

Detection of Ostreid Herpesvirus 1 (OsHV-1) DNA in seawater by PCR: influence of water parameters in bioassays

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ABSTRACT: Since 1991, herpesvirus infections have been reported among larvae and juveniles of various bivalves. Most of the studies focused on detection of viral infections of economically important species. However, the persistence of bivalve herpesviruses in the marine environment is poorly documented. The present study concerns the role of seawater parameters in Ostreid Herpesvirus 1 (OsHV-1) detection by polymerase chain reaction (PCR). Viral DNA extracted from purified particles or virions present in infected oyster larvae were detected by PCR after storage in different media at different temperatures. The lowest detection threshold was found using distilled water or Tris EDTA buffer. In seawater, the threshold was higher. The use of sterile media permitted detection of viral DNA stored over a longer period. Storage temperature also had a significant influence on detection, with lower temperatures promoting DNA detection over a longer period. In summary, water parameters such as temperature influenced detection of OsHV-1 DNA by PCR. However, the PCR technique may also be successfully applied to samples in natural seawater. Indeed, the PCR technique permitted detection of naked viral DNA at 100 ng l⁻¹ in seawater in bioassays.

KEY WORDS: Oyster Herpesvirus 1 · Viral DNA · Seawater · Detection · PCR · Temperature

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INTRODUCTION

Shellfish are cultured increasingly all around the world, but only a few bivalve species, including oysters, mussels, clams and scallops, are produced commercially. Infectious diseases are likely to prove an important limiting factor for large-scale shellfish culture. Different factors have contributed, and still contribute, to the appearance and spread of infections: (1) intensive production, (2) focus on a small number of species and (3) animal transfers. Mortalities have been reported in several bivalve species, and some have been associated with the presence of viruses belonging to various families. The first description of a virus was reported in adult eastern oysters *Crassostrea virginica* with the detection of particles related to the Herpesviridae family (Farley et al.

1972). Other viruses described in bivalves include agents related to Birnaviridae, Iridoviridae, Papovaviridae, Paramyxoviridae, Reoviridae, Retroviridae and Togaviridae (Farley 1978, Elston 1997, Renault 1998, Susuki et al. 1997, 1998). In 1991, viruses interpreted as belonging to the Herpesviridae were associated with high mortality rates of hatchery-reared larval *C. gigas* in France (Nicolas et al. 1992) and New Zealand (Hine et al. 1992). Since 1992, sporadic high mortality rates of larval *C. gigas* have regularly been reported in some commercial French hatcheries, occurring each year during the summer period in association with detection of herpes-like viruses (Le Deuff et al. 1996, Renault et al. 1994a,b, 2001a,b). The pathogenicity of the virus was demonstrated by experimental transmission of the infection to healthy *C. gigas* larvae (Le Deuff et al. 1994). Additionally,

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outbreaks of mortality reported among *Ostrea edulis* and *C. gigas* spat were associated with detection of a herpes-like virus in France (Comps & Cochenne 1993, Renault et al. 1994a,b, 2000a). Herpes-like virus replication has also been observed in *O. angasi* adults in Australia (Hine & Thorne 1997) and in larval *Triostrea chilensis* in New Zealand (Hine 1997, Hine et al. 1998). Herpes-like viruses thus appear ubiquitous in bivalves. They are detected in different parts of the world, at different stages of development in different species. Moreover, herpes-like virus infections in bivalves are often associated with substantial mortalities. These observations highlight the importance of using a range of efficient diagnostic methods in order to assess the causative role of these viruses in bivalve mortalities. Using viral DNA extracted from infected *C. gigas* larvae (Le Deuff & Renault 1999), different molecular techniques, such as the polymerase chain reaction (PCR), have been developed (Renault & Lipart 1998, Renault et al. 2000b). PCR is used successfully to detect viral DNA during productive infections in oyster larvae and spat (Renault et al. 2000b). An *in situ* hybridisation technique has also been described (Lipart & Renault 2002). This technique may confirm the presence of herpes-like viruses in histological sections and localise viral DNA in different tissues and organs of infected individuals.

Although these molecular techniques are now used to detect herpes-like viruses in marine bivalves when abnormal mortalities occur, no data are available about the detection of virus infections in marine water samples. Diagnostic techniques such as PCR may be used to analyse seawater samples, in order to detect herpes-like virus DNA, and the information gathered may aid understanding and control of viral dissemination and transmission. The influence of water parameters on viral DNA detection by PCR needs first to be defined, in order to validate this detection procedure. A procedure based on 1-round PCR amplification of part of the genome of Ostreid Herpesvirus 1 (OsHV-1) from purified DNA or virions was used to examine the effects of storing samples in different media at different temperatures and to compare the persistence of naked viral DNA and viral DNA in virions.

MATERIALS AND METHODS

Naked viral DNA. OsHV-1 viral DNA was extracted from purified virus pellets (Le Deuff & Renault 1999). Briefly, purified virus particles were suspended in extraction buffer (100 mM NaCl; 10 mM Tris; 25 mM EDTA; 0.5% SDS; pH 8), and then Proteinase K (0.2 mg ml⁻¹) was added. DNA was purified using phenol/chloroform/isoamyl alcohol (24:23:1), precipitated with ethanol and suspended in TE buffer (10 mM Tris; 1 mM EDTA; pH 8). DNA concentration was determined using a Perkin-Elmer spectrophotometer at 260 nm. A working naked viral DNA suspension was prepared at 100 ng μ l⁻¹ in TE buffer.

Viral DNA in virions, infected ground oyster larvae. Infected *Crassostrea gigas* larvae originated from a French commercial hatchery (Vendée, France). Biological material was collected in 1995 and stored at -20°C; 50 μ g of frozen infected larvae were weighed in a 1.5 ml microtube and ground in 500 μ l of artificial seawater (Sigma) using a single-use pellet piston. Ground tissue was transferred to a sterile tube, and artificial seawater was added to a final volume of 20 ml. Samples were centrifuged at 150 \times g for 5 min at 4°C. Supernatants were recovered and immediately filtered through a 0.45 μ m filter (Polypropylene, Nalgene). Samples were stored at 4°C and contained viral DNA most likely in virions rather than free. (Note: 'Naked viral DNA' in the text corresponds to DNA extracted from purified particles. Viral DNA in larval samples is referred to as 'viral DNA in virions'.)

