TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC-TAMRA-3′), 1 μL of 25× real-time qPCR enzyme mix, and 5 μL of neat nucleic acid extract, resulting in a 25 μL total reaction volume. Purified nucleic acid from known OsHV-1 infected oysters was used for the positive control. Negative controls consisted of only the PCR mixture described above without any template DNA. Standards were created from plasmid *pOSHV1-Breg* (2×10^6 copies/μL) (University of Sydney, Camden, Australia) with a 10-fold dilution series (10^6–10^1 copies/μL) used to create the standard curve. All controls, standards and sample DNA were tested in duplicate. A real time thermocycler (Mx3000P, Stratagene, Agilent Technologies, Mulgrave, Australia) was used with a hot start activation phase of 10 min for 1 cycle at 95 °C, then for 45 cycles of denaturation for 15 s at 95 °C and annealing and extension for 45 s at 60 °C.

The second assay, used to analyse all samples in Experiments 2 and 3, was a SYBR® green assay using the oligonucleotide primers described above. The Quantitect SYBR® green QPCR master mix (Qiagen®, Chadstone, Australia) was used with a 25 μL total reaction. This reaction contained 12.5 μL of Quantitect SYBR® green QPCR master mix (2×), 0.5 μL of 10 μM OsHV1BF forward primer, 0.5 μL of 10 μM OsHV1B4 reverse primer, 6.5 μL of nuclease free water and 5 μL of sample template DNA. Control samples and standards were created as described above and tested in duplicate. This assay involved a hot activation phase for one cycle at 95 °C for 15 min, and 40 cycles of denaturation at 95 °C for 30 s, annealing

Table 1
Results of Experiment 1 displayed as a match-paired cross tabulation of pellet and unprocessed water. Numbers represent the number of samples where OsHV-1 DNA was detected ("positive") and not detected ("negative"). McNemar's chi sq. 22.00, $P < 0.0001$.

Unprocessed	Pellet		
	Positive	Negative	Total
Positive	32	23	55
Negative	66	127	193
Total	98	150	248

at 60 °C for 45 s, and extension at 72 °C for 45 s, followed by a dissociation curve.

Regardless of the assay used a valid PCR run was defined as one exhibiting no amplification of negative controls, amplification of both replicates of the positive control with a C_t within the range of the standard curve, a standard curve with $r^2 > 0.95$ and efficiency between 90 and 110%. The fluorescence threshold for each run was calculated using the amplification-based threshold algorithm (Stratagene) for the standard curves, and applied to all samples. Samples were defined as positive when one or both replicates exhibited an exponential increase in FAM or SYBR fluorescence signal and a cycle threshold of <40. For the SYBR® green assay a positive sample also had to have a dissociation curve with a melting curve (T_m) that conformed to that of the positive control. The quantitation limit of both assays was 12 viral copies per μL sample and the detection limit 3 viral copies per μL sample (Bustin et al., 2009).

2.6. Statistical analysis

McNemar's chi-square test for paired observations was used to compare the results of methods conducted on the same seawater samples (Motulsky, 1995).

3. Results

3.1. Experiment 1

After low-speed centrifugation of seawater, 39.5% of pellet samples tested positive for OsHV-1 by qPCR compared to 22.6% of unprocessed seawater samples ($P < 0.0001$) (Table 1). Thus testing a pellet after low speed centrifugation was almost twice as likely to result in detection of OsHV-1 compared to testing unprocessed water. C_t values were consistently below the limits of quantitation of the qPCR for both unprocessed water and pellets (C_t 34–40) indicating the amount of virus in the samples was <12 copies/μL. The consistency of results was better for pellets than for unprocessed water with 65% ($n = 98$) of pellet samples having both replicates positive by qPCR compared to 1.8% ($n = 55$) ($P < 0.01$) of unprocessed water (Table 3).

Table 3
Results of Experiment 2 displayed as a match-paired cross tabulation of pellet and supernatant samples. Data are the number of samples that were positive or negative by qPCR for OsHV-1. McNemar's chi sq. 1.98, $P = 0.15$.

Supernatant	Pellet		
	Positive	Negative	Total
Positive	12	26	38
Negative	36	174	210
Total	48	200	248

3.2. Experiment 2

There was no apparent difference between the results of testing pellets or supernatants after low speed centrifugation of samples, all of which had been frozen prior to testing. A similar proportion of pellets were positive (18.9%) compared to supernatants (15.3%) ($P = 0.15$) (Tables 2 and 3). C_t values were consistently below the limits of quantitation (C_t 34–40) of the assay.