Media for viral DNA dilution. Tris EDTA buffer (TE) (1 \times) was prepared from TE 50 \times (Sigma). Distilled water (Sigma) was used to prepare dilutions. Then, 3 types of seawater were tested: artificial seawater (Sigma), natural seawater collected at the IFREMER station (La Tremblade, Charente-Maritime, France) along the French Atlantic coast, and underground salty water collected at the same site (-100 m, depth beneath the land surface). The 3 seawater types were used as non-autoclaved and autoclaved media (20 min, 120°C). The pH, salinity and temperature were measured for each medium (Table 1).

PCR detection of viral DNA. PCR primers: PCR primers were derived from the OsHV-1 sequence. For-

Table 1. Physico-chemical parameters of the media used. TE: Tris EDTA buffer; a: autoclaved; na: non-autoclaved

	TE	Distilled water	Artificial seawater				Natural seawater				Underground seawater			
			Assay 1		Assay 2		Assay 1		Assay 2		Assay 1		Assay 2	
			na	a	na	a	na	a	na	a	na	a	na	a
pH	8	6	7.6	8.1	8.1	8.3	8.0	8.2	8.1	8.1	6.9	7.2	6.8	8.2
Salinity (‰)	0	0	32	32	34	34	31	30	28	28	30	30	27	28

ward primers (C5 and Gp4) and reverse primers (C13 and Gp3) have been described previously and provided 2 primer-pair combinations (C13/C5 and Gp3/Gp4) with expected PCR products of 765 and 698 bp, respectively. The primer sequences were: C13 (5'-CCT CGA GGT AGC TTT TGT CAA G-3') and C5 (5'-CCG TGA CTT CTA TGG GTA TGT CAG-3'), and Gp3 (5'-GGT TGT GGG TTT GGA AAT GT-3') and Gp4 (5'-GGC GTC CAA ACT CGA TTA AA-3'). The Gp locus (at approximately 134 000 bp in the genome) encodes part of a putative glycoprotein, and the C locus (present twice in the genome at 4500 and 178 500 bp) encodes parts of 2 proteins of unknown functions. The primers were supplied by Eurogentec (Belgium).

PCR protocol: The protocol used has been described previously (Renault et al. 2000b). Thermal cycling was carried out using a Crocodile III thermal cycler (Applied Biosystems). The 50 μ l PCR reactions were carried out using Goldstar Polymerase (Eurogentec), each containing the appropriate reaction buffer (Eurogentec), 0.05 mM of each dNTP, 100 ng of each primer, 2.5 mM $MgCl_2$, 2.5 U of DNA polymerase and 1 μ l of template DNA. After heating samples for 2 min at 94°C, 35 cycles were performed, followed by a final elongation step of 5 min at 72°C. Each of the 35 cycles consisted of a DNA melting step at 94°C for 1 min, a primer annealing step for 1 min at 50°C and a primer elongation step at 72°C for 1 min. The sizes of PCR products (10 μ l) were estimated by electrophoresis on 1% agarose gels, using SmartLadder molecular size markers (Eurogentec). Gels were stained with ethidium bromide (0.5 μ g ml^{-1}).

Quantification of viral DNA by PCR: The quantity of viral DNA in filtered supernatants from ground oyster larvae was determined using a semi-quantitative PCR procedure (Arzul et al. 2002). A deleted DNA fragment served as an internal standard in PCR reactions. The primer pair combination C2/C6 (C2: 5'-CTC TTT ACC ATG AAG ATA CCC ACC-3'; C6: 5'-GTG CAC GGC TTA CCA TTT TT-3') was used to amplify a 690 bp fragment from OsHV-1 genomic DNA and a 613 bp fragment from the internal standard. The internal standard comprised a plasmid containing a naturally deleted form of the 690 bp fragment. Semi-quantitative PCR was performed under the conditions described above, with 10-fold serial dilutions of the internal standard (350 to 3.5 $pg \mu l^{-1}$) and oyster samples.

Limit of naked viral DNA detection by PCR: Genomic viral DNA was diluted in various media (TE, distilled water, artificial seawater, natural seawater and underground seawater). Then, 30 μ l of 10-fold dilutions of genomic viral DNA (1 $ng \mu l^{-1}$ to 1 $fg \mu l^{-1}$) was prepared from the working solution (100 $ng \mu l^{-1}$) and analysed immediately. Both primer-pair combina-

tions (C13/C5 and Gp3/Gp4) were used. The limit of detection, which corresponded to the last dilution of viral DNA allowing detection of the expected amplicon, was defined using triplicate reactions.

Persistence of viral DNA detection by PCR: A total of 30 μ l of viral DNA dilutions (naked viral DNA or viral DNA from infected ground larvae) was prepared in microtubes. For each PCR, 1 μ l of viral DNA dilution was analysed using primer-pair C13/C5.

Naked viral DNA: Two experiments were carried out using four 10-fold serial dilutions of viral DNA according to the limit of viral DNA detection defined previously. In the first assay, chosen dilutions were: (1) 1 dilution corresponding to the limit of detection, (2) 1 dilution lower than the limit of detection and (3) 2 dilutions higher. In the second assay, the first dilution used corresponded to the limit of detection and served to prepare three 10-fold dilutions. Viral DNA dilutions were incubated in different media (TE, distilled water, artificial seawater, natural seawater and underground seawater) at 4, 11 and 20°C. The tubes incubated at 20°C were covered with parafilm to avoid evaporation. Serial dilutions of viral DNA were analysed over time (Days 0, 1, 2, 6, 9, 12, 16, 22, 29, 44, 51, 65 and 79), and the lowest viral DNA concentration allowing the production of the expected amplicon was determined.

Viral DNA in virions: Viral DNA was quantified in infected ground larvae. Then, 30 μ l of viral DNA concentrations (1.9 and 9.5 $pg \mu l^{-1}$) was prepared in non-autoclaved artificial seawater, non-autoclaved natural seawater and autoclaved underground seawater, and incubated at 4, 11 and 20°C. The test was based on PCR amplification in triplicate on Days 0, 1, 2, 6, 9, 12, 16, 22, 30, 44 and 51.