3.3. Experiment 3 water filtration

Overall the results of filtration in Experiment 3 were inconclusive with respect to removal of OsHV-1 from seawater, and provided no clear pattern of OsHV-1 particulate attachment (Table 4). All 25 samples used in this analysis returned PCR quantities below the limit of quantitation, hence only mean C_t values for replicates and a presence or absence result could be reported. The number of samples with positive results varied from 0 to 8 for filtrates and from 0 to 3 among membranes. C_t values were relatively

Table 2
Number of positive samples and consistency of response among replicates in Experiments 1 and 2.

Sample date	Experiment	Number tested	Number of samples positive by qPCR			% Positive samples where only one of the two replicates were positive		
			Un-processed	Pellet	Supernatant	Un-treated	Pellet	Supernatant
21-Nov-12	1	54	5	3	–	100.0	100.0	–
29-Nov-12	1	71	8	23	–	100.0	78.3	–
10-Jan-13	1	71	32	44	–	100.0	52.3	–
24-Jan-13	1	52	10	28	–	90.0	82.1	–
10-Jan-13	2	71	–	4	3	–	100.0	66.7
24-Jan-13	2	52	–	6	15	–	100.0	66.7
29-Jan-13	2	40	–	17	10	–	58.8	90.0
15-Feb-13	2	1	–	No C_t	No C_t	–	No C_t	No C_t
20-Feb-13	2	6	–	2	1	–	50.0	100.0
26-Feb-13	2	11	–	3	No C_t	–	100.0	No C_t
28-Feb-13	2	5	–	3	2	–	33.4	50.0
6-Mar-13	2	5	–	2	1	–	100.0	100.0
7-Mar-13	2	3	–	1	No C_t	–	100.0	No C_t
14-Mar-13	2	13	–	No C_t	1	–	No C_t	100.0
19-Mar-13	2	6	–	No C_t	1	–	No C_t	100.0
20-Mar-13	2	10	–	1	No C_t	–	100.0	No C_t
28-Mar-13	2	2	–	1	No C_t	–	100.0	No C_t
2-Apr-13	2	6	–	1	2	–	100.0	100.0
3-Apr-13	2	11	–	5	1	–	100.0	100.0
11-Apr-13	2	6	–	2	1	–	100.0	100.0

No C_t = OsHV-1 DNA not detected; – = not included/compared in this experiment.

Table 4
Results of Experiment 3 in which water samples were filtered. Data shown are the mean C_t values for replicates.

Sample	Filtrates ($n = 25$)						Membranes ($n = 14$)[a]				
	Control (non-filtered)	IND/series 5.00 μm	Series 0.45 μm	Series 0.22 μm	IND 0.45 μm	IND 0.22 μm	IND/series 5.00 μm	Series 0.45 μm	Series 0.22 μm	IND 0.45 μm	IND 0.22 μm
1	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
2	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
3	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
4	37.91	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
5	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
6	No C_t	35.78	No C_t	No C_t	No C_t	38.44	No C_t	35.41	No C_t	33.79	34.42
7	No C_t	38.88	No C_t	No C_t	36.32	37.79	No C_t	No C_t	No C_t	No C_t	No C_t
8	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
9	No C_t	38.94	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
10	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
11	No C_t	35.90	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
12	No C_t	34.86	No C_t	No C_t	No C_t	37.81	37.48	No C_t	No C_t	No C_t	No C_t
13	No C_t	No C_t	No C_t	37.86	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
14	No C_t	36.43	No C_t	No C_t	No C_t	No C_t	38.57	No C_t	No C_t	37.66	36.44
15	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
16	No C_t	37.4	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
17	37.97	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
18	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
19	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
20	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
21	37.20	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	39.56	No C_t
22	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	–	–	–	–	–
23	No C_t	No C_t	No C_t	No C_t	34.02	No C_t	38.79	No C_t	No C_t	No C_t	No C_t
24	35.78	36.36	No C_t	35.80	37.86	No C_t	No C_t	34.15	No C_t	No C_t	No C_t
25	No C_t	No C_t	No C_t	No C_t	No C_t	36.76	No C_t	No C_t	No C_t	No C_t	No C_t
Total # positive	4	8	0	2	3	4	3	2	0	3	2

IND = individual; – = not tested.

[a] Sample membranes were tested only where the untreated water sample or one or more filtrates tested positive by qPCR.

high ranging from 34.02 to 38.94 for filtrates and from 33.79 to 39.56 for membranes. No obvious pattern consistent with concentration of the viral nucleic acid signal was observed for the 0.45 μm or 0.22 μm filter treatments either in series or individually. There is a possibility that 5.00 μm filtration may improve OsHV-1 detection by qPCR as the greatest number of positive filtrate samples was obtained by this method (Table 4), however no significant difference was found between the non-filtered control and the 5.00 μm filtrate (McNemar's chi-sq. 2.5, $P = 0.100$).

4. Discussion

This study is the first to assess detection of OsHV-1 based on its partitioning in natural seawater samples collected from an open estuarine environment. Detection by qPCR in seawater from estuaries containing infected oysters was improved two fold ($P < 0.0001$) by testing the pellet obtained by low speed centrifugation, compared to testing unprocessed seawater samples. This provides a simple method suitable for testing large numbers of water samples that will be applicable to epidemiological studies of OsHV-1. In addition, this observation supports the hypothesis that OsHV-1 can associate with particles. Simple filtration of seawater samples through low protein binding filters did not prove to be an appropriate method for assessing OsHV-1 association and/or attachment to particles of different sizes, probably due to dynamic interactions of the virus within the sample matrix itself as well as the filter membranes. This finding therefore needs to be explored further to identify the true nature of the viral interaction with particles as this will be critical to understanding the distribution and transmission of OsHV-1 in natural environments.

The distribution of OsHV-1 in seawater is unknown. Previous studies involving seawater samples from 25 L aquaria in which experimentally infected oysters were housed, or water-based media used for storage of oyster tissues have shown that OsHV-1 can be detected by PCR, in concentrations between 1×10^1 and 1×10^3 DNA copies/μL for aquaria samples (Vigneron et al., 2004; Schikorski et al., 2011a). These data are unlikely to be useful predictors of natural OsHV-1 distribution in open marine environments due to the huge differences in scale, and hence dilution, that exist between small laboratory tanks and open estuaries. Furthermore natural seawater is a far more complex medium in comparison to the filtered seawater or artificial seawater typically used in laboratory studies (Schikorski et al., 2011a).

The water samples used in this study consistently had viral nucleic acid concentrations much lower than those typically seen in host oyster tissues affected by disease due to OsHV-1, and lower concentrations than those reported from experimental infection trials in which aquarium tank water had been tested (Sauvage et al., 2009); in such studies dilution effects are minimal. In all the experiments conducted in this study, viral copy number per μL was below the limits of quantitation of the qPCR (<12 copies/μL). Furthermore a large percentage of the samples with positive results had only one of two replicate DNA extracts from the sample yield a positive result, which is consistent with a low concentration of template DNA molecules in the DNA extract (Bustin et al., 2009).

Honjo et al. (2010) investigated a quantification method for *Cyprinid herpesvirus-3* (CyHV-3) that combined viral concentration methods and quantitative PCR. A concentration method was necessary because the amount of CyHV-3 in water was always lower than that in host tissues. A cation-coated filter and ultrafiltration method were used. CyHV-3 DNA was detected in 60% of the environmental water samples that were examined; the highest concentration detected being 2×10^5 copies/L. Like filtration, ultracentrifugation has been shown to be a useful tool for separating viral assemblages from large volumes of natural water samples. However this method typically involves many steps, including 0.22 μm filtration or buoyant density separation of the sample prior to centrifugation. This is too tedious, time consuming and expensive for the analysis of large numbers of water samples (Lawrence and Steward, 2010). A simpler method for increasing OsHV-1 concentration in seawater prior to testing would therefore be highly beneficial.