RESULTS

Limit of naked viral DNA detection by PCR

For both primer pairs (C13/C5 and Gp3/Gp4), the limit of detection of naked viral DNA diluted in TE or distilled water was 100 and 10 $fg \mu l^{-1}$, respectively. After 48 h at -20°C, 100 $fg \mu l^{-1}$ of naked viral DNA were detected using both primer pairs C13/C5 and Gp3/Gp4 in TE and distilled water (data not shown). After freezing and thawing (5 times) OsHV-1 viral DNA in distilled water, the limit of detection was 10 and 100 $pg \mu l^{-1}$ using C13/C5 and Gp3/Gp4 primer pairs, respectively (data not shown). In artificial and natural seawater (autoclaved and non-autoclaved), the lowest quantity of viral DNA detected was 100 $fg \mu l^{-1}$ using both primer pairs. In non-autoclaved and autoclaved underground seawater, the limit of viral DNA

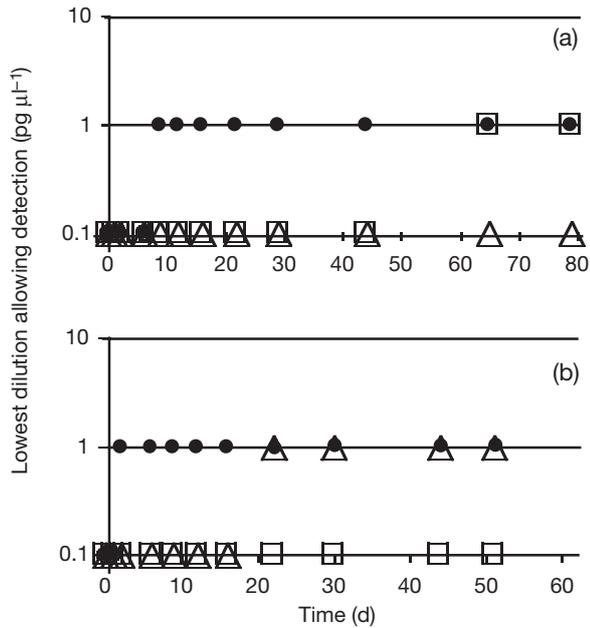


Fig. 1. Kinetic of genomic viral DNA detection in Tris EDTA. (a) First assay, (b) second assay, at 4 (□), 11 (Δ) and 20°C (●)

detection was 100 fg μl^{-1} and 1 pg μl^{-1} using C13/C5 and Gp3/Gp4, respectively.

Persistence of naked viral DNA detection in TE

In the first assay at 11°C, 100 fg of naked viral DNA (the limit of detection defined previously) were detected after 79 d, but no amplification was observed for 10 fg (Fig. 1a). In the same medium at 4 and 20°C, 100 fg were detected until 44 and 6 d, respectively. Subsequently, specific amplicons were obtained only from 1 to 10 pg μl^{-1} until 79 d (Fig. 1a). In the second assay, there was no decrease of viral DNA detection at 4°C until 51 d (Fig. 1b). At 11°C, 100 fg were detected until 16 d. Then, amplicons were observed only from 1 to 100 pg μl^{-1} until 51 d (Fig. 1b). At 20°C, 1 pg of naked viral DNA was needed to obtain specific amplicons from 2 to 51 d (Fig. 1b).

Persistence of naked viral DNA detection in distilled water

In the first assay at 11°C, 10 fg of naked viral DNA (the limit of detection defined previously) were detected for up to 29 d, but no amplification was observed for 1 fg (Fig. 2). Subsequently, no amplification was reported for any dilutions tested (1 fg μl^{-1} to

1 pg μl^{-1}). In the same medium, 10 fg of naked viral DNA was also detected for 44 d at 4°C (Fig. 2). At 20°C, 10 and 100 fg of naked viral DNA were detected for up to 12 and 44 d, respectively. After 44 d, all dilutions tested (1 fg μl^{-1} to 1 pg μl^{-1}) failed to show specific amplification. In the second assay, 10 fg to 10 pg of naked viral DNA was detected for 51 d at 4, 11 and 20°C in distilled water.

Temperature and naked viral DNA detection in non-autoclaved seawaters

Artificial seawater

In the first assay, detection of 10 pg of naked viral DNA was observed over 16 d at 4°C, 9 d at 11°C and 2 d at 20°C in artificial seawater (Fig. 3a). In the second assay, 100 fg of naked viral DNA (the limit of detection previously defined) was detected over 6, 2 and 1 d at 11, 4 and 20°C, respectively (Fig. 3b). Subsequently, no amplification was reported for this dilution. After 29 d, specific amplicons were only obtained using 100 pg of naked viral DNA at 4 and 20°C (Fig. 3b). Amplification was reported with 10 pg at 11°C, from Days 16 to 51 (Fig. 3b).

Natural seawater

In the first assay, detection of 10 pg of naked viral DNA was observed over 16 d at 4°C, 9 d at 11°C and 1 d at 20°C in natural seawater (Fig. 4a). In the second assay, 100 fg of naked viral DNA (the limit of detection defined previously) was detected on Day 2 at 11°C, and no amplicons were obtained at 4 or 20°C (Fig. 4b). After 9 d, 10 and 100 pg of naked viral DNA were amplified at 11°C, and only 100 pg at 4 and 20°C (Fig. 4b). A total of 100 pg of genomic viral DNA was detectable over 51 d at 4, 11 and 20°C in natural seawater (Fig. 4b).