In Experiment 1, detection of OsHV-1 in the pellet fraction after low speed centrifugation of seawater was twice as likely as in unprocessed seawater. This simple approach improves OsHV1-1 detection in seawater. Samples were centrifuged at only $1000 \times g$, which is far too low a force to pellet free virus or viral DNA (Bostrom et al., 2004; Lawrence and Steward, 2010). Centrifugal forces as low as $1000 \times g$ are capable of pelleting relatively large particles suspended within a sample, but not particles as small as free viruses. The particles would have to be approximately the same size or larger than plant or animal cells, such as human red blood cells (7–12 μm in diameter), to pellet at this force in only 20 min (Shimizu et al., 2006; Lawrence and Steward, 2010; Sigma–Aldrich®, 2011). This suggests that at least some OsHV-1 virions were attached to particles capable of being centrifuged at low speed, or were present in viral aggregates of this size. These results support the hypothesis first presented by Paul-Pont et al. (2013), which was based entirely on observations of the natural distribution of the disease, that OsHV-1 may be attached to particles in seawater.

The results of Experiment 2 shed more light on the distribution, and/or nature of OsHV-1 in seawater. Viral DNA was detected equally well in both pellet and supernatant after low speed centrifugation. This suggests that OsHV-1 virions were not only attached to particles, of the size mentioned above, but were also present as free virus, virus associated with smaller particles, or as free DNA. A caveat is that the samples in Experiment 2 had been stored at −20 °C prior to analysis and it is possible that the freeze–thaw steps could have caused cell or particle lysis, releasing OsHV-1, and changing the distribution of OsHV-1 within the sample. However, it is reasonable to assume that this would have increased the proportion of samples in which supernatant also contained OsHV-1 DNA, making it harder to recognise the occurrence of particle attachment.

It is unknown whether OsHV-1 exists naturally in seawater as free virus, flocculated or aggregated virus, virus adsorbed to particles, or various viral components, including free DNA, at different stages of the replication process. Bitton (1975) noted that viruses may adsorb to bacteria in marine environments in a two-step process involving both a reversible and an irreversible phase. Open estuarine environments are known to contain highly complex ecological communities containing a vast array of both biotic macro and micro organisms and abiotic compounds (Suthers et al., 2009). These communities are constantly growing, changing, and cycling over both the spatial and temporal scale (Malone and McQueen, 1983; Kingsford and Suthers, 1994; Rissik et al., 2009; Tang et al., 2011) hence it is reasonable to assume that while some viral particles are attached, others may not be intact or exist free in seawater.

In the water filtration experiment (Experiment 3) C_t values were consistently high and within a limited range (34.02–38.94 for filtrates and 33.79–39.56 for membranes) (Table 4) across all stages, regardless of the membrane pore size (5.00, 0.45, 0.22 μm). Cliver (1968) illustrated that viral adsorption to filters is governed by 3 key factors: the chemical composition of the membrane, the ratio of membrane pore size to virus size and the presence of inhibitors. Low protein binding filters were used in this study to try and minimise OsHV-1 adsorption to filter membranes. However, it is apparent that some retention of virus or viral DNA still occurred, but it was not possible to distinguish retained virus from flocculated virus or virus adsorbed to particles. Other types of filter or

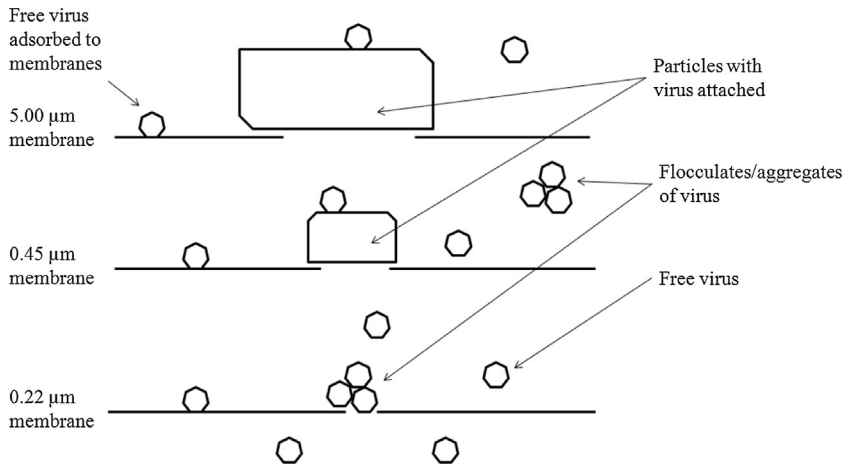

Fig. 2. Schematic representation of possible mechanisms by which OsHV-1 DNA could be detected both in filtrates and on membranes in series filtration. Free DNA not shown.

filter membrane may be more useful (Roberts, 1997; Lukasik et al., 2000).