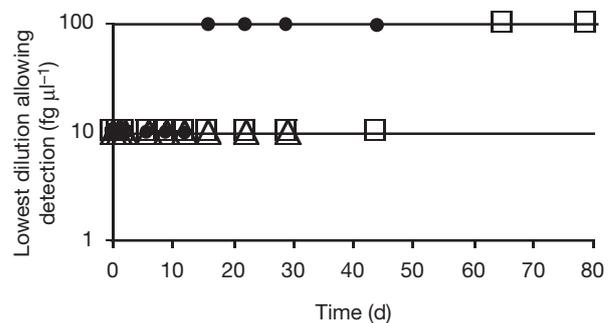


Fig. 2. Kinetic of genomic viral DNA detection in distilled water, at 4 (□), 11 (Δ) and 20°C (●)

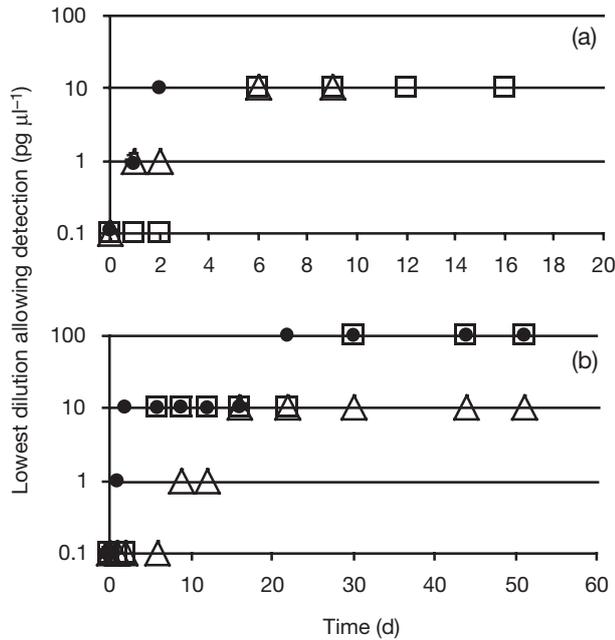


Fig. 3. Viral genomic DNA detection in artificial seawater. (a) First assay, (b) second assay), at 4 (□), 11 (△) and 20°C (●)

Underground seawater

In non-autoclaved underground seawater, optimal detection was observed at 4°C; 1 pg of viral DNA was detected over the entire course of both assays (Fig. 5). At 11°C, 10 pg was needed to obtain amplicons after Day 9 in the first assay and after Day 22 in the second (Fig. 5). A total of 10 pg of naked viral DNA was detected over 9 d in both assays at 20°C; after 12 d, 100 pg was needed to obtain specific amplicons (Fig. 5).

Effect of seawater autoclaving on detection of naked viral DNA

Artificial seawater

In the first assay, 10 pg of naked viral DNA (the highest amount tested) was not detected in non-autoclaved artificial seawater after Day 9 at 20°C and after Day 16 at 4°C (Fig. 6a). On Day 79, 10 and 1 pg were detected in autoclaved artificial seawater at 20 and 4°C, respectively (Fig. 6b). In the second assay, 10 pg of DNA was not detected in non-autoclaved artificial seawater on Day 22 at 20 or 4°C (Fig. 6c). Subsequently, 100 pg was detected at both temperatures. The lowest quantity of DNA detected in autoclaved artificial seawater at 20 and 4°C was 10 pg on Day 51 (Fig. 6d).

Natural seawater

In the first assay, 10 pg of viral DNA was detected over 16 d at 4°C and 1 d at 20°C in non-autoclaved natural seawater (Fig. 7a). The same quantity of DNA was detected over 44 d at 20°C in autoclaved natural seawater (Fig. 7b). A total of 100 fg of DNA yielded amplicons until Day 6 in autoclaved seawater at 4°C. No amplification was reported for this amount of DNA in seawater without autoclaving (Fig. 7a). In the second assay, the lowest quantity of DNA detected was 100 pg in non-autoclaved seawater on Day 51, in contrast to 10 pg in autoclaved medium (Fig. 7c,d).

Underground seawater

In the first assay at 20°C, 100 pg was needed to obtain amplicons on Day 79 in non-autoclaved underground seawater instead of 10 pg in autoclaved medium (Fig. 8a,b). On the same date, PCR products were obtained at 4°C with 1 pg of viral DNA in non-autoclaved underground seawater and with 10 pg in autoclaved medium (Fig. 8a,b). In the second assay, the DNA quantities needed to obtain PCR amplification in non-autoclaved water at both temperatures were similar to those in the first assay (Fig. 8c,d). Amplicons were obtained with higher amounts of

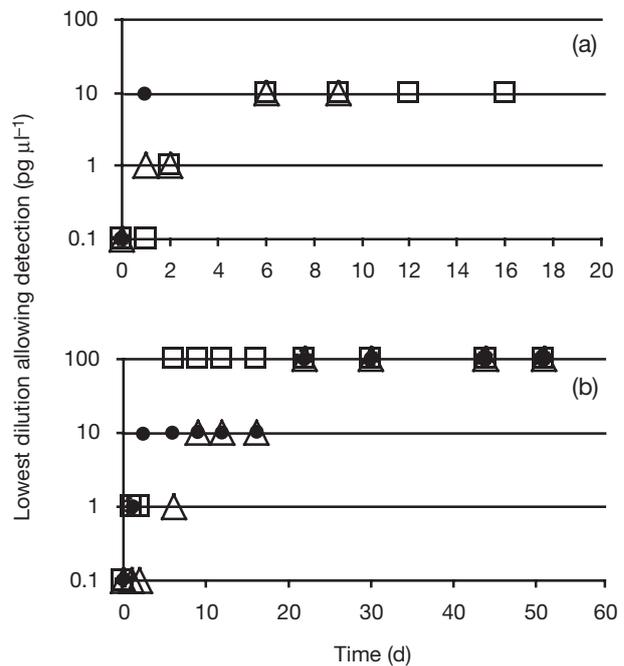


Fig. 4. Viral genomic DNA detection in natural seawater. (a) First assay, (b) second assay), 4 (□), 11 (△) and 20°C (●)

viral DNA in non-autoclaved underground seawater than in autoclaved medium at 20°C from Days 12 to 44 (Fig. 8c,d).

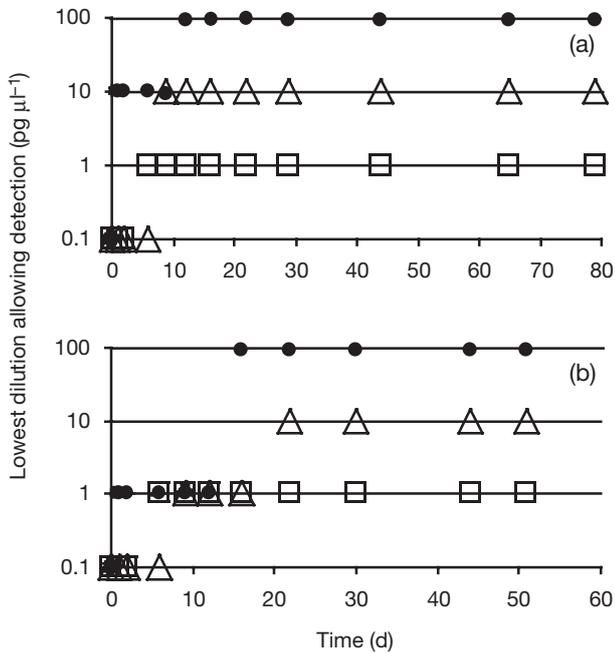


Fig. 5. Viral genomic DNA detection in underground seawater. (a) First assay, (b) second assay, at 4°C (□), 11°C (△) and 20°C (●)

Detection of viral DNA from infected ground larvae

Quantification of viral DNA in supernatants from infected ground larvae was carried out using a semi-quantitative PCR performed in triplicate. According to internal standard amplification, the quantity of viral DNA was 19 pg µl⁻¹.

In the first assay, viral DNA (1.9 pg µl⁻¹) was detected over 9 d at 4°C in all media tested, whereas detection at 20°C was successful only during Day 1 (Table 2). In the second assay, viral DNA was detected in the 3 media tested over the entire course of the experiment (22 d) at 4°C. At 20°C, no PCR products were obtained on Day 22 in non-autoclaved artificial seawater and autoclaved underground seawater; the last detection was on Day 12 in non-autoclaved natural seawater (Table 2).

DISCUSSION

Detection limits of naked OsHV-1 DNA by PCR

As a first step, the limit of detection of OsHV-1 DNA by PCR was defined in different media using 2 primer pairs (C13/C5 and Gp3/Gp4). Both primer pairs have already been extensively used for OsHV-1 diagnostic purposes (Arzul et al. 2001a,b, Renault et al. in press).

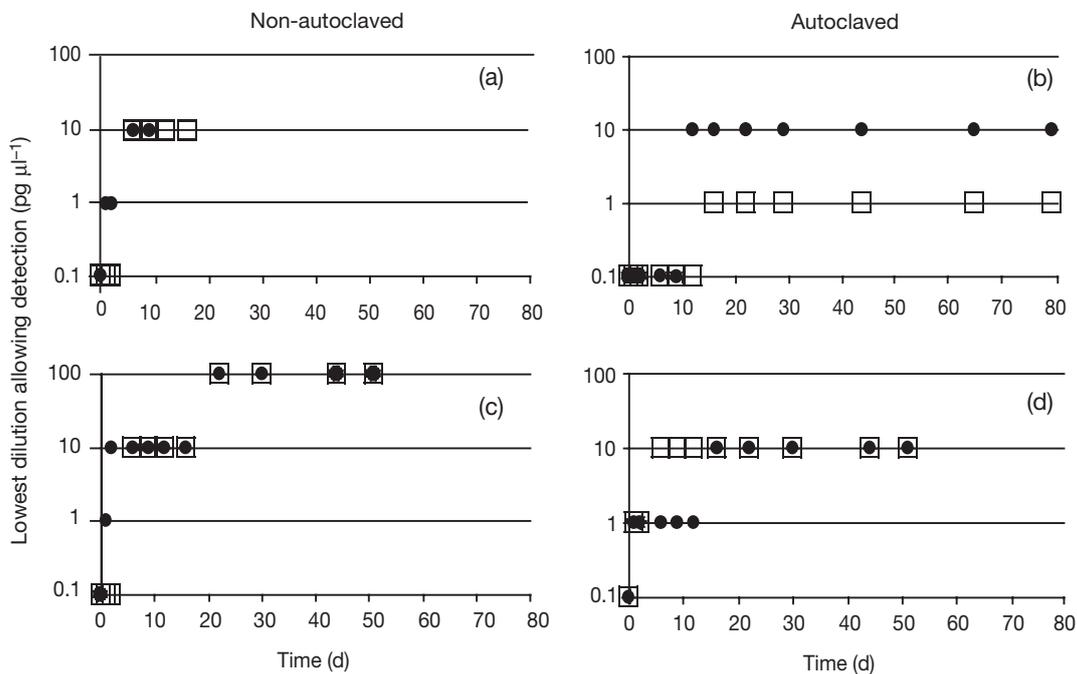


Fig. 6. Viral genomic DNA detection in artificial seawater. (a) First assay non-autoclaved, (b) first assay autoclaved, (c) second assay non-autoclaved, (d) second assay autoclaved, at 4°C (□) and 20°C (●)

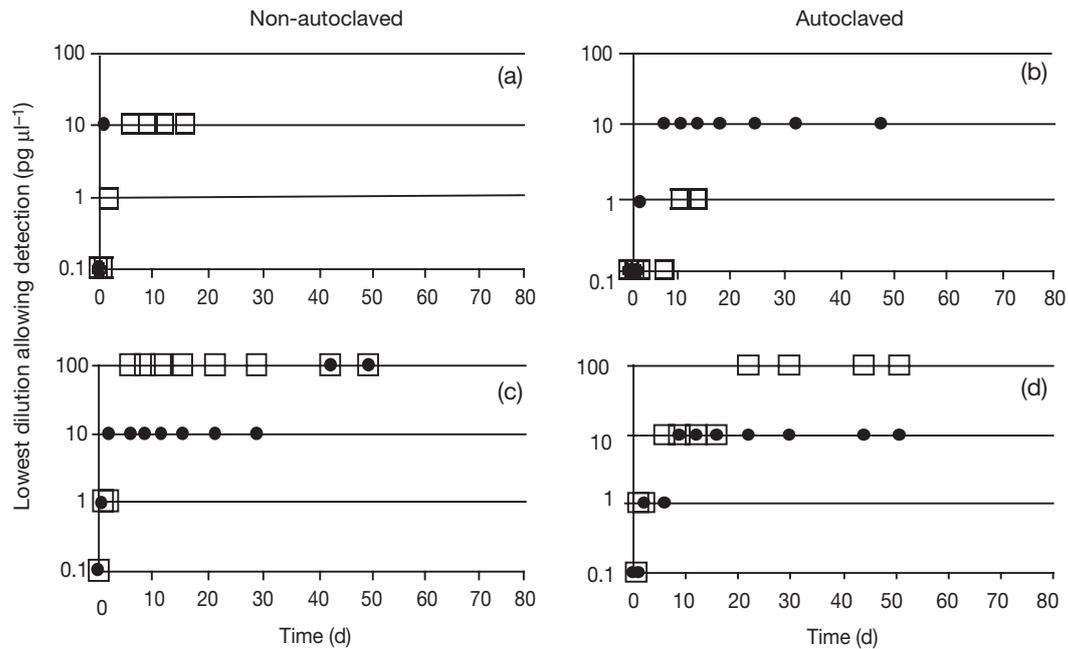


Fig. 7. Viral genomic DNA detection in natural seawater. (a) First assay non-autoclaved, (b) first assay autoclaved, (c) second assay non-autoclaved, (d) second assay autoclaved, at 4 (\square) and 20°C (\bullet)