Filtration methods are commonly used in virological analysis (Cliver, 1968; Maruyama et al., 1993; Roberts, 1997; Haramoto et al., 2007, 2009). Filtration is typically used to concentrate viruses from samples in order to enhance the detection of that virus by downstream techniques such as PCR. Methods involving adsorption of viruses onto either a positively or negatively charged filter membrane are most commonly used (Katayama et al., 2002). Using filtration as a method for assessing viral association with or adsorption to particulates within water samples is much less common. The results of the present work demonstrate that filtration cannot be easily used to assess the potential for OsHV-1 particulate attachment by attempting to collect virus attached to particles larger than virions in seawater. Nor can it be used to improve OsHV-1 detection rate. No discernible pattern of OsHV-1 depletion from filtrate or attachment to membranes could be seen in Experiment 3 when seawater samples were filtered. Whilst difficult to interpret, there are a number of plausible explanations for both filtrates and membranes testing positive. Firstly, low DNA copy number would impose stochastic variation, as the probability of every subsample from a dilute sample containing template DNA is variable (Bustin et al., 2009).

Secondly, the viral DNA that was detected by qPCR could exist in different states: free viral DNA, free virions, free flocculated or aggregated virions, virus or DNA attached to large and/or small particles, and virus or DNA adsorbed to membranes (Fig. 2). In this conceptual analysis the presence of free virus or viral DNA at each stage leads to filtrates testing positive, but the presence of any or all of these other states could lead to membranes also testing positive. Previous studies have suggested that Koi herpesvirus (KHV) is not able to survive in the environment for more than several days, but free KHV-DNA is detectable for much longer periods (Haramoto et al., 2009). Shimizu et al. (2006) suggested that KHV loses its infectivity within approximately 3 days in natural environmental water above 15 °C, however they also noted that KHV infectivity could be maintained for more than 7 days in filter-sterilised or autoclaved water. The overall stability of OsHV-1 in natural seawater or water samples is unknown, and as such the effect of cold storage on OsHV-1 in water samples is unknown. It is possible that the low viral quantities observed in water in this study are a consequence of OsHV-1 stability in water and/or the effects of refrigerating, freezing and thawing samples. The refrigeration or freeze/thaw process could lyse waterborne particles or organisms and might also have caused some virus to dissociate from particles, increasing the percentage of free OsHV-1 in a sample, or even damage the virus in such a way that it becomes undetectable by qPCR. Furthermore, as it is unknown what other organisms or compounds are present in the water sample at the time of collection and what effects these components may have on OsHV-1 stability and survival, particularly in the interim period between collection and processing. Thus it is also possible that these sample matrix components play a key role in the overall detectability of OsHV-1 and the state in which it is detected. Studies investigating OsHV-1 stability and survival in seawater samples and the effects of cold storage on seawater samples are required to address these issues.

Thirdly, the need to shake vigorously the water samples during processing to resuspend particulates may have caused flocculation or viral aggregation or dissociation from particulates which may have altered interactions between the virus and the filter membranes. Furthermore, Lukasik et al. (2000) suggested that salts such as sodium chloride and aluminium chloride can increase viral adsorption to filter membranes by promoting hydrophobic interactions between virions and a filter or substrate. They noted that salt concentration can have both direct and indirect effects on the filtration of viruses, including the formation of salt bridges between virions and filters, alterations to filter charge, changes to pH, and flocculation formation. As natural saline estuarine water samples were analysed in the present study it is possible that the salt concentration may have played some role in membrane interaction.

5. Conclusion

The current study is the first to focus specifically on (1) evaluating simple centrifugation methods to prepare seawater samples for detection of OsHV-1 by qPCR, for the purpose of testing large numbers of samples efficiently, and (2) using filtration for the assessment of potential viral adsorption to particles in seawater. Centrifugation of water samples at $1000 \times g$ for 20 min and then testing the pellet almost doubled the rate of detection of OsHV-1 by real-time qPCR compared to testing untreated water. The results suggest that OsHV-1 may exist as both free virus and as virus attached to particulates in the order of 10 µm which are large enough to be pelleted at $1000 \times g$, and also may be attached to smaller particles. These results support the hypothesis proposed by Paul-Pont et al. (2013), that the distribution and transmission of OsHV-1 in natural seawater might reflect attachment to some form of particulate matter, either abiotic or biotic.

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