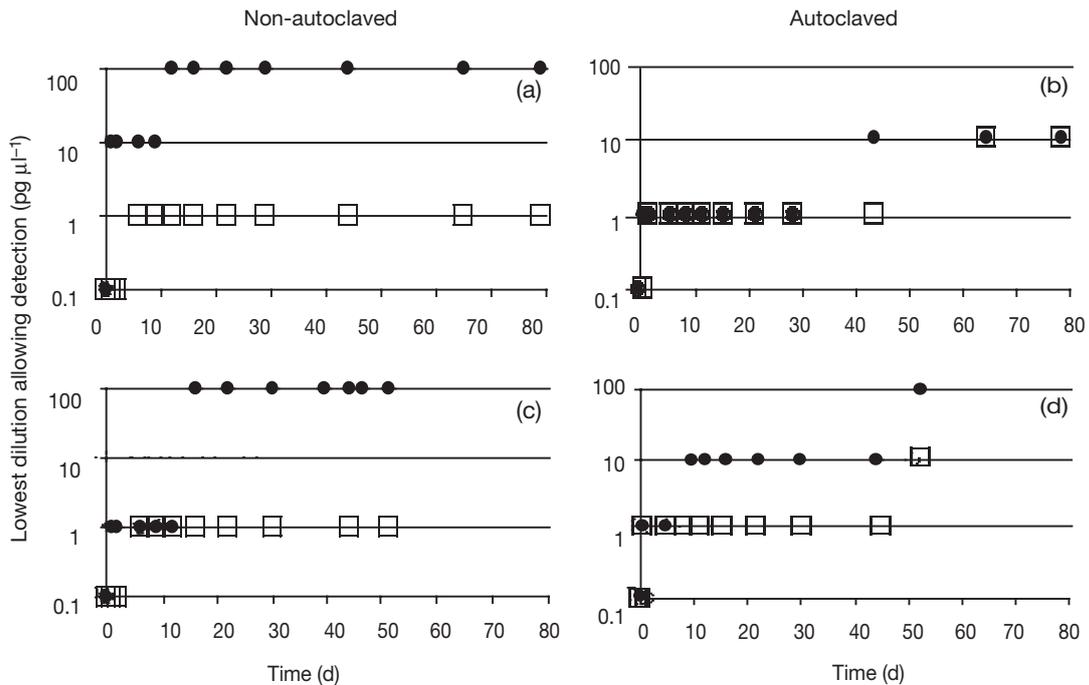


Fig. 8. Viral genomic DNA detection in underground seawater. (a) First assay non-autoclaved, (b) first assay autoclaved, (c) second assay non-autoclaved, (d) second assay autoclaved, at 4 (\square) and 20°C (\bullet)

The primer pair C13/C5 was selected on the basis that it targets the C locus present twice in the OsHV-1 genome. Moreover, the C locus presents polymorphism, including several single nucleotide substitu-

tions and, more notably, a deletion. A region of the inverted repeat of at least 2.8 kbp is absent in a OsHV-1 variant (Arzul et al. 2001a,b). The C locus appears suitable to identify viral variants. The Gp locus appears

Table 2. Detection of viral DNA from samples of infected ground larvae samples analysed in triplicate (scores are on the basis of 3 assays by temperature and time of incubation). Viral DNA was prepared in non-autoclaved artificial seawater (Table 1, Assay 1), non-autoclaved natural seawater (Table 1, Assay 1), or autoclaved underground seawater (Table 1, Assay 1). The first assay was prepared with $1.9 \text{ pg } \mu\text{l}^{-1}$ and the second with $9.5 \text{ pg } \mu\text{l}^{-1}$. nd: not determined

Assay Time	4°C		20°C	
	1st assay	2nd assay	1st assay	2nd assay
Artificial seawater				
Day 0	3	3	3	3
Day 1	3	3	3	3
Day 2	3	3	0	3
Day 6	2	3	0	1
Day 9	3	nd	0	nd
Day 12	0	3	0	1
Day 16		2		3
Day 22		2		0
Natural seawater				
Day 0	3	3	3	3
Day 1	3	2	3	3
Day 2	3	3	0	3
Day 6	1	3	0	3
Day 9	3	nd	0	nd
Day 12	0	1	0	1
Day 16		3		0
Day 22		3		0
Autoclaved underground seawater				
Day 0	3	3	3	3
Day 1	3	3	2	3
Day 2	3	3	0	3
Day 6	3	3	0	3
Day 9	3	nd	0	nd
Day 12	0	3	0	3
Day 16		3		3
Day 22		3		0

more conserved. Examinations of the kinetics of viral DNA detection were then carried out on the basis of the previously defined detection limit. Both primer pairs, which amplify different parts of the viral genome, showed similar results. The most sensitive detection of viral DNA was found using distilled water ($10 \text{ fg } \mu\text{l}^{-1}$). Other media may contain PCR inhibitors or substances that are able to degrade DNA (nucleases). Seawater and TE contain salts, which are well known to be PCR inhibitors, and 35 to 45% of nucleic acids contained in water samples can be destroyed by nucleases (Bettarel et al. 2000). Moreover, marine waters contain a variety of potential antiviral factors, including native marine micro-organisms such as autochthonous bacteria.

The pH and salinity of the seawater samples used in our studies varied, but these factors alone do not explain the observed differences in viral DNA detection. Some authors (Gantzer et al. 1998) have shown

that salinity does not have any importance in the stability of viruses, although salinity was associated with detection efficiency.

Persistence of OsHV-1 DNA detection by PCR

Two examinations into the persistence of naked viral DNA detection by PCR were carried out and yielded similar results. Underground seawater, which contains fewer micro-organisms, allowed detection of viral DNA over a longer period than natural or artificial seawater. When the media were autoclaved, viral DNA detection was more sensitive and occurred over a longer period. Some authors (Gerba & Schaiberger 1975) obtained similar results with a bacteriophage (T2). Also, human enteric viruses remained stable for 7 d in filtered seawater ($0.22 \text{ } \mu\text{m}$), whereas they were readily inactivated in seawater that had been either filtered through $2 \text{ } \mu\text{m}$ membrane filters or unfiltered (Girones et al. 1989). Moreover, viruses were unaffected when the seawater was autoclaved (Girones et al. 1989). As expected, DNA in a medium lacking micro-organisms has been shown to be less degraded by endonucleases and exonucleases (Sobsey 1993, Tsai et al. 1995). Finally autoclaving may destroy a great variety of active substances (proteolytic and lipolytic enzymes), in addition to killing the autochthonous microflora.

Assays carried out on samples stored at 4, 11, or 20°C demonstrated that detection of OsHV-1 DNA decreased as the temperature of incubation increased. This is in accordance with previous reports. Poliovirus RNA is more easily detected at 4°C than at 23°C (Tsai et al. 1995), and human astrovirus persists longer at 4°C than at 20°C (Bosch et al. 1997). At 6°C, poliovirus persists significantly longer (4 to 25 d) than at 20°C (1 to 3 d) (Wait & Sobsey 2001), and the enveloped viral haemorrhagic septicaemia virus (VHSV) is more stable at 4°C than at 20°C (Parry & Dixon 1997). Temperature appears to be among the most important factors affecting virus survival in seawater. The activity of the aquatic microflora responsible for the virucidal action in seawater is somewhat inhibited by low temperature (Dahling & Safferman 1979).

Two assays were also carried out using viral DNA in virions from infected ground oyster larvae in the 3 kinds of seawater. The genomic viral DNA extracted from purified particles is naked, whereas DNA in larval extracts probably had 2 protective coats, capsid and envelop. A comparison between the stability of naked viral DNA and viral DNA in ground larvae showed differences. In autoclaved underground salty water, naked viral DNA was detected for 28 d longer than viral DNA from ground larvae, at all temperatures

assayed. These results may be explained by the presence of biological contaminants or other compounds (Sobsey 1993) in ground larvae (moribund animals) affecting viral structure, by viral DNA persistence or by PCR efficiency. However, useful information may result from detecting only DNA. The lack of cell lines permissive to OsHV-1 does not allow the measurement of viral infectivity.

Global viral abundance (with all viruses counted) has been estimated to be in the range of 2.6 to $300 \times 10^9 \text{ l}^{-1}$ in estuarine surface waters (Wait & Sobsey 2001) and 1 to $30 \times 10^9 \text{ l}^{-1}$ in oyster ponds (Montanié et al. 2002). Among recognised viruses, some may be OsHV-1 particles, and may represent evidence for viral dissemination in the environment from infected oysters. Diagnosing OsHV-1 in seawater could thus provide information about potential risks for aquaculture. De Flaun et al. (1987) reported that dissolved DNA (size $< 0.2 \mu\text{m}$) ranged from 10 to $19 \mu\text{g l}^{-1}$ in estuarine waters. However, encapsid viral DNA might represent only 8 to 15% of the dissolved DNA (0.8 to $2.85 \mu\text{g l}^{-1}$), with the remaining DNA of eubacterial and eukaryotic origin potentially comprising 50% free DNA and 35 to 42% DNA bound to uncharacterised colloids (Jiang & Paul 1995). PCR allows detection of at least $0.1 \mu\text{g l}^{-1}$ of OsHV-1 genomic DNA in natural seawater ($100 \text{ fg } \mu\text{l}^{-1}$), corresponding to 4.4×10^8 genomic copies or 4.4×10^8 particle equivalents. Consequently, OsHV-1 may be detectable in field samples by PCR if OsHV-1 DNA represents more than 3.5 to 12.5% of the total viral DNA contained in the sample. In the case of epizootics with spreading of newly synthesised virions in seawater, viral concentration may reach 6 to $7 \times 10^9 \text{ l}^{-1}$ (several $\text{pg } \mu\text{l}^{-1}$ of DNA). PCR detection of OsHV-1 in natural seawater thus appears to be a realistic objective. However, detection depends on temperature and time of storage: PCR amplification of 100 fg of viral DNA is possible within 2 d at 11°C and 1 d at 20°C . Assays with DNA from infected ground larvae confirmed detection of genomic DNA at 11°C (data not shown) for up to 2 – 6 d and only for the first day at 20°C . Although the quantities of OsHV-1 viral DNA may be sufficient for PCR detection in natural samples, *in situ* viral decay due to high temperature may be a limiting factor.

Interestingly, samples could be stored at 4°C without loss of detection for between 2 and 9 d. As a conservative measure, we recommend that PCR analysis be carried out within 48 h. Moreover, no loss of OsHV-1 detection is observed within 48 h when viral DNA is frozen at -20°C in TE and distilled water. However, successive freezing/thawing cycles result in lower OsHV-1 DNA detection by PCR. In subsequent work, it would be useful to investigate the dynamics of OsHV-

1 in marine habitats such as oyster ponds and to optimise the quantification of PCR detection in order to clarify the natural infection cycle of this herpesvirus of marine bivalves.

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LITERATURE CITED

- Arzul I, Nicolas JL, Davison AJ, Renault T (2001a) French scallops: a new host for ostreid herpesvirus-1. *Virology* 290:342–349
- Arzul I, Renault T, Lipart C, Davison AJ (2001b) Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. *J Gen Virol* 82:865–870
- Arzul I, Renault T, Thebault A, Gerard A (2002) Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. *Virus Res* 84:151–160
- Bettarel Y, Sime-Ngando T, Amblard C, Laveran H (2000) A comparison of methods for counting viruses in aquatic systems. *Appl Environ Microbiol* 66:2283–2289
- Bosch A, Pinto RM, Villena C, Abad FX (1997) Persistence of human astrovirus in fresh and marine water. *Water Sci Technol* 35:243–247
- Comps M, Cochenec N (1993) A herpes-like virus from the European oyster *Ostrea edulis*. *J Invertebr Pathol* 62: 201–203
- Dahling DR, Safferman RS (1979) Survival of enteric viruses under natural conditions in a subarctic river. *Appl Environ Microbiol* 38:1103–1110
- De Flaun MF, Paul JH, Jeffrey WH (1987) Distribution and molecular weight of dissolved DNA in subtropical estuarine and oceanic environments. *Mar Ecol Prog Ser* 38: 65–73
- Elston RA (1997) Special topic review: bivalve mollusc viruses. *World J Microbiol Biotechnol* 13:393–403
- Farley CA (1978) Viruses and virus-like lesions in marine molluscs. *Mar Fish Rev* 40:18–20
- Farley CA, Banfield WG, Kasnic JRG, Foster WS (1972) Oyster herpes-type virus. *Science* 178:759–760
- Gantzer C, Dubois E, Crance JM, Billaudel S, Kopecka H, Schwartzbrod L, Pommepuy M, Le Guyader F (1998) Influence of environmental factors on the survival of enteric viruses in seawater. *Oceanol Acta* 21:983–992
- Gerba CP, Schaiberger GE (1975) Effect of particulates on virus survival in seawater. *J Water Pollut Control Fed* 47: 93–103
- Girones R, Jofre J, Bosch A (1989) Natural inactivation of enteric viruses in seawater. *J Environ Qual* 18:34–39
- Hine PM (1997) Trends in research on diseases of bivalve molluscs. *Bull Eur Assoc Fish Pathol* 17:180–183
- Hine PM, Thorne T (1997) Replication of herpes like viruses in haemocytes of adult flat oysters *Ostrea angasi*: an ultra-structural study. *Dis Aquat Org* 29:189–196
- Hine PM, Wesney B, Hay BE (1992) Herpesviruses associated with mortalities among hatchery-reared larval Pacific oysters, *Crassostrea gigas*. *Dis Aquat Org* 12:135–142

- Hine PM, Wesney B, Besant P (1998) Replication of a herpes-like virus in larvae of the flat oyster *Tiostrea chilensis* at ambient temperatures. *Dis Aquat Org* 32:161–171
- Jiang SC, Paul JH (1995) Viral contribution to dissolved DNA in the marine-environment as determined by differential centrifugation and kingdom probing. *Appl Environ Microbiol* 61:317–325
- Le Deuff RM, Renault T (1999) Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. *J Gen Virol* 80:1317–1322
- Le Deuff RM, Nicolas JL, Renault T, Cochenne N (1994) Experimental transmission of herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas*. *Bull Eur Assoc Fish Pathol* 14:69–72
- Le Deuff RM, Renault T, Gerard A (1996) Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster, *Crassostrea gigas*. *Dis Aquat Org* 24:149–157
- Lipart C, Renault T (2002) Herpes-like virus detection in infected *Crassostrea gigas* spat using DIG-labelled probes. *J Virol Methods* 101:1–10
- Montanié H, Hartmann HJ, Crottereau C, Trichet C (2002) Virus-like particle analysis in a seston-rich coastal pond using transmission electron microscopy. *Aquat Microb Ecol* 28:105–115
- Nicolas JL, Comps M, Cochenne N (1992) Herpes-like virus infecting Pacific oyster larvae, *Crassostrea gigas*. *Bull Eur Assoc Fish Pathol* 2:11–13
- Parry L, Dixon PF (1997) Stability of nine viral haemorrhagic septicaemia virus (VHSV) isolates in seawater. *Bull Eur Assoc Fish Pathol* 17:31–36
- Renault T (1998) Infections herpétiques chez les invertébrés: détection de virus de type herpes chez les mollusques bivalves marins. *Virologie* 2:401–403
- Renault T, Lipart C (1998) Diagnosis of herpes-like virus infections in oysters using molecular techniques. *Eur Aquac Soc (Spec Publ)* 26:235–236
- Renault T, Cochenne N, Le Deuff RM, Chollet B (1994a) Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. *Bull Eur Assoc Fish Pathol* 14:64–66
- Renault T, Le Deuff RM, Cochenne N, Maffart P (1994b) Herpesviruses associated with mortalities among Pacific oyster, *Crassostrea gigas*, in France—comparative study. *Rev Med Vet* 145:735–742
- Renault T, Le Deuff RM, Chollet B, Cochenne N, Gerard A (2000a) Concomitant herpes-like virus infections in hatchery-reared larvae and nursery-cultured spat *Crassostrea gigas* and *Ostrea edulis*. *Dis Aquat Org* 42:173–183
- Renault T, Le Deuff RM, Lipart C, Delsert C (2000b) Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. *J Virol Methods* 88:41–50
- Renault T, Lipart C, Arzul I (2001a) A herpes-like virus infecting *Crassostrea gigas* and *Ruditapes philippinarum* larvae in France. *J Fish Dis* 24:369–376
- Renault T, Lipart C, Arzul I (2001b) A herpes-like virus infects a non-ostreid bivalve species: virus replication in *Ruditapes philippinarum* larvae. *Dis Aquat Org* 45:1–7
- Renault T, Arzul I, Lipart C (in press) Development and use of an internal standard for oyster herpesvirus 1 detection by PCR. *J Virol Methods*
- Sobsey MD (1993) The use of molecular techniques for the detection of viruses in environmental samples. In: Guerrero R, Pedros-Alio (eds) *Trends in microbial ecology*. Spanish Society for Microbiology, Barcelona, p 601–606
- Suzuki S, Nakata T, Kamakura M, Yoshimoto M, Furukawa Y, Yamashita Y, Kusuda R (1997) Isolation of birnavirus from Agemaki (jack knife clam) *Sinonovacula constricta* and survey of the virus using PCR technique. *Fish Sci* 63:563–566
- Suzuki S, Kamakura M, Kusuda R (1998) Isolation of birnavirus from Japanese pearl oyster *Pinctada fucata*. *Fish Sci* 64:342–343
- Tsai YL, Tran B, Palmer CJ (1995) Analysis of viral-RNA persistence in seawater by reverse-transcriptase PCR. *Appl Environ Microbiol* 61:363–366
- Wait DA, Sobsey MD (2001) Comparative survival of enteric viruses and bacteria in Atlantic Ocean seawater. *Water Sci Technol* 43:139–142

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